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The Bacteriostatic Action of Benzoic and Salicylic Acids

II. The Effect on Acetate Metabolism

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The effect of benzoic and salicylic acids on the acetate metabolism of resting cells of *Proteus vulgaris* was investigated by the Warburg technique.

The oxidation of acetate by washed cells previously grown in a medium containing glucose and amino acids was inhibited by both acids. Small amounts of succinate added simultaneously with the acetate in these respiration studies decreased the inhibition caused by benzoic acid considerably.

In similar experiments with cells previously grown in a medium with acetate as the sole carbon source, the effect of benzoic acid on the rate of acetate oxidation was much less, while that of salicylic acid was as before.

Both inhibitors had a tendency to increase the total amount of CO_2 evolved per mole of substrate added in experiments with washed cells previously grown in the acetate medium. Such an increase in CO_2 output from acetate induced by benzoic acid was further investigated in experiments with labelled acetate and shown to be derived mainly from the methyl group of the acetate molecule.

Neither of the acids had any appreciable effect on pyruvate dismutation with cell-free extracts of *Escherichia coli.*

In a previous investigation [1] the oxidation of glucose and pyruvate by resting cells of *Proteus vulgaris* was found to be completely inhibited at the oxidation level of acetate by 0.01 M benzoic acid or 0.003 M salicylic acid at pH 6.0. It was further shown that these concentrations of the two acids are necessary for complete suppression of growth of this organism at the pH mentioned. It was considered that the inhibition of acetate oxidation might have something to do with the bacteriostatic effect.

This paper is concerned with the influence of benzoic and salicylic acids on the oxidation of acetate by resting cells of *P. vulgaris* and with the effect of these acids on acetyl phosphate formation with extracts of *Escherichia coli.*

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EXPERIMENTAL

Organisms. The same strain of *Proteus vulgaris* was used as before. The extract in the experiments on acetyl phosphate formation was prepared from cells of *Escherichia coli.*

Growth conditions. Cell suspensions for respiration studies with *P. vulgaris* were prepared from cultures grown either in a fairly rich medium containing glucose and amino acids (medium A) or in a poor medium with acetate as the sole carbon source (medium B). In the case of medium B the inoculation was made with cells previously adapted to growth in this medium. Medium A: glucose, 10.0 g; casein hydrolysate (Difco vitamin-free casamino acids), 1.0 g; NH_4Cl, 1.0 g; nicotinamide, 0.2 mg; K_2HPO_4, 9.5 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; distilled water to 1 l; adjusted to pH 7.0. Medium B: $CH_3COONa \cdot 3H_2O$, 1.0 g; NH_4Cl, 1.0 g; nicotinamide 0.2 mg; $Na_2HPO_4 \cdot 2H_2O$, 6.0 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; distilled water to 1 l; adjusted to pH 7.0. The cultures were grown at 37°C in Kluyver flasks with sufficient aeration to ensure maximum growth rate or, in some experiments using medium A, in Erlenmeyer flasks without aeration or shaking. In some experiments the cells were harvested early in the logarithmic growth phase, and then the medium was inoculated with a heavy suspension of washed cells. When necessary, growth was followed by turbidity measurements in a Beckman spectrophotometer at 425 mμ.

Methods. Oxygen consumption and production of carbon dioxide was measured by the conventional Warburg technique as described in the first paper of this series. The data recorded in the figures and tables have been corrected for endogenous respiration. In the experiments with labelled acetate the oxidation was terminated by addition of 0.3 ml of 5 N H_2SO_4 when the rate of oxygen consumption had decreased to the value of the endogenous respiration. The cells were collected by centrifugation, washed three times with distilled water and dried at 110°C. The media and the washings were pooled, neutralized with 2 N NaOH and evaporated to dryness over night by freeze drying. The total amount of carbon in the cells and in the media was then converted into CO_2 by the wet decomposition method of van Slyke and Folch [2]. The CO_2 was collected as $BaCO_3$ in centrifuge tubes containing a saturated solution of $Ba(OH)_2$ in water. The precipitates were washed twice with distilled water, dried at 125°C and weighed. The respiratory CO_2, which was absorbed during the experiment in 10 % KOH in the center wells of the Warburg flasks was carefully transferred, together with the KOH, into a flask containing a known amount of K_2CO_3 dissolved in distilled water. The carbonate was collected as $BaCO_3$ by addition of 0.2 M $BaCl_2$, and the precipitate was washed, dried and weighed in the way described. The total amount of radioactivity in the three samples of $BaCO_3$, from cells, media and respiratory CO_2 was then determined by plating the carbonate on aluminium dishes and counting in a Geiger-Müller counter. In each experiment it was checked that the sum of the radioactivities recovered in the three samples of $BaCO_3$ corresponded to the amount added with the acetate.

Preparation of E. coli extract. The soluble enzyme system used in the experiments on acetyl phosphate formation was prepared from cells of *E. coli* grown in a medium containing lactose, meat extract, yeast extract, trypton and phosphate. The cells were harvested by use of a Sharples centrifuge, washed twice with 0.9 % KCl-solution and freeze dried. The extract was prepared from the dry powder in the way described by Korkes *et al.*[3]

Reagents. The reagents used were commercial preparations with the exception of acetyl phosphate, which was synthesized from *iso*propenyl acetate and phosphoric acid by the method of Stadtman and Lipmann [4]. DPN * and CoA were supplied by Pabst Laboratories; DPT and lactic dehydrogenase, by Sigma Chemical Company; and methyl and carboxyl labelled samples of sodium acetate, by The Radiochemical Centre, Amersham, England.

