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THE MECHANISM OF STIMULATION OF AEROBIC FERMENTATION IN YEAST BY A QUATERNARY AMMONIUM DETERGENT*

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Abstract—Of the various metabolic effects produced in yeast cells by the cationic detergent, benzalkonium, the most pronounced is the inhibition of the pathway of respiration of two-carbon compounds. Less sensitive is the fermentation of glucose and the respiration of three-carbon substances. Because glucose is normally respired via two pathways, the formation of two-carbon substances and the direct oxidation of pyruvate, benzalkonium disturbs the normal pattern of its metabolism. The R.Q. is markedly elevated. The "extra CO_2" production is paralleled by an appearance of ethyl alcohol. This action of benzalkonium on glucose can be largely duplicated by over-loading the system through addition of two-carbon compounds such as ethyl alcohol, acetaldehyde or acetate, or by excessive concentrations of glucose itself. The detergent also causes an increase in the total amount of O_2 consumed for a given amount of substrate and a change in the R.Q. for the respiration of alcohol or acetaldehyde. These actions are related to the inhibition of carbohydrate assimilation.

INTRODUCTION

BECAUSE of their importance as antiseptic agents, the action of quaternary ammonium detergents on microbial cells has been much investigated. Yeast cells have also served as convenient test organisms. A variety of inhibitory effects has been observed. Of these, the inhibition of growth is most sensitive, followed by the inhibition of respiration and finally by the lysis of the cells. The three inhibitory effects are apparently quite independent of each other.[1] Recently, the lytic effect has been quantitated in terms of a loss of K^+ from the cells, an inhibition of anaerobic glycolysis and the ability of cells to decarboxylate pyruvate under anaerobic conditions.[2]

In addition to the general inhibitory effects, a stimulation of respiration was observed at sharply defined, but very low concentrations of Zephyran.[1‡] More recently Scharff and Beck[3] have shown that certain concentrations of this agent induce in baker's yeast a substantial increase in aerobic fermentation, accompanied by only a small increase of oxygen consumption and of hexose uptake. A true inhibition of the Pasteur effect seems to be involved, as opposed to the simple switch-over from respira-

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‡ Zephyran is a mixture of high molecular weight alkyl-dimethylbenzyl-ammonium chlorides, commonly called benzalkonium chloride. For convenience, this preparation will be denoted in this paper as benzalkonium, or in the figures as B.Z.

tion to glycolysis that occurs with respiratory inhibitors such as cyanide and carbon monoxide.

The action of the cationic detergents involves at least in parts a binding at the cell surface. The lytic action itself implies a breakdown of the cell membrane. Furthermore, the lytic action on yeast can be prevented by uranyl ions[4, 5] which do not penetrate, but combine firmly with anionic groups of the cell surface.[6] The possibility of metabolic effects by agents acting at the cell surface is supported by evidence[7–9] pointing to the location of glycolytic enzymes in the periphery of the cell. In view of these facts, it seemed profitable to investigate further, the action of benzalkonium on the metabolism of yeast using a variety of substrates, in an attempt to localize the exact point of action in the metabolic sequence.

EXPERIMENTAL AND RESULTS

Fresh baker's yeast (Standard Brands, Incorporated) was used in all experiments. The cells were thoroughly washed several times by suspending in water and centrifuging. The speed of the centrifugation was low, so that colloidal material and cell debris was discarded. After aeration for several hours the cells were centrifuged and resuspended in water or buffer. The final concentration of the suspension was checked by photometric measurement of its turbidity. The cells were incubated at 26 °C in the presence of appropriate additions, and air or pure nitrogen, respectively, were bubbled through the suspension to achieve mixing and aerobic or anaerobic conditions. For chemical determinations samples were taken out, centrifuged at high speed (10,000 **g** and analyses were carried out on the supernatant.

Ethanol was determined by the alcohol-dehydrogenase method according to Bücher and Redetzki.[10]

Glucose was determined by the glucose oxidase method, according to a modification of the method of Saifer *et al.*[11]

Potassium was determined by means of the flame photometer (Beckman attachment with a model DU Beckman spectrophotometer).

