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The Bacteriostatic Action of Benzoic and Salicylic Acids

IV. The Effect on Oxidation of TCA Cycle Intermediates, Lactate and Gluconate

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Resting cells of *Proteus vulgaris* oxidize succinate, fumarate, malate and oxalacetate at similar rates, whereas α-ketoglutarate is attacked more slowly. Exogenous citrate is not metabolized at pH 6.0 by resting cells.

The rate of oxidation of the dicarboxylic acids involved in the TCA cycle is inhibited to a varying extent by benzoic and salicylic acids according to the conditions prevailing during growth of the cells. The total amount of oxygen consumed per mole of substrate is mostly somewhat increased by the inhibitors studied.

The oxidation of lactate (in contrast to the oxidation of pyruvate) is not blocked at the acetate level by benzoic and salicylic acids.

Streptomycin, at concentrations arresting growth, does not affect the oxidation of pyruvate by resting cells of *P. vulgaris*.

Most of the experiments reported in this series have been concerned with the influence of benzoic and salicylic acids on the oxidation of glucose, pyruvate and acetate. Interest has been focused primarily on the effect of these inhibitors on the metabolism of the acetyl group, especially its entrance into the TCA cycle.

Conclusive proof of the occurrence of the TCA cycle in *Proteus vulgaris* has as yet not been produced, in contrast to what has been shown for the other organisms used in this work (*Escherichia coli*, *Pseudomonas fluorescens* and baker's yeast). From a general point of view, however, this metabolic cycle most likely occurs also in *P. vulgaris*. This is indicated by the fact that this organism can be adapted to growth on acetate as the sole carbon source and can oxidize acetate at least almost completely to CO_2 and H_2O.

The present investigation is concerned with the ability of resting cells of *P. vulgaris* to oxidize members of the TCA cycle and the effect of benzoic and salicylic acids on these oxidations. In addition, the effects of benzoic acid on pyruvate and lactate oxidation were compared in respiration studies with labelled substrates. Some experiments with streptomycin were included in these studies owing to certain similarities between the influence of this inhibitor and that of benzoic acid on resting cell metabolism.

EXPERIMENTAL

Organism. The same strain of *Proteus vulgaris* was used as before. The ability of this strain to grow in simple media, especially with acetate as the sole source of carbon, seems to have diminished somewhat in the course of this investigation. No change affecting the results obtained with the inhibitors studied has, however, occurred.

Medium and growth conditions. The cells were cultured at 35 – 37°C with aeration either with glucose as carbon source or with pyruvate and succinate. In the former case the cells were harvested in the stationary phase, in the latter case early in the logarithmic phase. The growth conditions have been described in detail previously [1].

Methods. The procedure adapted in the preparation of cell suspensions and in respiration measurements was essentially the same as previously outlined. In the studies with labelled substrates the respiratory CO_2, absorbed in KOH during the experiment, was precipitated as $BaCO_3$ after dilution with a known amount of K_2CO_3. The precipitate was washed and dried and the radioactivity determined in a Geiger-Müller counter after plating the carbonate on aluminium dishes. The total amount of radioactivity supplied to the cells was determined in the same way after conversion of an aliquot of the substrate into CO_2 by the wet decomposition method of van Slyke and Folch [2].

Reagents. All reagents used were commercial preparations. The pyruvic acid was redistilled before use. Samples of sodium pyruvate-2-^{14}C and sodium DL-lactate-2-^{14}C were supplied by The Radiochemical Centre, Amersham, England. Streptomycin was obtained as the sulfate from AB Kabi, Stockholm, Sweden.

RESULTS

The effect of benzoic and salicylic acids on the oxidation of TCA cycle intermediates. The capacity of washed *Proteus vulgaris* cells to oxidize citrate and the dicarboxylic acids of the TCA cycle is shown in Table 1. Somewhat lower Q_{O_2}-values were generally observed in experiments with cells pre-grown without aeration. In no case was citrate metabolized at the pH in question (6.0). The dicarboxylic acids were oxidized at nearly identical rates with the exception of α-ketoglutaric acid.

Table 1. Rate of oxygen consumption during oxidation of TCA cycle intermediates by washed cells of *P. vulgaris*.

