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# The Effects of Salicylic Acid on Metabolism and Potassium Ion Content in Yeast (39146)

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Numerous studies have shown that salicylates markedly alter metabolism, enzymic activity, and cell growth (1–5). In both human erythrocytes (6) and molluscan neurones (7), salicylate increases the permeability of the cell membrane to $K^+$ ion; in the latter study it was shown that salicylate simultaneously lowered the permeability to chloride ion. In the neuronal studies (7), these changes resulted in an increased potential difference across the cell membrane and the authors postulated that salicylate analgesia may result from an impaired ability of the neurone to conduct impulses along the hyperpolarized cell.

The present paper deals with the metabolic and membrane effects of salicylic acid on commercial baker's yeast. While corroborating earlier studies (2) of the inhibitory effects of the compound on glucose metabolism, we present new information that salicylic acid increases endogenous fermentation and causes a leakage of $K^+$ ions from the cells. The first and last mentioned effects are reversed by washing the cells free of salicylic acid.

*Methods.* Experiments were done on suspensions of nongrowing baker's yeast as reported earlier (8). Suspensions were maintained at appropriate pH by use of a tris-succinate-tartrate (TST) buffer consisting of equimolar concentrations of succinic and tartaric acids adjusted to pH with tris-hydroxymethylaminomethane. With the exception of the experiment shown in Fig. 3, no $K^+$ was added to the experiment. Any $K^+$ found in the medium came through release of this ion by the cells. In all experiments, nitrogen gas was used to maintain anaerobic conditions and to keep suspensions from settling. $K^+$ efflux experiments were done via a modification of the cell column technique of Rothstein and Bruce (9); in our experiments we replaced the sintered glass filters used by these investigators with a 0.45-$\mu$m microfilter 47 mm in diameter. Eluting fluid was forced through the yeast column under 5–6 psi of nitrogen. Gas exchange was measured via standard manometric techniques. Experiments were conducted at 30° or 6°.

Estimations of glucose in diluted, filtered suspending medium were made either by means of a commercial glucose oxidase preparation (Worthington Biochemical Corp.) or the reducing sugar method of Nelson (10). Alcohol was determined quantitatively by use of a commercial alcohol dehydrogenase preparation (Worthington) and was identified as ethyl alcohol through gas chromatography.[1] $K^+$ in the medium, a measure of $K^+$ lost from the cell, was determined on filtrates of cell suspensions through direct flame photometry. Yeast concentrations are expressed in milligrams wet weight of cells per milliliter of suspension. For convenience, the total concentration of the salicylate–salicylic acid will be referred to in the text as "salicylate." Through calculation, the actual concentrations of the two species can be determined in each case from total "salicylate" concentration, the pH and the $pK_a$ of salicylic acid (3.0).

*Results.* In Table I are shown the effects of 5 m*M* salicylate on glucose utilization and net $K^+$ loss at different pH values. At pH 5.5, where undissociated acid is present at about 0.3% of the total concentration, little effect was seen on either glucose utilization or net $K^+$ loss. As undissociated acid concentration was raised through lowering external pH, both $K^+$ loss and inhibition of glucose utilization rose sharply until at pH 3.5 glucose utilization was inhibited 98%, and net $K^+$ loss in the presence of salicylate

[1] The authors thank Dr. John A. Nicholson of this department for the gas chromatographic identification of ethanol.

was approximately 8.5 times the loss in the control suspension. This finding suggests that the undissociated form of salicylic acid was needed in the production of the "salicylate" effects.