Buffer solutions. Triethylamine-succinate-tartrate buffer at pH 4·5 was used in all experiments where the use of buffer is indicated.[12]

Gas exchanges were measured by standard Warburg manometry with air or nitrogen, respectively, in the gas phase.

The effects of benzalkonium were found to be proportional to the concentration of cells, at least in the ranges used in the present experiments (from 10 to 40 mg cells wet wt./ml of suspension). For this reason, the amounts of detergent used in particular experiments are always expressed in terms of μg/mg wet weight of cells. The concentration of cells is given for each experiment so that the concentration of detergent can be readily calculated.

One of the striking effects of benzalkonium is the stimulation of aerobic fermentation.[3] In studying this response, it was observed that even in untreated baker's yeast the rate of aerobic fermentation was very high, and that a primary factor is the glucose concentration (Fig. 1). At levels below 0·01 M, the aerobic fermentation (measured as "extra CO_2" above R.Q. of 1·0) was minimal, but at relatively high concentrations of glucose, the rate of "extra CO_2" production was almost half as high as the rate of respiration (R.Q. of 1·5). The glucose concentration chosen for most experiments was

0·025 M, sufficiently high to give a maximal rate of respiration initially, but sufficiently low to induce minimal aerobic fermentation.

With the lowest effective concentration of detergent (1·5 μg/mg wet wt. of yeast) the rate of respiration of glucose was not altered during the first 2 hr, but, in contrast to the control, remained linear for the subsequent 2 hr. Consequently, the

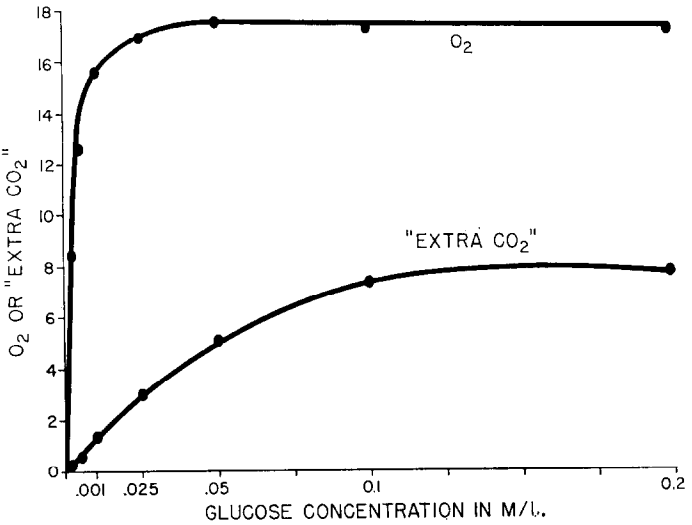


FIG. 1. The dependence of the rate of respiration and of the rate of "extra CO_2" production on the glucose concentration; 20 mg/ml yeast wet weight in 0·02 M triethylamine–succinate–tartrate buffer pH 4·5, containing 0·002 M KCl and the indicated concentrations of glucose.

total O_2 consumption was considerably increased (Fig. 2). In contrast to the unaltered rate of respiration, the initial rate of CO_2 production increased from 19·5 μl/mg per hr to 40·0 μl/mg per hr, indicating that aerobic fermentation was enhanced (in Fig. 2 the effects are expressed in terms of the "extra CO_2" over an R.Q. of 1·0). With 1·5 μg/mg of detergent the "extra CO_2" production was considerable, but transient. It reached a peak in 30 min and thereafter had a negative value, so that by 6 hr the "extra CO_2" was virtually zero. The action of the detergent can also be expressed in terms of the R.Q. which was considerably greater than 1·0 during the first 30 min, and was less than 1·0 thereafter.

With a higher concentration of detergent (3 μg/mg of yeast) the rate of respiration was inhibited to the extent of about 50 per cent, but, in contrast to the control, the rate was nearly constant for 6 hr, rather than for only 2 hr. The action on aerobic fermentation was more prolonged. With 6 μg/mg of yeast, the respiration was depressed to the endogenous level and little aerobic fermentation was observed. The endogenous respiration itself is insensitive to these concentrations of detergent.

