

EFFECT OF CARBON DIOXIDE AND AGING ON THE DIHYDRO-STREPTOMYCIN INHIBITION OF PYRUVATE FERMENTATION IN *ESCHERICHIA COLI*¹

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Received for publication August 27, 1952

Barkulis (1953) demonstrated that resting cells of a strain of *Escherichia coli* ferment pyruvate in bicarbonate buffer chiefly by way of the phosphoroclastic reaction and that pyruvate fermentation is strongly inhibited by low concentrations of dihydrostreptomycin. When this work was almost completed, Umbreit and Oginsky (1951) found that anaerobic pyruvate breakdown in their strains of *E. coli* is inhibited by streptomycin only in the presence of carbon dioxide and bicarbonate.

These experiments were carried out to determine the effect of carbon dioxide on the dihydrostreptomycin inhibition of pyruvate fermentation in the strain *E. coli* studied by Barkulis (1953). Dihydrostreptomycin had almost no effect on pyruvate fermentation in freshly prepared resting cellular suspensions when carbon dioxide was excluded from the test system. This finding confirms the observation of Umbreit and Oginsky (1951) with respect to fresh cellular suspensions. However, when the cells were aged at 2 C, the dihydrostreptomycin inhibition in the absence of carbon dioxide rapidly increased, and within one to two weeks dihydrostreptomycin almost completely prevented all anaerobic pyruvate removal, both in the presence and in the absence of carbon dioxide. These data suggest the existence of at least two main pathways of pyruvate fermentation in fresh cellular suspensions of *E. coli* with different carbon dioxide requirements and different susceptibilities to dihydrostreptomycin.

METHODS

The normal dihydrostreptomycin sensitive strain of *E. coli* designated by Barkulis (1953)

¹ From a thesis submitted by Eugene Zebovitz to the Faculty of the Division of Biological Sciences of the University of Chicago in partial fulfillment of the requirements for the degree of Master of Science, March, 1952.

as *E. coli*, strain N, was used in all experiments. Cells were grown, harvested, washed, and adjusted to proper concentration as described by Barkulis (1953). The concentration of cells in the final resting cellular preparation was critical. Suspensions containing 5 to 8 mg bacterial nitrogen per ml were most suitable. Less dense suspensions fermented pyruvate too slowly while denser suspensions were not so strongly inhibited by dihydrostreptomycin. The resting cellular suspensions were stored in stoppered pyrex tubes at 2 C.

Pyruvate fermentation was measured in terms of the rate of pyruvate disappearance in two standard test systems, one with carbon dioxide and one without. Both systems were prepared in Warburg flasks and contained in a volume of 1 ml, 0.2 ml cellular suspension, 0.01 M sodium pyruvate, and 0.001 M adenosine-5-phosphate. For fermentation in the presence of carbon dioxide, 0.02 M sodium bicarbonate also was added and the atmosphere was 5 per cent carbon dioxide-95 per cent nitrogen, giving a pH of 7.0 at 37 C. When carbon dioxide was to be excluded, 0.01 M phosphate buffer, pH 7.0, was added, the atmosphere was 100 per cent nitrogen, and 0.1 ml 20 per cent potassium hydroxide was placed on a roll of filter paper in the center cup of each flask. A single concentration of dihydrostreptomycin, 50 µg per ml, always was used. The flasks were shaken at 37 C, and samples for pyruvate determination were deproteinized with 9 volumes of 10 per cent trichloroacetic acid at 10 minute intervals. Pyruvate was measured by the method of Friedemann and Haugen (1943) as modified by Speck *et al.* (1946).

For other experimental details see Barkulis (1953).