RESULTS

The effect of benzoic acid on acetate oxidation by washed cells previously grown in medium A. Preliminary respiration studies showed that the acetate oxidiz-

* Abbreviations: DPN = diphosphopyridine nucleotide, CoA = coenzyme A and DPT = diphosphothiamine.

Table 1. Influence of growth conditions, increasing physiological age of the cells at the time of harvesting and the presence of small amounts of succinate on the rate of acetate oxidation in respiration studies with washed cells of *P. vulgaris.*

Expt. No.	Growth at time of harvesting, mg dry weight/ml growth medium	Growth medium and growth phase at time of harvesting	Oxygen consumption during acetate oxidation, µl/mg dry weight and hour (Q_{O_2})		b/a
			a without succinate	b with addition of small amounts of succinate	
1	1.50	Medium A. Stationary phase. No aeration.	0	0	—
2	0.72	Medium A. Log phase. Aeration only during one generation of growth before harvesting.	0	0	—
3	0.14	Medium A. Log phase. Aerated.	30	34	1.1
4	0.23	Medium A. Log phase. Aerated.	22	31	1.4
5	0.56	Medium A. Log phase. Aerated.	17	29	1.7
6	1.30	Medium A. Very slow growth. Aerated.	8	26	3.2
7	2.00	Medium A. Stationary phase. Aerated.	1.5	15	10.0
8	0.17	Medium B. Log phase. Aerated.	125	125	1.0

Each vessel contained 5—10 mg dry weight of washed cells, 133 µmoles of potassium phosphate, 7 µmoles of sodium acetate and 0.2 µmoles of sodium succinate (when present). Temp. 37°C; pH 6.0. In these experiments the rate of oxygen consumption generally became constant about 20 min after addition of substrate. The Q_{O_2} values recorded are based on measurements made after this initial period. In all experiments the oxygen consumption due to succinate oxidation was completed about 15 min after addition of substrate as determined in separate flasks where only succinate was added. This oxygen uptake could thus have no influence on the Q_{O_2} values shown in the table. Moreover, the theoretical amount of oxygen for complete oxidation of 0.2 µmole of succinate is only 15.7 µl (generally about 10 µl was consumed in these experiments) whereas the total oxygen consumption due to acetate oxidation during the experimental period was more than 200 µl.

ing capacity of *P. vulgaris* cells, isolated from the growth medium by centrifugation and washed as described in the experimental part, varies with the growth conditions and with the physiological age of the cells at the time of harvesting. The influence of such factors in the pre-history of the cells on the rate of oxygen consumption after addition of acetate to washed cell suspensions is shown in Table 1 (see also Table 6). In these tables the increase in the rate of acetate oxidation, caused by the addition of small amounts of succinate together with the acetate is clearly demonstrated. It is probable that this stimulating

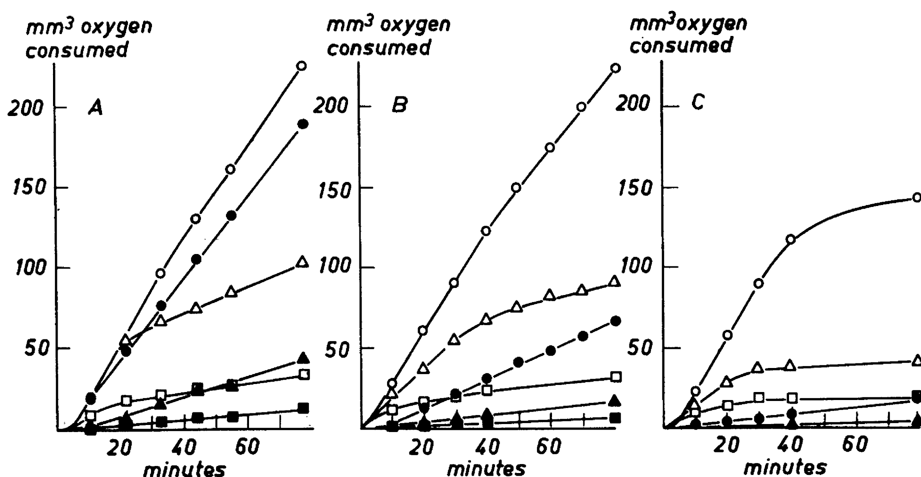

Fig. 1. Influence of benzoic acid on acetate oxidation by washed *P. vulgaris* cells previously grown with aeration in medium A. A — cells harvested in the logarithmic growth phase, B — cells harvested early in the stationary phase, and C — cells harvested after 8 h in the stationary phase. The vessels contained besides 7 mg dry wt. of cells, 133 μmoles of potassium phosphate and 10 μmoles of sodium acetate: ●———● no further additions; ▲———▲ 3.5 mM sodium benzoate; ■———■ 7.0 mM sodium benzoate; ○———○ 0.2 μmoles of sodium succinate; △———△ 0.2 μmoles of sodium succinate and 3.5 mM sodium benzoate; □———□ 0.2 μmoles of sodium succinate and 7 mM sodium benzoate. Temp. 37°C; pH 6.0. The values recorded have been corrected for the oxygen uptake attributable to the succinate oxidation (10—15 μl).

effect is due either to conversion of succinate to oxalacetate, which serves as a condensing partner for oxidation of acetate through the Krebs cycle or to the functioning of succinate as a readily utilizable source of energy for the ATP-generated activation of acetate.

