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THE NATURE OF THE INHIBITORY ACTION OF H^+ AND FLUOROACETIC ACID ON METABOLISM IN YEAST CELLS

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Abstract—The concentration of intracellular citrate of non-dividing yeast cells has been shown to fluctuate with variations in environmental pH and glucose concentration. Environmental conditions which lead to a low intracellular citrate favor poisoning by fluoroacetic acid (HFA), but a high concentration of citrate does not always protect against HFA toxicity.

It is suggested that yeast cells possess two metabolic pathways in which citrate occurs as an intermediate, but only one of these is blocked by HFA. A high H^+ ion concentration limits the input into one of these pathways and therefore limits the total amount of citrate that accumulates in HFA poisoned cells. While the addition of K^+ ions stimulates citrate production, this does not appear to take place in the HFA blocked pathway.

INTRODUCTION

Suspensions of non-proliferating yeast cells that have been exposed to fluoroacetic acid (HFA) consume oxygen at a very much reduced rate in media buffered at pH 2·2; but at pH 5·5 the rate of respiration is the same as that of an untreated control suspension.[1] This phenomenon was observed as long as the cells were suspended in a glucose-containing medium. When suspended in a medium containing acetate as the extracellular substrate, the same degree of respiratory depression was seen at either extreme of pH. Thus the effect of raising the pH appeared to have been one of altering the metabolic route of glucose oxidation from one which had been blocked by exposure to HFA to one which had not. Since HFA was presumed to be a specific inhibitor of the tricarboxylic acid (TCA) cycle[2] certain deductions were made in an attempt to interpret these findings in terms of the relation between intracellular biochemical reactions.

Subsequent work on this problem has brought facts to light which cast some doubt not only upon our previous interpretations but also upon the assumed nature of the metabolic block induced by HFA in yeast cells.

Measurements of respiratory activity are very indirect criteria on which to base the identity of intracellular reactions and therefore a less circumstantial type of evidence was sought. The classical work of Peters and the co-workers has pointed to the TCA cycle as the site of HFA poisoning[2] and with this in mind it was decided to analyze both normal and HFA-poisoned cells for their content of citric acid. Citrate may be determined very precisely at a micro-chemical level[3] and since it does not penetrate the yeast cell[4] there is little likelihood of "leakage" from the cell during manipulative procedures.

MATERIALS AND METHODS

In the majority of experiments to be reported, the cells from commercial yeast in cake form were freed from filler and washed twice with distilled water. The cells were then given a two-hour aeration in 50 mM NaH_2PO_4 containing 5% glucose. In experiments carried out towards the end of the program it was found that the reproducibility of results could be improved by aerating the washed cells in liquid growth medium[5] for 10 hr before submitting them to aeration in the NaH_2PO_4—glucose medium.

The cells from either preparative procedure were washed twice and suspended in the medium whose environmental effects were under investigation. Since no source of nitrogen was present, these cells were in the "resting" or non-proliferating condition. All experiments were carried out in McIlvaine's citric acid-phosphate buffer, but not until it had been established that the citric acid in the buffer played no part in the intracellular events measured.

Treatment with HFA was carried out by exposing the cells to 5 mM HFA for 30 min and then washing them twice with cold distilled water.

In preparation for citrate analyses suitable aliquots of the cell suspension were rapidly centrifuged and the sedimented cells washed twice with cold distilled water prior to freezing them solid. After thawing, the cell paste was film frozen and thawed twice and extracted with 8% trichloroacetic acid. Precipitated protein was removed by centrifugation and an aliquot of the extract analyzed for citrate following the method of Taylor.[6] All values in this report have been expressed in terms of micromoles citrate per g (dry weight) of cells. The reproducibility of the extraction procedure was determined by submitting six different volumes (ranging from 1 to 10 ml) of a 2% cell suspension to the freezing-thawing procedure and analyzing for citrate. The mean value of 27·31 μM/g showed a standard error of $\pm$ 0·832 despite the wide range of sample size.

All experiments were carried out at room temperature 21–23° with the exception of respiratory measurements which were made at 25° in the conventional Warburg respirometer.