RESULTS

The effect of carbon dioxide on freshly harvested cellular suspensions. As observed by Umbreit and

Oginsky (1951), carbon dioxide and bicarbonate may have a profound effect on dihydrostreptomycin inhibition of pyruvate fermentation in *E. coli*. As shown in figure 1, pyruvate fermentation by freshly harvested cells in bicarbonate buffer was inhibited 60 to 70 per cent by 50 μ g dihydrostreptomycin per ml, while fermentation of pyruvate by the same cellular suspension in phosphate buffer (no carbon dioxide) was scarcely affected at all. It was observed also that in the absence of dihydrostreptomycin, cells in bicarbonate buffer show an induction period (Barkulis, 1953), but the cells in phosphate buffer attack pyruvate at a rapid, almost linear rate until all of the pyruvate has disappeared.

To interpret these results properly, it became necessary next to decide whether the presence of bicarbonate or the absence of phosphate was necessary for the dihydrostreptomycin effect. Since Kalnitsky and Werkman (1943) had shown that inorganic phosphate was necessary for the phosphoroclastic split of pyruvate in cell-free preparations of *E. coli*, it was conceivable that the presence of phosphate might facilitate the breakdown of pyruvate and thus account for the lack of dihydrostreptomycin inhibition in freshly harvested cells. This possibility was tested by replacing the phosphate buffer with a glycyl-glycine buffer, pH 7, but fresh cellular suspensions were also dihydrostreptomycin insensitive in glycyl-glycine. In another experiment, both 0.01 M phosphate, pH 7.0, and 0.02 M bicarbonate were added to the test system which was gassed then with 5 per cent carbon dioxide-95 per cent nitrogen. Pyruvate removal in the presence of the mixture of phosphate and bicarbonate was inhibited by dihydrostreptomycin to the same extent as in the presence of bicarbonate alone. Therefore, it appears that the presence of carbon dioxide and bicarbonate is required for inhibition of pyruvate fermentation in freshly harvested cellular suspensions of *E. coli*, strain N, and that inorganic phosphate does not influence the dihydrostreptomycin sensitivity of these preparations.

The effect of aging the resting cellular suspension.

The response of washed suspensions of *E. coli* to dihydrostreptomycin was altered profoundly during storage at 2 C. Figure 1 shows the effect of aging the cellular suspension for 10 days in distilled water. In bicarbonate buffer pyruvate was fermented at the same rate by fresh and aged suspensions, and the extent of dihydrostrepto-

mycin inhibition was approximately the same. However, in phosphate buffer there was now an initial lag in pyruvate fermentation and the rate was almost identical to that in bicarbonate buffer. Furthermore, dihydrostreptomycin strongly

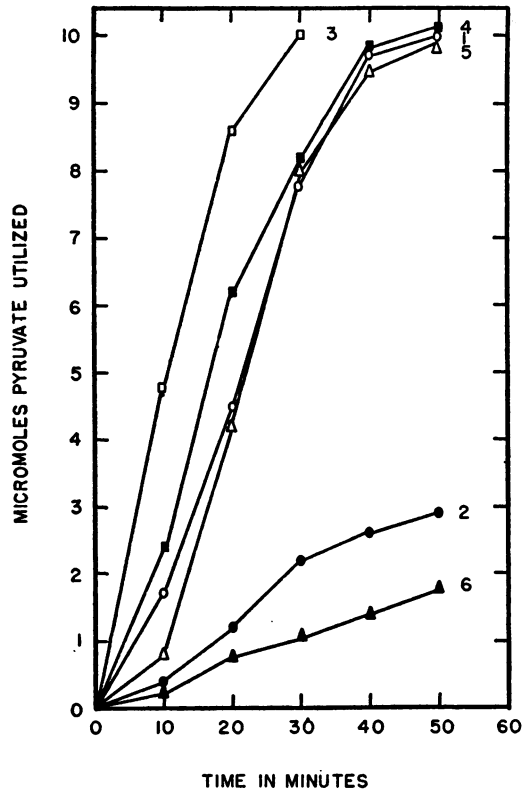


Figure 1. The effect of carbon dioxide and aging on the dihydrostreptomycin inhibition of pyruvate fermentation in a single suspension of *Escherichia coli*. 1. Bicarbonate buffer, no dihydrostreptomycin, cells either fresh or aged; 2. Bicarbonate buffer, 50 μ g dihydrostreptomycin per ml, cells either fresh or aged; 3. Phosphate buffer, no dihydrostreptomycin, fresh cells; 4. Phosphate buffer, 50 μ g dihydrostreptomycin per ml, fresh cells; 5. Phosphate buffer, no dihydrostreptomycin, cells aged 10 days; 6. Phosphate buffer, 50 μ g dihydrostreptomycin per ml, cells aged 10 days.