It is evident from the table that only cells pre-grown with adequate aeration oxidize acetate to any appreciable extent and at a rate decreasing with increasing age of the cells when harvested (Expts. Nos. 3—7). Cells, harvested after growth without aeration, or with aeration for only one or two generations, did not oxidize acetate (Expts. Nos. 1—2). The great acetate oxidizing capacity of washed cells pre-cultivated in medium B is apparent from Expt. No. 8.

For the cultivation of cells in medium B, the Kluyver flasks were inoculated with cells previously adapted to the growth medium employed, but unaccustomed to excessive aeration. Therefore, in order to obviate any consequent delay in growth (Dagley, Dawes and Morrison [5] or Lwoff and Monod [6]), the medium was inoculated with a fairly large amount of cells.

In some experiments *P. vulgaris* was studied with regard to its ability to oxidize other fatty acids, such as propionic and butyric acids. No oxygen consumption attributable to such an oxidation was, however, noticed, not even in the presence of succinate.

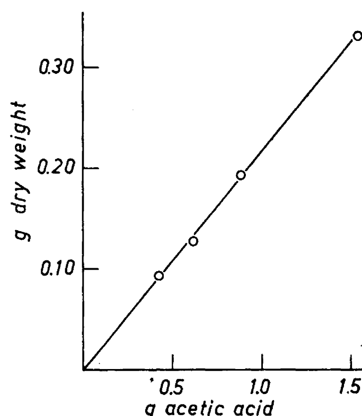

Fig. 2. Total growth of *P. vulgaris* in medium B as a function of the amount of acetic acid available.

As is shown in Fig. 1 A, B and C benzoic acid completely inhibited acetate oxidation by washed *P. vulgaris* cells, pre-grown in medium A, at concentrations previously found not to influence the initial rate of glucose and pyruvate oxidation by this organism. The degree of inhibition was approximately the same whether the cells were harvested in the logarithmic or in the stationary phase. Simultaneous oxidation of a comparatively small amount (0.2 μmole) of succinate markedly reduced the inhibitory effect of 3.5 mM benzoic acid for the first 20—30 min. In parallel experiments with omission of acetate the same amount of succinate was oxidized with consumption of 10—15 μl of oxygen during this period, after which the rate of the oxygen uptake decreased to that of the endogenous respiration (see the text to Table 1). The succinate oxidation was not inhibited by benzoic acid in the concentrations used.

In a similar study (unpublished) with salicylic acid, which in a concentration of about 1—2 mM completely inhibited any acetate oxidation, addition of succinate did not markedly reduce the inhibition produced. Salicylic acid in the same concentration range was previously found [1] to block glucose and pyruvate oxidation at the acetate level in experiments where the initial rate of glucose oxidation was actually stimulated.

Adaptation of P. vulgaris to growth on acetate as the sole carbon source. The comparatively strong inhibitory effect of benzoic acid on acetate oxidation as revealed by the experiments discussed above was further investigated, but now with cells grown on acetate as the sole carbon source (apart from CO_2). The same strain of *P. vulgaris*, but acetate-adapted, was used as before. The strain was adapted to acetate by successive inoculation in test tubes with medium B containing decreasing amounts of glucose or some other readily utilizable carbon source. pH values above 6.5 proved best for the adaptation process which resulted in a rather slow growth, even of fully adapted cells, owing to lack of aeration. The adapted cells behaved in the same way as the original cells in bacteriological standard tests and were, as before, dependent on addition of nicotinamide for growth. Cells for respiration studies were cultured in Kluyver flasks inoculated with adapted cells and adequately

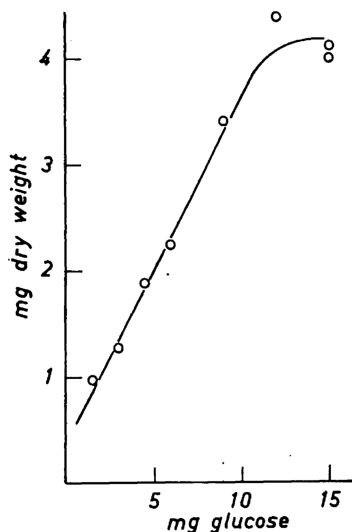

Fig. 3. Total growth of acetate adapted *P. vulgaris* in medium B containing butyrate instead of acetate and supplemented with varying amounts of glucose. When growth had ceased in the different flasks the content of volatile acids in the culture media was determined by steam-distillation and paper chromatography of the distillates. Butyric acid was quantitatively recovered from all the distillates. In flasks where relatively much glucose had been added, additional amounts of volatile acids (probably acetic and formic) were found.

