INHIBITION OF THE ANAEROBIC PYRUVATE METABOLISM OF ESCHERICHIA COLI BY DIHYDROSTREPTOMYCIN[1, 2, 3]

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Streptomycin inhibits the bacterial oxidation of a number of amino acids and of intermediates in carbohydrate metabolism. Geiger (1947) believed that in *Escherichia coli* streptomycin prevents the utilization of an intermediate in fumarate metabolism which is necessary for the rapid oxidation of a number of amino acids. Umbreit and his associates further studied the effect of streptomycin on terminal oxidation in *E. coli* and concluded that the antibiotic specifically inhibits the condensation of pyruvate and oxalacetate, thus preventing a variety of substrates from being oxidized via a tricarboxylic acid cycle (Oginsky *et al.*, 1949; Umbreit *et al.*, 1951). Streptomycin also interferes with anaerobic dissimilations. Henry *et al.* (1948) found that streptomycin interferes with pyruvate fermentation in *Bacillus cereus* and *Shigella sonnei*, but the nature of the inhibited reactions was not determined.

The present paper describes the effect of dihydrostreptomycin on the anaerobic metabolism of pyruvate in a strain of *E. coli* and in two mutant strains derived from it, one resistant to dihydrostreptomycin and the other dependent upon the antibiotic for growth. When normal cells of *E. coli* from a glucose synthetic medium fermented pyruvate in bicarbonate buffer, the main reaction fitted the over-all equation for the phosphoroclastic split of pyruvate into acetate and formate (Kalnitsky and Werkman, 1943). Low concentrations of dihydrostreptomycin strongly inhibited this fermentation. The fermentation of pyruvate by the resistant and dependent mutants was much slower than by the parent strain and was not influenced by dihydrostreptomycin.

All the experiments described here were carried out in the presence of carbon dioxide. The relation of carbon dioxide to dihydrostreptomycin inhibition of pyruvate fermentation in the same strain of *E. coli* is considered in another paper (Zebovitz and Moulder, 1953).

METHODS

Dihydrostreptomycin sulfate and streptomycin sulfate were obtained from Charles Pfizer and Company and used in aqueous solution. All the experiments reported here were performed with dihydrostreptomycin. Preliminary work indicated, however, that the results are obtainable with either form. Crystalline sodium pyruvate was prepared by the method of Lipmann (1944). Synthetic diphosphothiamine was supplied by Merck and Company, Inc. Adenosine mono-, di-, and triphosphates were obtained from the Sigma Chemical Company.

A typical strain of *E. coli*, designated strain N, isolated from feces was used throughout the work. Two mutants were isolated from *E. coli*, strain N, in the presence of 100 μg dihydrostreptomycin per ml. One was a dihydrostreptomycin resistant mutant, *E. coli*, strain R, which grew well in all concentrations of dihydrostreptomycin tested (up to 400 μg per ml). The other was a dihydrostreptomycin dependent mutant, *E. coli*, strain D, which grew only in dihydrostreptomycin concentrations between 10 and 200 μg per ml and always more slowly than *E. coli*, strains N and R. Stock cultures of the strains were maintained on nutrient agar slants with the addition of 100 μg dihydrostreptomycin per ml for the mutants. For metabolic experiments 2 liter Erlenmeyer flasks almost completely filled

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[2] A preliminary account of a portion of this work has been published (Barkulis, 1951*a*).

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with glucose synthetic medium (Anderson, 1946) were seeded with an inoculating needle from nutrient agar slant cultures. *E. coli*, strains N and R, were harvested by centrifugation after 18 to 24 hours at 37 C, while the slower growing *E. coli*, strain D, was harvested after 60 to 70 hours. The cells were washed twice in distilled water and suspended in water to a concentration of approximately 4.5 mg bacterial nitrogen per ml on the basis of a curve relating bacterial nitrogen to turbidity reading in the Klett-Summerson colorimeter. Each Warburg flask received 0.1 ml of this suspension or 0.45 mg bacterial nitrogen. The resting cellular suspension was stored at 2 C for 1 to 7 days before use. The effect of the age of the suspension on dihydrostreptomycin inhibition of pyruvate fermentation is considered elsewhere (Zebovitz and Moulder, 1953). When tested in bicarbonate buffer, the cells showed little change in activity after as long as 2 weeks at 2 C.