inhibited pyruvate fermentation in phosphate buffer by these aged cells.

This change in the character of pyruvate fermentation in aged suspensions of *E. coli* has been observed with seven different preparations. Figure 2 gives the mean per cent inhibition of anaerobic pyruvate utilization by 50 μ g per

ml dihydrostreptomycin when the seven preparations were tested in both phosphate and bicarbonate buffer after storage of 0 to 15 days at 2 C. Five fresh suspensions were completely insensitive to dihydrostreptomycin, while pyruvate removal in the other two was inhibited by 28 and 38 per cent. As the cells were aged at 2 C, the degree of dihydrostreptomycin inhibition

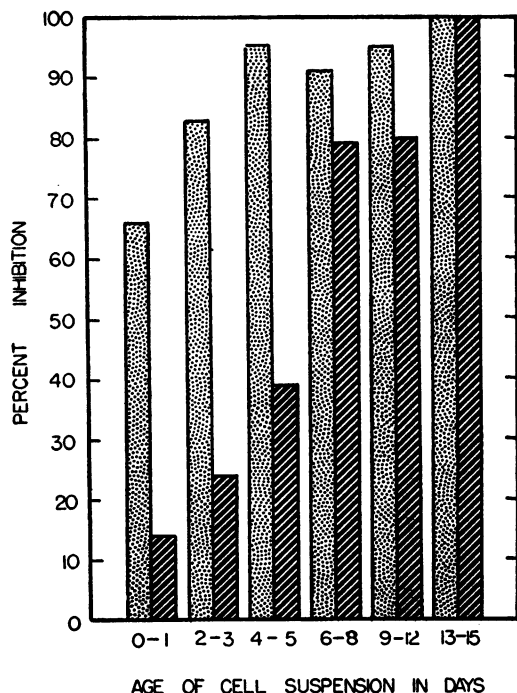


Figure 2. The effect of age of the resting cellular suspension on dihydrostreptomycin inhibition of pyruvate fermentation by *Escherichia coli* in bicarbonate and phosphate buffers. —bicarbonate buffer. —phosphate buffer. The bars represent the mean inhibition of anaerobic pyruvate removal in 7 different cellular suspensions. The concentration of dihydrostreptomycin was 50 μ g per ml.

increased when pyruvate fermentation was measured in either bicarbonate or phosphate buffer. In the bicarbonate test system, the dihydrostreptomycin inhibition of anaerobic pyruvate utilization increased from 68 to 100 per cent in 15 days. In the phosphate test system, the same cells exhibited a remarkable increase in dihydrostreptomycin sensitivity. From an average of 14 per cent immediately after harvesting, the dihydrostreptomycin inhibition

of pyruvate fermentation rapidly increased and reached 100 per cent in 13 to 15 days. It is of great importance that dihydrostreptomycin may prevent completely all anaerobic pyruvate removal, for this means that all anaerobic pathways of pyruvate breakdown may be dihydrostreptomycin sensitive.