EXPERIMENTAL AND RESULTS

1. *The effect of glucose concentration on poisoning by HFA*

Preliminary experiments carried out in connection with work already published[1] indicated that although it was impossible to poison cells with HFA in a glucose medium at pH 5·5, it was possible to poison them in the absence of glucose at this pH. This finding suggested that HFA and a product of glucose metabolism might compete for an enzyme surface. Belief that poisoning by HFA had resulted in a block of the TCA cycle led to an examination of the citrate content of cells at pH 5·5, since a high citrate concentration might have been the factor responsible for the inability to poison cells in 5% glucose.

Experiments were therefore designed to follow the changes in intracellular citrate in three types of cell preparation: (a) cells aerated for 30 min at pH 2·2 in 5% glucose, washed and resuspended in buffer at pH 5·5, (b) the same re-suspended in 5% glucose at pH 5·5, and (c) cells exposed to HFA in 5% glucose pH 2·2 for 30 min, washed and re-suspended in 5% glucose at pH 5·5. The cell suspensions were aerated and at various time intervals, sampled for citrate determinations. Fig. 1 records the data of a number of such experiments where, it should be noted, the results show changes in

citrate content of cells after taking them from an environment of pH 2·2 and placing them in one at pH 5·5.

A comparison of curves A and B shows that the rise and fall in citrate concentration at this pH is associated with the addition of an extracellular substrate. In poisoned cells citrate not only accumulates to a much higher concentration than in normal cells but this process continues for the duration of the experimental period. It is pertinent therefore to recall that under these environmental conditions the oxygen consumption of the treated cells in curve C is the same as that in normal cells, curve B[1]. The previously-noted recovery of respiration therefore takes place without a concomitant reversal of the effect of HFA on citrate accumulation. From this observation it follows that an increase in pH of the medium to 5·5 must alter the route of glucose metabolism from one that is blocked by HFA to one that is not.

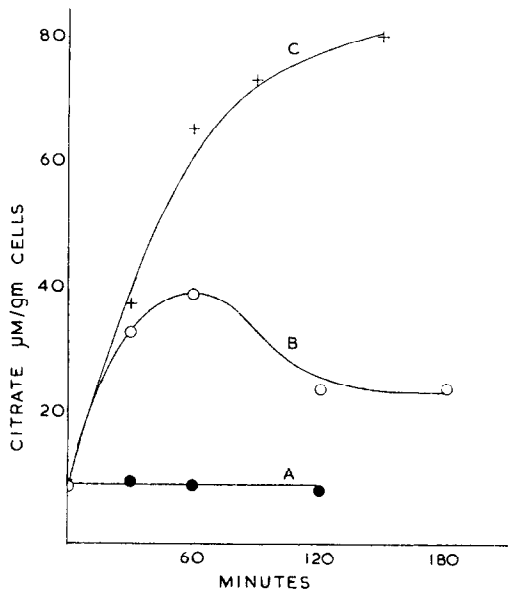

FIG. 1. The citrate content of cells as a function of time of aeration at pH 5·5. A, normal cells no substrate; B, normal cells in 5% glucose, C, HFA poisoned cells in 5% glucose.

From Fig. 1 it is apparent that normal cells accumulate citrate to a considerable degree during the first hour of the experiment and it is therefore possible that the previously noted inability to poison cells under these environmental conditions might have been due to a competition between the products of glucose metabolism (citrate) and HFA or the products of its metabolism (fluorocitrate). To explore this possibility cells were exposed for 30 min to a constant concentration of HFA in media containing various concentrations of glucose and buffered at pH 5·5. Following this exposure they were washed twice and each re-suspended in buffer, pH 5·5, containing 10 mM acetate instead of glucose. Aeration was commenced, samples were taken immediately and again one hour later for citrate analyses. The use of acetate as the extracellular

substrate was dictated by the fact that if oxygen consumption in addition to citrate content was to be used as a criterion of HFA poisoning, respiratory measurements made in glucose would have shown the same rate for all samples. The results of a number of experiments in this series are shown in Table 1.

TABLE 1. CITRATE CONTENT OF CELLS FOLLOWING EXPOSURE TO HFA IN VARYING CONCENTRATIONS OF GLUCOSE AT pH 5·5. CITRATE ACCUMULATION MEASURED IN 10 mM ACETATE pH 5·5

Glucose present at time of exposure to 5 mM HFA mM	Citrate content μM/g ± standard error	
	Initial	Final
0 0	39·35 ± 3·45	47·42 ± 5·63
0·5	87·41 ± 5·52	111·50 ± 9·97
0·10	42·28 ± 8·59	68·22 ± 14·08
0·50	26·10 ± 7·16*	29·35 ± 2·98*
0·100	23·01 ± 2·91*	28·76 ± 2·66*

*N.S.D.