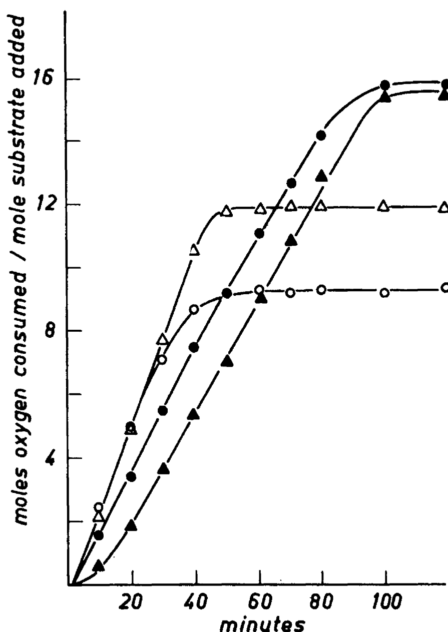

Fig. 4. Effect of benzoic acid on pyruvate and acetate oxidation by washed *P. vulgaris* cells previously grown in medium B. Each vessel contained 2.2 mg dry wt. of cells and 133 μmoles of potassium phosphate. ○——○ 7 μmoles of sodium pyruvate; ●——● 7 μmoles of sodium pyruvate and 8 mM sodium benzoate; △——△ 7 μmoles of sodium acetate; ▲——▲ 7 μmoles of sodium acetate and 8 mM sodium benzoate. Temp. 37°C; pH 6.0; final volume 2.5 ml.

aerated. As mentioned above, it was necessary to start with heavy inoculation to avoid unduly long lag phases. The cells were therefore allowed to multiply for 24—48 h before aeration was started.

During these conditions the total yield of bacterial growth obtained varied with the amount of acetate added as shown in Fig. 2. The mean generation time during aeration was 3—4 h. Attempts to adapt the strain to growth on butyrate as the sole carbon source proved unsuccessful. Fig. 3 shows an experiment demonstrating the inability of acetate adapted cells to utilize butyrate when grown in a butyrate medium containing various amounts of glucose to support growth.

The effect of benzoic acid and salicylic acid on acetate and pyruvate oxidation by washed cells pre-grown in the acetate medium. The highest rate of oxygen

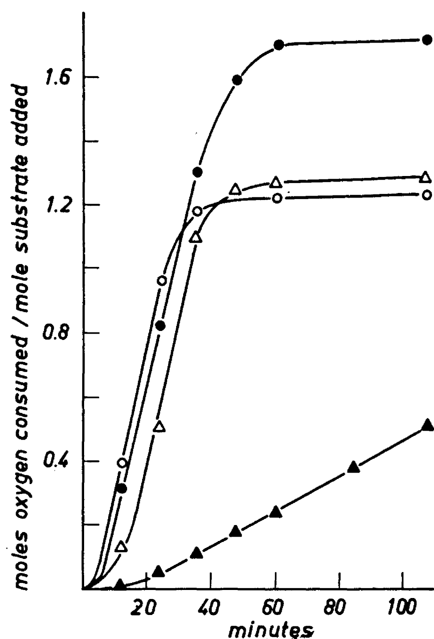

Fig. 5. Effect of salicylic acid on pyruvate and acetate oxidation by washed *P. vulgaris* cells previously grown in medium B. Each vessel contained 3.6 mg dry wt. of cells and 133 μmoles of potassium phosphate. ○——○ 7 μmoles of sodium pyruvate, ●——● 7 μmoles of sodium pyruvate and 2 mM sodium salicylate, △——△ 7 μmoles of sodium acetate, ▲——▲ 7 μmoles of sodium acetate and 1 mM sodium salicylate. Temp. 37°C; pH 6.0; final volume 2.5 ml.

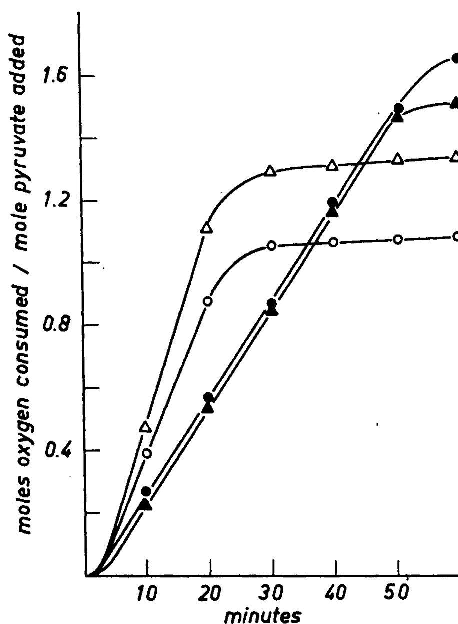

Fig. 6. Effect of benzoic acid on pyruvate oxidation in presence and absence of ammonium ions by washed *P. vulgaris* cells previously grown in medium B. Each vessel contained 3.2 mg dry wt. of cells, 133 μmoles of potassium phosphate and 7 μmoles of sodium pyruvate. ○——○ no further additions, ●——● 8 mM sodium benzoate, △——△ 20 μmoles of ammonium chloride, ▲——▲ 20 μmoles of ammonium chloride and 8 mM sodium benzoate. Temp. 37°C; pH 6.0; final volume 2.5 ml.

consumption during acetate oxidation with washed cells previously grown on glucose and amino acids was about 30 μl oxygen per mg dry weight and hour (Table 1). The corresponding values found in similar experiments in which the cells had been grown on acetate and harvested in the logarithmic phase generally ranged between 100 and 200. These latter experiments also showed (Figs. 4 and 5) that the rate of oxygen consumption was approximately the same whether acetate or pyruvate was used as substrate; the total amount of oxygen taken up per mole substrate was somewhat greater for acetate.

