ACTA CHEMICA SCANDINAVICA 13 (1959) 803–813

The Bacteriostatic Action of Benzoic and Salicylic Acids

I. The Effect on the Oxidation of Glucose and Pyruvic Acid by *Proteus vulgaris*

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The effect of benzoic and salicylic acids on glucose and pyruvate oxidation by *Proteus vulgaris* was studied by means of Warburg experiments. Neither benzoic nor salicylic acid had any inhibitory influence on the rate of oxygen consumption until the oxidation level of acetate was reached. Further oxidation was specifically stopped in the presence of 0.01 M benzoic, or 0.003 M salicylic acid, at pH 6.0. These concentrations are close to those required for a bacteriostatic effect.

The antimicrobial action of benzoic and salicylic acids has long been known and studied in various respects. Much work [1–17] has been done concerning the effect of these acids on different enzymes, or complex of enzymes, isolated or in intact cells, but the ultimate cause of the growth-inhibiting effect, in terms of metabolic reactions, is still open to discussion. The present work is an attempt to trace the mode of action somewhat further by determining what reactions, vital to the cell, are susceptible to the inhibitors at concentrations suppressing the growth of microorganisms. The experiments presented below are respiration studies with resting cells of *Proteus vulgaris*, in which the conventional Warburg technique has been used.

EXPERIMENTAL

Organism. A strain of *Proteus vulgaris* was selected as testorganism, mainly on account of its rapid growth on different carbon sources in synthetic media. Washed cells of this organism readily oxidize members of the tricarboxylic acid cycle and related compounds, besides glucose and pyruvic acid, especially when aerated during cultivation and harvested in the logarithmic growth phase. Moreover, such suspensions have a very low endogenous respiration, a fact of great value in the present studies. The culture was maintained on nutrient agar and transferred to new agar slants every month.

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Medium and growth conditions. The cultivation medium had the following composition, unless otherwise stated: glucose, 10.0 g; NH_4Cl, 1.0 g; K_2HPO_4, 9.5 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; casein hydrolysate (Difco vitamin-free casamino acids), 1.0 g; nicotinamide, 0.2 mg; distilled water to 1 l; adjusted to pH 7.0. The proper amount of glucose was dissolved in part of the water and autoclaved as a separate solution. Cultures were grown at 37°C, either in Erlenmeyer flasks without shaking, or in Kluyver flasks with sufficient aeration to ensure maximum growth rate. The growth media were inoculated with actively growing cells from a medium of the same composition. In most experiments the cells were harvested at the end of the logarithmic phase, or just after cessation of growth. In these cases, the growth was followed 1—2 h before harvesting by optical density measurements in a Beckman spectrophotometer at 425 mμ with sterile culture medium in the blank cell. Units of cell mass were determined by means of a standard curve for the organism, relating dry weight to optical density. In some experiments the cells were harvested at the beginning of the logarithmic phase of growth. In these cases, the culture medium was inoculated with a heavy suspension of washed cells, the optical density measurements being immediately started and the cells harvested, as soon as the logarithmic phase was established.

Preparation of cell suspensions. The cells were harvested by centrifugation and washed once by resuspension in the approximate growth volume of M/15 phosphate buffer of the same pH as in the actual experiment, usually 6.0. After repeated centrifugation, the cells were again suspended in the buffer, and the cell concentration adjusted to appropriate density. Repeated washings had no effect on cell activity, or endogenous respiration. Of this suspension 2.0 ml were added to each Warburg vessel.

Respiration studies. Oxygen consumption was measured by the conventional Warburg technique. The cell suspension was added to the main compartment of the Warburg vessels, and neutralized solutions of inhibitors and substrates to the side arms. The center well of the flasks contained 0.2 ml of 10 % KOH, absorbed on filter paper. The additions of substrate were so limited that the oxygen uptake could be followed until it had decreased to the value of the endogenous respiration. When necessary, cell activity was terminated by the addition of 0.3 ml of 5 N H_2SO_4 at the end of the experiment. CO_2 evolution, when determined, was measured according to the direct method of Warburg. The data recorded in the figures and tables have been corrected for the endogenous respiration which in no case was more than 10 % of that obtained in the presence of added substrate. All respiration measurements were made at 37°C and pH 6.0, if not otherwise stated.

