THE ACTION OF STREPTOMYCIN

V. The Formation of Citrate

W. W. UMBREIT, P. H. SMITH, AND E. L. OGINSKY

Merck Institute for Therapeutic Research, Rahway, New Jersey

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Previous papers (Umbreit, 1949; Oginsky, Smith, and Umbreit, 1949) have presented data showing that one of the actions of streptomycin on a sensitive *Escherichia coli* strain was to inhibit the oxalacetate-pyruvate reaction. It was admitted that the exact nature of this reaction was not known and that methods for its measurement were somewhat indirect. At about the time these papers were published, three developments altered the general concepts of the nature of this reaction. Stern and Ochoa (1949) demonstrated that in cell-free extracts of pigeon liver a condensation occurred between acetate and oxalacetate to yield citrate. The hypothesis of the "three-point landing" was proposed (Ogston, 1948) to account for asymmetry in the citrate formed. There was the demonstration by Potter and Heidelberger (1948) that the citrate formed was indeed asymmetrical. These three factors emphasized the position of citric acid in the "tricarboxy acid cycle." It is, therefore, now generally agreed that the product of condensation of acetate and oxalacetate is citrate.

It has been known for years that pyruvate can be oxidized to acetate (or "active acetate") so that the pathway pyruvate → active acetate → citrate was indeed a probable one, particularly since, in the presence of trace quantities of oxalacetate, pyruvate would tend to be oxidized to completion. By way of a wide variety of studies there was conclusive evidence that pyruvate could be oxidized by way of the citric acid cycle (cf. Krebs, 1943; Martius and Lymen, 1950). Since, therefore, pyruvate could enter the citric acid cycle via acetate, there was no longer any need to assume a "direct" pyruvate-oxalacetate condensation.

Since previous data have shown that streptomycin acts somewhere in this complex of enzymes, its effect should be evident on the citrate-forming system. Drs. Stern and Ochoa very kindly tested streptomycin on the isolated system from liver carrying out this reaction, but found no appreciable inhibition.[1] Nevertheless, it was still possible that either (1) the acetate-oxalacetate condensation in the susceptible bacteria differed in some subtle way from that in the animal or (2) that streptomycin acted upon this reaction by inhibiting another step, for example, the formation of some cofactor that was added in the experiments of Stern and Ochoa. The first possibility is quite unlikely since, although the features of the reaction susceptible to streptomycin inhibition may differ from the animal to the bacteria, they have in common the streptomycin-inhibited step, since animal tissues as well as *E. coli* show streptomycin inhibition of this reaction (Umbreit and Tonhazy, 1949).

[1] Personal communication.

It was therefore of interest to study the influence of streptomycin upon the reaction system forming citrate. This was done by measuring citrate formation in the "resting cell," without additions of cofactors, etc., so that any effect of streptomycin might be evident either on the citrate-forming enzyme itself or on the subsidiary reactions necessary for its operation.

The occurrence of citrate as an intermediate in the terminal respiration system of *E. coli* is quite unsettled at the moment, particularly since citrate, *cis*-aconitate, and *alpha*-keto-glutarate are oxidized only slowly, if at all, by most strains of *E. coli*. Nevertheless, this type of evidence may not be critical since it is possible that these compounds do not penetrate the living cell. With *alpha*-keto-glutarate, this seems to be the case since glutamate is oxidized readily and virtually to completion and *alpha*-keto-glutarate is known to be an important intermediate in its oxidation. It is therefore still possible that citrate is an intermediate in the complete oxidation of pyruvate in the presence of oxalacetate. It was hoped that determinations of citrate might throw some light on this broader problem.

If citrate were an intermediate in terminal oxidation, it might not accumulate in detectable amount, and, therefore, this method of approach would not be applicable. Experimentally, however, measurable amounts of citrate were found to accumulate, so that the problem could be studied in this manner.