Anaerobic CO_2 production was not stimulated by the detergent, but only inhibited. At 3 μg/mg, the inhibition was 33 per cent and at 5 μg it was 82 per cent.

The dramatic changes in the relationship between O_2 consumption and CO_2 production, induced by the detergent, first a production of "extra CO_2" and then a decrease in "extra CO_2", suggested that the response might be due to the accumulation

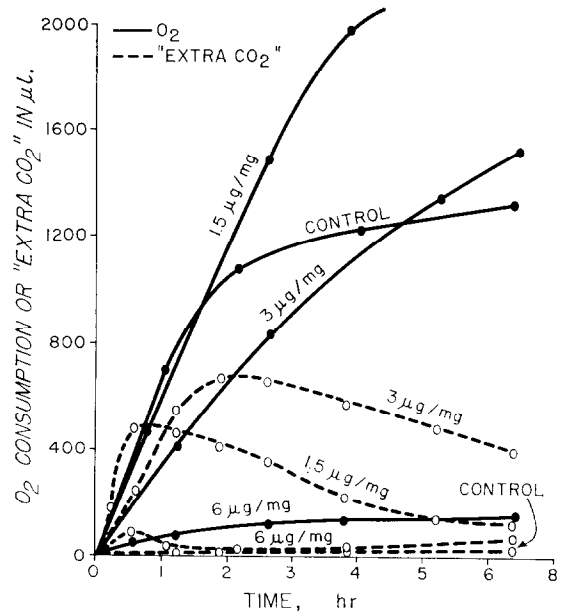


FIG. 2. The effect of benzalkonium on the respiration and "extra CO_2" production from glucose; 20 mg/ml of yeast in distilled water with 0·025 M glucose and benzalkonium as indicated.

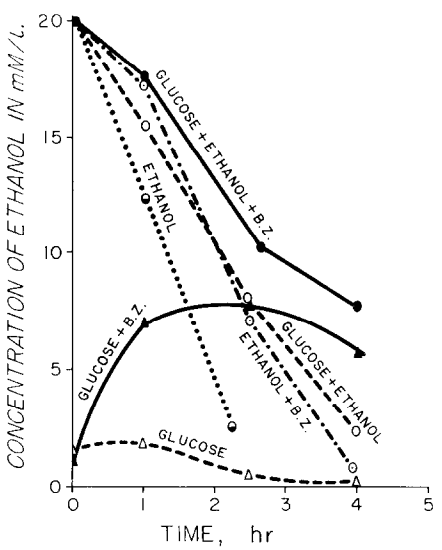


FIG. 3. The influence of benzalkonium on the alcohol content of the medium with glucose or alcohol or mixtures as substrates; 20 mg/ml yeast in distilled water with 0·025 M glucose and/or ethanol and 3 µg/mg benzalkonium where indicated.

of a metabolic intermediate which is in a reduced state relative to glucose, followed at a later time by the oxidation of this intermediate. Transient accumulations of ethyl alcohol have been reported in yeast presented with high concentrations of glucose.[13] A series of experiments was performed with glucose and ethyl alcohol alone and in mixtures (Fig. 3). With glucose alone (concentration of 0·025 M) little alcohol was

found. With glucose plus detergent, however, a large output of alcohol was found which was parallel to the "extra CO_2" shown in Fig. 2. With alcohol added initially, its rate of disappearance was decreased by about 40 per cent by either the addition of detergent or of glucose and was decreased about 60 per cent if both glucose and detergent were present.

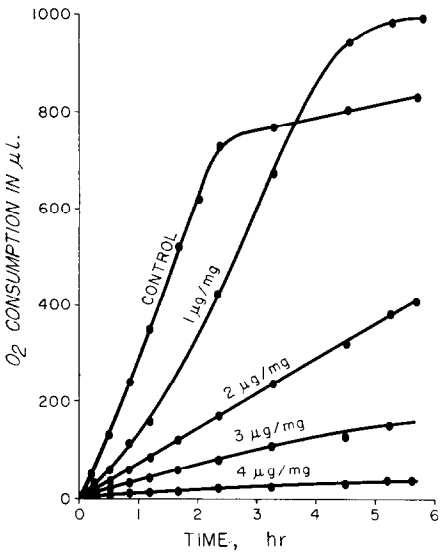


FIG. 4. The effect of benzalkonium on the respiration of acetate; 20 mg/ml yeast in 0·02 M buffer, pH 4·5 with 0·025 M acetate, and indicated concentrations of benzalkonium.

