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Mechanisms of Fatty Acid Toxicity for Yeast

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Abstract

Neal, A. L. (Rutgers, The State University, New Brunswick, N.J.), Joan O. Weinstock, and J. Oliver Lampen. Mechanisms of fatty acid toxicity for yeast. J. Bacteriol. **90**:126–131. 1965.—The internal *p*H of stationary- and log-phase yeast cells dropped quite rapidly when the cells were exposed to acetate buffers at *p*H 4 and 3, whereas no, or much less, acidification occurred with pyruvate or phosphate. Although inhibition of respiration and glycolysis was almost instantaneous when the cells were exposed to 0.2 M acetate at *p*H 4, the effect was not permanent and could be reversed by washing them with water or phosphate buffer. Irreversible inhibition did occur, however, at 0.5 M acetate under the same conditions; there was a marked decrease in several glycolytic enzyme systems, which undoubtedly contributed to the irreversible nature of the inhibition. In cell-free homogenates, various low-molecular-weight monocarboxylic acids exhibited about the same inhibitory effect on glycolysis; structural differences such as branching or unsaturation did not cause a marked change in their inhibitory effect. Also, glycolysis was much more sensitive to dicarboxylic acids such as succinate and phthalate than to acetate; phthalate was more inhibitory than succinate. This is in contrast with the noninhibitory nature of succinate and phthalate to whole cells, even at *p*H 4. Pyruvic acid decarboxylation was inhibited by phthalate but not by succinate. The greater toxic effect of phthalic acid may be due to the fixed steric configuration of its carboxyl groups, as compared with those of succinic acid.

Fatty acids are known to exert a fungistatic and fungicidal action which is maximal at low *p*H. With intact cells, this activity is probably dependent on the concentration of the undissociated molecule, at least at *p*H values below neutrality (Rahn, 1945; Prince, 1959).

Proposals concerning the mechanism by which inhibition occurs have emphasized either a possible fall in internal *p*H as a result of uptake of the free acid (with resultant metabolic inhibition) or a direct inhibitory action of the acid on critical enzymes of the cell. In qualitative studies with *p*H indicators, Maesen and Lako (1952) obtained evidence for an internal acidification of yeast cells in fatty acid solutions. Suomalainen and Oura (1955) measured the *p*H of yeast plasmolysates obtained by freezing (in liquid air) and thawing; acetic (but not citric) acid produced a fall in the internal *p*H. In contrast, Samson, Katz, and Harris (1955) found that the fatty acids were inhibitory to glycolysis by yeast extracts. Pyruvate kinase and possibly enolase were sites of inhibition in this system. Sorbic acid (2,4-hexadienoic acid) also inhibits enolase (Azukas, Costilow, and Sadoff, 1961), which was considered to be the primary site of sorbic acid inhibition of alcoholic fermentation. Palleroni and de Pritz (1960) also suggested that sorbate interferes with citric acid formation in yeast, by forming a sorbyl-coenzyme-A complex. Hydroxylation or carboxylation of the fatty acids (e.g., hydroxybutyric or succinic acids) essentially eliminates their inhibitory action on the intact cell (Samson et al., 1955), probably because of lack of penetration (Suomalainen and Oura, 1955).

The present experiments were designed to provide information on the relative importance of the several potential mechanisms of inhibition, particularly as a function of the specific acid or concentration of acid used. A preliminary report on a part of this work has been presented (Lampen and Weinstock, 1962).

Materials and Methods

Organisms. Log-phase and stationary-phase cells of *Saccharomyces cerevisiae* LK2G12 were grown in Wickerham's medium with 1% glucose as described by Marini, Arnow, and Lampen (1961). The cells were washed twice in 0.02 M potassium phosphate buffer (*p*H 6.8) before use. Commercial baker's yeast was generously provided by Anheuser-Busch, Inc., Old Bridge, N.J., in the form of a washed filter cake.

Manometric techniques. To measure glycolysis

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and respiration of intact cells, each flask received ca. 2 mg (dry weight equivalent) of cells, and the following (μmoles per milliliter): 300 glucose or 100 ethyl alcohol, 60 KCl, 60 NH_4Cl, 20 $MgCl_2$, and 60 Na-K phosphate buffer and the anion under test, both at the *p*H values indicated below. For glycolysis, the gas phase was 7% CO_2 and 93% N_2; for respiration, air was used. The temperature was 30 C.