The citrate content of the cells, determined immediately on placing them in the acetate medium and again 60 min later, shows the greatest accumulation to occur at the lower concentrations of glucose in those flasks where it was present during exposure to HFA. The initial values represent the accumulation during the exposure to HFA, whereas the final values are a measure of the further accumulation in acetate. If one examines the initial values it is apparent that citrate accumulation decreases as the glucose concentration at the time of exposure to HFA is raised from 5 to 100 mM. This effect reaches a minimum somewhere in the range 10–50 mM. One hour later citrate is observed to have accumulated only in those samples exposed to HFA where the glucose concentration was 10 mM or lower, suggesting that only in these samples was a metabolic block induced. Thus if citrate accumulation were to be used as an indication of HFA inhibition, one would conclude that the cells of the last two lines of Table 1 (i.e. exposed to HFA in 50 and 100 mM glucose) were not poisoned.

From the experiments in this series, it would appear that at pH 5·5 there is a competition between HFA and glucose, or a product of its metabolism, such that 10–50 mM glucose affords maximum "protection" against HFA.

The data reported in an earlier paper[1] suggested that at pH 2·2 the route of glucose metabolism was different from that occurring at pH 5·5. It therefore became of interest to follow the citrate changes in normal and HFA-poisoned cells at this acid pH and to determine whether glucose and HFA exhibited the same type of competition as that depicted in Table 1.

Fig. 2 records the time course of citrate accumulation in (a) normal cells with no external substrate (b) in normal cells with 5% glucose added and (c) in HFA-poisoned cells with 5% glucose added. The preparation of these cells differed from those used in the experiment of Fig. 1 only in that the final suspension medium was buffered at pH 2·2. The difference in magnitude of the changes in citrate compared to those of Fig. 1 is seen in the expanded scale of the ordinate.

As at pH 5·5 there is practically no change in the citrate content of normal cells when no external substrate is added. The addition of glucose results in a fall in the level of intracellular citrate such that an asymptotic value is reached in about three hours.

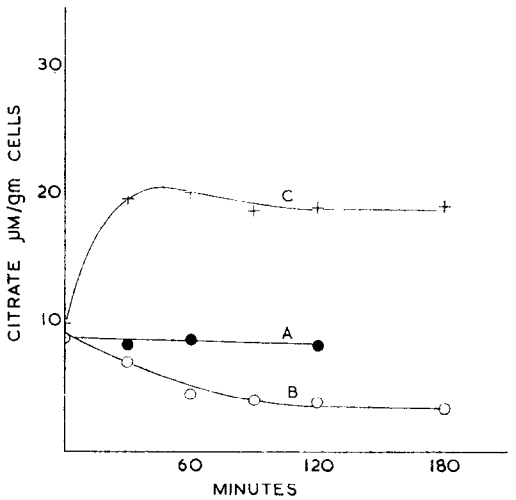

FIG. 2. The citrate content of cells as a function of time of aeration at pH 2·2. A, normal cells no substrate; B, normal cells in 5% glucose; C, HFA poisoned cells in 5% glucose.

In HFA poisoned cells the citrate content is doubled within the first thirty minutes but there is no further increase during the remainder of the experimental period. Thus, although an extracellular substrate is present citrate synthesis does not continue as it does at pH 5·5. It is clear that HFA treatment is not the factor responsible for the cessation of citrate synthesis because this process takes place readily in poisoned cells at either pH. At pH 2·2 however the extent to which this continues is obviously limited. It would appear therefore that at low pH the amount of product from which citrate arises (i.e. a citrate precursor) is limited, and this suggestion would also account for the observation that in normal cells the citrate content decreases with time to very low values (Curve B). The fact that it decreases at all indicates that pH does not influence the disposal of citrate; and in other experiments it has been found possible to deplete intracellular citrate from very high values (50 μM/g) to less than 10 μM/g merely by aerating them in buffer at pH 2·2 to which glucose had been added.