METHODS

The bacterial cultures used were "Gratia" streptomycin-sensitive and streptomycin-resistant strains of *E. coli*. The methods for cultivation and treatment of the organisms were the same as those previously described (Oginsky, Smith, and Umbreit, 1949). Chemical analyses were performed on filtrates obtained after trichloroacetic acid precipitation of the reaction mixtures. Citric acid was determined by the method described by Lardy (1949). This procedure is specific for citric acid, and will not detect isocitric, *cis*-aconitic, or *trans*-aconitic acids. In preliminary experiments we used a rather nonspecific method for citrate (Saffran and Denstedt, 1948) in order to measure the amount of all the tricarboxy acids. However, oxalacetate interfered considerably with this method so that it was of little value in the measurement of citrate formation from pyruvate and oxalacetate. Keto acids were determined by the method of Friedemann and Haugen (1943).

RESULTS

The end product of the acetate-oxalacetate condensation is citrate. If streptomycin prevents any step in this reaction it should prevent the formation of citrate. In the animal, at least, citrate is oxidized further via *cis*-aconitic, oxalosuccinate, α-keto-glutarate, etc. If streptomycin interferes with any further oxidation of citrate, this substance should accumulate in the presence of streptomycin.

The data obtained by incubating cells, pyruvate, and oxalacetate with and without streptomycin are given in table 1. In experiment I, with oxalacetate and pyruvate, streptomycin had no effect on citrate formation. In the presence of

fluoroacetate (in an attempt to increase citrate accumulation[2]) streptomycin had no effect. In experiment II, with fluoroacetate present in all cases it may first be noted that no citrate was formed from either pyruvate or stearate. Oxalacetate alone produced 4.91 μM; with streptomycin, 4.83 (98 per cent as much). Oxalacetate and pyruvate produced 6.98; with streptomycin, 6.28 (90 per cent as much).

TABLE 1

Citrate formation by E. coli, Gratia strain

Experiment I

	CITRATE FORMED μM 60 MIN	O_2 UPTAKE μL, 165 MIN
Oxalacetate, 250 μM + pyruvate, 50 μM		
(a) No additions	3.88	566
(b) + streptomycin, 250 μg	3.88	212
(c) + fluoroacetate, 10 mg	7.58	—
(d) + fluoroacetate, 10 mg + streptomycin, 250 μg	7.58	—

Experiment II

SUBSTRATE	250 μG STREPTOMYCIN	10 MG FLUOROACETATE	CITRATE FORMED 60 μM MIN
Pyruvate, 50 μM	+	−	0
Pyruvate, 50 μM	+	+	0
Oxalacetate, 100 μM	+	−	4.91
Oxalacetate, 100 μM	+	+	4.83
Both	+	−	6.98
Both	+	+	6.28
Stearate, 50 μM	+	−	0*
Stearate, 50 μM	+	+	0*

Experiment I: 2.5 ml cell suspension containing 1.0 mg nitrogen per ml, 0.3 ml 0.1 M phosphate buffer pH 7, 0.5 ml 0.1 M pyruvate, 0.5 ml 0.5 M oxalacetate, 0.7 ml streptomycin (calcium chloride complex) containing 364 μg free base per ml, 0.5 ml fluoroacetate containing 20 mg per ml, H_2O to make final volume 5 ml. Oxidation data based on one-fifth the amount of cells and substrate.

Experiment II: 2.5 ml cell suspension containing 1.0 mg nitrogen per ml, 0.3 ml 0.1 M phosphate buffer pH 7, 0.5 ml 0.1 M pyruvate, 0.5 ml 0.2 M oxalacetate, 0.5 ml 0.1 M stearate, 0.7 ml streptomycin (calcium chloride complex) containing 364 μg free base per ml, 0.5 ml fluoroacetate containing 20 mg per ml, H_2O to make final volume 5 ml.