Analytical procedures. Analyses of the contents of the Warburg vessels were made after removal of the cells by centrifugation. Pyruvic acid was determined as total keto acids with 2,4-dinitrophenylhydrazine, according to Friedemann and Haugen [18]. In the present experiments this method proved to give the same results as polarographic analysis. Acetic acid was determined as volatile acid by fractional steam-distillation. The titration was carried out with 0.1 N NaOH by means of a micrometer syringe. Benzoic acid, when present, distilled over together with acetic acid, and was corrected for by measuring the optical density of the distillate at 230 mμ in a Beckman DU spectrophotometer.

RESULTS

Growth experiments

The first step in this investigation was to determine the concentration of benzoic and salicylic acids required to prevent growth of *P. vulgaris*. As in the case of all weak acids, the biological activity of these compounds depends on the amount of substance present as neutral molecules, probably because these alone, not the ions, can penetrate the semipermeable membrane assumed to surround the bacterial cell. Thus, the activity of a given acid will depend on pH in a predictable manner, increasing with decreasing pH. Most of the

experiments in this investigation were carried out at pH 6.0, since a lower pH in itself would have led to unfavourable conditions for the organism.

The growth-inhibiting effect of benzoic acid on *P. vulgaris* was studied in the medium adjusted to pH 6.0 (Table 1). The main effect seemed to be a lengthening of the lag phase, but at concentrations approaching those required for a bacteriostatic effect, the growth rate in the logarithmic phase was also influenced. A characteristic feature in these experiments was the fact that benzoic acid, even at rather low concentrations, reduced the total amount of growth as compared with the amount obtained without inhibitor. This is easily accounted for by the formation of acid during growth, which resulted in an increasing effect of the benzoic acid and ended with growth being completely suppressed. In fact, a calculation of the actual concentrations of undissociated benzoic acid present at the moment when growth ceased in cultures with varying total amounts of the inhibitor gave the same value in all cases. In cultivation media where pH did not change during the experiment, no effect of benzoic acid on the total amount of growth could be demonstrated. It is evident that benzoic acid at concentrations about 0.01 M completely inhibited growth of *P. vulgaris* at pH 6.0. The same experiment with salicylic acid proved this inhibitor, producing bacteriostasis at concentrations about 0.002 M, to be more effective. However, the good correspondence between the amounts of undissociated acid present when growth ceased, as shown for benzoic acid in Table 1, did not appear when salicylic acid was used as inhibitor.

Table 1. Effect of benzoic acid on the growth of *P. vulgaris*. 50 ml of growth medium in 100-ml Erlenmeyer flasks were inoculated with cells actively growing in medium of the same composition without inhibitor. Different inocula were used in the two experiments. Initial pH, 6.0. Temp. 35°C.

Expt. No.	Total concn. of benzoic acid, M × 10^3	Growth	pH-values of the culture media when growth had ceased	Calculated * concn. of undissociated benzoic acid when growth had ceased, M × 10^3
1	—	+	4.51	—
	0.7	+	4.65	0.18
	1.7	+	5.09	0.19
	3.5	+	5.63	0.13
	5.3	+	5.82	0.12
	7.0	+	5.86	0.15
	10.5	—	5.98	—
2	—	+	4.62	—
	0.7	+	4.80	0.14
	1.7	+	5.28	0.13
	3.5	+	5.66	0.12
	5.3	+	5.81	0.13
	7.0	+	5.91	0.13
	10.5	—	5.98	—

* $10^{-4.20}$ has been used as dissociation constant for benzoic acid in this calculation.