If a high concentration of H^+ does limit the amount of citrate precursor that is formed from glucose it follows that at pH 2·2 there should be no demonstrable competition between glucose and HFA; for under these conditions pH, rather than glucose concentration, should determine the amount of precursor formed. Table 2 shows the results of a number of experiments designed to test this supposition. These experiments were carried out in the same manner as those in Table 1 except that all media were buffered at pH 2·2.

Two particular points concerning these data are important to note: Firstly the citrate content of the cells is independent of the concentration of glucose that was

present during exposure to HFA and in this respect the results are quite different from those of Table 1. Moreover the change in citrate content which occurs over the subsequent hour is hardly significant and is the same for all samples. Secondly, poisoning with HFA must have taken place because the absolute values for citrate are characteristic of poisoned cells at this pH (See Curve C, Fig. 2).

TABLE 2. CITRATE CONTENT OF CELLS FOLLOWING EXPOSURE TO HFA IN VARYING CONCENTRATIONS OF GLUCOSE AT pH 2·2. CITRATE ACCUMULATION MEASURED IN 10 mM ACETATE pH 2·2

Glucose present at time of exposure to 5 mM HFA mM	Citrate content μM/g $\pm$ standard error	
	Initial	Final
0·0	22·56 $\pm$ 4·22	34·20 $\pm$ 5·53
5	23·90 $\pm$ 3·66	30·10 $\pm$ 5·23
10	26·90 $\pm$ 2·65	32·50 $\pm$ 7·06
50	22·83 $\pm$ 3·77	28·63 $\pm$ 6·82
100	23·66 $\pm$ 3·48	31·33 $\pm$ 5·48
Mean	23·97 $\pm$ 5·90	31·53 $\pm$ 7·41

It would thus appear that HFA poisoning, judged by the accumulation of intracellular citrate, takes place only when the concentration of a product of glucose assimilation falls below some critical value. At pH 5·5 the concentration of this product is directly related to the concentration of extracellular glucose; but at pH 2·2 the inhibitory effect that H^+ appears to have on the formation of this product results in its concentration being independent of the extracellular glucose concentration.

This suggested action of H^+ on glucose metabolism was examined in more detail since at this point in the investigation there was no reason to suspect that HFA produced its inhibitory action in any manner other than that described by Peters, i.e. by inhibiting the TCA cycle at the aconitase stage.

If H^+ does indeed limit the amount of citrate precursor that is formed from glucose, then under conditions of constant glucose concentration, intracellular citrate should increase as the pH of the environment is increased. In order to test this suggestion cells were poisoned with HFA in the usual manner at pH 2·2 in 5% glucose. During the washing procedure the cell suspension was divided into eight aliquots and the cells resuspended as follows: one each in buffer at pH 2·2, 3·5, 4·5 and 5·5 respectively, and one each in the same media but containing 5% glucose. Samples for citrate analyses were taken immediately from both series, aeration of all samples was commenced, and one hour later samples from both series were again taken. Fig. 3 shows the results of a number of such experiments and demonstrates that citrate formation does increase in the manner expected. (Curve C). The behaviour of the cells in the absence of glucose reveals several important features of the phenomenon under investigation. Since the cells were all derived from the same original population the citrate contents at zero time of cells both in the presence and absence of glucose were pooled to provide the data for Curve A. This mean value of 25·8 μM/g showed a standard error of 0·731. These cells would presumably all contain the same amount of citrate precursor. One hour later these samples reached the values shown by Curve B.

Although there appears to be a tendency for the citrate content to increase with increasing pH, the standard errors of the means are large enough to suggest that the differences are not significant. The mean of the pooled values of Curve B is 38·8 ± a standard error of 0·84. If this interpretation of the data is correct, it follows that the synthesis of citrate from its precursor is not influenced by extracellular pH over this range. This conclusion has already been stated in connection with Figs. 1 and 2.