TABLE 1. INFLUENCE OF BENZALKONIUM ON THE RESPIRATION OF VARIOUS SUBSTRATES

(Yeast, 20 mg/ml in water; substrates, 0·03 M; benzalkonium, 3 μg/mg of yeast.)

Substrate	O_2		
	Control	B.Z.	Inhibition %
Pyruvate	8·8	5·9	33
Lactate	8·8	5·9	33
Glucose	18·0	8·0	55
Ethanol	19·5	4·5	77
Acetaldehyde	13·4	2·7	80
Acetate	13·4	1·5	89
Glucose and acetate	19·5	1·5	92

The rates are in μl/mg per hr for the first hour.

Eaton and Klein[13] and Holzer[14] suggested that a fraction of glucose is respired via glycolysis and subsequent oxidation via the two-carbon pathway and the Krebs cycle. The accumulation of alcohol in the presence of benzalkonium and the inhibition of its oxidation would suggest that the agent acts somewhere in the two-carbon pathway. A series of tests was carried out using a variety of substrates. A typical set of data is

given in Fig. 4 for acetate. As in the case of glucose, the rate of respiration was reduced, but the rate was maintained for a longer period than in the case of the control. Comparative data for a variety of substrates are given in Table 1.* The respiration (O_2 consumption) of the two-carbon compounds was the most sensitive to the agent in the order: acetate, acetaldehyde, ethanol (from 89 to 77 per cent inhibition). The respiration of the three-carbon compounds, lactate and pyruvate, was much less sensitive (33 per cent inhibition), a finding which is in line with the fact that the metabolism of these substances does not go via the two-carbon pathways.[15] The respiration of glucose (O_2 consumption) was intermediate in sensitivity (55 per cent inhibition). However, the disappearance of glucose, and the anaerobic CO_2 production were of lesser sensitivity (from 32 to 33 per cent inhibition). These observations are compatible with the concept that part of the respiration of glucose proceeds via the two-carbon pathway and that this component is most sensitive to the detergent, whereas the glycolytic path is relatively insensitive.

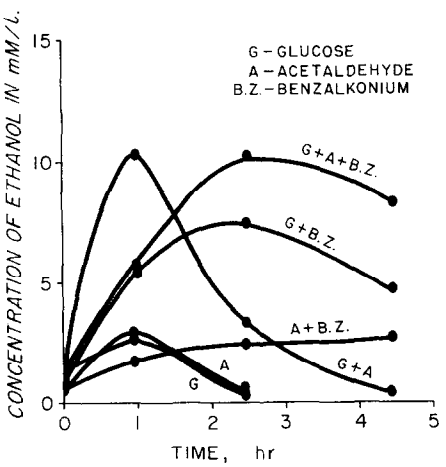


FIG. 5. The effect of benzalkonium on alcohol production from glucose, from acetaldehyde or from both substrates; 20 mg/ml yeast in distilled water with 0·025 M glucose and/or acetaldehyde and 3 μg/mg benzalkonium where indicated.

If the detergent blocks at the two-carbon level, its action on glucose metabolism should be imitated by "loading" the two-carbon pathway with exogenous two-carbon substrates. A series of experiments were therefore carried out with glucose, two-carbon substrates, and detergent, alone, and in mixtures. The data of Fig. 5 indicate that acetaldehyde, like the detergent, induced a large yield of alcohol from glucose. The effect with acetaldehyde was transient because this substance is used as a substrate, whereas the effect with detergent was prolonged. That acetaldehyde does not itself act as a major source of the alcohol is indicated by the fact that glucose is essential. Acetaldehyde alone, or together with detergent, gave only a low yield of

