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THE EFFECT OF MONOFLUOROACETIC ACID UPON THE GLUCOSE METABOLISM OF *SACCHAROMYCES CEREVISIAE*

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Abstract—(1) The effect of monofluoroacetic acid (HFA) upon the glucose metabolism of resting suspensions of yeast cells has been investigated.

(2) The degree of inhibition of respiration, ethanol production and polysaccharide synthesis and glucose uptake in the presence of HFA at pH 2·2 and pH 6·8 has been compared.

(3) A greater inhibition of these parameters at pH 2·2 than at pH 6·8 has been found.

(4) It is argued that the primary effect of HFA appears to be upon the glucose uptake and that effects upon respiration, ethanol production and polysaccharide synthesis are largely a consequence of the effects upon glucose uptake.

(5) The results are argued to be consistent with the existence in HFA treated cells of an alternative oxidative route at pH 6·8 which is either absent or largely inhibited at pH 2·2.

ALDOUS[1, 2] has reported a differential effect of monofluoroacetic acid (HFA) upon resting cell suspensions of baker's yeast (*Saccharamyces cerevisiae*) at acid and neutral pH values. Their respiration in the presence of glucose was found to be inhibited when suspended at both pH 2·2 and pH 6·8 but was more inhibited at pH 2·2 (~80%) than at pH 6·8 (~15%). As a result of investigations of this phenomenon it has been argued that at neutral pH values an alternative metabolic route is available for the oxidation of glucose which is not inhibited by HFA. At acid pH values it is argued that this alternative route is not available and the HFA shows its full effects. In animal tissues HFA is believed to act via a lethal synthesis of monofluorocitric acid which inhibits aconitase (E.C.4.2.1.3 citrate (isocitrate) hydrolyase) and hence blocks the complete oxidation of 2C fragments via the tricarboxylic acid cycle.[3] When this phenomenon occurs it is possible to detect a large increase in the citric acid level in the tissue.[3] Recent work in heart tissue[4, 5, 6] and in yeast cells[7] has shown that citric acid, together with ATP, is a powerful inhibitor of the enzyme phosphofructokinase (E.C.2.7.1.11.ATP: D-Fructose-6-phosphate 1-phosphotransferase) from these sources. It could be argued then, that in addition to the inhibition of aconitase, HFA could cause, via a feedback mechanism, the inhibition of phosphofructokinase in these cells. Such an inhibition in turn could lead to the accumulation of hexose monophosphates in the cells. This has been shown to occur in rat heart.[15] If such a feedback mechanism occurs then HFA might be expected to exert an effect upon such parameters as glucose uptake, ethanol production and polysaccharide synthesis in addition to the observed effects upon oxygen. uptake. Examination of these parameters might then

lead to an understanding of the nature of the alternative mechanisms said to be available at neutral pH which are not available at acid pH.

METHODS

(a) *Preincubation of cells with HFA*

5 g of baker's yeast (D.C.L.) was washed twice with distilled water to free it from filler material and a 25 mg wet weight/ml suspension prepared using 0·1 M HCl/KCl buffer[17] at pH 2·2. Four 20 ml aliquots of this suspension were taken and to two of these sufficient HFA (BDH) was added to yield a final concentration of 5 mM. The remaining two aliquots had no additions and acted as controls. All four were then incubated at 25° in a shaking waterbath for 30 min. Each of the aliquots was centrifuged at 18,000 *g* for 15 min, the supernatant discarded, and the cells washed twice with the HCl/KCl buffer at pH 2·2.

(b) *Respiration of cells*

The cells, prepared as before, were made up either in 20 ml of the HCl/KCl buffer or in 20 ml of a double phosphate buffer[18] at pH 6·8. The oxygen uptake of these cells was then measured at 25°, with a final concentration of 0·3 M glucose, for a minimum of 2 hr.

(c) *Measurement of glucose uptake, ethanol production and polysaccharide synthesis*

The HFA poisoned and control cells were made up either in HCl/KCl buffer at pH 2·2 containing 0·3 M glucose or in the double phosphate buffer at pH 6·8 containing 0·3 M glucose. All suspensions were adjusted to give 25 mg wet wt./ml of cells. Immediately the suspensions were prepared 2 ml samples were taken from each to give a zero time level. These samples were filtered through membrane filters (Oxoid.), the filtrate was collected and later used to determine reducing sugar by the 2·5 dinitrosalicylate method[11] and to determine ethanol by the alcohol dehydrogenase method.[10] The cells on the membrane were washed with 50 ml of the buffer in which they had been incubated and then, together with the membrane, were transferred to 5 ml of 0·5 M trichloroacetic acid. This extraction was continued overnight and then the cells in the extractant were made up to 50 ml with distilled water. Aliquots of this solution were than taken for total carbohydrate determination by the anthrone method.[9]

The original aliquots after removal of the zero time sample were placed in a shaking waterbath at 25° and further samples were taken from each at 2 hr and 4 hr intervals. Analyses, similar to those described for the zero time sample, were carried out on these.

