Biochemical Pharmacology, Vol. 20, pp. 1071–1077. Pergamon Press, 1971. Printed in Great Britain

EFFECT OF 3-DEOXY-3-FLUORO-D-GLUCOSE ON GLYCOLYTIC INTERMEDIATES AND ADENINE NUCLEOTIDES IN RESTING CELLS OF *SACCHAROMYCES CEREVISIAE*

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(*Received* 23 *March* 1970; *accepted* 30 *October* 1970)

Abstract—The effects of 3-deoxy-3-fluoro-D-glucose (3FG*) upon the levels of glycolytic intermediates, adenine nucleotides and inorganic phosphate in resting cell suspensions of *Saccharomyces cerevisiae* have been investigated.

Significant decreases in the intracellular levels of UDPG, PGAs, ATP and inorganic phosphate were detected whilst increases in ADP, G-1-P and F-6-P were observed. The changes in ATP, ADP and G-1-P only became apparent after the changes in the other intermediates had occurred. It is argued that 3FG acts at least in part as a phosphate trap thereby occasioning a shift in the energy balance of these cells.

IN EARLIER reports[1,2] it was shown that the preincubation of resting cell suspensions of *Saccharomyces cerevisiae* with 5mM 3FG produced contrasting effects on the subsequent metabolism of glucose or of galactose. Thus with glucose, whilst the respiratory rate was only transiently affected, both glucose uptake and the synthesis of polysaccharide was inhibited. In contrast, with galactose, long term respiration was inhibited, sugar uptake was not affected and polysaccharide synthesis was stimulated. These results indicate the possibility of an inhibitory form of the analogue acting on either phosphoglucomutase (α-D-Glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) or UDPG pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9) in a similar fashion to that suggested for 2DOG.[3] In an attempt to localize such possible sites of action the present study of glycolytic intermediate levels, together with those of adenine nucleotides and inorganic phosphate, was undertaken.

METHODS

(a) *Growth of cells*

Cells grown overnight at 30° on agar slopes, made up as: 2% glucose, 0·3% yeast extract (Difco, London), 0·04 M potassium phosphate buffer pH 6·8 and 1·2% Oxoid agar No. 3,[3] were removed into sterile water and a suitably diluted suspension used to inoculate 75 ml of similar aqueous medium in a 250-ml conical flask at a starting level

* Abbreviations used: 3-deoxy-3-fluoro-D-glucose (3FG); 2 deoxy-D-glucose (2DOG.); Uridine diphosphate glucose (UDPG); glucose-1-phosphate (G-1-P); glucose-6-phosphate (G-6-P); fructose-6-phosphate (F-6-P); 2 and 3-phosphoglyceric acids (PGAs); phosphoenolpyruvic acid (PEP); adenosine-5′-triphosphate (ATP); adenosine-5′-diphosphate (ADP); adenosine-5′-monophosphate (AMP).

equivalent to 50 μg dry wt. of cell/flask. Growth at 30° and with shaking at 250 rev./min on a gyrorotatory shaker (Gallenkamp Ltd. London), was continued for 20 hr at which stage the culture had entered the stationary phase. The cells were harvested by centrifugation, washed twice with 100 ml of 0·04 M phosphate buffer at pH 5·2 and 5° and then resuspended in the same buffer to a final concentration equivalent to 3·5 mg dry wt./ml.

(b) *Incubation with 3FG*

After equilibration of this suspension to 30°, 3FG was added to a final concentration of 5mM and incubation continued with shaking (250 rev./min) for 90 min. Samples for intermediate estimation were removed at intervals as indicated below.

(c) *Extraction of intermediates*

Intracellular intermediates were extracted by the method described by Maitra and Estabrook.[4] This involved taking aliquots of the cell suspension at intervals, adding perchloric acid to a final concentration of 0·5 M and allowing to stand at room temperature, with occasional shaking, for 30 min. The suspension was then neutralized with 40% potassium hydroxide and the resulting precipitate spun down. Samples of the clear supernatant were then used in estimations described below. This method has been reported to be less effective for adenine nucleotides than a trichloracetic acid extraction.[5] However experiments in which known amounts of ATP or of ADP were added to the cell suspensions immediately before perchloric acid treatment gave recoveries of better than 95 per cent after correction of the values for the intracellular level measured in parallel control experiments. In addition, the values obtained for the intracellular adenine nucleotide levels were of similar magnitude to those reported by the latter workers. These observations together with the experimental design, in which a comparison with control cells was carried out in parallel to the 3FG treated cells, make it unlikely that this source of error was significant.