The effect of benzoic and salicylic acids on glucose oxidation

Preliminary respiration studies at pH 6.0, with glucose as substrate, showed that neither inhibitor, at the concentrations mentioned above, had any effect on the rate of oxygen consumption during the first stage of glucose oxidation, and that lower concentrations generally increased the rate somewhat. The results, however, varied with growth conditions and the age of the cells at the time of harvesting.

Experiments with cells grown without shaking or aeration. The cultures were harvested in the stationary phase, generally within 1—2 h after growth had ceased. Warburg experiments with such cells showed that oxygen consumption during glucose oxidation proceeded at a constant rate until a sudden break occurred, after which it continued at a lower, though still constant rate, which, however, was significantly higher than that of the endogenous respiration. At the time of the break, all added glucose had disappeared from the medium. Pyruvate accumulated during the first part of the oxidation and disappeared again after the break (Fig. 1). The maximum amount of pyruvate in the medium, always appearing simultaneously with the break, varied in magnitude with the cell preparations according to some unknown factor, although the experimental conditions were kept as constant as possible.* In

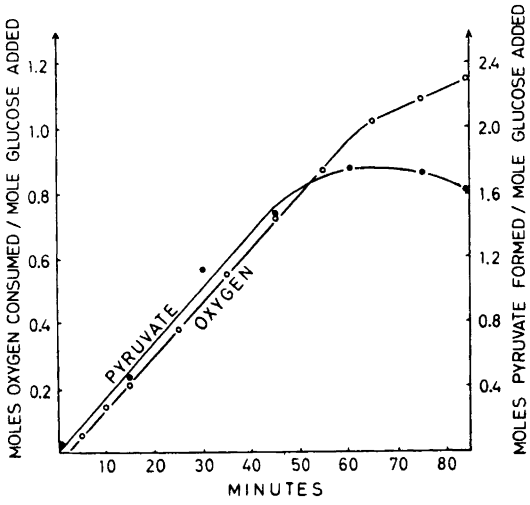

Fig. 1. Pyruvate accumulation during glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 2.5 μmoles of glucose and 133 μmoles of potassium phosphate in a total volume of 2.5 ml.

* After these experiments were performed, related results have been reported by Jackson[19] who investigated the accumulation of pyruvate in cultures of *P. vulgaris* grown with limiting concentrations of nicotinic acid. Although the concentrations of nicotinamide (0.2 μg/ml) used in the present work hardly can have had a limiting effect on growth, it is still possible that this factor might have been of some significance even in this case.

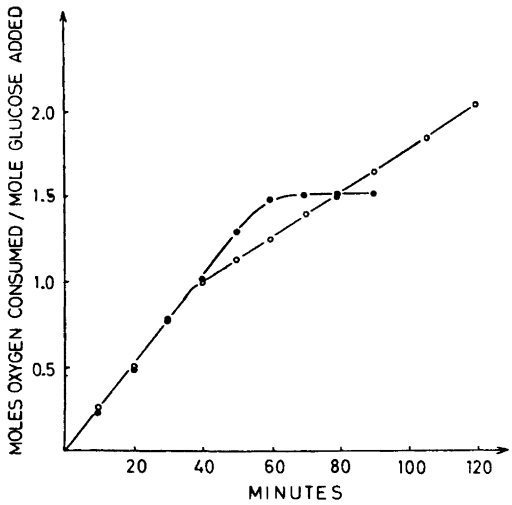

Fig. 2. Effect of benzoic acid on the oxygen consumption during glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 2.5 μmoles of glucose and 133 μmoles of potassium phosphate. O———O no benzoate, ●———● 0.01 M sodium benzoate. Final volume 2.5 ml.

some experiments, about 90 % of the maximum amount of pyruvate possible, as calculated from the amount of glucose added, had accumulated before the break. In such cases, the break occurred when almost exactly one mole of oxygen had been consumed per mole of glucose added, which seemed to indicate that the oxidation took place in two separate steps, first a conversion of all glucose added into pyruvate, and next a further oxidation of this metabolite.