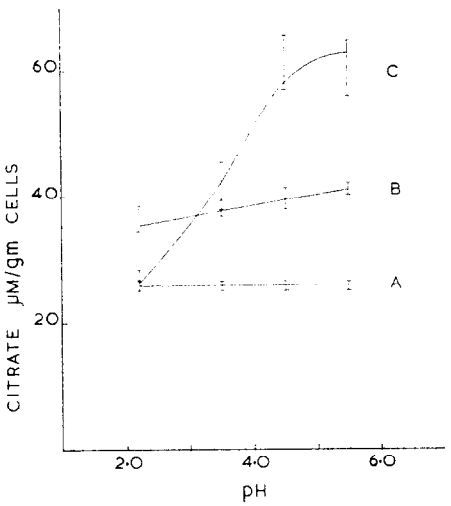

FIG. 3. The effect of pH on citrate accumulation in HFA treated cells. A, citrate content at zero time; B, after 60 min aeration with no substrate; C, after 60 min aeration in 5% glucose. Height of bar indicates standard error of mean.

It is to be noted that at pH 2·2 the citrate content is lower in the presence of glucose (Curve C) than it is in its absence. (Curve B). The significance of this observation will be discussed later.

2. *The effect of potassium on citrate accumulation and HFA poisoning.*

In an investigation into the effect of cations on the metabolic activity of yeast cells, Rothstein[7] noted that the H^+ and K^+ had a reciprocal effect upon fermentation of glucose. Thus in the absence of K^+ fermentation was progressively lowered by decreasing pH but in the presence of K^+ the rate was independent of pH. In these experiments the stimulating effect of K^+ on fermentation disappeared as a pH of 4·5 was approached.

Since our observations have revealed a somewhat similar effect of pH on glucose metabolism, it was of interest to inquire whether K^+ would alter the citrate content of cells suspended in glucose media at pH 2·2 where there should be a maximum stimulation of fermentation and at pH 5·5 where the stimulating effect should be absent. The data of Table 3 derived from normal cells aerated for one hour in glucose with and without the addition of K^+ show that these expectations are borne out.

If a high concentration of intracellular citrate at pH 5·5 were the factor which prevented poisoning by HFA at this pH then anything that would increase intracellular citrate at pH 2·2, such as the addition of K^+, should alter the degree of poisoning. Table 4 shows the results of adding various amounts of KH_2PO_4 to the medium

during exposure to HFA, and after washing, measuring the rate of oxygen consumption in 5% glucose at pH 2·2. Very obviously the presence of K^+ at this time does not alter the degree of poisoning, and therefore one might conclude that citrate was not the product that competed with HFA to prevent poisoning at pH 5·5. This conclusion would not necessarily follow from the experimental observations if the citrate that is increased by K^+ arose in a metabolic pathway other than that blocked by HFA.

TABLE 3. THE EFFECT OF POTASSIUM ON CITRATE CONTENT OF CELLS AT TWO EXTREMES OF pH. GLUCOSE (5%) PRESENT IN ALL EXPERIMENTS

Environmental conditions		Citrate content μM/g. ± standard error of mean
pH 2·2	K^+ absent	7·04 ± 0·645
	20 mM KH_2PO_4	12·27 ± 0·063
		Difference in means: t = 8·07, p<0·001
pH 5·5	K^+ absent	16·56 ± 0·355
	20 mM KH_2PO_4	19·43 ± 1·472
		Difference of means: t = 1·895, p = > 0·10

TABLE 4. RESPIRATION OF CELLS MEASURED IN 5% GLUCOSE, pH 2·2, AFTER HAVING BEEN EXPOSED TO HFA WITH VARYING AMOUNTS OF KH_2PO_4 PRESENT IN THE MEDIUM

Concentration of KH_2PO_4 present during exposure to HFA	Oxygen consumption μl/15 min ± standard error
mM	
0	20·7 ± 0·36
1	19·5 ± 0·74
5	20·6 ± 0·64
20	20·2 ± 0·69

TABLE 5. THE OXYGEN CONSUMPTION OF CONTROL AND HFA TREATED CELLS FOLLOWING THE ADDITION OF BUFFER OR KH_2PO_4. RESPIRATION MEASURED IN 5% GLUCOSE, pH 2·2

Concentration of KH_2PO_4 mM	Oxygen consumption μl/15 min ± standard error	
	Control	Treated
0	38·05 ± 1·11*	19·21 ± 0·66
20	46·18 ± 0·95	35·76 ± 1·58*

* not significantly different.

