METABOLISM OF 3-DEOXY-3-FLUORO-D-GLUCOSE BY *PSEUDOMONAS FLUORESCENS*

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Received 15 October 1970
Original figures received 30 October 1970

1. Introduction

As part of a programme of investigations concerned with the metabolic effects of monofluorinated deoxysugars on yeast [1] and certain microorganisms, we have recently examined the effect of 3-deoxy-3-fluoro-D-glucose* on *Pseudomonas fluorescens*. This organism is considered to initiate glucose catabolism via a cytochrome oxidase system within the cytoplasmic membrane [2] to D-gluconic and 2-keto-D-gluconic acid. Our previous *in vitro* studies indicated that 3FG could act as a rather poor substrate for yeast hexokinase [3] and this prompted us to examine the effect of 3FG on the glucokinase and hexokinase-free system of *Ps. fluorescens*.

It is the purpose of this communication to report that (a) with increasing concentrations of 3FG as a substrate for resting cell suspensions of *Ps. fluorescens* in 0.067 M buffer, 3-deoxy-3-fluoro-D-gluconic acid* is the primary oxidation product which accumulates with subsequent loss of cell viability; (b) in the same system as above but with 0.67 M buffer, 3FGA is the primary oxidation product which accumulates without significant loss of cell viability; (c) when 3FGA is a substrate for resting cells of *Ps. fluorescens* in 0.67 M buffer, a further oxidation product is formed which is tentatively assigned the structure 3-deoxy-3-fluoro-2-keto-D-gluconic acid*; (d) with 3FG or 3FGA as substrates there is no apparent cleavage of the C–F bond.

* *Abbreviations*:
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.

2. Methods and materials

Crystalline 3FG was prepared as previously reported by us [1]. A culture of *Ps. fluorescens*, A.3.12, obtained from N.C.I.B. was routinely maintained on glucose/mineral salts agar slopes. Respirometric studies were made with washed whole cell suspensions grown in glucose mineral salts and harvested after 18 hr at 30° in a Gallenkamp orbital shaker. After two washes at room temperature in either 0.067 M Sorensen-phosphate buffer or 0.67 M buffer, both at pH 7, the cells were resuspended in one of the above buffers at the equivalent of 20 mg dry wt./ml. Oxygen uptake was followed by the manometric method [4] in a Warburg respirometer at 30° and a total liquid volume of 2 ml, (see figs. 1 and 2). Cell viability was determined by serial dilution and colony counts on nutrient agar plates after 48 hr (see tables 1 and 2). Large scale incubations for the isolation of 3FGA were carried out in 1 litre Ehrlenmeyer flasks on a rotary shaker at 30° containing equiv. to 1000 mg dry wt. of washed resting cell suspensions in phosphate buffer of the appropiate molarity and 1 g of 3FG in a total volume of 200 ml. The progress of the incubation was monitored by withdrawing a 1 ml aliquot at zero time, shaking in the Warburg apparatus and determining the extent of oxidation. When the incubation was complete, the contents of the flasks were centrifuged at 6,200 *g* for 15 min and the supernatant immediately freeze-dried.

Glucose determinations on cell supernatants were by the Glucostat reagent (Hoffmann-La Roche and Co. Ltd., Basle, Switzerland). Fluoride anion was

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measured by a fluoride electrode (Orion Research Inc., Cambridge, Mass., U.S.A.) on both culture supernatants and alkali hydrolysed perchloric acid cell extracts as described elsewhere by us [6]. Total carbohydrate determinations were made by the phenol–sulphuric acid method [7] on both culture supernatants and trichloroacetic acid extracts of the cells.