This picture was completely changed when the oxidation took place in the presence of benzoic acid (Fig. 2). The essential effect of this drug consisted in postponing the break, after which the oxygen consumption was strongly inhibited. This inhibition was complete at the same concentration of benzoic acid as that required to prevent growth of the organism at the pH in question. Under these conditions, accumulation of pyruvate was prevented.

Experiments with cells aerated during growth. The two characteristic features of glucose oxidation with old cells of *P. vulgaris*, the break in the rate of oxygen consumption at a point where about one mole of oxygen had been taken up per mole of glucose added, and the accumulation of pyruvate, seemed to be intimately linked with some factors in the growth conditions. These signs of a disturbed oxidative ability did not appear in cells aerated during growth and harvested in the logarithmic growth phase. The oxygen consumption during glucose oxidation by such cells, and the effect of varying concentrations of benzoic acid, are illustrated in Fig. 3. 0.0035 M benzoic acid slightly stimulated the rate of oxygen uptake, while higher concentrations decreased the rate. The results obtained indicate a specific effect of benzoic acid on the later stages of glucose oxidation, and the amount of oxygen consumed in presence

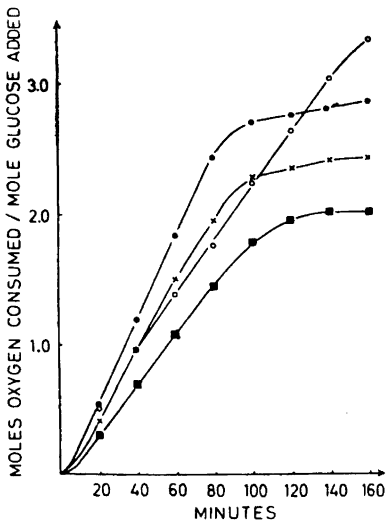

Fig. 3. Effect of benzoic acid on the glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 2.5 μmoles of glucose and 133 μmoles of potassium phosphate. O——O no benzoate, ●——● 0.0035 M, ×——× 0.0070 M, ■——■ 0.0140 M sodium benzoate. Final volume 2.5 ml.

of 0.014 M benzoic acid, about 2 moles per mole glucose added, suggests that the sensitive point lies at the oxidation level of acetate. Higher concentrations of benzoic acid inhibited the rate of oxygen consumption still more, but had no influence on this minimum amount of oxygen taken up per mole glucose. Essentially the same results were obtained with salicylic acid. This inhibitor, however, was more effective, reducing the oxygen consumption per mole glucose to 2 moles at concentrations about 0.003 M (Fig. 4). The close resemblance between these figures for the two acids and the bacteriostatic concentrations given on page 00 should be noticed. Large amounts of volatile acid were demonstrated in vessels where the oxidation occurred in the presence of benzoic or salicylic acid, whereas only traces were found in vessels without inhibitor.

The inhibition of ammonia assimilation during glucose oxidation by benzoic and salicylic acids

It has already been shown by Bernheim and DeTurk [10] that salicylic acid, benzoic acid, and related compounds, inhibit ammonia assimilation during succinate oxidation in *Pseudomonas aeruginosa*, more than they inhibit the oxidation of succinate itself. A similar specific effect on ammonia assimilation seemed to exist even in *P. vulgaris* (Table 2) when glucose was used as an energy source. Benzoic acid, at a concentration of 0.007 M, actually had a stimulating effect on the rate of oxygen consumption during glucose oxidation in the absence of ammonium ions. The degree of this stimulation varied with the age of the cells, being greater the older the cells were at the time of harvesting. The main results of the four experiments with benzoic acid