The observation that K^+ does not affect the degree of poisoning by HFA would suggest that its site of action does not involve the metabolic pathway that HFA blocks. Therefore when K^+ stimulates respiration, as Rothstein observed, one should see an increase in respiration in HFA poisoned cells upon the addition of K^+ to them. Table 5 records the results of experiments in which K^+ was added to both control and HFA treated cells suspended, after washing, in 5% glucose at pH 2·2. The addition of K^+ almost doubles the rate of respiration in the poisoned cells and although the rate

attained is not equal to that of the corresponding control sample, it is not significantly different from the control in the absence of K^+.

These observations would suggest that when K^+ stimulates glucose metabolism the products of the augmented process do not enter the metabolic pathway that is blocked by HFA. Thus although H^+ and K^+ may appear to exert reciprocal effects, in actual fact the inhibitory action of H^+ is maintained and K^+ merely stimulates some other pathway which is also characterized by the consumption of oxygen and the production of citrate. The significance of this suggestion will be discussed later.

TABLE 6. THE RESPIRATORY CHARACTERISTICS OF CELLS FOLLOWING EXPOSURE TO HFA IN VARYING CONCENTRATIONS OF GLUCOSE AT pH 2·2 AND 5·5. RESPIRATION MEASURED AT THE SAME pH AS THAT AT WHICH EXPOSURE TO HFA WAS MADE

Glucose present at time of exposure to 5 mM HFA	Mean respiratory rate μl/10 min			
	pH 2·2		pH 5·5	
mM	No substrate	10 mM acetate	No substrate	10 mM acetate
0·0	2·46	3·45	1·39	1·36
5	5·57	5·95	2·42	3·38
10	9·40	10·8	8·93	25·15
50	13·3	12·8	12·57	28·88
100	15·0	15·5	11·01	23·85
control (untreated) cells	9·12	34·3	4·18	24·30

3. *The relation between HFA poisoning and acetate metabolism*

Experiments designed to follow the changes in citrate content of normal and HFA treated cells at pH 2·2 and 5·5 with 10 mM acetate present instead of 5% glucose, yielded curves almost identical to those of Figs. 1 and 2. The fact that citrate is high in normal cells at pH 5·5 (see Curve B, Fig. 1) means that citrate cannot be the material with which HFA competes because exposure to HFA results in a degree of poisoning which is the same at either extreme of pH when acetate is the intracellular substrate.[1] This suggestion agrees therefore with the data obtained from the addition of K^+ in the previous section. The changes in citrate content observed in an acetate medium being identical to those occurring in glucose raises the question as to whether acetate is in fact being converted to citrate, or whether it is merely acting to catalyse the conversion of a precursor already present in the cell. Evidence in support of this suggestion has been obtained from an examination of the oxygen consumption of cells suspended in an acetate medium. For example, Table 6 records the mean rate of oxygen consumption of the cells used in the experiments of Tables 1 and 2. In these experiments the cells were exposed to HFA at pH 2·2 and 5·5 in varying concentrations of glucose and, after washing, were resuspended in buffer or acetate-containing buffer at the same pH as that at which exposure to HFA had been carried out. An untreated control sample was run under each environmental category.

The last line of Table 6 shows that the addition of acetate to untreated cells results in a considerable stimulation of respiratory rate. In HFA-treated cells, however, a stimulation of respiration is seen only in those samples where exposure was made at pH 5·5 in glucose solutions of 10 mM and greater. These samples therefore appear not to have been poisoned—a conclusion supported for the most part by the data of Table 1.

At pH 2·2 the rate of respiration varies with the concentration of glucose that had been present at the time of exposure to HFA. H^+ therefore does not interfere with the uptake of glucose from the extracellular environment. Since extracellular glucose had been removed from these cells, it is obvious that the respiratory activity in each case is due to the metabolism of assimilated glucose or one of its products of metabolism. This suggestion is supported by the fact that when the data of Table 6 (Column 1) are plotted to show oxygen consumption as a function of time, a series of "decay" curves is obtained as in Fig. 4. Thus not only is the initial rate of respiration related to the amount of glucose present during exposure to HFA, but so also is the length of time over which a rate greater than endogeneous is maintained (curve A).