Carbon dioxide evolution was measured by conventional manometric methods. When the sum of metabolic carbon dioxide plus carbon dioxide due to acid formation in bicarbonate buffer was measured, the cells were suspended in 0.02 M $NaHCO_3$ to give a pH of 7.0 at 37 C in an atmosphere of 5 per cent carbon dioxide-95 per cent nitrogen. When the formation of metabolic carbon dioxide alone or of hydrogen was determined, the cells were suspended in 0.01 M phosphate buffer, pH 7.0, and the gas phase was 100 per cent nitrogen. Initial and final bound carbon dioxide was estimated by tipping in acid from the side arm. In such experiments the cellular suspensions were of such an age that pyruvate fermentation appeared to follow the same course in phosphate as in bicarbonate buffer (*see* Zebovitz and Moulder, 1953). To test for hydrogen evolution, the carbon dioxide evolved was absorbed by 0.1 ml of 20 per cent potassium hydroxide.

When end products of fermentation were measured, 100 to 2,000 micromoles of glucose or pyruvate were fermented in bicarbonate buffer. The following analytical procedures were used: glucose (Nelson, 1944), pyruvate (Friedemann and Haugen, 1943), lactate (Barker and Summerson, 1941), and volatile acids (Speck *et al.*, 1946). Acetate and formate were identified by Duclaux distillations (Gillespie and Walters, 1917) and by the lanthanum nitrate color test for acetate and the chromotropic acid color test for formaldehyde after reduction with Mg and HCl (Feigl, 1943).

RESULTS

It was found that dihydrostreptomycin in concentrations of 10 μg per ml completely inhibited the growth of *E. coli*, strain N, in nutrient broth under either aerobic or anaerobic conditions. This observation suggested that dihydrostreptomycin inhibited some step in the anaerobic metabolism of *E. coli* in addition to interfering with oxidative pathways (Oginsky *et al.*, 1949).

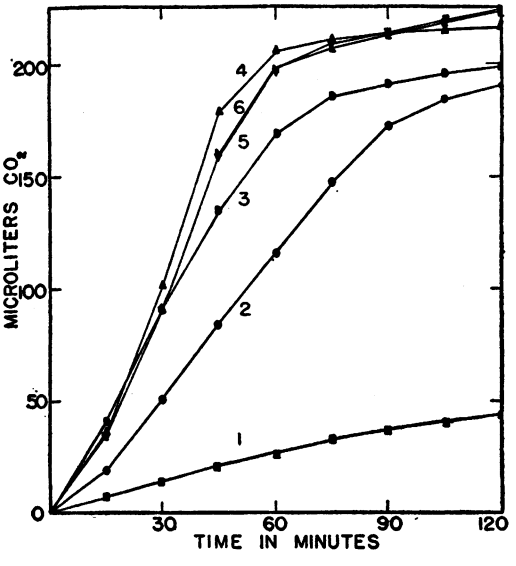

Figure 1. The effect of cofactors on pyruvate fermentation in *Escherichia coli*, strain N. (1) No pyruvate and no cofactors. (2–6) 10 μM pyruvate and: (2) no cofactors, (3) 1 mg yeast extract per ml, (4) 0.002 M adenosine monophosphate, (5) 0.002 M adenosine diphosphate, (6) 0.002 M adenosine triphosphate.