In view of the marked effect of carbon dioxide on dihydrostreptomycin inhibition in freshly harvested cells, the absorption of carbon dioxide from the atmosphere or slow endogenous carbon dioxide production by the resting cells during storage at 2 C might account for the increased sensitivity of the aged preparations to dihydrostreptomycin in the supposed absence of carbon dioxide. If this were true, removal of carbon dioxide from the aged cells should restore their original insensitivity to dihydrostreptomycin in the phosphate buffer test system. However, when an aged cellular suspension was gassed with carbon dioxide-free nitrogen for one hour at room temperature, pyruvate fermentation in phosphate buffer was still strongly inhibited by dihydrostreptomycin. In another experiment, freshly harvested cells were tested in phosphate buffer to which different concentrations of sodium bicarbonate had been added. In order to obtain appreciable inhibition of pyruvate fermentation by dihydrostreptomycin in the fresh cells, at least 0.01 M bicarbonate had to be added to the phosphate test system. The results of these two experiments make it very unlikely that the increased dihydrostreptomycin inhibition of pyruvate fermentation by aged cells in a carbon dioxide-free test system can be attributed to the accumulation of carbon dioxide in the cellular suspension during storage at 2 C.

DISCUSSION

The important question raised by this work is why freshly harvested cells require carbon dioxide for dihydrostreptomycin inhibition of pyruvate fermentation while aged cells are dihydrostreptomycin sensitive both in the presence and absence of carbon dioxide. This observation strongly suggests that carbon dioxide may be fixed by fresh suspensions of *E. coli*, and that when, and only when, this fixation occurs, pyruvate fermentation is inhibited by dihydrostreptomycin. However, it is difficult to correlate this hypothetical fixation reaction with any of the known mechanisms of pyruvate fermentation in

E. coli. It is conceivable that pyruvate might fix carbon dioxide to form oxalacetate which then could condense with pyruvate. This would involve the dihydrostreptomycin sensitive reaction postulated by Oginsky *et al.* (1949), but it is hard to see how such a mechanism could lead to the fermentation balances obtained by Barkulis (1953).

The general relationship of carbon dioxide and age of the resting cellular suspension to dihydrostreptomycin inhibition of pyruvate fermentation may be explained if we assume that two separate enzyme systems for anaerobic pyruvate breakdown exist in *E. coli*, each competing with the other.

System I. This system is dihydrostreptomycin sensitive and is effective both in the presence and absence of carbon dioxide. It remains stable upon aging.

System II. This system is dihydrostreptomycin insensitive, is inhibited by the presence of carbon dioxide, and rapidly deteriorates upon aging.

In freshly harvested cells fermenting pyruvate in the presence of carbon dioxide, system I will function while system II will be inactive. Since system I is dihydrostreptomycin sensitive, fermentation will be inhibited. In the absence of carbon dioxide, both systems can function in fresh cells, and since system II is not affected by dihydrostreptomycin, no inhibition of pyruvate breakdown will be observed. When the cellular suspension is aged, system II is inactivated, leaving only the dihydrostreptomycin sensitive system I. Therefore, in aged suspensions, pyruvate fermentation is inhibited both in the presence and absence of carbon dioxide. Other similar explanations are possible, but at least two pathways of pyruvate breakdown, differing in dihydrostreptomycin sensitivity and carbon dioxide requirement, are always demanded. It is highly desirable to identify these purely formal systems with actual mechanisms of pyruvate metabolism, but it does not appear possible to do so at present.

SUMMARY

Dihydrostreptomycin inhibited the fermentation of pyruvate in freshly harvested cellular suspensions of *Escherichia coli* only in the presence of carbon dioxide.

When the cells were aged at 2 C, the dihydrostreptomycin inhibition in the absence of carbon dioxide rapidly increased. Within one to two weeks aging, dihydrostreptomycin almost completely prevented all anaerobic pyruvate removal, both in the presence and in the absence of carbon dioxide.

It was concluded that there are at least two pathways of pyruvate fermentation in *E. coli*. One is probably dihydrostreptomycin sensitive, unaffected by carbon dioxide, and stable upon aging, while the other is insensitive to dihydrostreptomycin, inhibited by carbon dioxide, and unstable upon aging.

It was not possible to identify either of these postulated systems with known mechanisms of pyruvate fermentation.

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