For both experiments, streptomycin was added 30 minutes, fluoroacetate 10 minutes, before substrates. Incubation time was 60 minutes at 37 C. Reaction was stopped with 0.1 ml 100 per cent trichloroacetic acid; 3.5 ml supernatant were analyzed for citrate.

* Incubation time: 120 minutes.

Table 2 presents the data on the formation of citrate from oxalacetate and pyruvate over the course of 3.5 hours. Two observations may be noted: first, streptomycin increased somewhat the accumulation of citrate, but this effect

[2] We are indebted to Dr. John O. Hutchens, Toxicity Laboratory of the University of Chicago, for the sodium mono-fluoroacetate used in these experiments. The use of fluoroacetate for this purpose has been discussed by Potter (1950) and Elliot and Kalnitsky (1950).

later disappeared; second, over the course of the 3.5-hour period there was a continued increase in citrate formation. These observations suggest that citrate is not an intermediate continually being formed and continually being broken down, but that it accumulates as a by-product, which is perhaps never broken down at all.

The data so far presented are capable of supporting the following statements:

(1) Citrate was formed in *E. coli* in detectable quantity when oxalacetate, or oxalacetate and pyruvate, were supplied. It was not formed from pyruvate in the absence of oxalacetate.

(2) Streptomycin had little effect upon citrate formation. What effect was observed was actually always an *increase* of doubtful significance in citrate in the presence of streptomycin compared to the controls without streptomycin. If streptomycin were inhibiting an additional reaction involving pyruvate and

TABLE 2

Effect of length of reaction time on citrate formation from oxalacetate and pyruvate

(Citrate formed, μM)

TIME, MIN	WITHOUT STREPTOMYCIN	WITH STREPTOMYCIN
0	0	0
15	2.49	2.38
30	2.98	3.01
90	4.09	5.65
150	5.50	6.99
210	6.69	7.28
270	7.28	6.99

A 1.3 ml cell suspension containing 1.0 mg nitrogen per ml, 0.3 ml 0.1 M phosphate buffer pH 7, 0.5 ml 0.1 M pyruvate, 0.5 ml 0.2 M oxalacetate, 0.7 ml streptomycin (calcium chloride complex) containing 364 μg per ml, H_2O to give final volume to 5.0 ml.

oxalacetate, thus making more pyruvate and oxalacetate available for the acetate-oxalacetate reaction, this type of result might be obtained.

(3) The citrate tended to increase in the system with time, as if it were the accumulation of a side reaction rather than an intermediate in oxidation. Streptomycin apparently did not block the further utilization of citrate since no more accumulated in its presence.

Comparative studies were then made on the course of citrate formation, oxygen uptake, and keto acid disappearance in the three types of cells listed in table 3. The first type of cell is that obtained when the sensitive strain is grown in flasks almost completely filled with medium ("deep grown"). The properties of these cells have been previously described (Oginsky, Smith, and Umbreit, 1949). The second type of cell is that obtained when the sensitive strain is grown in shallow layers of media in a shaking machine ("air grown"). Such cells, in contrast to the "deep grown," have the ability to oxidize acetate to completion (reaction 4, table 3) by unknown mechanisms that are not inhibited by streptomycin. It was hoped that these cells might permit the accumulation of greater

amounts of citrate since they so actively oxidize acetate. Finally, resistant cells were studied since these had previously been shown to lack the "pyruvate-oxalacetate" condensation (Smith, Oginsky, and Umbreit, 1949) and thus should not form citrate if the "pyruvate-oxalacetate" reaction resulted in citrate formation. Citrate formation (reaction 3, table 3) was found in all cases as described in the following paragraphs.