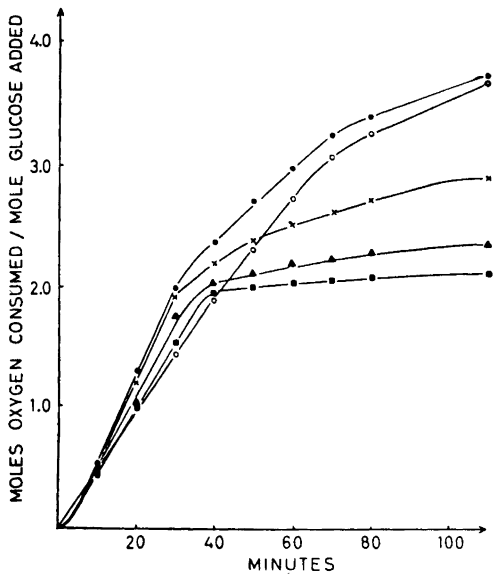

Fig. 4. Effect of salicylic acid on the glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 5 μmoles of glucose and 133 μmoles of potassium phosphate. O———O no salicylate, ●———● 0.00125 M, ×———× 0.00188 M, ▲———▲ 0.00313 M, ■———■ 0.00500 M sodium salicylate. Final volume 2.5 ml.

were the same, being independent of the growth conditions, *i.e.* the increase in oxidation rate accompanying the presence of ammonium ions was completely abolished. Similar results were obtained with salicylic acid.

The effect of benzoic and salicylic acids on pyruvate oxidation

Bacteria from aerated cultures were used in these experiments. The cells were harvested at the end of logarithmic phase, or just after they had reached

Table 2. Influence of benzoic acid on the rate of glucose oxidation in the presence and absence of ammonium ions (four experiments). Each vessel contained 133 μmoles of potassium phosphate, 5 μmoles of glucose and 5 μmoles of $(NH_4)_2SO_4$ (when added). The final concentration of sodium benzoate was 0.007 M when present.

Additions	Relative rates of oxygen consumption. Rates with glucose as the only addition = 1			
	I	II	III	IV
Glucose	1	1	1	1
Glucose + $(NH_4)_2SO_4$	1.5	1.4	1.6	1.5
Glucose + sodium benzoate	1.3	1.1	1.2	1.4
Glucose + $(NH_4)_2SO_4$ + sodium benzoate	1.3	1.1	1.2	1.4

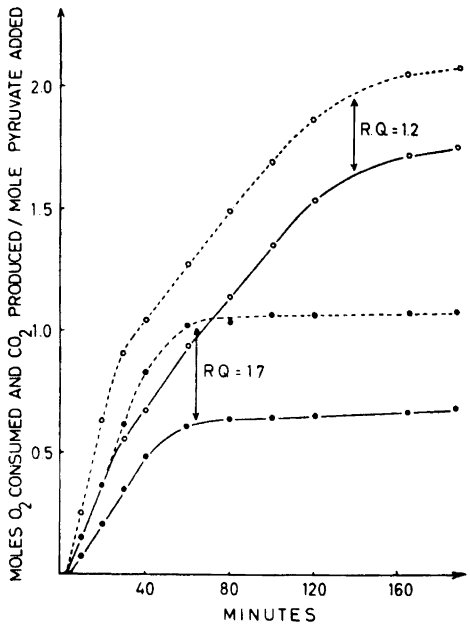

Fig. 5. Effect of benzoic acid on the pyruvate oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 5 μmoles of sodium pyruvate and 133 μmoles of potassium phosphate. Final volume 2.5 ml. O———O no benzoate, ●———● 0.0083 M sodium benzoate. Solid line, oxygen; dash line, carbon dioxide.