* The organic acids are respired only if free acid is present in the medium.[20] Because the pH tends to rise during their metabolism, it is necessary to use buffers. The buffer system chosen was triethlamine–succinate–tartrate.[12] Although cations in general have a protective action against the cationic detergents (see later section) the effect of triethlamine in the concentration used was small and did not change the relative effects on the different substrates.

alcohol. A similar response is also found in terms of "extra CO_2" production (Fig. 6). With acetaldehyde as a substrate, less CO_2 was produced than O_2 consumed (theoretical R.Q. is 0·84). With glucose alone, a small surplus of CO_2 was found. With a mixture of glucose and acetaldehyde, however, the surplus of CO_2 was considerably but temporarily increased. The detergent produces a similar but more permanent increase.

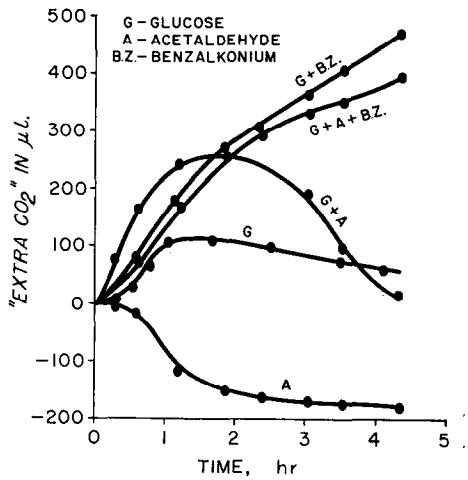


FIG. 6. The effect of benzalkonium on the "extra CO_2" production from glucose, from acetaldehyde, or from both substrates. Identical to Fig. 5.

Acetate also behaved like acetaldehyde, increasing the output of alcohol and of "extra CO_2" from glucose.*

In Table 1, it was demonstrated that benzalkonium in appropriate concentrations inhibited the respiration of all of the substrates tested. In addition to this inhibition, the relationship of O_2 consumption to CO_2 production was altered. The results, in the case of ethyl alcohol, are given in terms of the R.Q. (Fig. 7). Although the theoretical value is 0·67, the control values were considerably lower (about 0·3) for about 1 hr, and somewhat higher (about 0·8) thereafter. However, at the end of 5 hr, the average R.Q. was 0·59, reasonably close to theoretical. The rate of respiration was relatively linear for the whole period, but the CO_2 production lagged initially, and finally caught up. Apparently alcohol is initially converted in part to a metabolite in a higher state of oxidation which accumulates for a time, but which is finally respired. With benzalkonium present, the pattern was quite different; the R.Q. started at 1·08 and gradually drifted to about 0·7. The average R.Q. for the 5-hr period was greater than theoretical. It must be kept in mind, however, that the rate of respiration in this case was only about 30 per cent of normal, so that the endogenous metabolism represents about 30 per cent of the total respiration (compared with less than 10 per cent in the control).

The pattern for R.Q.'s for acetaldehyde was similar. The theoretical value is 0·84, but during the first hour, the observed value was less than 0·7. In the presence of

* However, the inhibitory effect of detergent on the respiration of glucose was markedly enhanced in the presence of acetate, increasing from 55 to 92 per cent (Table 1). None of the other two- or three-carbon compounds had this effect, nor did acetate have any such effect in the absence of detergent.

detergent, the value was slightly over 1·0. In the case of acetate, on the other hand, the observed R.Q. was close to the theoretical (1·0) during the whole period of metabolism and the addition of detergent had little effect.

The action of benzalkonium is reported to involve a binding to anionic sites on the cell surface.[4] For this reason, cations in the medium will protect by competing for the binding sites, provided that they are added prior to, or at the same time as the detergent.[20] In the present studies cations were found to exert a strong protective action