Reversibility of the effects of salicylic acid on glucose metabolism and $K^+$ loss are shown in Table II. After a 2-hr incubation with salicylate, an average of approximately 83% inhibition of glucose utilization was observed in salicylate treated yeast suspensions. In addition, about a 13.5-fold loss of $K^+$ over the control loss was seen in the salicylate suspension. The total $K^+$ loss in the presence of salicylate amounted to about 15% of the original $K^+$ content of the cells. After each suspension was washed four times through repeated centrifugation, decantation, and resuspension in distilled water (the volume in each case being equal to the original suspension volume), the suspensions were reconstituted in buffer and glucose. $K^+$ appearance in the medium was measured and it can be seen that both the salicylate and control suspensions now showed the same net $K^+$ loss after 2 hr of

TABLE I. pH VERSUS SALICYLATE EFFECTS ON METABOLISM AND $K^+$ LOSS IN YEAST.[a]

| | Glucose utilization | | | | | | Net $K^+$ loss | |
|---|---|---|---|---|---|---|---|---|
| | Control | | | 5 m*M* salicylate | | | Control | 5 m*M* salicylate |
| pH | Time (min) | Glucose (μmoles/ml) | Glucose used (μmoles/ml) | Glucose (μmoles/ml) | Glucose used (μmoles/ml) | Inhibition by salicylate (%) | $K^+$ In medium (m*M*) | $K^+$ In medium (m*M*) |
| 5.5 | 0 | 97 | — | 94 | — | — | 0.06 | 0.08 |
| | 60 | 49 | 48 | 48 | 46 | 4 | — | 0.02 |
| | 90 | 29 | 68 | 30 | 64 | 6 | 0.01 | 0.01 |
| 4.5 | 0 | 92 | — | 95 | — | — | 0.06 | 0.06 |
| | 60 | 47 | 45 | 90 | 5 | 89 | — | 0.21 |
| | 90 | 30 | 62 | 88 | 7 | 89 | 0.02 | 0.20 |
| 3.5 | 0 | 91 | — | 95 | — | — | 0.20 | 0.20 |
| | 60 | 50 | 41 | 95 | 0 | 100 | — | 0.84 |
| | 90 | 36 | 55 | 94 | 1 | 98 | 0.15 | 1.30 |

[a] Experiments were run under nitrogen for 90 min with 0 or 5 m*M* salicylate, 0.095 *M* glucose, 0.03 tris-succinate-tartrate buffer at pH 3.5, 4.5, or 5.5. Yeast concentration was 29 mg/ml. $pK_a$ salicylic acid is 3.0. Temperature, 30°C.

TABLE II. REVERSIBILITY OF THE EFFECTS OF SALICYLATE ON $K^+$ LOSS AND GLUCOSE METABOLISM.[a]

| Suspension | Time (hr) | $K^+$ in medium (m*M*) | Glucose in medium (m*M*) | Glucose (μmoles used/ml) | Glucose used (% of control) |
|---|---|---|---|---|---|
| Control | 0 | 0.027[b] | 98[b] | — | — |
| | 2 | 0.020 | 52 | 46 | 100 |
| 5 m*M* salicylate | 0 | 0.060 | 100 | — | — |
| | 2 | 0.270[c] | 92 | 8 | 17 |
| Washed control[d] | 0 | 0.020 | 97 | — | — |
| | 2 | 0.020 | 50 | 47 | 100 |
| Washed salicylate[d] | 0 | 0.018 | 94 | — | — |
| | 2 | 0.016 | 55 | 39 | 83 |

[a] Suspensions were initially incubated for 2 hr under nitrogen with 0 or 5 m*M* salicylate in 0.03 *M* TST buffer at pH 4.5. Original glucose concentration was 100 m*M*.

[b] All values are averages of two experiments.

[c] This concentration represents less than 15% of initial total cellular $K^+$.

[d] After initial incubation, each suspension was centrifuged, decanted, resuspended in distilled water to make total volume equal to that of original suspension, and centrifuged, and the supernate was discarded. Each suspension was washed four times in this way, then resuspended and incubated in buffer and glucose, but no salicylate.

incubation. Moreover, glucose utilization in the suspension washed free of salicylate reached at least 83% of that of the washed control suspension. No inorganic phosphate or protein, indicators of membrane damage, were detected in the medium of either original control or salicylate suspensions. We concluded that the effects of salicylate on either the cell membrane or cell metabolism were reversible under the given conditions.