the stationary phase. The growth phase at harvesting did not seem to be critical for the results. As in the case of glucose oxidation, the results (Fig. 5) strongly suggest that the main effect of benzoic acid is close to the oxidation of acetate, or some »active» form of it. The theoretical amounts of oxygen consumed and carbon dioxide produced at a conversion of pyruvate into acetate (or some of its »active» forms) are 0.5 and 1.0 mole per mole pyruvate, respectively. As shown in Fig. 5, the actual values obtained in the presence of 0.0083 M benzoic acid agreed well with these figures. Determination of accumulated volatile acid in the vessels, after the oxygen consumption had practically ceased, supported this interpretation of the data. The results of one such determination are shown in Fig. 6 B, and the corresponding manometric data in Fig. 6 A. Similar experiments with salicylic acid gave corresponding results. The distillation constant calculated from the experimental data agreed with that obtained by distilling a similar solution with known amounts of acetic and benzoic acids, and acetate was demonstrated in the distillate by a method described by Feigl [20]. In other experiments the contents of the Warburg vessels were analysed by chromatographic methods. Apart from benzoic and salicylic acids, only acetic acid could be demonstrated. When the oxidation was carried out without inhibitor, only traces of acid were found (Fig. 6 C).

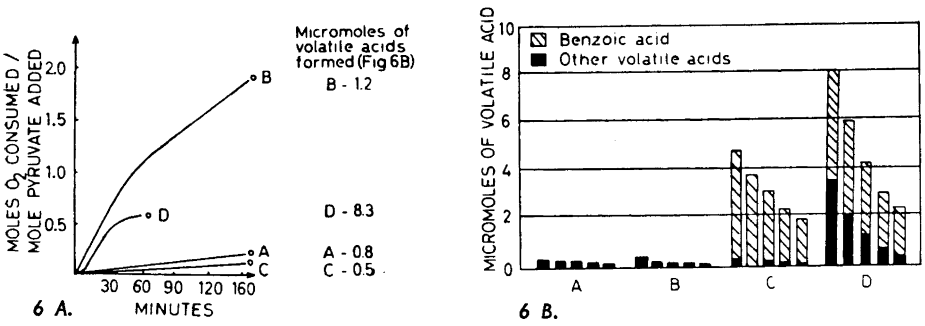

Fig. 6 A. Effect of benzoic acid on oxygen consumption and formation of volatile acids during pyruvate oxidation by *P. vulgaris*. The vessels contained besides 2.0 ml of cell suspension and 133 μmoles of potassium phosphate: A, no further additions; B, 10 μmoles of sodium pyruvate; C, 0.01 M sodium benzoate; D, 10 μmoles of sodium pyruvate and 0.01 M sodium benzoate. Final volume 2.5 ml. The oxidation was stopped, at points indicated, by addition of H_2SO_4 and the amounts of volatile acids determined as shown in Fig. 6B.

Fig. 6 B. Formation of volatile acids during pyruvate oxidation by *P. vulgaris*. Each group of columns represents the amount of volatile acids in the first five fractions of distillate from one Warburg vessel. Indications as in Fig. 6 A. The volume of the fractions was equal to that of the distilling solution.

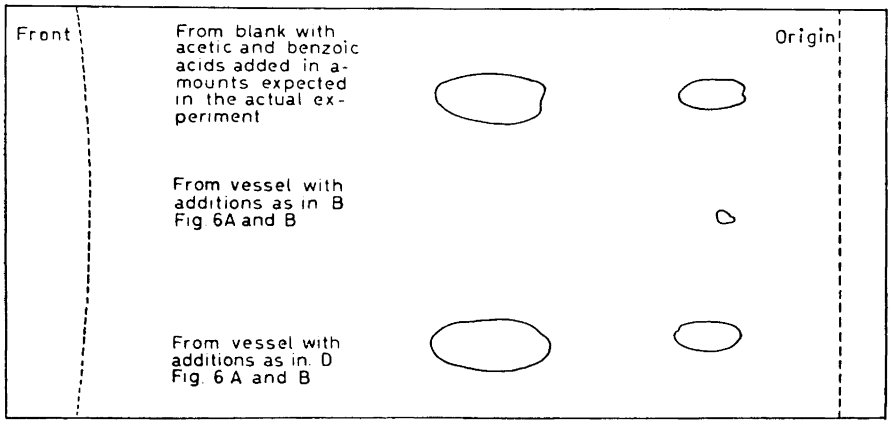

Fig. 6 C. Chromatographic separation of reaction products after an experiment similar to that in Fig. 6 A. The oxidation was stopped by addition of H_2SO_4, the contents in the vessels neutralized and concentrated by evaporation, acidified and extracted with *n*-butanol. Descending solvent: water-saturated *n*-butanol.