Actively glycolyzing homogenates were prepared by grinding cells at 0 to 10 C with glass beads (1.5 volumes) in 0.05 M phosphate buffer (*p*H 6.8, 1.5 volumes) containing 0.1 mg of 2,3-mercapto-1-propanol per ml and 4.1 mmoles of nicotinamide per ml. Beads and debris were removed by centrifugation for 20 min at 900 × *g*. A particulate-free preparation was obtained by centrifugation at 100,000 × *g* for 90 min at 2 to 4 C. For measurement of glycolysis, flasks contained (μmoles per 3 ml): 10 adenosine triphosphate, 10 nicotinamide adenine dinucleotide, 10 thiamine pyrophosphate, 246 niacinamide, 60 $MgCl_2$, 30 NH_4Cl, 30 KCl, 60 phosphate buffer (*p*H as indicated), 300 glucose, 0.5 ml of the homogenate, and the acid under test (*p*H and concentration as indicated).

The homogenates varied considerably in activity; therefore, to obtain a more uniformly active material an acetone powder was prepared from lyophilized yeast by the method of Hochster and Quastel (1951). For assay, each flask contained 20 mg of the acetone powder, 60 μmoles of β-glycerophosphate as buffer (in place of the inorganic phosphate), 10 μmoles of pyruvate, and other salts and cofactors as for the homogenates. The pyruvate was needed to initiate maximal CO_2 production (Fig. 1), and was prepared fresh each day.

Measurement of intracellular pH. The intracellular *p*H of yeast cells was measured on the basis of Kotyk's (1962) demonstration that only the un-ionized form of 2,4-dinitrophenol (DNP) can penetrate the cell membrane. At equilibrium, the concentration of the un-ionized form should be identical inside and outside the cell; the distribution of total DNP is thus dependent upon the fraction ionized within and without the cell and hence upon both external and internal *p*H. In a medium of known *p*H and DNP content, the concentration of un-ionized form (AH) can be calculated by the equation:

$$pH = pK_a + \log [A^-]/[AH]$$

The pK_a of DNP is ca. 4.1 (Kotyk, 1962). Since [AH] inside (i) = [AH] outside (o), one can calculate the apparent internal *p*H (if the total cellular DNP is known) by reapplying the above equation. The relation can also be conveniently expressed in the form:

$$pH_i = pH_o + \log [A^-]_i/[A^-]_o$$

Optimal conditions for determining intracellular *p*H were to incubate 20 to 30 mg of cells per ml at 37 C in phosphate buffer (*p*H 4.0) containing

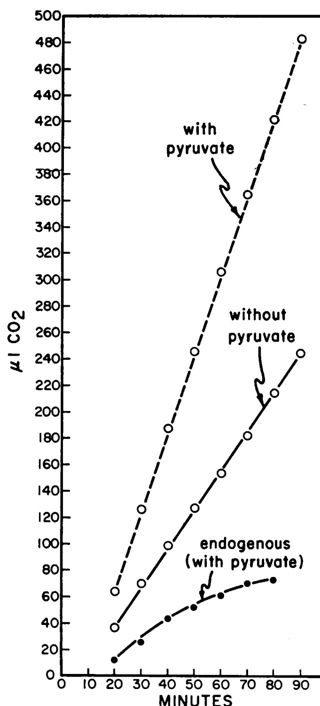

FIG. 1. *Priming effect of pyruvate on glycolysis by an acetone powder prepared from yeast extract.*

K^+, NH_4^+, and Mg^{++} (as for the manometric tests) and 10^{-4} M DNP. At various times, 5-ml samples were rapidly filtered on Millipore filters (0.45-μ porosity). The filtrate was diluted tenfold with 0.2 M Na_2CO_3, and the extinction was measured at 420 mμ with a Zeiss PMQ II spectrophotometer. Since DNP was usually concentrated 20-fold to 100-fold by the cells, a reasonable estimate of cellular DNP could be obtained by sucking excess medium from the cells on the filter pad and extracting the cells and pad with 5 ml of 0.2 M Na_2CO_3 at 60 C for 4 min. The sample was centrifuged, and its extinction at 420 mμ was compared with a standard curve of DNP in Na_2CO_3.