Fermentation of pyruvate in E. coli, strain N. The anaerobic breakdown of pyruvate in *E. coli*, strain N, then was investigated. Figure 1 shows the effect of various cofactor additions on the rate of pyruvate fermentation. In a concentration of 1 mg per ml, Difco yeast extract greatly accelerated the fermentation. However, 0.002 M additions of the adenine nucleotides were able to replace yeast extract in this respect and actually brought about a greater stimulation than yeast extract alone. Adenosine monophosphate was most effective, and maximum stimulation was achieved with concentrations of

0.00075 to 0.002 M. When yeast extract and adenosine monophosphate were added together, the rate of pyruvate fermentation was not higher than with the nucleotide alone, suggesting that adenosine monophosphate is the active factor in yeast extract. This effect by adenosine monophosphate may be related to the observation of Chantrenne and Lipmann (1950) that the adenine nucleotides inhibit the fixation of

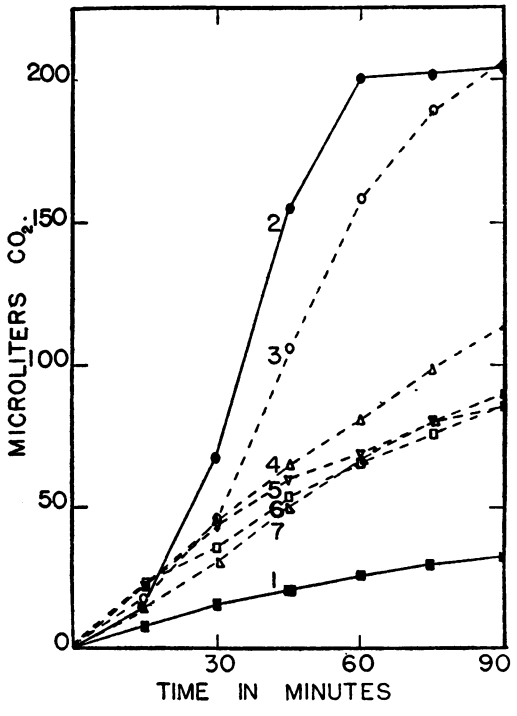

Figure 2. The effect of different dihydrostreptomycin concentrations on pyruvate fermentation in *Escherichia coli*, strain N. (1) No pyruvate. (2–7) 10 μM pyruvate and: (2) no dihydrostreptomycin, (3) 8 μg dihydrostreptomycin per ml, (4) 16 μg dihydrostreptomycin per ml, (5) 28 μg dihydrostreptomycin per ml, (6) 50 μg dihydrostreptomycin per ml, (7) 100 μg dihydrostreptomycin per ml.

formate into pyruvate by *E. coli* extracts, the reverse reaction of the phosphoroclastic split of pyruvate. The nature and concentration of the end products of pyruvate fermentation were not altered by the addition of adenosine monophosphate. No competitive relationship between adenosine monophosphate and dihydrostreptomycin was evident. In the following experiments, either yeast extract or adenosine monophosphate was added to all fermentation mixtures.

Diphosphothiamine, manganese, magnesium, or phosphate ions did not affect the rate of pyruvate fermentation either singly or in combination, probably because they were already present in adequate concentration.

The effect of different concentrations of dihydrostreptomycin on pyruvate fermentation is shown in figure 2. As little as 8 μg dihydrostreptomycin per ml inhibited, the degree of inhibition increased with concentrations up to 28 μg per ml, and higher dihydrostreptomycin concentrations produced no further inhibition. These results are comparable to experiments on the effect of dihydrostreptomycin on the growth of *E. coli*, strain N, in which its bactericidal action was first noted at 3 μg per ml and increased to a maximum at about 20 μg per ml. The inhibition of pyruvate fermentation by dihydrostreptomycin was noncompetitive with respect to pyruvate.