Data on the first type of cell suspension (sensitive, "deep grown") are given in figure 1. These cells did not oxidize acetate but showed a marked streptomycin effect upon the oxidation of oxalacetate and pyruvate. The oxygen uptake curves and the keto acid data are entirely typical and comparable to those previously discussed (Oginsky, Smith, and Umbreit, 1949). The reason for keto acid disappearance without concomitant oxygen uptake was shown previously to be probably due to the oxidation of pyruvate to acetate with the simultaneous reduction

TABLE 3

Summary of the reactions present in the strains employed

REACTION	SENSITIVE TO STREPTOMYCIN		RESISTANT
	"Deep grown"	"Air grown"	
1. "Pyruvate-oxalacetate" condensation, inhibited by streptomycin	Present	Possibly present	Absent
2. Pyruvate to acetate	Present	Present	Present
3. Citrate formation	Present	Present	Present
4. Complete oxidation of added acetate	Absent	Present	Present†
5. Decarboxylation of oxalacetate*	Present	Present	Present
Example	Figure 1	Figure 2	Figure 3

* This reaction could be removed in varying degrees by holding cell suspensions in the refrigerator. In the resistant strains it was usually found to be lower than in sensitive strains.

† In the resistant strain this reaction is also dependent, as in the sensitive, upon growth conditions. It is absent in "deep grown" resistant cells but present, at least to a measurable extent, in "air grown" resistant cells.

of oxalacetate to malate, and, as subsequent figures will show, this reaction complex is possessed by all three types of cells here studied regardless of their oxygen uptake on these substrates. This reaction is not inhibited by streptomycin.

However, with "deep grown" cells (figure 1) three points are evident with respect to citrate. First, only a small portion of the keto acid disappearing appeared as citrate (7 to 8 μM out of 150 μM substrate added). Second, the citrate accumulation was not influenced to any great extent by streptomycin. Third, even after all the substrate had been oxidized (in the absence of streptomycin at 4.5 hours) citrate remained and was apparently used only slowly if at all.

The second type of cell studied was the sensitive "Gratia" strain of *E. coli* grown with aeration. In these cells, the system oxidizing acetate is present and oxidizes this substrate virtually to completion by an unknown mechanism that