DISCUSSION

The experiments described show that the presence of benzoic or salicylic acid during glucose and pyruvate oxidation by *P. vulgaris* causes an accumulation of acetate in the medium. The utilization of this compound, as acetyl-

S-CoA, or in some other activated form, in synthesis of cell material and as an energy source is thereby prevented. The central position of such intermediates in the metabolism of living cells and the fact that benzoic and salicylic acids in the present experiments caused the accumulation of acetate at the same concentrations as those required to suppress growth of *P. vulgaris* suggest that the bacteriostatic effect, at least in part, may be due to an interference with the metabolism of the acetyl group.

Although the net result of the action of the two acids on the oxidation processes studied is relatively clear, the data presented give no information as to the way in which the inhibition is accomplished. Nevertheless, the results may be considered in view of what is known about the pyruvate metabolism in *P. vulgaris*. Apart from the enzyme system transforming pyruvate into metabolic active C_2-compounds, this organism seems to possess a pyruvate oxidase of quite another type. This enzyme has been thoroughly investigated by Stumpf [21] and by Moyed and O'Kane [22] and shown to give rise to acetate from pyruvate, without intermediate formation of any detectable acyl compound. The only cofactors required for the action of this enzyme are divalent ions and cocarboxylase. If the normal way of pyruvate metabolism in *P. vulgaris* is sensitive to benzoic and salicylic acids, and the acetate generating system is not, the presence of these acids during oxidation should lead to the withdrawal of great amounts of C_2-compounds as acetate from the normal metabolic route.

Irrespective of the mechanism of the acetate accumulation, the mere fact that it occurs indicates that the oxidation of acetate in the present experiments is much more sensitive to benzoic and salicylic acids than that of glucose and pyruvate. As the first step in acetate oxidation involves activation in an energy-requiring process, it is possible that the inhibition of this oxidation by benzoic and salicylic acids is related to the effect of these acids on another energy-requiring process, *e.g.* assimilation of ammonium ions. The work by Bernheim and DeTurk [10] already referred to and the experiments described in this paper show that benzoic and salicylic acids specifically inhibit the increased rate of substrate oxidation observed during simultaneous assimilation of ammonium ions. Bernheim and DeTurk suggest that their results are due to an inhibition of the formation, or utilization, of high energy compounds in the presence of salicylic or benzoic acid. In the present experiments such an interpretation would agree well with the stimulation of glucose oxidation, caused by the two acids, as an increase in oxidation rate is often observed in the presence of substances disturbing the energy conservation process. In recent years, inhibition of oxidative phosphorylation in isolated mitochondria by salicylic acid (Penniall, Kalnitsky and Routn [23]) and by salicylic and benzoic acids (Bosund [24]) has been reported. These findings, especially in the case of salicylic acid, bear out the hypothesis that some of the growth-inhibiting effect may be due to an interference with the formation of high energy compounds.

This work is part of an investigation supported by the *Swedish Natural Science Research Council* and the *Swedish Technical Research Council.*

I am greatly indebted to Professor G. Lindeberg, Agricultural College of Norway, Vollebekk, Norway, for introducing me into this field of investigation and for his

patient advice and encouragement throughout the work. I also wish to thank Professor E. Adler, Chalmers University of Technology, Gothenburg, Sweden, for continued interest and helpful criticism.

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Received January 8, 1959.