RESULTS

(a) *The effect of HFA upon glucose respiration*

The respiration rates of cells treated as described are shown in Table 1. It is apparent that the control rate of respiration is very similar at the two pH values and that there is a marked difference in the degree of inhibition in poisoned cells. Thus at pH 6·8 the inhibition is 16 per cent but at pH 2·2 it is as high as 66 per cent. Although the inhibition at pH 6·8 agrees well with reported values[1] the pH 2·2 level is not as great. The difference may well be due to the suspending buffer. At pH 2·2 a KCl/HCl buffer

systems is used whereas K+ions were absent in the system used by Aldous. Rothstein[8] has reported that K+ions exert a stimulatory effect on glucose respiration at acid pH. In one experiment where the respiration with poisoned cells was compared

TABLE 1. THE EFFECT OF HFA UPON THE RESPIRATION RATE OF GLUCOSE IN YEAST CELLS SUSPENDED AT pH 2·2 AND pH 6·8

	C* 2·2	H* 2·2	C* 6·8	H* 6·8
$\bar{x}$	7·3†	3·2†	7·3†	6·1†
n	7	7	6	6
SEM	0·77	0·39	0·65	0·53
		% Inhibition of respiration		
		66%		16%

* = C 2·2 control at pH 2·2.
H 2·2 HFA treated cells at pH 2·2 etc.
† = average values in μl/min/25 mg wet wt. of cells.

at pH 2·2 in (a) KCl/HCl, and (b) NaCl/HCl it was found that the degree of inhibition was greater in NaCl/HCl (77%) .The oxygen uptake curves appeared linear up to 2 hr in all cases and the extent of the inhibition remained the same after 2 hr as immediately after the addition of glucose.

(b) *The effect of HFA upon glucose uptake*

Comparison of the effect of HFA upon the respiration rate with its effect upon glucose uptake (Table 2) shows certain differences. There may be a difference in the

TABLE 2. THE EFFECT OF HFA UPON THE GLUCOSE UPTAKE IN YEAST CELLS SUSPENDED AT pH 2·2 AND pH 6·8

Time (min)		C 2·2	H 2·2	C 6·8	H 6·8
120	x	76*	42	90	70
	n	3	3	3	3
	S.E.M.	2·3	2·1	1·5	1·5
240	$\bar{x}$	88	47	169	131
	n	3	3	3	3
	S.E.M.	2·9	5·5	16·5	11·5
% Inhibition of Glucose uptake					
120			45%		22%
240			46%		22%

* = average values in μM/glucose/25 mg wet wt. of cells.

rate of glucose uptake in the early stages between the controls ($P > 0·1$) and this difference becomes very apparent after 4 hr. This is largely due to a slowing down of the uptake at pH 2·2 after 2 hr, a phenomenon which is not apparent at pH 6·8. The same type of behaviour is to be seen in poisoned cells but in addition an inhibition of uptake is apparent and, like the respiratory pattern, it is greater at pH 2·2 (45%) than at pH 6·8 (22%).

(c) *The effect of HFA upon ethanol production*

The conditions under which the cells are incubated (high glucose and cell concentra-

tion) are similar to those which have been reported to lead to the onset of aerobic fermentation[13] and considerable amounts of ethanol have been found to accumulate. The amounts measured are shown in Table 3. The progress of the accumulation is similar to that seen in the glucose uptake data. Thus, although the levels at 2 hr are not significantly different in the two controls ($P > 0{\cdot}10$) after 4 hr it becomes highly significant ($P < 0{\cdot}001$). Again like the progress of glucose uptake, ethanol production slows down in the controls at pH 2·2 after 2 hr but continues at much the same rate at pH 6·8. Again this behaviour is reflected in that of the poisoned