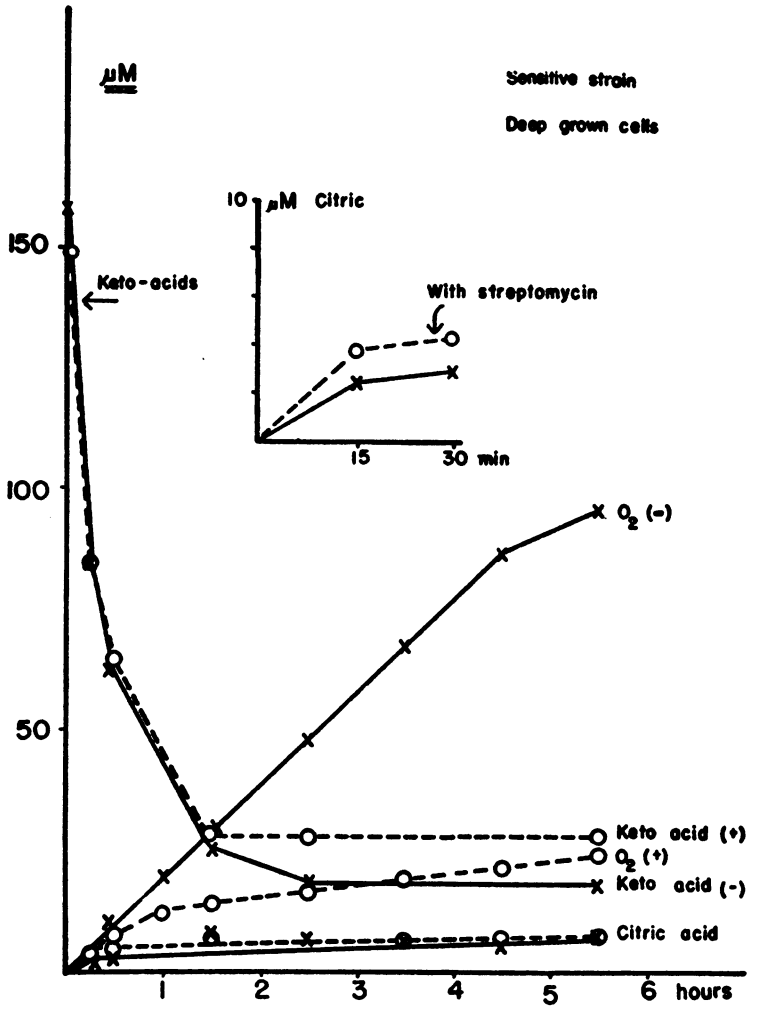

Figure 1. Citrate formation from pyruvate and oxalacetate by resting cell suspensions of "deep grown" sensitive strain *E. coli*. X——X = without streptomycin; 0- - -0 = with streptomycin.

Conditions: A. Oxidation studies—0.5 ml cell suspension containing 1.0 mg nitrogen per ml, 0.1 ml 0.1 M phosphate buffer pH 7, 0.17 ml 0.1 M pyruvate, 0.17 ml 0.2 M oxalacetate, 0.23 ml streptomycin hydrochloride containing 10 mg per ml, H_2O to give final volume of 3 ml. B. Citrate and keto acid studies—1.5 ml same cell suspension, 0.3 ml 0.1 M phosphate buffer pH 7, 0.5 ml 0.1 M pyruvate, 0.5 ml 0.2 M oxalacetate, 0.7 ml streptomycin hydrochloride containing 10 mg per ml, H_2O to give final volume of 5 ml.

Figures for oxygen uptake thus represent data on one-third the amount of cells and substrate in citrate and keto acid experiments, but the ratio of cells to substrate to streptomycin is the same in all experiments.

is not inhibited by streptomycin. The oxidation of oxalacetate and pyruvate, therefore, may proceed by either of two pathways.

First, the pyruvate may be oxidized to acetate and the acetate oxidized to completion via the acetate-oxidizing mechanism developed in these cells. Since neither the oxidation of pyruvate to acetate nor the acetate-oxidizing mechanism