In an experiment using unbuffered yeast suspensions adjusted to pH 4.5 at the outset we found that 3 m*M* salicylate induced a rise in pH of the medium from pH 4.5 to 5.0 in about 20 min. Control suspensions did not show a rise in pH. Therefore, at least a part of the $K^+$ loss due to salicylate appeared to involve a $K^+$–$H^+$ exchange mechanism. This finding agrees with the earlier studies of Rothstein and Demis (11) and Rothstein and Bruce (12), who established that $K^+$ efflux from cells during active metabolism of exogenous substrate increased with decreasing pH of the suspending medium in a $K^+$–$H^+$ exchange. The control data for $K^+$ loss in Table I are also in agreement with the concept of $K^+$–$H^+$ exchange.

In another set of experiments (Fig. 1), five concentrations of salicylate were added to otherwise identical yeast suspensions. Samples were taken for $K^+$ analysis at 0, 1, and 2 hr. The figure shows that net $K^+$ loss after 1 or 2 hr did not continue to increase with increasing salicylate concentration, but showed a maximum value at about 2 m*M* salicylate. This general finding of a maximum $K^+$ loss at 2 m*M* salicylate was seen repeatedly. In Fig. 2, for example, samples taken after 10 and 30 min of incubation showed the same maximum in experiments conducted at 30°; at 6°, on the other hand, no maximum value was seen and the net $K^+$ loss was much lower than at 30°.

In another experiment, approximately 11 m*M* KCl was added initially to suspensions. No detectable net uptake of $K^+$ was seen in salicylate or control suspensions (Fig. 3). With 100 m*M* acetic acid at the same pH, net uptake of $K^+$ did occur. The latter finding had been reported by us earlier (8, 13).

The failure of yeast to take up appreciable amounts of $K^+$ in the presence of salicylic acid (Fig. 3) suggests that the maximum in $K^+$ loss at 2 m*M* salicylate is not a consequence of changes in influx rates in the presence of salicylate. We next did experiments in which yeast was used as an ion exchange column (9). Eluting solutions containing different concentrations of buffered salicylate were passed through the columns. Eluates were tested for $K^+$. Since no appreciable buildup of $K^+$ could occur in the flowing eluting fluid, influx back into the yeast could not readily occur. Hence,

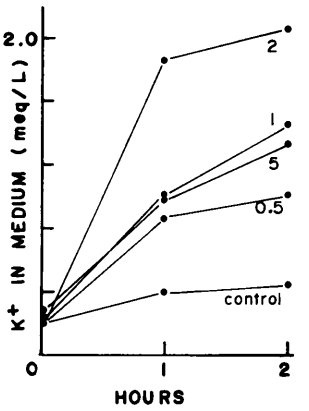


FIG. 1. $K^+$ loss vs salicylate concentration. Yeast: 50 mg/ml. Buffer: 0.03 MTST, pH 4.5. Temperature: 30°. Salicylate: none or 5 m*M*, added at zero time. Each point represents an average from three experiments. Numbers accompanying curves refer to millimolar concentrations of salicylate.

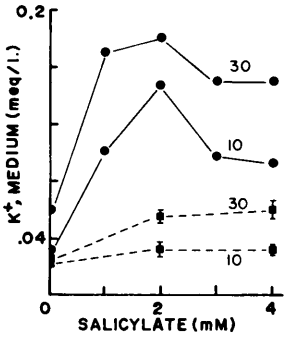


FIG. 2. Temperature vs net $K^+$ loss from yeast cells in the presence of salicylic acid. Yeast: 15 mg/ml. Buffer: 0.005 MTST, pH 4.5. Salicylate: 0 or 5 m*M*. Solid lines: 30°. Broken lines: 6°. Numerals accompanying each curve represent time in minutes of samples for that curve. Vertical bars in 6° curves represent range for two suspensions.

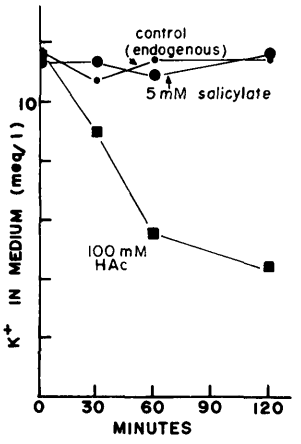


FIG. 3. Net uptake or loss of $K^+$ in yeast suspensions treated with salicylate or acetic acid. Yeast: 100 mg/ml. Buffer: 0.03 MTST, pH 4.5. Salicylic acid: 0 or 5 m*M*. Acetic acid: 0 or 100 m*M*. KCl: 0.11 *M* at outset of experiment. Temperature: 30°.