TABLE 3. THE EFFECT OF HFA UPON THE ETHANOL PRODUCTION FROM GLUCOSE IN YEAST CELLS SUSPENDED AT pH 2·2 AND pH 6·8

Time (min)		C 2·2	H 2·2	C 6·8	H 6·8
120	$\bar{x}$	46*	19	54	41
	n	3	3	3	3
	SEM	2·7	3·0	3·4	1·3
	%†	60%	45%	60%	59%
240	$\bar{x}$	68*	24	109	83
	n	3	3	3	3
	SEM	4·4	3·5	6·6	2·4
	%†	77%	51%	64%	63%
% Inhibition of ethanol production					
120			59%		24%
240			65%		24%
% Inhibition of the route to ethanol					
120			25%		0%
240			44%		0%

* = average values in μM glucose equivalent/25 mg wet wt of cells.
† = % of glucose taken up appearing as ethanol

cells and again the inhibition in the early stages is higher at pH 2·2 (59–65%) than at pH 6·8 (24%).

In general then it can be seen that ethanol production closely parallels the behaviour of the cells with respect to glucose uptake. Differences do exist however, when the results are presented in terms of the proportion of glucose taken up that appears as ethanol. These proportions are a measure of the extent to which the metabolic route to ethanol is utilized in the four conditions and it can be seen that in addition to the inhibitory effects of HFA upon glucose uptake there is a more direct effect of HFA upon ethanol production at pH 2·2. Thus whereas at pH 6·8 the same proportion of glucose appears as ethanol in the poisoned cells as appears in the controls, at pH 2·2 the proportions are changed in the poisoned cells compared with the controls. This can be interpreted in that whereas the route to ethanol is used to the same extent in poisoned cells as in controls at pH 6·8 and the reduction in ethanol production in the poisoned cells is simply a consequence of the reduced glucose uptake, at pH 2·2 in addition to the reduction in ethanol caused by reduced glucose uptake the actual route to ethanol is inhibited by HFA. The extent of this direct inhibition of the route appears to increase with time.

(d) *The effect of HFA upon polysaccharide synthesis*

At the high levels of glucose used during these incubations polysaccharide synthesis

has been shown to occur.[12] The influence of HFA upon this metabolic route has been examined at the two pH values. The absolute levels of anthrone reacting material are shown in Table 4 and the increase in level is shown in Table 5. Approximately the same behaviour is seen in these results as is seen in ethanol production and glucose

TABLE 4. THE EFFECT OF HFA UPON THE POLYSACCHARIDE LEVELS IN YEAST CELLS SUSPENDED AT pH 2·2 AND pH 6·8 AND IN THE PRESENCE OF GLUCOSE

Time (min)		C 2·2	H 2·2	C 6·8	H 6·8
0	x	75·9*	75·9	79·1	79·1
	n	27	27	30	30
	S.E.M.	0·80	0·80	0·97	0·97
120	x	104·8	81·7	105·6	90·2
	n	13	14	8	8
	S.E.M.	2·10	2·02	2·48	3·21
240	x	96·7	85·3	135·7	100·2
	n	15	13	10	9
	S.E.M.	1·62	2·10	3·04	1·69

* Average values in μg glucose equivalent/mg wet wt. of cells.

uptake. The levels continue to increase linearly up to 4 hr at pH 6·8 but again at pH 2·2 the increase falls away after 2 hr and a decrease in level is seen in the control cells after 4 hr ($P < 0{\cdot}001$). The incorporation in poisoned cells at pH 2·2 however, continues at a low rate up to 4 hr.

TABLE 5. THE EFFECT OF HFA UPON POLYSACCHARIDE LEVELS IN YEAST CELLS SUSPENDED AT pH 2·2 AND pH 6·8 AND IN THE PRESENCE OF GLUCOSE

Time (min)	C 2·2	H 2·2	C 6·8	H 6·8
120	4·00*	0·80	3·68	1·54
	(5·3†)	(1·9)	(4·1)	(2·2)
240	2·88	1·31	7·86	2·93
	(3·3†)	(2·8)	(4·6)	(2·2)
%Inhibition of polysaccharide synthesis				
120	80%			58%
240	55%			63%
% Inhibition of the route to polysaccharide				
120	64%			46%
240	15%			52%

* = average values in μM glucose equivalent/25 mg wet wt. of cells.
† = % of glucose taken up appearing as polysaccharide.

Consideration of the extent of inhibition (Table 5) shows some differences from the behaviour shown with glucose uptake and ethanol production. Firstly, the extent of the inhibition is greater at both pH values. Secondly, the difference in the extent of inhibition at the two pH values is much less. This is largely due to the inhibition at pH 6·8 being disproportionately increased.