Ascending thin-layer chromatography (TLC) was carried out on 20 X 20 cm plates using Silica-gel G according to Stahl (Shandon Scientific Co. Ltd.) and ethyl acetate–acetic acid–water (3:3:1, v/v) as solvent. After the solvent was removed from the developed plate it was sprayed with sulphuric acid–ethanol (60:40, v/v) and heated to 100° for 10 min. Alternatively, free sugars and aldonic acids were detected by benzidine–periodate [5]. All preparative plates (20 X 40 cm) were prepared with Silica-gel PF_{254} (Shandon Scientific Co. Ltd.). After the solvent had been removed from the developed plate it was examined under UV light (254 nm) and the different zones eluted with methanol or ethanol. The freeze-dried residue obtained from the supernatants of large scale incubations was extracted with cold ethanol, concentrated *in vacuo* and applied to the plate as a band. Electrophoretic separations were carried out in a Miles High Voltage Unit (Miles Hivolt Ltd.) using Whatman No. 1 paper (pre-washed with buffer) at 6 kV for 2 hr in formic–acetic acid buffer (pH 2). Aldonic acids were detected with benzidine–periodate [5] and the mobilities recorded with reference to glucose (M_G).

3. Results and discussion

The chemical synthesis of 3FGA was based upon

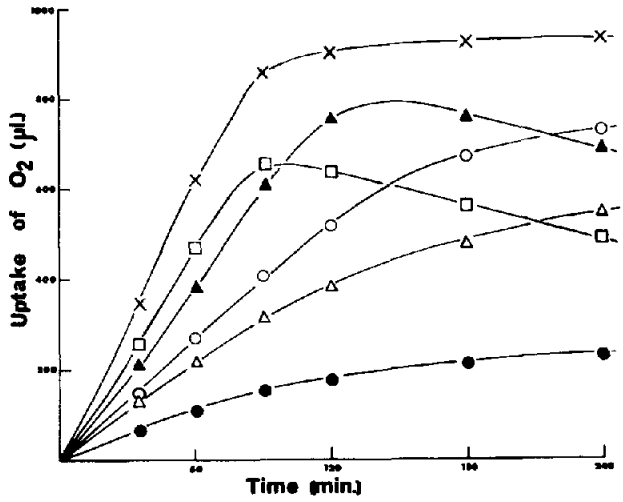

Fig. 1. Oxidation of 3FG by glucose grown washed cell suspensions of *Ps. fluorescens*. Warburg conditions: 30°, total volume, 2.0 ml gas phase, air. Each flask contains 20–100 µmoles substrate, 0.067 M buffer to 1.8 ml in main well, 0.2 ml KOH in centre well. Reaction was initiated by tipping 0.5 ml cell suspension (10 mg dry wt.) from side arm. x—x, 100 µmoles 3FG (endogenous respiration not subtracted); ▲—▲, 100 µmoles 3FG*; □—□, 80 µmoles 3FG*; ○—○, 60 µmoles 3FG*; △—△, 40 µmoles 3FG*; ●—●, 20 µmoles 3FG* (*endogenous subtracted, 450 µl in 4 hr).