Figs. 4 and 5 illustrate the effect of benzoic and salicylic acids on acetate and pyruvate oxidation by washed cells previously grown on acetate. The most striking feature in these results compared with those obtained in a previous study is the absence of any specific inhibiting effect on the pyruvate

Table 2. Influence of benzoic and salicylic acids on the extent and rate of pyruvate and acetate oxidation by washed *P. vulgaris* cells previously grown in medium B. The experiments were similar to those in Figs. 4 and 5.

Substrate	Benzoate experiments			Salicylate experiments			
	Moles of oxygen consumed per mole substrate (4 experiments)		Benzoate conc. giving 50 % inhibition of oxidation rate, mM	Moles of oxygen consumed per mole substrate (2 experiments)			Salicylate conc. giving 50 % inhibition of oxidation rate, mM
	No inhibitor	8 mM benzoate		No inhibitor	0.4mM salicylate	4 mM salicylate	
Pyruvate	1.00	1.60	15	1.10	—	1.70	4
Acetate	1.25	1.60	10	1.25	1.35	—	0.4

oxidation at the oxidation level of acetate in the presence of benzoic or salicylic acid. On the contrary these acids considerably increase the *total amount* of oxygen consumed per mole of substrate. The magnitude of this increase is shown in Table 2 together with the approximate concentrations at which the two acids produced 50 % reduction in the *rate* of oxygen consumption. These figures show that the rate of acetate oxidation by washed cells grown in medium B is much more sensitive to salicylic acid than is that of pyruvate oxidation. The difference is also apparent from the experiment illustrated in Fig. 5 where 2 mM salicylic acid produced no demonstrable effect on the rate of pyruvate oxidation, whereas half this concentration caused 90 % inhibition of the rate of acetate oxidation. The influence of salicylic acid on the amount of oxygen consumed per mole of acetate could thus be studied only at rather low concentrations. At these concentrations no significant increase was observed.

The above effects of benzoic acid on the pyruvate and acetate oxidations were further studied in some experiments in which ammonium ions were added to the cell suspension simultaneously with the substrate. The presence of ammonium ions caused the oxygen consumption per mole pyruvate to decrease in the presence of benzoic acid and to increase in the absence of inhibitor (Fig. 6). A similar tendency, though less marked, was also noticed in some experiments with acetate.

The results suggested an enhanced release of CO_2 from pyruvate and acetate in the presence of benzoic acid and from pyruvate in the case of salicylic acid. This was demonstrated more directly by actual measurement of CO_2 production and furthermore in experiments with labelled acetate, which were carried out in Warburg vessels in the same way as those with unlabelled substrates. The total amount of radioactivity in cells, medium and respiratory CO_2 after oxidation of the same amounts of methyl and carboxyl labelled acetate was measured in the way described in the experimental part. Table 3

Table 3. Effect of benzoic acid on radioactivity of CO_2, medium and cells after oxidation of methyl and carboxyl labelled acetate by *P. vulgaris.*

Fraction	$^{14}CH_3COOH$ experiment Distribution of radioactivity in per cent of total		$CH_3{}^{14}COOH$ experiment Distribution of radioactivity in per cent of total	
	No inhibitor	With 6 mM benzoate	No inhibitor	With 6 mM benzoate
Respiratory CO_2	48	75	76	81
Medium	42	16	14	11
Cells	10	9	10	8

shows the result of a typical experiment with washed cells harvested from an actively growing culture in medium B. From the values of the distribution of radioactivity recorded in this table it can be calculated that the amount of CO_2 evolved per mole of acetate increased from 1.24 to 1.56 in the presence of benzoic acid. These values are in excellent agreement with those obtained by direct measurement of the CO_2 evolved. Such measurements, performed in separate experiments, showed that the molar amount of CO_2 evolved per mole of acetate increased from 1.25 in the absence to 1.60 in the presence of benzoic acid. It is noteworthy that the additional CO_2 production caused by benzoic acid originates almost entirely from the methyl group of the acetate molecule, and that the incorporation of both the methyl and carboxyl carbon in the cells is almost uneffected by benzoic acid.

Table 4 shows the distribution of methyl carbon in cells, media and respiratory CO_2 after acetate oxidation with and without addition of ammonium ions and the effect of benzoic acid on this distribution. In the absence of ammonium ions the quantitative values differ somewhat from those in Table 3, but the tendency is the same. Addition of ammonium ions resulted in increased incorporation of carbon in the cell, both in the presence and in the absence of benzoic acid. However, this increase was somewhat larger when the oxidation

Table 4. Effect of benzoic acid on radioactivity of CO_2, medium and cells after oxidation of methyl labelled acetate by *P. vulgaris* in presence and absence of ammonium ions.