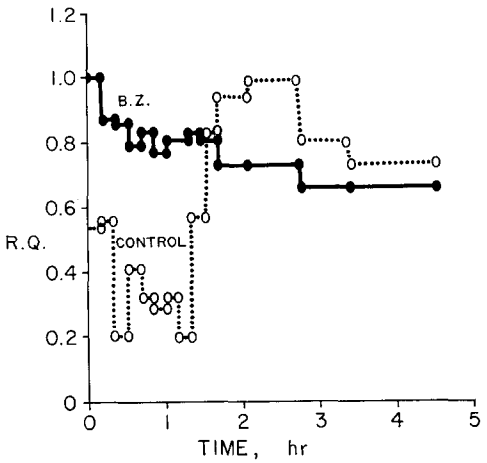


FIG. 7. The effect of benzalkonium on the R.Q. with alcohol as a substrate; 20 mg/ml yeast in 0·02 M buffer, pH 4·5 with 0·025 M ethanol and 3 μg/mg benzalkonium where indicated.

TABLE 2. PROTECTIVE ACTION OF Ca^{2+} AGAINST "EXTRA CO_2" PRODUCTION INDUCED BY BENZALKONIUM

(The yeast concentration was 20 μg/ml; glucose, 25 mM; benzalkonium, 3 μg/mg; and Ca^{2+} (as $CaCl_2$ adjusted to pH 4·5), 0·1 M.)

Time (hr)	Control	B.Z.	B.Z.	Ca^{2+}
0	0	0	0	0
0·5	20	120	150	20
1·0	20	280	320	20
1·0	No Additions		Ca added	B.Z. added
1·5	24	420	460	24
2·0	30	520	560	30
3·0	40	640	560	80
4·0	40	700	460	60
5·0	40	700	320	40
6·0	40	620	160	30

if added before the detergent, a slow but definite reversal if added after the detergent. For example, 0·1 M Ca^{2+} gave almost complete protection against "extra CO_2" production when added 1 hr before benzalkonium (Table 2). When the order of addition was reversed (B.Z. added 1 hr prior to Ca^{2+}), the action of the detergent was modified

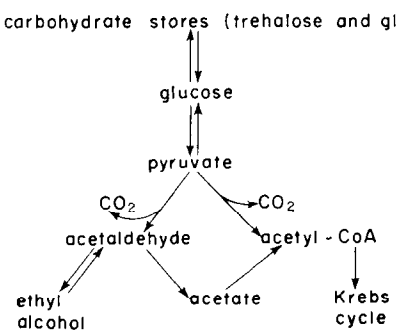
only after a delay period of from 1 to 2 hr. Five hours after the addition of Ca^{2+}, much of the "extra CO_2" had disappeared.

The inhibition of respiration induced by the detergent could also be modified by cations. If the cations were added before the detergent, complete protection could be afforded, but if the detergent was added first, the effect was only partially reversed and only after a delay of from 1 to 2 hr. Washing with solutions containing bivalent had little additional effect. No extensive tests were carried out with different cations, but from the available data, the relative efficacy of protection was in the order Na^+, K^+, triethylamine$^+$ $<$ Mg^{2+} $<$ Ca^{2+} $<$ UO^{2+}.

DISCUSSION

Among the multiple effects of benzalkonium on metabolism are: (1) the increase in total O_2 consumption from a given quantity of substrate; (2) the inhibition of respiration of various substrates; (3) the inhibition of anaerobic metabolism of glucose; (4) the stimulation of alcohol production and of "extra CO_2" from glucose; and (5) the changes in R.Q. for alcohol and acetaldehyde.

The interpretation of these phenomena and their interrelationships should be consistent with our knowledge of the pathways of glucose metabolism in yeast. A current picture is one summarized by Holzer.[14] Added to his scheme is the pathway of assimilation (formation of carbohydrate stores) which may account for from 20 to 40 per cent of substrate utilization, including that of two- or three-carbon compounds.[16, 17]



The relative importance of the two parthways of pyruvate degradation depends on its intracellular concentration; at low levels the pyruvic oxidase (right) is saturated first, due to its lower K_M: at higher levels the pyruvate overflows into the decarboxylative pathway (left), which is joined again to acetyl-CoA through the dehydrogenases and the aceto-CoA kinase. This picture is in agreement with the known facts and notably with the findings that the rate of fermentation is parallel to the intracellular concentration of pyruvate.[18, 19] It is also consistent with the appearance of aerobic fermentation only at higher glucose concentrations in the medium (Fig. 1) and with the previously mentioned results of Eaton and Klein[13] who found, using glucose-3, 4-^{14}C, that most of the label was in the CO_2 evolved initially, i.e., during the phase when ethanol temporarily accumulates in the medium.