TABLE 1

End products of pyruvate fermentation in Escherichia coli, strains N, R, and D

	mM PER 100 mM PYRUVATE FERMENTED			
	Escherichia coli, strain N		*Escherichia coli*, strain R	*Escherichia coli*, strain D
	No dihydrostreptomycin	100 μg dihydrostreptomycin per ml*	100 μg dihydrostreptomycin per ml	100 μg dihydrostreptomycin per ml
Total CO_2 produced in bicarbonate buffer	89.5	20.3	93.5	106.8
Metabolic CO_2	27.3	0.5	32.3	52.5
Volatile acids	135.8	24.5	137.8	134.3
Lactate	4.0	Traces	Traces	Traces
$\frac{\text{Total } CO_2}{\text{Pyruvate}}$	0.90		0.94	1.07
$\frac{CO_2 \text{ observed}}{CO_2 \text{ calculated}}$	0.90		0.92	0.90
Per cent carbon recovery	81.0		80.0	85.0

* The fermentation of pyruvate in the presence of dihydrostreptomycin was always very incomplete, and no balance was attempted. The accumulation of end products in the presence of dihydrostreptomycin represents amounts found in the time necessary to ferment 100 μM pyruvate in the uninhibited system and is thus a measure of the degree of inhibition.

Table 1 gives the balance for pyruvate fermentation in the presence and absence of 100 μg

dihydrostreptomycin per ml. In the uninhibited reaction, approximately one mole of carbon dioxide was formed in bicarbonate buffer from each mole of pyruvate utilized. Of this carbon dioxide, about one-fourth was metabolic carbon dioxide and the other three-fourths was liberated from bicarbonate by the formation of volatile acids. Duclaux distillation of the volatile acids showed that they could be only acetate, formate, or a mixture of the two. Qualitative color reactions revealed the presence of both of these acids. Cells from glucose-synthetic medium had no detectable hydrogenlyase, but when they were grown on a hydrogenlyase producing medium, somewhat less than one mole of hydrogen was formed from each mole of pyruvate. These results indicate that the main reaction in the anaerobic breakdown of pyruvate by *E. coli*, strain N, is the phosphoroclastic split to acetate and formate (Kalnitsky and Werkman, 1943). About 80 per cent of the pyruvate carbon was accounted for in the end product determination. The metabolic carbon dioxide probably arose from the formation of ethanol or succinate, or a mixture of both, although they were not measured. If it is assumed that equal amounts of each were formed, a near 100 per cent carbon recovery would be achieved and the oxidation-reduction balance would be satisfied. When dihydrostreptomycin was present during the fermentation, the phosphoroclastic reaction, as judged by volatile acid formation, was inhibited approximately 80 per cent. However, the production of metabolic carbon dioxide was almost completely inhibited as well (see table 1). Therefore, dihydrostreptomycin appears to block other pathways in pyruvate breakdown in addition to the phosphoroclastic reaction.

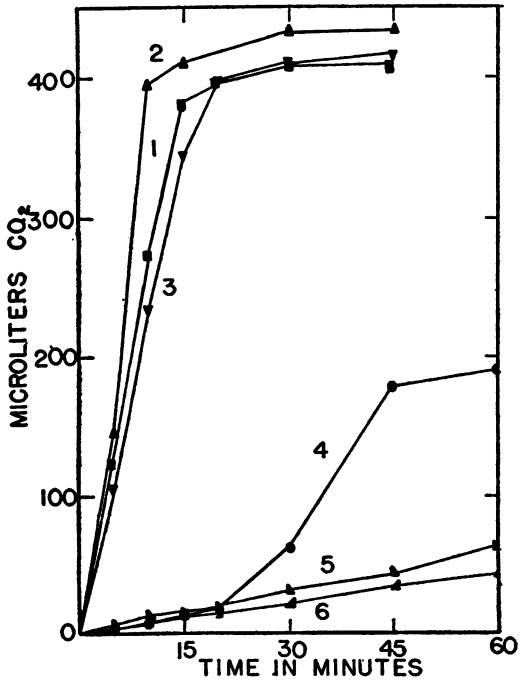

Figure 3. The fermentation of glucose and pyruvate in *Escherichia coli*, strains N, R, and D. (1–3) 10 μM glucose: (1) *Escherichia coli*, strain N, (2) *Escherichia coli*, strain R, (3) *Escherichia coli*, strain D. (4–6) 10 μM pyruvate: (4) *Escherichia coli*, strain N, (5) *Escherichia coli*, strain R, (6) *Escherichia coli*, strain D.