RESULTS

Metabolic effects on intact cells. The sensitivity of stationary-phase cells of *S. cerevisiae* LK2G12 to various acids is illustrated in Table 1. Similar results were obtained with log-phase cells or with commercial baker's or brewer's yeast. In phosphate buffer there was a high rate of glycolysis over the *p*H range 2.5 to 6.0, whereas the straight-chain fatty acids inhibited CO_2 production under conditions where most of the acid was in the un-ionized form. At *p*H 4.0, inhibition by 0.02, 0.05, and 0.2 M acetate was 22, 84, and 99%, respectively. Endogenous respiration and

TABLE 1. *Structural relations in fatty acid inhibition*

Acid	pK_a*	Glycolysis in 0.2 M buffer		
		*p*H 2.5	*p*H 4	*p*H 6
Phosphoric	1.96, 7.13, 12.32	1,630†	1,570	1,540
Formic	3.75	6	—	1,950
Acetic	4.76	—	5	1,100
Butyric	4.83	—	36	1,190
Trimethylacetic	ca. 4.8	—	8	378
Succinic	4.18, 5.55	800	920	1,520
Phthalic	2.90, 5.51	—	1,840	1,960

* *Handbook of Chemistry and Physics*, Chemical Rubber Publishing Co., Cleveland, 1958–59.
† Results are expressed as μliters of CO_2 evolved per hour. Stationary-phase cells of *S. cerevisiae* LK2G12.

TABLE 2. *Internal pH of yeast cells in various buffers**

Buffer	*p*H	Apparent internal *p*H	
		1 min	10 min
Acetate	6.2	6.1	6.2
Phosphate	6.2	6.2	6.3
Acetate	4.2	4.9	5.1
Phosphate	4.1	5.7	5.8
Acetate	3.0	4.3	4.4
Phosphate	3.0	6.5	5.5–6.5

* Separate experiments were performed at approximately *p*H 6, 4, and 3. DNP was 10^{-4} M; all buffers, 0.2 M. Stationary-phase cells of strain LK2G12 were employed.

oxidation of glucose or ethyl alcohol were similarly reduced. Trimethylacetic acid was toxic even at *p*H 6, but succinic or phthalic (or, in other experiments, malic or citric) acid produced at most a partial inhibition, even at acid *p*H. Pyruvic acid (pK_a ca. 2.6) was rapidly decarboxylated at *p*H 2.0.

Internal acidification by fatty acid buffers. As measured by DNP distribution, the apparent intracellular *p*H of normal washed log-phase or stationary-phase cells (Table 2) was 5.8 to 6.2 under the general conditions of the tests for glycolytic activity (0.2 M phosphate buffer, *p*H 4 to 6). This value is in reasonably good agreement with those reported by Conway and Downey (1950). For instance, with 26.7 mg (ca. 25 μliters) of cells per ml of phosphate buffer (*p*H 4.26), the DNP distribution after 1 min was 0.75 μmole per ml of cells and 0.019 μmole per ml of medium. Of this 0.019 μmole, there would be at *p*H 4.26 approximately 0.011 μmole of the ionized form per ml, and 0.008 μmole of the undissociated acid. Inside the cell there should then be 0.008 μmole of undissociated acid per ml and 0.742 μmole of ion; this is equivalent to an apparent internal *p*H of 6.1. Similar values were obtained with cells in *p*H 6 phosphate and 10^{-3} or 10^{-4} M DNP. In *p*H 3.0 phosphate and 10^{-4} M DNP, the level of DNP in the medium became too small for accurate determination; however, the indicated value for internal *p*H was still 5.5 to 6.5.

When cells were suspended in 0.2 M acetate buffer at *p*H 4, the intracellular fluid dropped rapidly to a *p*H of about 5; similar results were obtained with 0.1 M acetate buffers. In *p*H 3 buffer, an internal *p*H of 4.3 was quickly reached. No acidification occurred in 0.2 M pyruvate (*p*H 4.0).