It can be predicted from the above scheme that aerobic fermentation, that is, accumulation of ethanol and "extra CO_2" will occur whenever the rate of the

carboxylase reaction exceeds the rate of oxidation of the two-carbon intermediates. Three conditions producing this imbalance have been demonstrated:

(a) a high external concentration of glucose (Fig. 1);

(b) overloading of the oxidative system with exogenous two-carbon intermediates (Figs. 3, 5, 6);

(c) inhibition of the oxidation of one of the two-carbon intermediates by benzalkonium (Figs. 2, 3, for example).

In all cases, the effect is transient. After the glucose concentration is reduced to levels which cannot maintain maximal rates of respiration, the accumulated alcohol is respired and in consequence, the "extra CO_2" disappears.

This explanation of the benzalkonium-induced alcohol accumulation or "extra CO_2" production is consistent with the fact that the respiration of two-carbon substances is more sensitive to benzalkonium than is the respiration of glucose or of three-carbon compounds, or the fermentation of glucose. It is not possible to pinpoint the exact site of inhibition. The reaction alcohol $\leftrightharpoons$ acetaldehyde is not blocked, or alcohol would not accumulate. But either the reaction acetaldehyde to acetate, or acetate to acetyl-CoA, must be more sensitive to benzalkonium than any reactions in the glycolytic sequence or in the pyruvate-to-Krebs cycle-to-cytochrome sequence. With higher concentrations of the agent, reactions in the glycolytic path and in the respiratory pathway are also blocked.

The increase in total O_2 consumption induced by benzalkonium from each of the substrates tested, suggests a diminished assimilation (formation of carbohydrate stores). A few direct measurements of the changes in the total intracellular carbohydrates using the anthrone method[20] confirmed this suggestion. The inhibition of the assimilation process also provides an explanation for the R.Q. patterns with alcohol (Fig. 7). In the control, the initial low values (about 0·4) probably represent formation of carbohydrate from alcohol. The higher values in the presence of benzalkonium are consistent with the suggestion that assimilation is blocked or that carbohydrate stores are mobilized. The pattern with acetaldehyde is similar but the changes are smaller because the acetaldehyde is already at a higher state of oxidation than is alcohol. With acetate, no changes in R.Q. were observed because it is in the same state of oxidation as carbohydrate.

Basic dyes behave in some respects like benzalkonium. They bind on anionic groups of the cell surface and produce an irreversible effect from which protection is afforded by inorganic cations.[22–24] The basic dyes cause disruption of the cell membrane as a permeability barrier so that intracellular constituents such as K^+ leak out and the cells are stained. The response is all or none for individual cells.[24] Benzalkonium, at high concentrations, can produce a similar, all-or-none, breakdown of the cellular membrane as measured by loss of K^+, or staining, or release of carboxylase.[2] However, the reported specific actions on the metabolism of two-carbon compounds are apparently independent of the lytic action itself. First, with basic dyes, which also cause lysis, the "extra CO_2" production was not observed.[22, 23] Second, the effects on endogenous respiration associated with the lytic action, require higher concentrations of detergent than those on the exogenous metabolism.[5] Third, the concentrations of detergent required for the lytic effect are higher than those which produce "extra CO_2". Scharff and Maupin,[2] using the same yeast and detergent as in the present experiments,

studied the release of K^+ in detail. The lytic effect was minimal below a concentration of benzalkonium of 4 to 5 μg/mg of yeast, but increased rapidly with higher concentrations. In the present experiments no K^+-loss was induced by detergent at concentrations of 1·5 or 3·0 μg/mg, but K^+-loss was 30 per cent in a few hours at 6·0 μg/mg (in good agreement with Scharff and Maupin[2]). In view of the fact that the metabolic effects reported in this paper predominated at concentrations of 1·5 and 3·0 μg/mg, they are presumably independent of the lytic effect.

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