Fermentation of pyruvate in E. coli, strains R and D. Figure 3 compares the rate of pyruvate fermentation by suspensions of *E. coli*, strain N, *E. coli*, strain R, and *E. coli*, strain D, grown at the same time and tested at the same cellular concentration. While glucose was broken down at essentially the same rate by each of the three strains, *E. coli*, strain R, and *E. coli*, strain D, attacked pyruvate much more slowly than *E. coli*, strain N. The rate of pyruvate fermentation in the two mutant strains was completely unaffected by 100 μg dihydrostreptomycin per ml.

The pyruvate fermentation balances for *E. coli*, strains R and D, are shown in table 1 along with the balance for *E. coli*, strain N, which has already been discussed. The mutant strains were tested only in the presence of 100 μg dihydrostreptomycin per ml. When pyruvate fermentation was allowed to go to completion, the end products were practically identical in all three strains. Thus, the dihydrostreptomcyin resistant and dependent mutants appear to ferment pyruvate by the same mechanism as the parent *E. coli*, strain N, only at a much slower rate.

These differences in rate of pyruvate fermentation were not apparent in anaerobic growth experiments with pyruvate as the sole energy and carbon source. Each of the three strains grew anaerobically as well in the pyruvate-synthetic medium as in the usual glucose-synthetic medium. Therefore, despite their inability to ferment pyruvate rapidly in manometric experiments, *E. coli*, strains R and D, were still able to utilize

pyruvate efficiently as the only energy and carbon source for anaerobic growth.

Fermentation of glucose. Figure 3 demonstrates that the normal, the dihydrostreptomycin resistant, and the dihydrostreptomycin dependent strains of *E. coli* fermented glucose at the same rate. Anaerobic glucose breakdown was also almost completely insensitive to dihydrostreptomycin in all three strains. In *E. coli* the fermentation was not inhibited until dihydrostreptomycin concentration reached 25,000 μg per ml. Glucose fermentation in *E. coli* was expected to be sensitive to dihydrostreptomycin since the anaerobic pyruvate breakdown was strongly inhibited, and presumably glucose is fermented via pyruvate as an intermediate. Because of this paradox, glucose fermentation in *E. coli*, strain N, was studied in more detail.

TABLE 2

End products of glucose fermentation in Escherichia coli, strain N

	mM PER 100 mM GLUCOSE FERMENTED	
	No dihydrostreptomycin	500 μg dihydrostreptomycin per ml
Total CO_2 produced in bicarbonate buffer	159.0	159.5
Metabolic CO_2	12.3	7.3
Lactate	52.8	52.3
Volatile acids	106.3	113.3
$\frac{\text{Total } CO_2}{\text{Glucose}}$	1.6	1.6
$\frac{CO_2 \text{ observed}}{CO_2 \text{ calculated}}$	0.93	0.93
Per cent carbon recovery	55.0	56.0

An analysis of the end products of anaerobic glucose dissimilation in the absence and presence of 500 μg dihydrostreptomycin per ml is given in table 2. Dihydrostreptomycin altered neither the rate of fermentation nor the nature of the end products. Only about 1.6 moles of carbon dioxide per mole of glucose was produced in bicarbonate buffer. This carbon dioxide was completely accounted for by lactate, volatile acids (acetate and formate), and a small quantity of metabolic carbon dioxide. However, these end products accounted for only 55 per cent of the glucose carbon. Only a small amount of ethanol or succinate could have been formed because the production of metabolic carbon dioxide was low. Consideration of the carbon and oxidation-reduction balance indicated that about 40 per cent of the glucose must have been converted to a reduced, nonacidic substance without the formation of carbon dioxide. Although glycerol has not been reported as a product of glucose fermentation by *E. coli*, the amount of glycerol necessary to satisfy the carbon balance would here give a proper oxidation-reduction balance. Since glycerol is fermented slowly by *E. coli*, strain N, and a positive acrolein test for glycerol may be obtained on the products of glucose fermentation, it is possible that glucose actually may be converted into glycerol.