Reversibility of inhibition. Inhibition of respiration or glycolysis in 0.2 M acetate buffer (*p*H 4.0) was almost instantaneous, but the effect was readily reversed by washing the cells in water or in phosphate buffer (Table 3). Only at very high buffer concentration (0.5 M) were the cells unable to resume metabolic activity or to reproduce. Phosphate buffer (*p*H 4.0) had some toxic action at 0.5 M, but this was minor in comparison with the effect produced by the fatty acid.

Rapid destruction of a variety of enzymes occurred when cells were suspended in 0.5 M acetate buffer of *p*H 4.0 (Table 4). There was an almost complete elimination of several glycolytic or oxidative enzymes. The exact amount of enzyme loss varied from one cell sample to another. Activities extracted from phosphate-treated cells were similar to those obtained from untreated yeast.

Inhibition of homogenate glycolysis by acids. Homogenates were used to study inhibition of glycolysis by acids, to eliminate the effect of cell permeability. Where 0.2 M acetate (*p*H 5.5) was used as the reference and the *p*H was lowered to 5.3, a 68% decrease in CO_2 production resulted. Glycolysis was much more sensitive to succinate than to acetate; at *p*H 5.9 and 5.5,

TABLE 3. *Reversibility of inhibition by acetic acid**

Buffer (*p*H 4.0)	O_2 uptake in buffer	Activity of cells resuspended in 0.1 M phosphate, *p*H 6	
		O_2 uptake	Viable count per ml
	µliters/hr	*µliters/hr*	
Acetate, 0.2 M	0	240	10.3×10^7
Acetate, 0.5 M	0	31	3×10^2
Phosphate, 0.2 M	504	312	9.4×10^7
Phosphate, 0.5 M	480	330	6.5×10^7

* Respiration by 9.7×10^7 stationary-phase cells of strain LK2G12 per milliliter in the indicated buffers was followed for 60 min, after which the cells were washed and resuspended in 0.1 M phosphate buffer (*p*H 6.0). Oxygen uptake in this buffer was determined under the standard conditions. For viable count, samples were plated on Wickerham's medium (plus 1% glucose) containing 100 µg of neomycin per ml to prevent bacterial growth.

succinate (0.2 M) decreased glycolysis 74 and 90%, respectively.

Kinetics. Increments of CO_2 evolution per 10 min by nonparticulate preparations in acetate, succinate, and phthalate buffers are shown in Fig. 2. The evolution of CO_2 in the presence of 0.1 and 0.13 M acetate (*p*H 5.6) was equivalent, and the maximal rate was attained after 30 to 40 min. Increasing the concentration of acetate to 0.2 M resulted in a slight lag period, although the maximal rate was the same as at the lower concentrations of buffer. At *p*H 5.2, however, the rate of evolution of CO_2 was less, even with 0.1 M buffer, and did not reach a maximum until after about 50 min. Succinate (0.2 M, *p*H 6.0) limited the rate of CO_2 production to a maximum of only about 24 µliters per 10 min, and this value remained constant from 30 to 60 min. Phthalate (0.2 M, *p*H 6.0) was extremely inhibitory to glycolysis; this acid permitted only a slight increment in CO_2 evolution during the first 20-min period, after which a gradual decline occurred until the value reached essentially zero after 60 min. This activity approximates the endogenous CO_2 production in the presence of 0.2 M acetate (*p*H 5.6), and may represent decarboxylation of organic acids in the enzyme preparation.

The inhibition of glycolysis by acetate, succinate, and phthalate at *p*H approximately 5.6 and 5.2 was determined. The value taken as 100% activity was the µliters of CO_2 produced with β-glycerophosphate (*p*H 5.6 to 5.7) as the buffer. There was no inhibition by 0.075 or 0.1 M acetate (*p*H 5.7); however, when the concentration of acetate was made 0.2 M, about a 15% decrease in CO_2 evolution occurred. Phthalate (*p*H 5.7, 10^{-3} M) was noninhibitory, and only slight inhibition was noted at 10^{-2} M. At 0.075 M, this acid decreased glycolysis about 60%. When the *p*H of 0.075 M phthalate was adjusted to 5.2, glycolysis was almost completely inhibited. Succinate at 0.075 M produced about half the inhibitory effect of phthalate, and at 0.2 M, *p*H 5.7, both succinate and phthalate completely inhibited (98%) CO_2 evolution.