Substrate	A *	B *
	μl oxygen consumed per mg dry wt. and hour	μl oxygen consumed per mg dry wt. and hour
Citrate	0	0
α-Ketoglutarate	20	20
Succinate	60	100
Fumarate	60	100
Malate	50	100
Oxalacetate	50	80

* A. Cells pre-grown in glucose-casein hydrolysate medium, aerated during growth and harvested in the stationary growth phase.
B. Cells pre-grown in pyruvate-succinate-casein hydrolysate medium, aerated during growth and harvested in the logarithmic growth phase.

Benzoic acid at concentrations lower than 8 mM did not appreciably suppress the oxidation of succinate, fumarate, malate or oxalacetate in experiments with resting cells grown as in B, Table 1. The degree of inhibition increased rapidly with increasing concentration of inhibitor and was total at 12—15 mM of benzoic acid. When the cells were cultured as in A, benzoic acid was somewhat more effective as an inhibitor. With both types of cells α-ketoglutarate oxidation was the most sensitive.

The dicarboxylic acids of the TCA cycle can easily be converted to pyruvate in most cells *via* oxidation to oxalacetate followed by decarboxylation of this substance. Such a transformation is, in fact, necessary if these acids are to be oxidized by way of the TCA cycle. Since the oxidation of pyruvate is blocked at the acetate level by benzoic and salicylic acids, these inhibitors might be expected to affect the oxidation of the TCA cycle intermediates in a corresponding way. This would imply that benzoic and salicylic acids would block the oxidation of succinate, fumarate, malate and oxalacetate at the acetate level, *e.g.* after consumption of 1.5, 1.0, 1.0 and 0.5 moles of oxygen per mole of substrate, respectively (Fig. 4). It was thus of considerable interest to investigate the effect of these inhibitors on the total amount of oxygen consumed per mole of acid. Fig. 1 shows an experiment with succinate, fumarate, malate and pyruvate. It is evident that benzoic acid did not affect the oxidation of the dicarboxylic acids in the predicted way. On the contrary, the amount of oxygen consumed was increased in the presence of benzoic acid. Similar results were obtained in experiments with cells pre-grown in glucose-casein hydrolysate medium and in studies with salicylic acid.

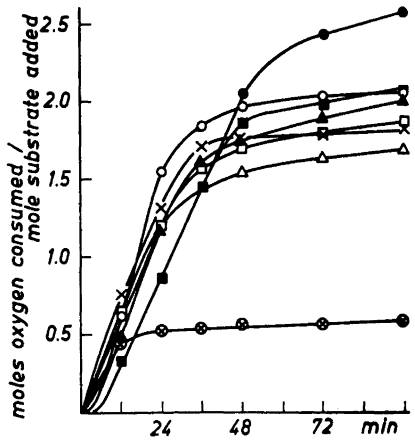

Fig. 1. Effect of benzoic acid on oxidation of succinate, fumarate, malate and pyruvate by washed *P. vulgaris* cells grown as in B, Table 1. Each vessel contained 4.5 mg dry wt. of cells and 133 μmoles of potassium phosphate. ×——× 5 μmoles of sodium pyruvate, ⊗——⊗ 5 μmoles of sodium pyruvate and 8 mM benzoic acid, O——O 5 μmoles of sodium succinate, ●——● 5 μmoles of sodium succinate and 8 mM benzoic acid, □——□ 5 μmoles of sodium fumarate, ■——■ 5 μmoles of sodium fumarate and 8 mM benzoic acid, △——△ 5 μmoles of sodium malate, ▲——▲ 5 μmoles of sodium malate and 8 mM benzoic acid. Temp. 37°C; pH 6.0; final volume 2.4 ml.

Table 2. Effect of benzoic acid on the total amount of oxygen consumed during oxidation of pyruvate and fumarate. The oxidation was carried out with washed cells of *P. vulgaris* pre-grown in glucose-casein hydrolysate medium, aerated during growth and harvested in the stationary phase. Each vessel contained 1/15 M phosphate buffer at pH 6.0 and substrate and inhibitor as shown. Gas phase air; temp. 30°C.

Substrate	Total amount of oxygen consumed, µl	
	without inhibitor	with 10 mM benzoic acid
5 µmoles of pyruvate	210	70
5 µmoles of fumarate	275	285
5 µmoles of pyruvate 5 µmoles of fumarate	455	365

These results seem to indicate that pyruvate formed within the cell during oxidation of TCA cycle intermediates is not freely interchangeable with pyruvate entering the cell from the external medium. Table 2 shows another experiment illustrating the same point. The figures recorded in this table show that the effect of benzoic acid on the total amount of oxygen consumed during oxidation of exogenous pyruvate persists even when an equal amount of fumarate is oxidized simultaneously. The amount of oxygen consumed per mole of fumarate was not influenced by benzoic acid in this experiment.