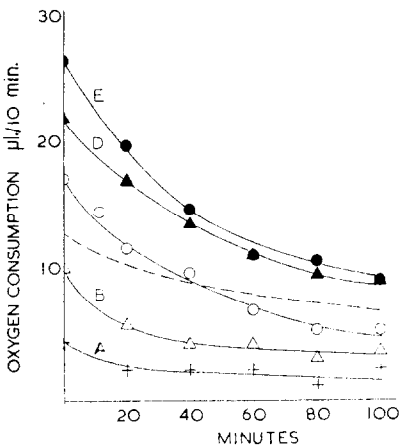

FIG. 4. Oxygen consumption of cells exposed to HFA at pH 2·2 in varying concentrations of glucose. Respiration measured in buffer pH 2·2 Concentrations of glucose during HFA treatment A, O; B, 5 mM; C, 10 mM; D, 50 mM; E, 100 mM. Broken line indicates untreated controls.

The addition of acetate (Column 3, Table 6) does not alter the respiratory rate of the poisoned cells indicating that in each case poisoning by HFA must have been complete and therefore of the same magnitude. This is the same conclusion that was reached from the citrate accumulation data of Table 2.

DISCUSSION

The present investigation has centred upon two problems, namely the effect of H^+ upon glucose metabolism, and the nature of the metabolic block induced by HFA. In many respects these problems are inter-related because in using HFA as an investigational tool one assumes a specifically defined mode of action for it, and interprets the action of H^+ in conformity with this assumption.

It is clear that whatever the specific mechanism of action of HFA turns out to be, yeast cells exposed to it accumulate citrate. This accumulation could arise by virtue of the "lethal synthesis" of fluorocitrate which specifically blocks the action of aconitase on citrate. Yeast cells (*S. cerevisiae*) contain all the elements of the TCA cycle[8] and therefore possess the ability to induce a block in this metabolic pathway and to thereby exhibit an accumulation of citrate.

There are however two quite different reasons for believing this is not the mechanism by which HFA poisons yeast cells. Firstly, attempts to isolate fluorocitrate from HFA poisoned cells or to detect its presence therein yielded negative results.[9] Secondly, cells that have been poisoned by exposure to HFA will respire at a normal rate at pH 5·5 and at the same time accumulate citrate (Fig. 1). At this particular pH the poisoned cells grow normally—the concentration of HFA needed to reduce viability being nearly ten times greater.[10] It is difficult to see how the energy requirements for growth could be met and a normal rate of oxygen consumption maintained in cells in which the TCA cycle activity was blocked.

Citrate also occurs as an intermediate in the glyoxalate cycle[11, 12] the enzymic components of which have been identified in yeast cells.[13, 14] Entry of HFA into this cycle in place of acetate has been demonstrated by Dixon *et al.*[13] but whether the reduced rate at which it does so is a consequence of the initial condensation (as fluoroacetyl CoA) with glyoxalate or an inhibition at a post-condensation stage, is not clear. Blocking of this cycle at either stage might well result in the accumulation of citrate, particularly if there were no reduction in the input of two-carbon fragments. It may be pertinent to note in this connection that the present work, as well as that of others[15–17] points to the metabolism of acetate as being the function that HFA interferes with most consistently in yeast. The absence of a respiratory response to added acetate in HFA poisoned cells together with an accumulation of citrate are effects which might be anticipated were the metabolic block to have taken place in this manner.

The occurrence in yeast of both the TCA and the glyoxalate cycles means that there is probably a duplicity of intermediates, such as citrate, and microchemical analysis will not distinguish these, i.e. only total citrate will be measured. With this fact in mind an attempt has been made in Fig. 5 to set down a simple scheme which incorporates the concept of an inter-relation of two metabolic pathways, and which, for the most part, accounts for the observations arising out of the present work. It should be pointed out that the cycles have been identified as either A or B in order to differentiate two metabolic pathways which have certain intermediates in common. Evidence that cycle A might be the glyoxalate and B the TCA cycle will have to await further investigation; however these two pathways do appear to function concurrently in *Aspergillus niger*[18] and *Fusarium solani*[19]. The entry of acetate into these cycles has not been shown in Fig. 5, but from the observation that its metabolism is

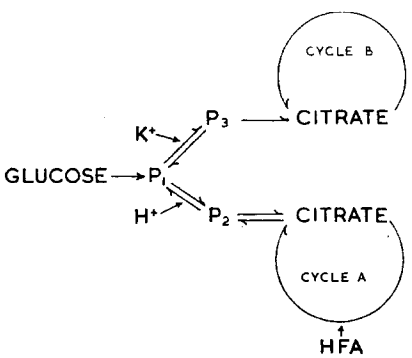

FIG. 5. Diagram to illustrate the inter-relation of reactions involving citrate metabolism and the probable site of action of H^+, K^+ and HFA.

independent of pH, one might presume this to occur at the level of P2 or later in cycle A.