(d) *Measurement of intermediates*

G-6-P, F-6-P and ATP were measured by the method of Hohorst *et al.*;[6] G-1-P in the same system with the addition of phosphoglucomutase (α-D-glucose-1,6-diphosphate; α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) subsequent to the G-6-P estimation; UDPG by the methods of Mills and Smith;[7] fructose-1,6-diphosphate and triose phosphates by the method of Bücher and Hohorst;[8] PGA, PEP and pyruvic acid by the method of Czok and Eckert[9] and ADP by the method of Adam.[10]

The extraction and estimation of inorganic and total phosphates was carried out by first filtering off a 20-ml sample of the cell suspension onto a membrane filter (Oxoid Ltd, London), washing the cells with 200 ml of distilled water at 5° and then extracting with 5 ml of 0·5 perchloric acid, at room temperature, for 30 min. This extract was then adjusted to pH 7·0 with 40% potassium hydroxide. An aliquot of the clear supernatant was taken to estimate inorganic phosphate by the method described by Clark.[11]

Total phosphate was measured by taking a 1-ml aliquot of the complete extract, hydrolysing it, by refluxing until clear with 1 ml of a mixture of 3 vol. analar H_2SO_4 to 2 vol. 70% perchloric acid, diluting to 25 ml with distilled water and estimating by

the method of Clark.[11] All assays were carried out using a Unicam SP800 spectrophotometer with scale expansion and recorder.

(e) *Effect of 3FG or its metabolites on intermediate measurements*

In measuring intermediate levels in 3FG treated cells the possibility of the results being influenced by 3FG or its metabolites was examined. Two different types of error could occur. In the first type it might be argued that 3FG or its metabolites inhibited the assay systems used and gave falsely low values.To check this, after the assay had gone to completion, a known amount of the natural intermediate was added to the cuvette and the extent of the renewed activity noted. In every case the recovery of this added material was complete.

The second type of error would have arisen if 3FG or its metabolites reacted with the assay system and would give falsely high values for the intermediate. It has been shown for instance, that a combination of hexokinase (ATP: D-hexose-6-phosphate transferase, EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate; NADP oxidoreductase, EC 1.1.1.49) can act upon 3FG *in vitro*.[1] However the *in vitro* studies required higher enzymes concentrations to get a detectable rate than those used in these assays, and since the latter were carried out at the same time as those on extracts from control cells and showed both similar rates and completion times to the control cell extracts, it is regarded as unlikely that the results have been influenced by such effects.

(f) *Materials*

Reagents were of Analar grade (B.D.H. Poole) where possible, and all enzymes and substrates were supplied by Boehringer Corporation (London) Ltd. with the exception of uridine-diphosphate-glucose dehydrogenase (UDP glucose: NAD oxidoreductase, EC 1.1.1.22) (Sigma Chemical Co. London) Crystalline 3FG was prepared as previously reported.[2]

RESULTS

(a) *The effect of 3FG on hexose monophosphate levels*

Figure 1 shows the changes in G-1-P (1a), G-6-P (1b) and F-6-P (1c) during the 90-min incubation.

As might be expected in cells without external glucose the levels of these intermediates are rather low. However some significant ($P < 0{\cdot}05$) differences between control and 3FG treated cells were detected. It would appear that there is little difference between the control and 3FG treated cells in either G-6-P or F-6-P levels but that a significantly greater level of G-1-P starts to appear in 3FG treated cells after 20 min incubation. There may be a tendency for the other monophosphates to increase above the control level at later stages of the incubation but this only becomes significant, in the case of F-6-P after 90 min.

(b) *The effect of 3FG on PGAs, PEP and pyruvate levels*

It is apparent from Fig. 2 that 3FG treatment initiates a rapid drop in the levels of PGAs and of PEP. A significant but less marked drop in pyruvate also occurs. Unlike

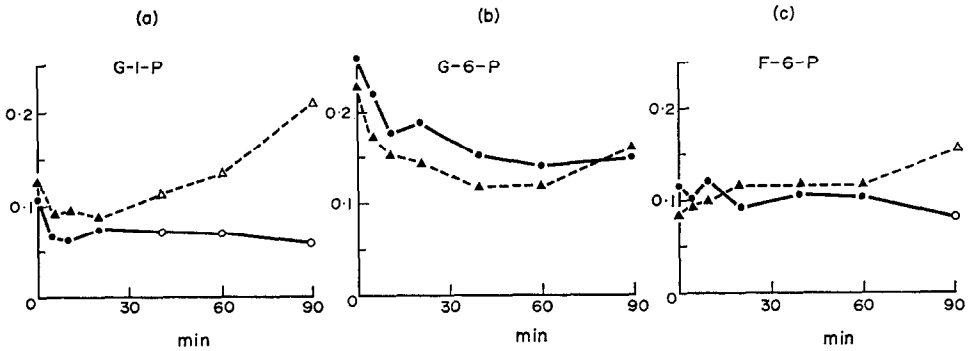

FIG. 1. The effect of 5 mM 3FG on the levels of (a) G-1-P; (b) G-6-P; (c) F-6-P. ●—○ control; ▲—△ 3FG; open symbols = sign. diff. *t*-test. $n = 3$. Units μM/g wet wt.