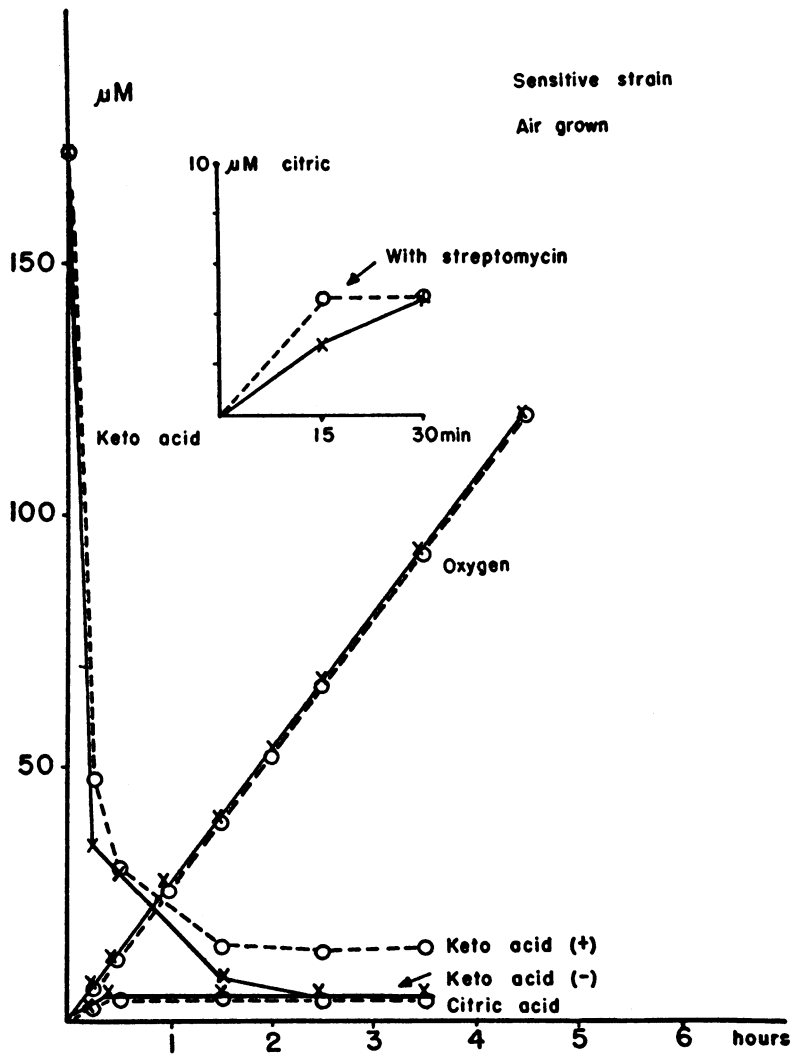

Figure 2. Citrate formation from pyruvate and oxalacetate by resting cell suspensions of the "air grown" sensitive strain of *E. coli*. X——X = without streptomycin; 0- - -0 = with streptomycin. Conditions as in figure 1.

is inhibited by streptomycin, pyruvate may be oxidized by these pathways without showing streptomycin inhibition. Second, the pyruvate may be oxidized by way of the streptomycin-inhibited "pyruvate-oxalacetate" condensation. If the capacity of the two systems are about equally balanced, streptomycin would

have little effect on oxygen uptake. Data of this type are given in figure 2 and will be discussed shortly. If the "pyruvate-oxalacetate" system predominates over that of the "acetate" system (as in "deep grown" sensitive cells, figure 1), streptomycin causes an inhibition of oxygen uptake. However, if the "acetate" system predominates over the "pyruvate-oxalacetate" system, streptomycin,