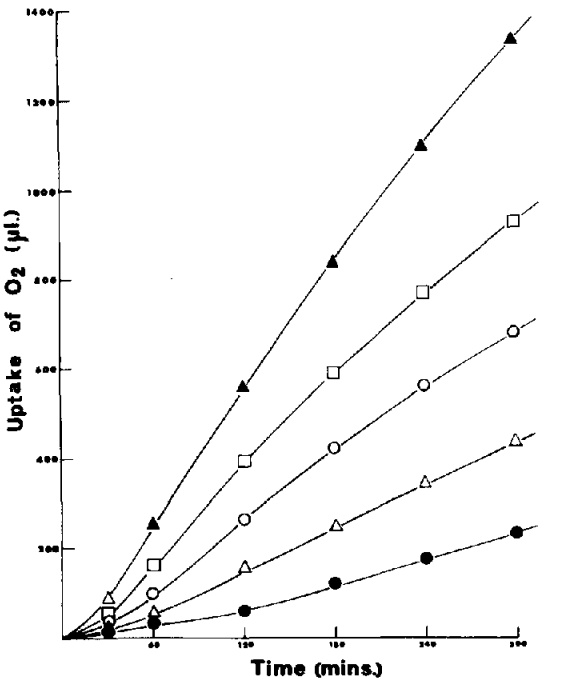

Fig. 2. Oxidation of 3FG by *Ps. fluorescens* cell suspensions in 0.67 M buffer. Warburg conditions: 30°, total volume, 2.0 ml, gas phase, air. Each flask contains 20–100 µmoles substrate, 0.67 M phosphate buffer to 1.8 ml in main well, 0.2 ml KOH in centre well. Reaction was initiated by tipping 0.5 ml cell suspension (8 mg dry wt.) from side arm. Endogenous respiration subtracted (260 µl in 6 hr). ▲—▲, 100 µmoles 3FG; □—□, 80 µmoles 3FG; ○—○, 60 µmoles 3FG, △—△, 40 µmoles 3FG; ●—●, 20 µmoles 3FG.

Table 1
Oxidation of 0.01–0.05 M 3FG by washed suspensions of *Ps. fluorescens* in 0.067 M buffer.

Substrate oxidised (μmoles)	Period of incubation (hr)	Rate of oxidation (moles oxygen/ mg dry wt./hr)	Net O_2 uptake ml endog. subtracted	Moles O_2/ mole substr. oxidised	Viable bacteria per ml ($\times 10^{-8}$)
–	0	–	–	–	170
–	4	–	–	–	172
3FG					
20	4	0.45	233	0.52	160
40	4	0.93	550	0.51	155
60	4	1.22	735	0.55	137
80	4	1.81	787	0.44	7
100	4	1.95	655	0.29	3

Cells, grown for 20 hr with aeration at 30° in glucose/mineral salts medium, were collected at 4040 *g* for 10 min, washed twice in 0.067 M phosphate buffer, pH 7, and resuspended in buffer of the same molarity and pH to 20 mg dry wt./ml. Their ability to oxidise 3FG was determined manometrically (fig. 1).

the electrolytic oxidation of 3FG by a modification of a method previously reported for gluconic acid [8], and the 3FGA isolated as the hydrated calcium salt, m.p. 198°, (Found: C, 29.6; H, 5.3; F, 4.1. $Ca(C_6H_{10}FO_6)_2 \cdot 3H_2O$ requires C, 29.7; H, 5.3; F, 4.2%). The free acid was obtained from the calcium salt, after passage through Amberlite IR 120(H) cation exchange resin, as a crystalline material, m.p. 119°, (Found: C, 36.3; H, 5.6; F, 9.0. $C_6H_{11}FO_6$ requires C, 36.3; H, 5.6; F, 9.6%). TLC gave R_f, 0.38 and electrophoresis M_G, 18.0×10^2. Full experimental details will be reported elsewhere.

The oxidation of 3FG, at different concentrations, by washed resting cell suspensions of *Ps. fluorescens* in 0.067 M and 0.67 M buffer (figs. 1 and 2 respectively) indicate that at the lower buffer concentration the extent of oxygen uptake falls sharply when the exogenous 3FG concentration is in the range 80–100

Table 2
Oxidation of 0.01–0.05 M 3FG by washed suspensions of *Ps fluorescens* in 0.67 M buffer.

Substrate oxidised (μmoles)	Period of incubation (hr)	Rate of oxidation (moles oxygen/ mg dry wt./hr)	Net O_2 uptake ml endog. subtracted	Moles O_2/ mole substr. oxidised	Viable bacteria per ml ($\times 10^{-7}$)
–	0	–	–	–	35
–	5	–	–	–	5
3FG					
20	5	0.27	230	0.51	18
40	5	0.53	432	0.48	7
60	5	0.87	675	0.50	5
80	5	1.13	922	0.51	29
100	5	1.57	1332	0.60	25

Cells were grown as in table 1. The cell pellet was washed twice in 0.67 M phosphate buffer, pH 7, and resuspended in buffer of the same molarity and pH to the equivalent of 16 mg dry wt./ml. Their ability to oxidise 3FG was determined manometrically (fig. 2).