Thus HFA appears to have a more extensive effect upon polysaccharide synthesis than upon glucose uptake particularly at pH 6·8 and there must be a greater net usage of other metabolic routes for the utilization of the glucose taken up, at least in

the poisoned cells. To compare the differential effect at the two pH values it is necessary again to consider the extent to which the route synthesising polysaccharide is utilised. Once again HFA inhibits specifically the route to polysaccharide above and beyond the extent expected merely by its action upon glucose uptake. In the case of this specific HFA effect upon polysaccharide synthesis there is little differential effect of pH. Thus the inhibition of the route to polysaccharide at pH 6·8 is very similar to the inhibition of that route at pH 2·2.

DISCUSSION

The response of the cells to HFA in terms of respiration, ethanol production and polysaccharide synthesis follows fairly closely the response in glucose uptake. It can be argued that the effects upon these parameters follow largely as a consequence of an interference in glucose uptake mechanisms. The feedback mechanism elaborated in the introduction could be used to explain the results at pH 2·2 particularly as the accumulation of hexose monophosphates might be expected to exert an inhibitory effect upon hexokinase (E.C.2.7.1.1.ATP: D-hexose-6-phosphotransferase) by product inhibition.[16] It is difficult however to argue that such a mechanism is the only one operating. Firstly, the work on phosphofructokinase has shown that ATP as well as citrate is an inhibitor and that AMP can stimulate the enzyme. In the normal cell it is likely that both citrate and ATP would be elevated under similar circumstances and there is no conflict in the operation of the dual inhibition. In HFA poisoned cells however it is likely that when the citrate is artificially elevated by aconitase inhibition there will not be a parallel increase but rather a decrease in ATP levels. The extent of feedback inhibition thus will be a resultant of the inhibition of the enzyme by increased citrate and its stimulation by increased AMP/ADP.

Secondly, the results reported for the condition at pH 6·8 do not fit a simple citrate feedback mechanism. At this pH the cells show little inhibition of glucose uptake but Aldous[14] has reported (and we have confirmed this) that citrate levels are much higher in HFA treated cells at pH 6·8 than at pH 2·2. If feedback occurs in poisoned cells at pH 2·2 then the greater level of citrate in poisoned cells at pH 6·8 would be expected to lead to a greater extent of feedback inhibition and a greater inhibition of glucose uptake. This is not the case. Citrate feedback could still be argued to occur in poisoned cells at pH 6·8, however, if an alternative route for the removal of hexosemonophosphates exists in these cells which is not available at pH 2·2, the removal of hexose monophosphates by this route would reduce the possibility of product inhibition of glucose uptake.

The results obtained for ethanol production and polysaccharide synthesis could be consistent with this hypothesis. The routes leading to the production of both these materials show inhibitory effects of HFA operative above and beyond the effects upon glucose uptake.

In the case of polysaccharide synthesis a citrate feedback leading to hexose monophosphate accumulation would make available increased levels of one of the necessary reactants for the synthesis to occur. That an increase in the utilization of this route does not occur may be due to the other component required, i.e. UTP and its precursor ATP, not being elevated but rather being reduced. Such an argument would hold for the results seen at pH 2·2. At pH 6·8 the argument is rather more complicated.

If the oxidation rate seen in poisoned cells is a consequence of a route that yields energy—it may be reasonable to suppose that the second component required for polysaccharide synthesis, i.e. UTP and ATP, would be present and that conditions would be suitable for an increased utilisation of this route. However, the mechanism leading to this energy production might of itself need hexose monophosphate and thus cause a situation where this material, which is a component required for synthesis, is no longer available at an increased level. If then the alternative oxidative route argued to be available at pH 6·8 in poisoned cells involves the removal of hexose monophosphates the results found in terms of polysaccharide synthesis at pH 6·8 might be expected.

In the case of ethanol production the different extent of utilisation of the route producing this material would suggest again that an alternative route for the production of ethanol precursors exists at pH 6·8 which is not available at pH 2·2. Thus a mechanism exists for the production of ethanol which is not inhibited at pH 6·8 above and beyond the primary inhibition of glucose uptake induced by HFA. This route is not available at pH 2·2 for inhibition above and beyond glucose uptake inhibition does occur in poisoned cells at this pH value.

The results reported here then may be consistent with the hypothesis that at pH 6·8 in HFA poisoned cells an alternative oxidation route for glucose is available which is absent or inhibited at pH 2·2. If feedback inhibition by citrate is the primary cause of the effects noticed then this alternative route must involve hexose monophosphate and lead to some yield of ATP. It would be expected that differences in these components would be found in cells exposed to the different environments and this is being investigated at the present time.

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