Fraction	$^{14}CH_3COOH$ experiment Acetate oxidation in absence of ammonium ions Distribution of radioactivity in per cent of total		$^{14}CH_3COOH$ experiment Acetate oxidation in presence of ammonium ions Distribution of radioactivity in per cent of total	
	No inhibitor	With 6 mM benzoate	No inhibitor	With 6 mM benzoate
Respiratory CO_2	54	71	47	63
Medium	29	14	21	14
Cells	17	15	32	23

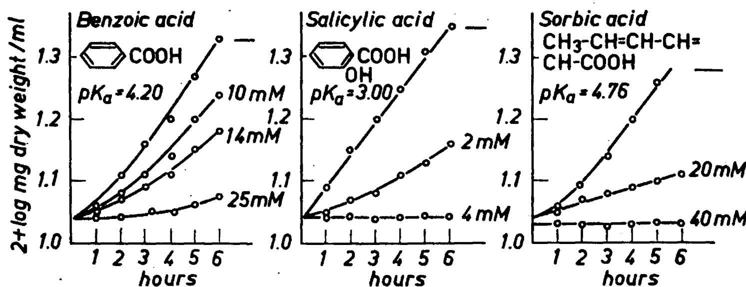

Fig. 7. Effect of benzoic, salicylic and sorbic acids on growth of *P. vulgaris* in medium B. Inhibitor concentrations as indicated. The experiments were performed at 30—35°C in aerated Kluyver flasks.

proceeded without inhibitor and the additional amount incorporated in this case was balanced by a decrease in the radioactivity of the medium.

Growth experiments with acetate adapted cells. * The results set forth above prompted investigations of the effect of benzoic and salicylic acids on the growth of *P. vulgaris* in medium B, in which all cell material must be synthesized from the acetyl group. Unfortunately these experiments had to be carried out at neutral pH values, at which the effect of benzoic and salicylic acids is rather low, since, as mentioned, the adapted strain did not grow rapidly in the acetate medium at pH values lower than 6.5. The experimental arrangement consisted of a battery of equally aerated Kluyver flasks containing 400 ml of the acetate medium at pH 7.0. The flasks were inoculated with adapted cells in the presence of varying amounts of inhibitor and the growth was followed turbidimetrically. Readings were taken continually for the first seven hours during which the pH rose from 7.0 to 7.3 in the absence of inhibitor. As might be expected from the experiments on acetate oxidation with adapted cells reported above, in acetate medium salicylic acid proved to be a much stronger inhibitor than benzoic acid. In this connection it was considered worthwhile to test the efficiency of some aliphatic fatty acid with growth inhibiting effect in the simple acetate medium under investigation. For this reason some experiments with sorbic acid were performed. As shown in Fig. 7, benzoic acid and sorbic acid seemed to be equally strong inhibitors, at least when compared without considering the difference in the dissociation constants, while salicylic acid proved much stronger.

In another series of experiments the effect of benzoic acid was studied on growth in medium A inoculated either with washed cells previously grown in this medium or with washed cells adapted to medium B. Since adaptation to acetate medium had strongly improved the ability of the cells to oxidize acetate in the presence of benzoic acid, it was tempting to imagine that the growth of such cells might be less sensitive to this inhibitor than the growth of unadapted cells, but no such difference could be demonstrated.

* This investigation was carried out in close collaboration with Miss Inger Erichsen, Swedish Institute for Food Preservation Research, who actually performed most of the experiments. For this valuable help I am greatly indebted.

The effect of benzoic and salicylic acids on pyruvate dismutation in extracts from E. coli. The preparation of extracts from *E. coli* and *Streptococcus faecalis* catalyzing the dismutation of 2 molecules of pyruvate to acetyl phosphate, carbon dioxide, and lactate in the presence of ortho-phosphate was described by Korkes *et al.* [3] The soluble enzyme system involved in this process seemed to be suitable for further investigation of the effect of benzoic and salicylic acids on pyruvate and acetate oxidation. The first preparations were made from cells of the *P. vulgaris* strain used in this investigation and resulted in extracts with much lower activity than those obtained by Korkes *et al.* from *E. coli.* However, since experiments (to be published) with resting cells of *E. coli*, similar to those with *P. vulgaris* previously reported, showed essentially the same effects of benzoic and salicylic acids on this organism, the present experiments on pyruvate dismutation could be performed equally well on extracts from *E. coli.* On the whole, there seems to be great similarities between the pyruvate metabolism in *E. coli* and in *P. vulgaris* (Razzell and Gunsalus [7]). The extracts were prepared in the way described above and assayed for activity in the system proposed by Korkes *et al.* The activity of our preparations ranged between 230 and 260 units per ml of dialyzed extract (1 unit = 1.0 mm^3 of CO_2 per hour at pH 6.0 and 25°C), compared with 270 units for that of Korkes *et al.* The influence of benzoic and salicylic acids on the evolution of CO_2 and the formation of acetyl phosphate during pyruvate dismutation was studied at pH 6.0. The enzyme activity at this pH was only about 60 % of that obtained at pH 7.4, but the lower pH was nevertheless preferred because of the known increase of metabolic activity of weak acids with decreasing pH.