$K^+$ efflux (and not influx) was of primary importance at 30° in these experiments. Data in Fig. 4 show that a maximum occurred in the efflux curve, a maximum similar to those shown in Figs. 1 and 2 for net $K^+$ loss.

Salicylate stimulated endogenous metabolism at 30°; at 6° this did not occur. In Fig. 5, a comparison of $CO_2$ and alcohol production at 30° for yeast cells in the presence of varying salicylate concentrations and in the absence of exogenous glucose shows that alcohol and $CO_2$ were produced in a 1–1 ratio. The alcohol was determined to be ethanol through gas chromatographic analysis. Earlier studies done by us of the effects of other penetrating acids (8, 13) showed conclusively that such compounds are able to dissimilate carbohydrate stores of the yeast cell to alcohol and $CO_2$. In this regard salicylic acid shows a similar, but reduced capacity. Therefore, in the present study no attempt was made to measure carbohydrate content of the cell before and after incubation in the salicylate since the error involved would preclude our obtaining reliable results. Based on the stoichiometry involved in $CO_2$–alcohol production, the identification of ethanol by at least one means, and our earlier studies with other acids (13), we concluded that salicylic acid also dissimilated yeast cell carbohydrate stores to the final fermentation products.

The actual rates of $CO_2$ and alcohol production were greatest shortly after addition of salicylate to the suspensions, the rates after 2 hr declining to low values. Furthermore, the rates of endogenous fermentation brought about by the highest concentrations of salicylate were about 25-fold less than are encountered when exogenous glucose is used as substrate. Hence it would be expected that even in glucose-utilizing suspensions which are inhibited by addition of salicylate, the remaining $CO_2$ production might mask the small evolution of $CO_2$ occurring through breakdown of the carbohydrate stores of the cell.

*Discussion.* Salicylate elicits $K^+$ loss in at least three disparate types of eukaryotic cells–human erythrocytes (6), molluskan

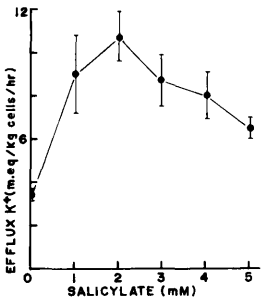


FIG. 4. Rate of $K^+$ efflux vs salicylate concentration. Yeast: 1500 mg. Salicylate: 0–5 m*M*. Buffer: 0.005 MTST, pH 4.5. Temperature: 30°. Nitrogen pressure: 5–6 psi. Vertical lines: ±SE. Each point represents mean of 3–5 determinations.

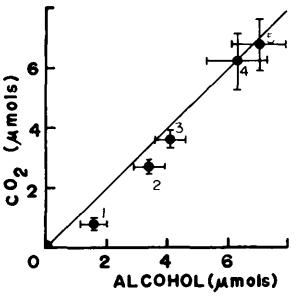


FIG. 5. Production of alcohol and carbon dioxide in yeast in the presence of varying concentrations of salicylate. Yeast: 150 mg/ml. Buffer: 0.03 MTST, pH 4.5. Temperature: 30°. Time of incubation: 2 hr. Salicylate: 0–5 m*M*, corresponding to numbers accompanying points on graph. Each point represents the average value obtained for three suspensions ±SE. Any point on diagonal line represents equal $CO_2$ and alcohol produced.

neurones (7), and yeast cells. We suggest that salicylate ion or undissociated salicylic acid has the ability to produce reversible metabolic and membrane effects which lead to leakage of $K^+$ from many types of cells—that perhaps $K^+$ loss is a fundamental characteristic of the action of salicylate on cells.