In this scheme glucose is visualized as being taken up by the cell and converted to P1. Since extracellular pH does not influence this process the concentration of P1 will be determined by the concentration of extracellular glucose. At pH 2·2 when reaction P1 to P2 is largely inhibited P1 may be converted to P3 and metabolized by Cycle B. The data of Table 6 and Fig. 4 support this view since the observed oxygen consumption is seen to vary with the concentration of glucose originally present in the medium yet, as Table 2 shows, this variability is not reflected in the citrate contents of the HFA poisoned cell. The citrate that does accumulate in the HFA poisoned cells must have been derived from P2 (citrate precursor) whose concentration is independent of P1 because of the inhibiting action of H^+. In non-poisoned cells citrate will decrease with time because of the limited amount of P2 present (Fig. 2, Curve B) and will result in a generally low concentration of intermediates in this cycle. If HFA competes with one of these intermediates then it should do so most effectively when the intermediate is at a low level, e.g. 5% glucose pH 2·2. If the addition of K^+ to normal or to poisoned cells at pH 2·2 were to augment the conversion of P1 to P3 which would then be oxidized in Cycle B (see Table 5) this action would not increase the level of intermediates in Cycle A and therefore should not influence the degree of poisoning by HFA (Table 4). Potassium does increase the citrate content of the cell at low pH but this is probably Cycle B citrate.

When the extracellular pH is changed to 5·5 the inhibition of the formation of P2 from P1 is removed and the concentration of P2 is now determined by the concentration of extracellular glucose. If this exceeds 10–50 mM the concentration of intermediates in Cycle A is raised to the point where one of them effectively competes with HFA (Tables 1 and 6) thereby preventing poisoning from taking place. At high concentrations of glucose it is possible that P1 may be directed into both cycles simultaneously. In HFA poisoned cells citrate would accumulate in Cycle A and since the reaction P1 to P2 is not inhibited the accumulated citrate might cause a backing up of the intermediates to the point where P1 was increased. This excess would then be free to be fed into Cycle B via P3. This sequence of events would account for the recovery of the oxygen consumption of poisoned cells at pH 5·5 in 5% glucose.

It will be noted that in Fig. 1 the citrate contents of normal cells (Curve B) rises and then falls to a new level. This may be due to P2 increasing faster than it can be disposed of in Cycle A. Either an adaptation subsequently takes place in this cycle or the accumulated P1 and P2 spill over into Cycle B. Thus the total citrate measured might represent the sum of that arising in the two cycles concurrently. If this were to take place in HFA poisoned cells where the disposal of the citrate arising in Cycle A is blocked one should be able to demonstrate a fall in the total citrate as the excess is disposed of in the non-blocked pathway. Evidence of this type of change in HFA poisoned cells has been obtained several times in the present investigation. For example, in Fig. 3, it will be noted that at pH 2·2 the citrate content in the presence of glucose (Curve C) is lower than in its absence. (Curve B). After the data from this experiment were obtained the cells remaining in those samples whose citrate contents are depicted by Curves B and C were all washed with distilled water and resuspended in buffer at pH 2·2 containing 5% glucose. In this environment no further increase in citrate would be expected to take place but since an external substrate was present

the necessary conditions for citrate depletion would be met (See Fig. 2). Aeration of the samples was commenced and one hour later those in Curve B had all fallen to a mean value of 30·7 ± 2·47 μM/g. and those in C to 26·4 ± 2·2 μM/g. It is pertinent to note that in the latter samples which, before aeration, had contained quite different amounts of citrate, none had decreased below 20 μM/gm—the value characteristic of poisoned cells at this pH (see Fig. 2). These observations would support the suggestion that there are two sources of citrate in the cell and that one of the metabolic routes responsible for citrate disposal had been blocked by HFA. The possibility of distinguishing these two sources of citrate and identifying the cycles to which they belong is presently under investigation.

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