The influence of benzoic acid and streptomycin on the oxidation of pyruvate and lactate. In another series of experiments the effect of benzoic acid on pyruvate oxidation was compared with its effect on lactate oxidation. It is difficult to imagine any other way of oxidizing lactate in a living cell than *via* pyruvate; consequently, one would expect the lactate oxidation to be strongly inhibited by benzoic acid after consumption of 1.0 mole of oxygen per mole of lactate. But as shown in Fig. 2, no such blocking of the lactate oxidation at the acetate level seemed to occur. This result is supported by the experiments with pyruvate-2-^{14}C and lactate-2-^{14}C summarized in Tables 3 and 4. According to Table 3, benzoic acid almost completely inhibits the occurrence of the keto carbon atom of pyruvate in the respiratory CO_2. Table 4 indicates that the extent of oxidation of the corresponding atom in the lactate molecule to CO_2 is only slightly affected. It may be noted that the rate of lactate oxidation in the latter experiment was strongly inhibited by benzoic acid and that in the presence of 8 mM of the inhibitor the oxidation was stopped before the rate of oxygen consumption had decreased to the value of the endogenous respiration.

Some years ago Umbreit *et al.*[3,4] studied the influence of streptomycin on the oxidative metabolism of resting cells of *Escherichia coli* and observed effects somewhat resembling those obtained with benzoic acid in this work. They reported that under certain conditions streptomycin prevented pyruvate from entering the terminal respiration system and suggested that the inhibited reaction might be a "direct condensation" of oxalacetate and

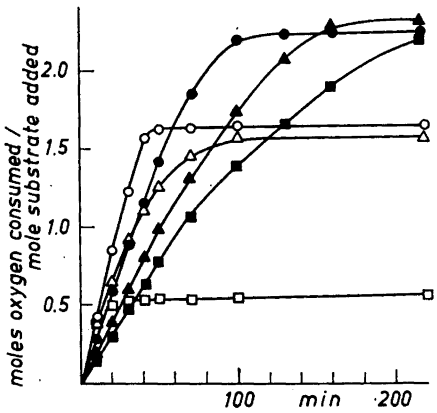

Fig. 2. Effect of benzoic acid on pyruvate and lactate oxidation by washed *P. vulgaris* cells grown as in B, Table 1. Each vessel contained 5.2 mg dry wt. of cells and 133 μmoles of potassium phosphate. O———O 7 μmoles of sodium pyruvate, △———△ 7 μmoles of sodium pyruvate and 4 mM benzoic acid, □———□ 7 μmoles of sodium pyruvate and 8 mM benzoic acid, ●———● 7 μmoles of sodium lactate, ▲———▲ 7 μmoles of sodium lactate and 4 mM benzoic acid, ■———■ 7 μmoles of sodium lactate and 8 mM benzoic acid. Temp. 37°C; pH 6.0; final volume 2.4 ml.

pyruvate to a 7-carbon intermediate. They did not exclude the possibility of formation of a transitory 2-carbon intermediate from pyruvate before the condensation with oxalacetate, but showed later [5] that formation of citrate was not influenced by streptomycin. Since the reaction

$$\text{pyruvate} + 0.5\ O_2 \rightarrow \text{free acetate}$$

Table 3. Effect of streptomycin and benzoic acid on the amount of $^{14}CO_2$ produced during oxidation of $CH_3{}^{14}COCOOH$. The oxidation was carried out with washed cells of *P. vulgaris* pre-grown in pyruvate-succinate-casein hydrolysate medium, aerated during growth and harvested in the logarithmic growth phase. Each vessel contained 1/15 M phosphate buffer, 7 μmoles of sodium pyruvate-2-^{14}C (with a total activity of 11 600 cpm.), 5.5 mg dry wt. of cells, and inhibitor as indicated. Gas phase air; pH 6.0; temp. 30°C.