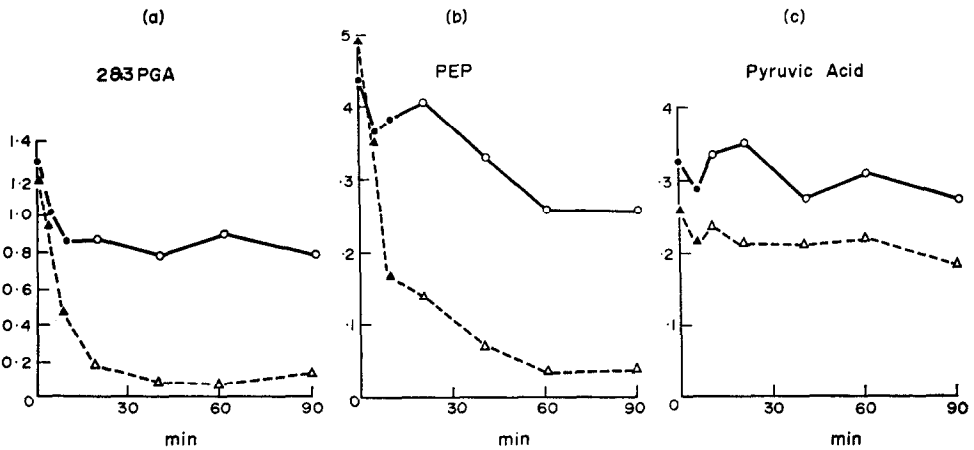

FIG. 2. The effect of 5 mM 3FG on the levels of (a) 2 and 3 PGA; (b) PEP; (c) Pyruvic acid. Legend as in Fig. 1.

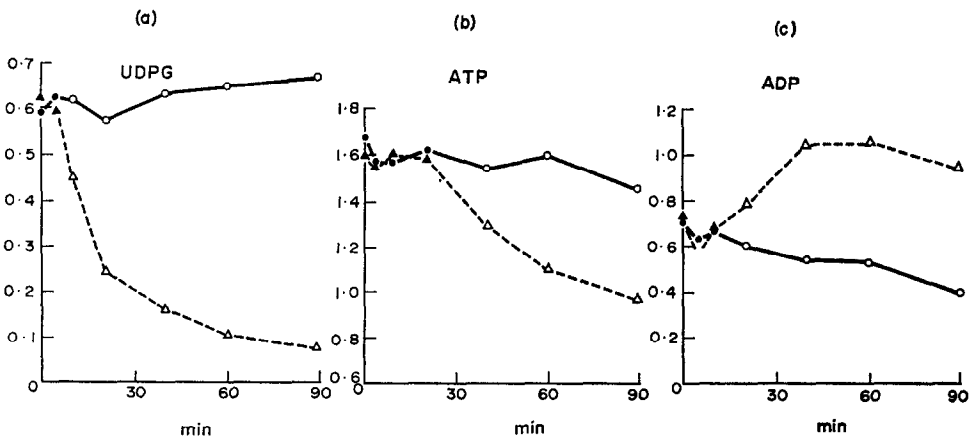

FIG. 3. The effect of 5 mM 3FG on the levels of (a) UDPG; (b) ATP; (c) ADP. Legend as in Fig. 1.

the sequence of change in the G-1-P level already described, the major decreases in both PGAs and PEP occur within 20 min and subsequent to this a low steady level appears to be established. This would suggest that 3FG produces changes in these intermediates prior to the changes in the hexose monophosphate levels.

(c) *The effect of 3FG on UDPG, ATP and ADP levels*

From Fig. 3 (a) and (b), it can be seen that both UDPG and ATP levels decrease very markedly. ADP shows a marked rise (3c). The behaviour of UDPG would appear to be different to that of ATP in that the fall in its level occurs more rapidly, in a similar fashion to that shown by PGAs and PEP. Both the decrease in ATP and the parallel increase in ADP occur in major part after 20 min. The ADP method can be adapted to measure AMP[10] but gave unsatisfactory results in these cell extracts.

TABLE 1. THE EFFECT OF 3FG ON THE LEVELS OF TOTAL AND INORGANIC PHOSPHATE

	Total P		Inorganic P	
Time (min)	0	90	0	90
Control	172·0 ± 3·8	172 ± 4·7	25·4 ± 1·1	25·3 ± 1·0
3FG	171·0 ± 3·8	172 ± 6·2	25·9 ± 2·2	20·3 ± 1·2

Data given as μmoles/g wet wt. of cells. ± S.E.M.; $n = 8$.