In our studies of the effects of salicylate on yeast, in contrast to those of others on erythrocytes and neurones (6, 7), we found the compound to be active only at pH values below 5.5. The question is thus raised as to whether yeast cells absorb only the unionized acid while the other cells studied absorb either form of salicylate, or whether the latter cells show a markedly greater susceptibility to this compound, i.e., do these cells respond to the extremely low concentrations of undissociated salicylic acid present at the higher pH values used?

The reversibility of the effects of salicylate on yeast glucose metabolism and $K^+$ loss shows that no evident permanent effect on the membrane results under the conditions of our experiments. The $K^+$ leakage due to salicylate may result from the ensuing endogenous metabolism of carbohydrate when no external glucose is present, the endogenous metabolism substituting for glucose in any $K^+$ leakage connected with increased metabolic rates above resting endogenous rates. It has been known for many years that actively metabolizing cells show greater $K^+$ fluxes than do resting cells (8, 12, 14). Furthermore, this finding of marked increase in $K^+$ loss in the presence of salicylate at 30° but not at 6° is in agreement with the concept that at least a part of the salicylate effect on $K^+$ leakage from yeast may be related to metabolism. The possibility of direct effects of salicylate on the membrane, unrelated to metabolism, is by no means precluded by the experiments shown here, however.

Salicylic acid has an action in common with other penetrating acids (13), the dissimilation of cell carbohydrates. However, under the conditions of our experiments, salicylic acid, unlike other acids tested, does not appear to elicit $K^+$ uptake (Fig. 3) to any appreciable extent at 5 m*M* concentration. Solubility limitations preclude study of salicylate at much higher concentrations. Acetic acid at 100 m*M* is capable of causing $K^+$ uptake at pH 4.5 (Fig. 3) but at concentrations comparable to the salicylate concentrations used, acetic acid also was not able to bring about $K^+$ uptake.

Remaining unexplained is the maximum in the curves for $K^+$ efflux or net $K^+$ loss (Figs. 1, 2, and 4). It is evident that the maximum is a result of salicylate effects on $K^+$ efflux, but we can only speculate concerning the actual mechanism (or mechanisms) involved. It appears a strong possibility that a two-phase action of salicylate occurs on $K^+$ loss as the concentration of salicylate is raised—one effect to raise the rate of efflux, followed at higher salicylate concentration by an effect which reduces the loss of the ion.

Despite the fact that both adenyl cyclase and 3′,5′-cyclic adenosine monophosphate have been found in yeast (15), the possible role of a phosphorylase–cyclic nucleotide system in $K^+$ loss was not clarified by us in an experiment where we added 1 m*M* dibutyryl cyclic adenosine monophosphate to yeast in the presence or absence of salicylate. Under either condition, no changes ascribable to the cyclic compound were seen in $K^+$ loss. In any case, however, the effects ascribed to salicylate on $K^+$ loss appear to be associated in some unknown way with the effects of salicylate on metabolism.

*Summary.* Under anaerobic conditions, at low pH and 30°, commercial baker's yeast loses $K^+$ ion in the presence of salicylic acid. Glucose utilization is inhibited. In suspensions containing no glucose, carbohydrate stores of the cell are dissimilated to carbon dioxide and alcohol. The ion loss and inhibitory effects of salicylic acid on glucose utilization are reversed by washing the cells free of salicylate. The loss of $K^+$ appears to be due at least partly to a $K^+$–$H^+$ exchange process. An unexplained maximum is seen in the curves of either net $K^+$ loss or $K^+$ efflux versus salicylic acid concentration. At 6° the effects of salicylic acid on both endogenous metabolism and net $K^+$ loss are minimal. Furthermore, no maximum is seen in the $K^+$ loss–salicyclic concentration curve at this temperature. It is generalized that salicylic acid or salicy-

late may elicit $K^+$ leakage from many types of cells, i.e., a fundamental action of this compound may be its ability to affect (reduce) $K^+$ content of the cell; furthermore, it appears that the salicylate effects on $K^+$ loss may be associated in an as-yet-unknown manner with the metabolic effects of this compound. The effects of salicylate on $K^+$ loss in yeast may not be unique for this compound, since no experiments of this nature have been done with other penetrating undissociated acids.

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