Inhibitor	Mole O_2 / mole pyruvate	Mole CO_2 / mole pyruvate	*R.Q.*	Fraction of the amount of radioactivity supplied recovered in respiratory CO_2	Fraction of the amount of substrate carbon recovered in respiratory CO_2
—	1.37	1.70	1.24	0.41	0.57
Streptomycin 8 mg/ml	1.36	1.84	1.35	0.40	0.61
Benzoate, 8 mM	0.56	1.02	1.82	0.08	0.34

Table 4. Effect of benzoic acid on the amount of $^{14}CO_2$ produced during oxidation of $CH_3{}^{14}CHOHCOOH$. The oxidation was carried out with washed cells of *P. vulgaris* pre-grown in pyruvate-succinate-casein hydrolysate medium, aerated during growth and harvested in the logarithmic growth phase. Each vessel contained 1/15 M phosphate buffer, 7 μmoles of sodium DL-lactate-2-^{14}C (with a total activity of 12 100 cpm.), 5.5 mg dry wt. of cells, and inhibitor as indicated. Gas phase air; pH 6.0; temp. 30°C.

Inhibitor	$\frac{\text{Mole } O_2}{\text{mole lactate}}$	$\frac{\text{Mole } CO_2}{\text{mole lactate}}$	*R.Q.*	Fraction of the amount of radio-activity supplied recovered in res-piratory CO_2	Fraction of the amount of substrate carbon recovered in respiratory CO_2
—	2.05	2.04	0.99	0.66	0.68
Benzoate, 5 mM	1.83	1.80	0.99	0.56	0.60
Benzoate, 8 mM	1.45	1.40	0.97	0.39	0.47

as well as the subsequent oxidation of acetate seemed to be completely insensitive to streptomycin, the effect of this inhibitor was demonstrable only on cells with a very low capacity to oxidize acetate. In the work of Umbreit *et al.* such cells were obtained by growing the culture in a rich medium without aeration and keeping the washed cells at refrigerator temperatures for some days before the experiments.

According to this study, the effect of streptomycin on the pyruvate oxidation by *P. vulgaris* cells pre-grown with heavy aeration, as in this investigation, should be very small, if any, since the first rapid step in the pyruvate oxidation by such cells is apparently the formation of free acetate from pyruvate. Nevertheless it was of interest to compare the effects of benzoic acid and streptomycin on cells aerated during growth. The data from such an experiment are given in Table 3. It is evident that streptomycin had no influence on the oxidation of pyruvate even in a final concentration of 8 000 μg/ml. The effect was not enhanced by incubation of the cells with streptomycin for one hour before addition of pyruvate. Oxidation of lactate was equally insensitive to streptomycin as was pyruvate oxidation by resting cells grown with acetate as the sole carbon source (resting cells pre-grown in this way apparently [6] have very little tendency to form free acetate during pyruvate oxidation). It may be argued that the experimental pH (6.0) was too low to produce a pronounced streptomycin effect, but growth experiments showed that the strain of *P. vulgaris* used was sensitive to streptomycin at a concentration of 25 μg/ml at this pH.

The effect of benzoic and salicylic acids on the oxidation of gluconate. In recent years it has become increasingly evident that many microorganisms in addition to dissimilating glucose *via* the classical scheme of Embden-Meyerhof may utilize another enzymatic mechanism. This pathway, usually called the hexosemonophosphate pathway, starts with glucose-6-phosphate, which *via* 6-phospho-gluconate is transformed to pentose phosphate in two oxidative

steps (the second accompanied by decarboxylation). From this intermediate glucose-6-phosphate can be regenerated through a series of anaerobic reactions. Consequently glucose can be oxidized completely to CO_2 and H_2O by this pathway without the participation of the TCA cycle.

Since benzoic and salicylic acids block glucose oxidation at the acetate level by inhibiting the function of the TCA cycle, it was considered of interest to investigate the effect of these inhibitors on the oxidation of some intermediate in the hexosemonophosphate pathway. As phosphorylated substances do not readily penetrate the cell membrane of intact cells, these experiments were performed with gluconate. Preliminary studies showed that washed cells of *P. vulgaris* pre-grown in glucose-casein hydrolysate medium and harvested in the logarithmic growth phase oxidized glucose and gluconate at approximately the same rate but in the case of gluconate only after an initial lag phase. The total amount of oxygen consumed per mole of gluconate was generally about 3.5 moles. As is shown in Fig. 3, salicylate inhibited the rate of gluconate oxidation rather strongly, whereas the rate of glucose oxidation was as usual considerably increased at these salicylate concentrations. No clear effect was observed on the oxygen consumption per mole of gluconate. Corresponding results were obtained with benzoic acid.