(d) *The effects of 3FG on total and inorganic phosphate levels*

It can be seen from Table 1 that no changes in total phosphate occurred in either control or 3FG treated cells over the period of the experiment and that the level was the same in both types of cell. However in the case of the inorganic phosphate levels, although these remain constant in the control cells, a 20 per cent decrease is apparent in the 3FG treated cells. Since these cells showed no change in total phosphate it must be assumed that this decrease was due to a fraction of the inorganic phosphate becoming bound during the 3FG incorporation into the cell.

DISCUSSION

The reduction of the inorganic phosphate level, in the context of a constant total phosphate level, in cells treated with 3FG, suggests that 3FG causes a binding of inorganic phosphate, possibly as 3FG phosphates.

If it is assumed that the endogenous metabolism of these resting cells is supported, at least in part, by the breakdown of reserve carbohydrate,[12] then the increased level of G-1-P could be argued to indicate an interference by 3FG metabolites with glycogen catabolism at the phosphoglucomutase (α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate transferase, EC 2.7.5.1) stage. However an inhibition at this stage should lead to a reduction in the level of the immediate product, G-6-P, and this does not occur. In addition the small increase in G-1-P only occurs after the more extensive decreases found in the levels of UDPG, PGAs and PEP. Thus the change in G-1-P level appears to reflect a consequential, rather than a primary, effect.

It has been known for some time from studies of the regulation of glycolysis[13] that

cells can react to changes which might alter the energy balance of the cell by shifting the control of regulatory enzymes so as to maintain this balance. Thus changes in the adenine nucleotide balance are known to alter the activity of yeast phosphofructokinase (ATP: D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11).[14,15]

The results reported here suggest that such a response occurs in 3FG treated cells. The breakdown of glycogen requires inorganic phosphate at both the phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1) and triosephosphate dehydrogenase/phosphoglycerate kinase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12) stages and the binding of inorganic phosphate in 3FG treated cells may be sufficient to reduce the rate of breakdown of glycogen. Some evidence for this arises from a consideration of the changes in the levels of PGAs, PEP and pyruvate. Two interpretations of the fall in PGAs and PEP levels can be put forward. Either it is due to activation of the enzyme pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) in the presence of an equal or increased flux of substrate through the glycolytic sequence, or the activity of the enzyme remains the same, and the changes are due to a reduced substrate supply. These alternatives can be distinguished by considering the changes in the product, in this case pyruvic acid. Clearly the decrease detected in the pyruvate level favours the second alternative, of a reduced substrate supply. This interpretation is reinforced by our unpublished observations that 3FG causes a small but consistent reduction in the endogenous respiration rate.

Such an inhibition of glycogen degradation would be expected to lead to a reduction in ATP formation. Under these circumstances, the rapid fall in UDPG level, whether arising from a direct inhibition of its synthesis by a fluorinated analogue, or from a reduction in the supply of UTP, could be seen to be an ATP conserving response. By contrast the fall in PGAs and PEP could be argued to be an ATP producing response, through the operation of pyruvate kinase. The maintenance of the ATP level throughout these changes can thus be explained. However such responses must be limited in their effectiveness, for under the continued pressure of 3FG uptake after 20 min, the ATP level cannot be maintained and, under the conditions of reduced inorganic phosphate level, the ADP content rises.

Both the binding of inorganic phosphate and the reduction of ATP level in cells treated with 3FG, together with the effects of the analogue on glucose and galactose metabolism previously reported,[2] are similar to the effects of 2DOG on yeast cells. Thus 2DOG has been shown to interfere with polysaccharide synthesis.[3] In addition to inhibiting this by the formation of UDP-2-deoxy-D-glucose, it has been shown recently that incorporation of the deoxy sugar into 2,2′dideoxy-α-α′-trehalose can occur.[16] In terms of its effects on energy balance, Maitra and Estabrook,[17] reported a decrease in ATP, coupled with an increase in ADP, in yeast cells treated with 2DOG, and correlated these changes with the binding of inorganic phosphate. Other nucleotide triphosphates have been shown to be reduced in animal cells treated with 2DOG.[18] From these results therefore it appears that 3FG acts, in a similar manner to 2DOG, as a phosphate trap and produces a shift in the energy balance of the resting cell suspension. Whether the binding of phosphate by the analogue is sufficiently tight to continue to operate during the metabolism of external glucose or whether, like 2DOG, metabolites directly inhibitory to enzymes are produced is not yet known but it is hoped to report on these alternatives as a result of further studies.

Acknowledgements—Part of this work was supported by a Science Research Council grant (NFT). The authors wish to thank Mrs. B. Hunt for her expert assistance in the preparation of crystalline 3FG. One of us (B.W.) acknowledges receipt of an S.R.C. research studentship.

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