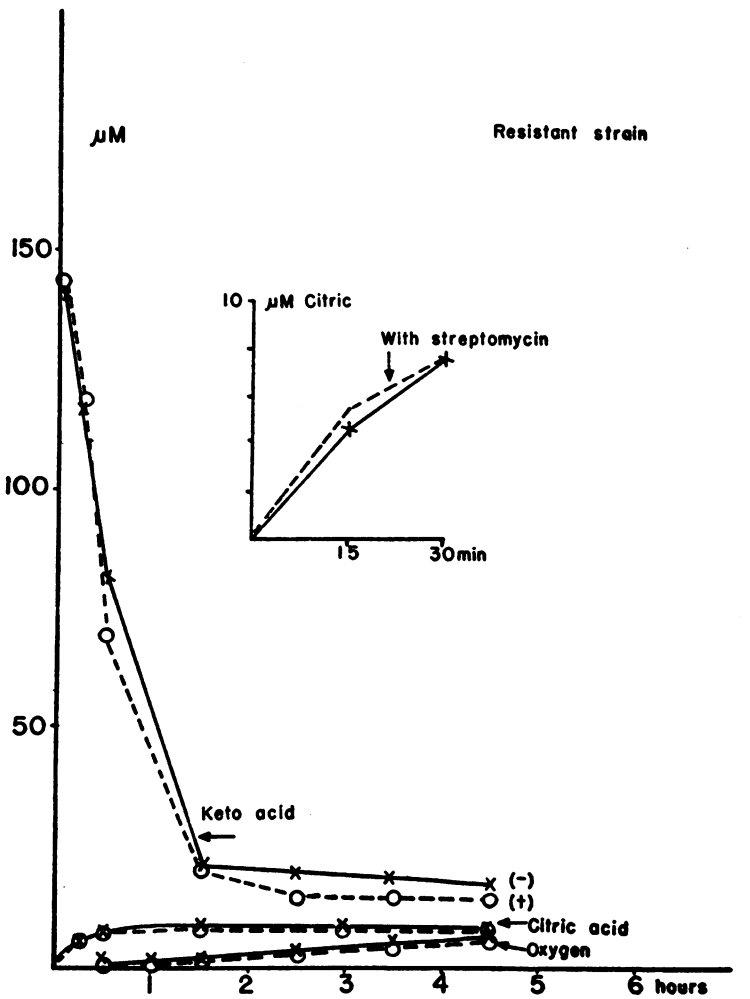

Figure 3. Citrate formation from pyruvate and oxalacetate by resting cell suspensions of a streptomycin-resistant strain of *E. coli*. X——X = without streptomycin; 0- - -0 = with streptomycin. Conditions as in figure 1.

by removing the competition for the pyruvate by the latter system, can actually cause an increase in oxygen uptake. We have considered that differences in the capacities of the two competing systems might be the cause of the increased oxygen uptake occasionally reported because of the addition of streptomycin (for example, Benham, 1947).

Data on "air grown" cells are given in figure 2. Streptomycin had no effect on oxygen uptake in these cells. Keto acid disappeared and citrate accumulated. However, streptomycin had very little effect on any of these processes. According to the theory described above, the two pathways would appear to be in equilibrium in these cells.

The strain rendered resistant to streptomycin was the third type of cell investigated. As previously demonstrated, when measured by oxygen uptake, these cells do not possess the "pyruvate-oxalacetate" reaction. Data on such cells are given in figure 3. First, oxygen uptake was extremely low; yet keto acid disappearance and citrate formation was comparable to that of the other cells. All of these reactions were uninfluenced by streptomycin.

DISCUSSION

By the direct analysis of citrate formed from pyruvate and oxalacetate, it was found that citrate formation was not inhibited by streptomycin. It thus appears that the "acetate-oxalacetate" condensation yielding citrate is not the reaction involved in streptomycin inhibition. Another reaction, inhibited by streptomycin, must therefore exist, and has been called the "pyruvate-oxalacetate" reaction. It was shown that in "deep grown" sensitive cells (illustrated in figure 1) most of the pyruvate entering terminal respiration goes through the "pyruvate-oxalacetate" reaction. Since streptomycin also inhibits similar oxidations in animal tissues (Umbreit and Tonhazy, 1949), the pyruvate-oxalacetate reaction must also occur in animal tissues and, because of the degree of inhibition achieved (90 to 100 per cent) in animal tissues, this reaction must constitute a rather large part of the pathways available for the oxidative removal of pyruvate. This conclusion is at variance with most contemporary studies in which it is assumed that "acetate-oxalacetate" condensation is the major pathway for pyruvate removal. From the data obtained with streptomycin, however, it would appear that the latter assumption is unwarranted. However, much as one might desire a simple and unified mechanism of entrance of pyruvate into the tricarboxy acid cycle, the data on streptomycin emphasize that the picture is more complicated than the mechanisms represented by the "acetate-oxalacetate" condensation. There is, therefore, still a need to postulate a "pyruvate-oxalacetate" reaction, but there is, from the data available, no reason why both reactions could not occur simultaneously. In fact, there would seem to be direct evidence that both do occur and compete with each other for available substrate.

SUMMARY AND CONCLUSIONS

Citrate formation from oxalacetate and pyruvate was not inhibited by streptomycin in a streptomycin-sensitive strain of *Escherichia coli*, yet oxidation of a mixture of these substrates was inhibited. A streptomycin-resistant strain of this organism, although incapable of appreciable oxidation of pyruvate and oxalacetate, was found to form comparable amounts of citrate. These facts would indicate that the "acetate-oxalacetate" condensation cannot be the sole or even the major pathway by which pyruvate enters the terminal respiration system of this organism. The streptomycin-inhibited reaction apparently does not proceed

through citrate. A "pyruvate-oxalacetate" reaction not forming citrate must be postulated to account for the results obtained, and it is this reaction that is inhibited by streptomycin.

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