Several low-molecular-weight acids and 10-undecenoic acid were tested for their effect on glycolysis, and were compared with β-glycerophosphate as the control. *N*-valeric and *i*-valeric acids, 10^{-2} M, were equally inhibitory (about 20%) at both *p*H 5.9 and 5.3 when compared with their respective controls, and β-β-dimethyl acrylic and tiglic acids, 10^{-2} M, were only slightly more inhibitory (28%). At 0.1 M and *p*H 5.9,

TABLE 4. *Enzyme destruction in acetate buffer*[a]

Enzyme assay	Extract	Activity (units/g) of cells incubated in	
		Phosphate	Acetate
Alcohol dehydrogenase[b]	I	16,000	0
	II	18,900	2,000
Aldolase[c]	I	74,000	0
	II	85,000	6,700
Malic dehydrogenase[c]	I	94,000	1,000
	II	88,000	32,000
Glucose-6-phosphate dehydrogenase[d]	I	15	0
	II	38	0
Pyruvate decarboxylase[e]	I	695	187
Glycolysis (µliters of CO_2/hr)	I	338	17
	II	1,445	38

[a] Commercial baker's yeast (7 g) was shaken in 30 ml of 0.5 M sodium-potassium phosphate or sodium acetate buffer (*p*H 4.0) for 30 min at 30 C. The cells were then washed in 0.1 M phosphate (*p*H 7.0) and disrupted by grinding with glass beads. The homogenates were tested for glycolytic activity and assayed for the individual enzymes. (When necessary, the extracts were dialyzed overnight at 0 C against 0.06 M sodium pyrophosphate (*p*H 8.5) to reduce the level of endogenous substrates.

[b] Racker (1955).

[c] Scholz et al. (1959).

[d] Kornberg and Horecker (1955).

[e] Singer (1955).

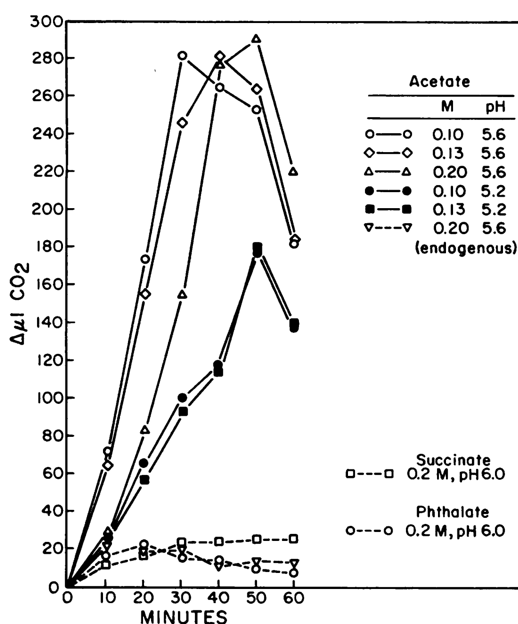

FIG. 2. *Rate of CO_2 production by particulate-free yeast homogenates.*

tiglic acid was slightly more inhibitory than *n*-valeric, *i*-valeric, or *β*-*β*-dimethyl acrylic acids. In general, there was no striking difference among the effects of these acids on glycolysis. Of the acids tested, 10-undecenoic acid was the most inhibitory; at *p*H 6.3 and concentrations of 10^{-3} and 5×10^{-3} M, this acid caused a decrease in the evolution of CO_2 of 38 and 100%, respectively. When the extent of this inhibition is compared with that of phthalic acid, it is to be noted that 10^{-2} M phthalate was not inhibitory at *p*H 6.3 and produced only a 20% inhibition at *p*H 5.3.