DISCUSSION

As mentioned above, there is no reason to question the occurrence of the TCA cycle in cells of *Proteus vulgaris* even if, as shown here, citrate (and presumably also the other tricarboxylic acids of the cycle) is oxidized at a

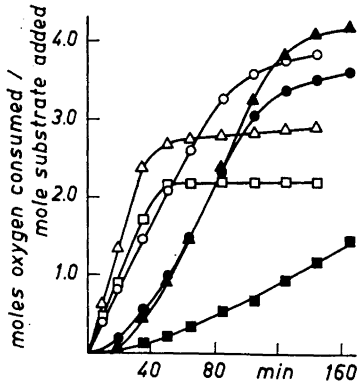

Fig. 3. Effect of salicylic acid on oxidation of glucose and gluconate by washed *P. vulgaris* cells grown in glucose-casein hydrolysate medium, aerated during growth and harvested in the logarithmic growth phase. Each vessel contained 1.6 mg dry wt. of cells and 133 μmoles of potassium phosphate. ○———○ 2.5 μmoles of glucose, △———△ 2.5 μmoles of glucose and 0.75 mM salicylic acid, □———□ 2.5 μmoles of glucose and 3.00 mM salicylic acid, ●———● 2.5 μmoles of sodium gluconate ▲———▲ 2,5 μmoles of sodium gluconate and 0.75 mM salicylic acid, ■———■ 2.5 μmoles of sodium gluconate and 3.00 mM salicylic acid. Temp. 37°C; pH 6.0; final volume 2.4 ml.

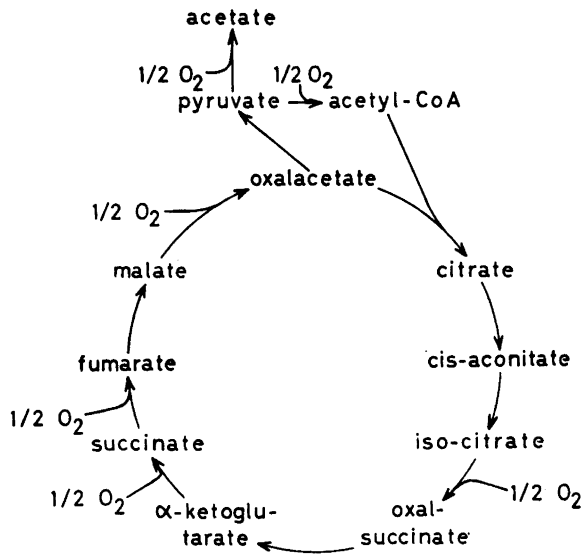

Fig. 4. Oxidative steps in the TCA cycle.

very low rate or not at all when added to suspensions of intact cells. This failure to attack intermediates of the cycle is certainly, as in analogous cases, due to impermeability of the cell membrane.

It was also pointed out that in order to oxidize TCA cycle intermediates beyond the oxalacetate stage, the cell must decarboxylate the oxalacetate to pyruvate (Fig. 4). The further metabolism of this pyruvate is, however, obviously not blocked at the acetate level by benzoic or salicylic acid, since the amount of oxygen consumed during oxidation of TCA cycle intermediates is increased by these inhibitors (Fig. 1). The reason may be that pyruvate formed from oxalacetate does not come into contact with the acetate producing pyruvate oxidase occurring in *P. vulgaris*. Anyhow, the results given in Fig. 1, Fig. 3 and Table 2 suggest that pyruvate originating from TCA cycle intermediates is not freely interchangeable with exogenous pyruvate or pyruvate formed from glucose. Such an interpretation is supported by the general conception that the TCA cycle enzymes, functioning more or less as a unit, are held together by some rigid structure, while the soluble glycolytic enzymes are spread throughout the cell protoplasm. It is a matter of controversy what the rigid structure might be like in a bacterial cell, which can obviously not contain anything as complex as the mitochondria of an animal cell. Evidence seems, however, to be accumulating [7,8] that the function of the TCA cycle in bacteria is closely associated with the cell membrane.

The difference in the action of benzoic acid on pyruvate and lactate oxidation (Fig. 2) is more difficult to interpret. It is possible, however, that better knowledge of the localisation in the cell of the enzymes involved might result

in the same line of reasoning as that set forth above. It may be significant that Mitchell and Moyle [8] reported that 80—95 % of the lactic dehydrogenase in *Staphylococcus aureus* is associated with the cell membrane. Similar results were obtained by Storck and Wachsman [9] in experiments with *Bacillus megaterium*. They showed after carefully controlled disintegration of the cells that the enzymes responsible for lactate oxidation were associated with the cell membrane fraction, while the enzymes responsible for pyruvate and glucose oxidation were localized in the supernatant.