Table 5 shows the influence of rather large amounts of benzoic and salicylic acids on pyruvate dismutation in cell free extracts. It is evident that the effect is weak, especially since the bacterial cell membrane usually believed

Table 5. Effect of benzoic and salicylic acids on pyruvate dismutation in cell free extracts of *E. coli.*

Additions of inhibitor	μmoles of CO_2 formed a	μmoles of acetyl phosphate formed b	b/a
—	4.54	1.58	0.35
3.5 mM benzoate	3.89	1.42	0.36
7.0 mM benzoate	3.33	1.33	0.40
14.0 mM benzoate	3.22	1.15	0.36
3.5 mM salicylate	3.34	1.49	0.45
7.0 mM salicylate	2.28	1.49	0.65
14.0 mM salicylate	2.31	1.85	0.80

Each vessel contained: 500 μl dialyzed extract, 50 μmoles of potassium phosphate, 2.4 μmoles of $MgCl_2$, 1.6 μmoles of $MnCl_2$, 0.1 μmoles of DPT, 50 μg of CoA, 0.15 μmoles of DPN and 2 μl of lactic dehydrogenase in the main compartment and 50 μmoles of sodium pyruvate together with the inhibitor in the side arm. Final volume 1.22 ml. Temp. 30°C; pH 6.0; gas phase, nitrogen. CO_2 evolution was measured 20 and 40 min after addition of pyruvate and inhibitor to the enzyme extract.

to prevent penetration of the inhibitors studied had been removed. The evolution of CO_2 from pyruvate in nitrogen atmosphere with intact cells at pH 6.0 is much more sensitive to benzoic and salicylic acids as revealed by experiments (not shown here) with washed cells identical with those from which the extracts were prepared. It is further clear from Table 5 that benzoic acid had no influence on the amount of acetyl phosphate formed per mole of CO_2 produced. On the other hand, salicylic acid seemed to increase this value. This effect was not further investigated because of the relatively high concentrations of inhibitor required.

Experiments with acetyl phosphate and intact cells. The sensitivity of acetate oxidation (at least by intact cells) to benzoic and salicylic acids and the protective effect of small amounts of succinate suggested that the inhibition might be overcome by simultaneous energy producing reactions. With this in mind, acetyl phosphate, in itself an energy-rich compound, should be a better starting point for acetate oxidation than the acetate molecule. However, when acetyl phosphate was added to intact cells of *P. vulgaris* or baker's yeast, the rate of oxygen consumption was very slow. Parallel experiments showed that the oxygen consumed could be ascribed to oxidation of acetate after chemical decomposition of the acetyl phosphate added.

DISCUSSION

Addition of acetate to a suspension of washed *Proteus vulgaris* cells in phosphate buffer is followed by increased oxygen consumption due to oxidation of the acetate. The rate of this oxygen consumption is determined by the pre-history of the cells. In the present work the influence of three factors in this pre-history on the acetate oxidizing ability has, to some extent, been investigated: 1. the composition of the growth medium employed in cultivating the cells, 2. aeration during growth, and 3. the physiological age of the cells at the time of harvesting. It is important to note that the term "acetate oxidizing ability" used above, refers to the rate of the oxygen consumption following the addition of acetate to cells isolated from the growth medium by centrifugation and washing. A survey of the experiments discussed is shown in Table 6.

This investigation shows that the rate of acetate oxidation by washed *P. vulgaris* cells varies considerably with the conditions prevailing during cultivation of the cells. The ability to oxidize acetate, at least by cells grown in a glucose-casein hydrolysate medium (medium A), thus seems to be dependent upon aeration during growth. When such a medium is inoculated with a small amount of cells and aerated, a lag phase occurs before growth begins. It is possible that the ability to oxidize acetate at any appreciable rate is acquired during this period. If large inocula are used, the lag phase is almost completely abolished and no acetate oxidizing power seems to be acquired, at least not by the first generation (Table 6:II).

The rate of acetate oxidation by washed cells previously grown in medium A decrease steadily with increasing age of the cells at the time of harvesting and becomes insignificant after some hours in the stationary phase (Table

Table 6. Summary of the effects of benzoic and salicylic acids on acetate and pyruvate oxidation by washed *P. vulgaris* cells.