Approximately one-fourth of the fermented glucose was converted to acetate and formate, probably by the phosphoroclastic reaction, yet an addition of 500 μg dihydrostreptomycin per ml was not inhibitory (table 2). The differential effect of dihydrostreptomycin on pyruvate and glucose also is apparent in aerobic experiments. At a concentration of 100 μg per ml, dihydrostreptomycin has no effect on glucose oxidation, while only 20 μg per ml is needed to inhibit pyruvate oxidation by more than 70 per cent (Barkulis, 1951*b*).

In spite of the insensitivity of glucose fermentation in resting cells of *E. coli*, strain N, to dihydrostreptomycin, the anaerobic growth of *E. coli*, strain N, with glucose as the sole carbon and energy source is completely inhibited by very low levels of dihydrostreptomycin.

DISCUSSION

In elucidating the mechanism of action of chemotherapeutic agents, it is desirable to learn whether the inhibition of a given reaction by the drug is directly responsible for its growth inhibiting action or is only the secondary effect of a more vital enzymic inhibition elsewhere in the metabolic scheme. It is usually impossible to make an unequivocal choice between the two alternatives. With respect to the dihydrostreptomycin inhibition of pyruvate fermentation in *E. coli*, strain N, several observations suggest that this effect of dihydrostreptomycin lies close to the primary site of action of the antibiotic. Approximately the same dihydrostreptomycin concentration was required to inhibit both pyruvate fermentation and growth; the inhibitory

effect of dihydrostreptomycin was apparent after only a few minutes contact with the fermenting cell; and the one step mutants *E. coli*, strains R and D, fermented pyruvate very slowly by mechanisms insensitive to dihydrostreptomycin. On the other hand, experiments on the effect of dihydrostreptomycin on growing cultures were not always consistent with the results of metabolic experiments. The fermentation of glucose by *E. coli*, strain N, was dihydrostreptomycin insensitive, but the bacteria were nevertheless unable to grow in glucose synthetic medium in the presence of dihydrostreptomycin. Also, *E. coli*, strains R and D, fermented pyruvate much slower than glucose, yet grew as well with pyruvate as the sole carbon source as with glucose. Nonetheless, it seems reasonable to assume that dihydrostreptomycin inhibits some as yet undisclosed reaction which is common to many anaerobic and aerobic pyruvate dissimilations in *E. coli*, strain N.

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SUMMARY

The effect of dihydrostreptomycin on the anaerobic metabolism of a strain of *Escherichia coli* (*E. coli*, strain N) was investigated. Two mutant strains also were studied, one resistant to dihydrostreptomycin (*E. coli*, strain R) and the other dependent on the antibiotic for growth (*E. coli*, strain D).

Resting cells of *E. coli*, strain N, fermented pyruvate mainly by the phosphoroclastic split of pyruvate into acetate and formate. Dihydrostreptomycin strongly inhibited this reaction but also inhibited pyruvate fermentation by other pathways.

Both mutants fermented pyruvate slowly as compared to *E. coli*, strain N, and dihydrostreptomycin had no inhibitory effect. The pyruvate fermentation balance was similar in all three strains, and *E. coli*, strains R and D, also were able to use pyruvate as the sole carbon and energy source for anaerobic growth.

E. coli, strain N, fermented glucose to lactate, acetate, and formate to the same extent with or without dihydrostreptomycin. However, it was unable to grow in glucose synthetic medium in the presence of dihydrostreptomycin.

It is concluded that dihydrostreptomycin inhibits some reaction common to the various pathways of pyruvate fermentation in *E. coli*.

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