Whatever hypothesis is put forward to account for the experimental data, it is evident that the oxidation of acetate is inhibited more than the oxidation of TCA cycle intermediates by benzoic and salicylic acids. This points to the activation of the acetate molecule as the primary site of action. Among the dicarboxylic acids of the TCA cycle the oxidation of α-ketoglutarate seems to be most sensitive to benzoic acid which agrees with results obtained by Kaplan, Kennedy and Davis [10] in experiments with rat tissue homogenates. It may be significant that the first step of this oxidation is CoA-dependent and the same type of reaction as the transformation of pyruvate to acetyl-CoA. The possibility that benzoic acid (and various other carboxylic acids) affects fatty acid metabolism in liver cells by interfering with the function of CoA has been suggested and investigated by Avigan, Quastel and Scholefield [11]. Their results show that various reactions of acetyl-CoA are specifically inhibited by acyl-CoA compounds such as benzoyl-CoA. The effects were not due to simple removal of free CoA. It is possible but still doubtful whether a similar interference occurs in bacterial cells.

The experiment illustrated in Table 3 shows that streptomycin, at concentrations far exceeding those necessary for inhibition of growth, has no influence on pyruvate oxidation by resting cells of *P. vulgaris* grown in the way described. In this respect, the effects of streptomycin and benzoic acid are clearly different. It is nevertheless true that under certain conditions both inhibitors prevent pyruvate from entering the terminal respiration system and that this effect may be closely associated with the inhibition of growth. As a matter of fact there seems to be other similarities in the action of streptomycin and benzoic and salicylic acids. In 1951 Bernheim and DeTurk [12] reported that streptomycin, benzoic acid and several members of the hydroxy benzoic acid series specifically inhibit the assimilation of ammonia during succinate oxidation by *Pseudomonas aeruginosa*. Fitzgerald and Bernheim [13] have presented data showing that streptomycin inhibits the formation of adaptive enzymes. Results indicating a similar effect by benzoic and salicylic acids have been obtained in this work and will be presented in a future paper. These effects may, of course, be secondary and due to an interference with the formation of high energy compounds, which is very probable in the case of salicylic acid. It may also be mentioned that both salicylic acid [14] and streptomycin [15] have been reported specifically to inhibit the synthesis of pantothenate.

The real significance of these apparent similarities can be made evident only by further research, and it is certainly true that, especially in the case of streptomycin, many facts not mentioned here have to be considered.

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REFERENCES

1. Bosund, I. *Acta Chem. Scand.* **14** (1960) 1231.
2. van Slyke, D. D. and Folch, J. *J. Biol. Chem.* **136** (1940) 509.
3. Umbreit, W. W. *J. Biol. Chem.* **177** (1949) 703.
4. Oginsky, E. L., Smith, P. H. and Umbreit, W. W. *J. Bacteriol.* **58** (1949) 747.
5. Umbreit, W. W., Smith, P. H. and Oginsky, E. L. *J. Bacteriol.* **61** (1951) 595.
6. Bosund, I. *Acta Chem. Scand.* **14** (1960) 111.
7. Weibull, C. and Bergström, L. *Biochim. et Biophys. Acta* **30** (1958) 340.
8. Mitchell, P. and Moyle, J. *Biochem. J.* **64** (1956) 19P.
9. Storck, R. and Wachsman, J. T. *J. Bacteriol.* **73** (1957) 784.
10. Kaplan, E. H., Kennedy, J. and Davis, J. *Arch. Biochem. Biophys.* **51** (1954) 47.
11. Avigan, J., Quastel, J. H. and Scholefield, P. G. *Biochem. J.* **60** (1955) 329.
12. Bernheim, F. and DeTurk, W. E. *J. Pharmacol. Exptl. Therap.* **103** (1951) 107.
13. Fitzgerald, R. J. and Bernheim, F. *J. Bacteriol.* **55** (1948) 765.
14. Ivanovics, G. *Z. Physiol. Chem. Hoppe-Seyler's* **276** (1942) 33.
15. Lichstein, H. C. and Gilfillan, R. F. *Proc. Soc. Exptl. Biol. Med.* **77** (1951) 459.

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