		I	II	III	IV
Pre-history of the cells used in the respiration studies	Cultivation	Growth in medium A. No aeration. ↓	Growth in medium A. No aeration ↓ Transfer of the washed cells to fresh medium A and aeration during the time required for the cell material to double ↓	Growth in medium A. Aeration started immediately after inoculation. ↓	Growth in medium B without aeration for 24–48 h ↓ Growth in medium B with aeration. ↓
	Harvesting and washing.	Harvesting in the stationary phase. Washing once in phosphate buffer.	Harvesting in the logarithmic growth phase. Washing once in phosphate buffer.	Harvesting at different ages of the cultures. Washing once in phosphate buffer. Increasing age of the culture ⟶	Harvesting in the logarithmic growth phase. Washing once in phosphate buffer.
Results of the respiration studies.	Q_{O_2} after addition of acetate (Table 1).	0	0	30 22 17 8 1.5	125
	Q_{O_2} after addition of acetate + small amounts of succinate (Table 1)	0	0	34 31 29 26 15	125
	Influence of benzoic acid on the oxidation of acetate.	—	—	Strong inhibition, partly abolished by small amounts of succinate.	The rate reduced to 50 % by 10 mM benzoic acid. Increased total substrate breakdown.
	Influence of benzoic acid on the oxidation of pyruvate.	—	—	Specific inhibition at the oxidation level of acetate (Bosund [1]).	The rate reduced to 50 % by 15 mM benzoic acid. Increased total substrate breakdown.
	Influence of salicylic acid on the oxidation of acetate	—	—	Strong inhibition, largely unaffected by succinate addition.	The rate reduced to 50 % by 0.4 mM salicylic acid. No increase in total substrate breakdown at this concentration.
	Influence of salicylic acid on the oxidation of pyruvate.	—	—	Specific inhibition at the oxidation level of acetate (Bosund [1]).	The rate reduced to 50 % by 4 mM salicylic acid. Increased total substrate breakdown.

6:III and Table 1). If the cultures are not too old when harvested, most of the activity can, however, be restored by adding a small amount of succinate together with the acetate in the respiration experiments. This seems to indicate that the decreasing oxidation rate is due either to lack of Krebs cycle intermediates or to a low level of energy-rich compounds in the cells. The latter explanation would be consistent with the marked increase in the rate of acetate oxidation observed after addition of succinate in the presence of benzoic acid if, as suggested in the first part of this series, this inhibitor interferes with the ATP-generated acetate activation. The absence of an equally marked effect in the case of salicylic acid may be due to the specific uncoupling of oxidative phosphorylation produced by this acid [8].

So far, only experiments with cells cultivated in medium A have been discussed. In such a fairly rich medium much of the energy requirements for growth can be met by ATP-generation at the substrate level. In medium B where acetate is the sole carbon source oxidative phosphorylation is of prime importance especially since the acetate molecule must be activated in an ATP-requiring process before it can be metabolized. It is therefore highly probable that the enzyme systems involved both in the acetate activation and in the generation of ATP from the acetate oxidation exist in larger amounts in cells grown in medium B than in cells grown in medium A. It may further be assumed that cells grown in medium B contain only small amounts of the acetate generating pyruvate oxidase (Stumpf [9], Moyed and O'Kane [10]), which significance in relation to the results previously obtained with cells grown in medium A was discussed in the first paper of this series.

It is consistent with this view that the characteristic feature of the action of benzoic and salicylic acids on cells grown in the glucose-casein hydrolysate medium, *i.e.* the blocking of pyruvate oxidation at the oxidation level of acetate, is not observed in experiments with cells grown in the acetate medium. Furthermore, the sensitivity of acetate oxidation to benzoic acid when the acetate is added directly as substrate is similarly less in experiments with cells grown in the acetate medium (the concentration of benzoic acid required for complete inhibition is increased 4—5 fold). It is noteworthy that this is true only with benzoic acid; salicylic acid in a concentration of 1—2 mM completely inhibits acetate oxidation by both kinds of cells although, as mentioned above, the blocking of pyruvate oxidation at the oxidation level of acetate is abolished, even in this case, by growing the cells in the acetate medium.

The most characteristic effect of benzoic and salicylic acids on acetate-adapted cells is the increased substrate breakdown induced in the presence of these acids. A stimulation of oxygen consumption and CO_2 production is generally observed in the presence of substances impairing the formation of energy-rich compounds and such an effect by salicylic acid agrees with the action of this substance on oxidative phosphorylation [8]. The similarity between the effects of benzoic and salicylic acids in the present experiments is noteworthy, though the inhibition of oxidative phosphorylation by benzoic acid is weak, at least in experiments with rat liver mitochondria [11].

Salicylate has for some years been known [12, 13] to produce effects on carbohydrate metabolism in the rat, which agrees well with the present find-

ings. In recent experiments with salicylate [14] an increased output of total CO_2 and $^{14}CO_2$ was observed in rats given acetate-2-^{14}C and a corresponding inhibition of isotope incorporation into liver glycogen and liver fatty acids.

The increase in the output of CO_2 from acetate observed in the presence of benzoic acid was further investigated in respiration studies with labelled acetate (Tables 3 and 4). These studies confirm the results obtained in the experiments in which the CO_2 evolution was actually measured and indicate that the increase in CO_2 production induced by benzoic acid is derived mainly from the methyl group of the acetate molecule. When acetate is oxidized in absence of inhibitor, a corresponding amount of methyl carbon accumulates in the medium. The incorporation of ^{14}C-compounds into the cells is largely uninfluenced by benzoic acid. The results could tentatively be explained by assuming that a keto acid, derived mainly from the methyl group of the original acetate molecule accumulates in the medium in the absence of benzoic acid. If the acetate oxidation occurs in the presence of ammonium ions, as in the experiment showed in Table 4, such a keto acid may be incorporated in the cell material after conversion to the corresponding amino acid. Further research is, however, necessary before any valid interpretation can be made on this point.

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