moles and that this also corresponds to a significant loss in cell viability (table 1). At higher buffer concentrations, however, this effect is not apparent (table 2) and the extent of oxidation of 3FG proceeds at all concentrations with the consumption of 1 atom of oxygen/mole of substrate. These results are consistent with the production of a fluorinated aldonic acid which leaks from the cell into the supernatant. At the lower buffer concentration (0.067 M) the accumulation of the acid alters the pH of the supernatant sufficiently to cause cell lysis and sudden change in oxygen consumption. This was confirmed when large scale incubations of 3FG at both 0.067 M and 0.67 M buffer concentrations allowed isolation of a fluoroaldonic acid from the washed cells and supernatant which had R_f, 0.38 and M_G, 18.0 X 10^2 identical with 3FGA (co-chromatography and electrophoresis). The extent and rates of oxidation of 3FG to 3FGA are shown in tables 1 and 2.

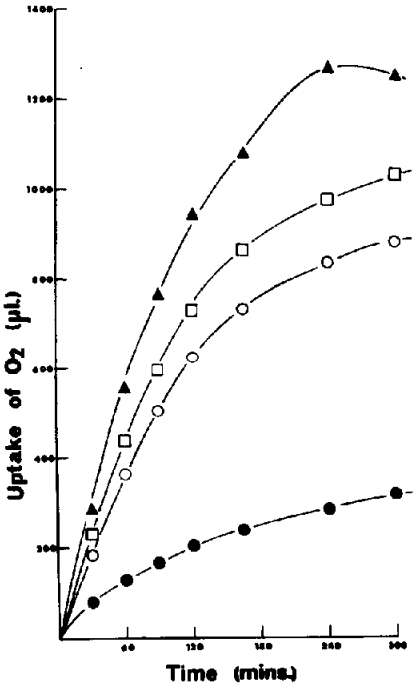

Fig. 3. Oxidation of 3FGA by washed resting cell suspensions of *Ps. fluorescens* in 0.67 M phosphate buffer. Warburg conditions: 30°, total volume, 2 ml, gas phase, air. Each flask contained 20–100 µmoles substrate, 0.67 M phosphate buffer to 1.8 ml in main well, 0.2 ml KOH in centre well. Reaction was initiated by tipping 0.5 ml cell suspension (15 mg dry wt.) from side arm. Endogenous respiration subtracted (144 µl in 5 hr). ▲—▲, 100 µmoles 3FGA; □—□, 80 µmoles 3FGA; ○—○, 60 µmoles 3FGA; ●—●, 20 µmoles 3FGA.

Fluoride determinations by the fluoride electrode on both cell supernatants and cell extracts [6] indicated that no detectable free fluoride anion was present at anytime during oxidation of the 3FG. Total carbohydrate determinations on both culture supernatants and trichloroacetic acid cell extracts indicated that unlike the effects of 3FG on the metabolism of *Saccharomyces cerevisiae* [1], there was no inhibition of polysaccharide synthesis. Although our results show that oxidation of 3FG by *Ps. fluorescens* is limited to the consumption of 1 atom of oxygen/mole of substrate, provision of this oxidation (3FGA) as an exogenous substrate for washed cell suspensions in 0.67 M buffer (fig. 3) show that further oxidation, with the consumption of another 1 atom of oxygen/mole of substrate, is possible to presumably 3-deoxy-3-fluoro-2-ketogluconic acid (3F2KGA). Although this acid has not yet been isolated and identified we have demonstrated that during the course of incubation with 3FGA there is no release of free fluoride anion and that a fluorinated reducing acid, shown by Benedicts reagent, is produced.

These results support our contention that certain monofluorinated analogous of carbohydrates and related compounds can act as biochemical pseudo-substrates [3] and further metabolic studies with 3FG and 3FGA are now in progress.

Acknowledgements

This work is supported by an S.R.C. Research Grant (NFT) and in part by a Grant from the S.R.C. Enzyme Chemistry and Technology Committee (NFT) jointly with Drs. R.Eisenthal and R.Harrison. One of us (FHW) thanks the Medical Research Council for the award of a Scholarship. We also wish to acknowledge the expert technical assistance of Mrs. B.Hunt.

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