Inhibition of pyruvate and fructose-1,6-diphosphate metabolism. Pyruvate decarboxylation was not decreased in the presence of 0.2 M acetate, succinate, or malate at either *p*H 5.4 or 5.0, whereas, when glucose was the substrate, CO_2 evolution was inhibited almost 100% by either succinate or malate. Phthalic acid (0.127 M and *p*H 5.4) also completely suppressed CO_2 formation when glucose was the substrate, but, in contrast with succinate and malate, CO_2 evolution was decreased by 63% when pyruvate was the substrate. This inhibition is comparable to that obtained with phthalate (0.1 M, *p*H 5.7) with fructose-1,6-diphosphate (FDP) as the substrate.

DISCUSSION

Acid inhibition of glycolysis in intact cells is dependent upon the rate of acid penetration, the subsequent decrease of internal *p*H, and the effect of *p*H and acid on specific enzyme systems. In general, acid inhibition of glycolysis is correlated with the amount of undissociated carboxyl groups as well as the structure of the acids; both of these properties play an important role with respect to permeability and site of enzyme inhibition. The extent of damage to the cell by acids is dependent upon these properties as well as the length of time the cell is subjected to the abnormal environment.

The importance of ionic species for the permeability of yeast cells to acetic acid is illustrated by data presented in Table 2. To be noted is the marked change caused by a decrease in *p*H from 6.2 to 3.0 of a 0.2 M acetate medium. Within a period of 1 min, this change in external *p*H resulted in a decrease of internal *p*H to 4.3. The extent of permanent injury to the cell after exposure to acetic acid at a given *p*H is undoubtedly a result of the combined effect of several factors, such as the influence of *p*H and specific acids on a particular enzyme system coupled with the combined effect of inhibition of synthesis and increasing proteolysis. Data presented show that exposure of cells to acetate at 0.2 M and *p*H 4.0 for 60 min did not cause irreversible damage, but, when the concentration of acetate was increased to 0.5 M, irreversible damage was noted. This difference may, in part, be explained by the destruction of several enzymes under the latter condition, notably, alcohol dehydrogenase, aldolase, and pyruvate decarboxylase. The action of the acids tested does not appear to be the same as that noted for sorbic acid (York and Vaughn, 1964); it perhaps results from the initiation of an autolytic process similar to that produced by the antifungal antibiotic nystatin at acid *p*H (Scholz et al., 1959).

Of the short-chain acids tested on cell homogenates, the position of the branched chain or olefinic bond did not appear to influence greatly the extent of inhibition by the acids; therefore, the greater inhibitory effect of *n*-valeric acid compared with *i*-valeric acid on intact cells must be due to the difference in cellular permeability. Hoffman, Schweitzer, and Dalby (1939) used intact cells and noted that the inhibitory effect of saturated acids decreased with increased branching, that unsaturated C4 and C5 acids were more inhibitory than the corresponding saturated acids, and that inhibition decreased as a side chain was moved nearer the carboxyl

group. These variations in activity with respect to structural differences of the acids are undoubtedly a reflection of cell permeability, since glycolysis in cell-free yeast preparations was not appreciably affected by branching or unsaturation in the low-molecular-weight acids.

Acetate, succinate, and malate apparently inhibit glycolytic CO_2 production at some stage between glucose and pyruvate, since pyruvate decarboxylation was not materially affected by the presence of these acids. More specifically, as CO_2 production was not appreciably decreased by succinate or malate when FDP was the substrate, these acids must inhibit enzyme systems between glucose and FDP. Phthalate, in contrast, inhibited the decarboxylation of pyruvic acid about 50 to 60%; this was approximately the same inhibition as was obtained with FDP as the substrate. This observation, plus the fact that phthalate inhibited glycolysis 100% when glucose was the substrate, indicate that phthalate exerts an inhibitory influence not only on the decarboxylation of pyruvic acid but also on enzyme systems between glucose and FDP.

The greater inhibitory influence of phthalate as compared with succinate may indicate that the steric configuration of the two acids is an important factor relative to their effect on glycolysis. Phthalic acid has its two carboxyl groups held in a fixed plane, whereas succinic acid does not. Also to be considered is the possible steric hindrance of the large benzene ring of phthalic acid. These two factors may account for the observation that phthalate inhibited pyruvate decarboxylation, whereas succinate did not.

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