EFFECT OF STREPTOLYSIN O ON THE UTILIZATION OF SOME KREBS CYCLE INTERMEDIATES BY AEROBACTER AEROGENES

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A preparation of streptolysin O partially purified from Streptococcus pyogenes strain C203S was shown to contain at least two distinct entities (Carlson et al., 1956): streptolysin O which brings about a marked loss of myocardial contractile power in isolated, perfused guinea pig, rabbit, and rat hearts (Kellner et al., 1956), and a diphosphopyridine nucleotidase which specifically attacks diphosphopyridine nucleotide at the nicotinamide-ribose linkage, consequently inhibiting oxygen consumption by mitochondrial preparations in the presence of various Krebs cycle intermediates (Carlson et al., 1957). The leukotoxicity of pyogenic streptococci may derive from the action of this latter enzyme (Bernheimer et al., 1957).

No study of possible metabolic effects of streptolysin O itself on bacterial cells has been reported. It would seem that pure cultures of bacteria, containing the enzyme systems chosen for study, would afford an excellent tool for a biochemical and physiological study of the various exotoxins. Ajl et al. (1958) subjected cell-free extracts of Escherichia coli, Proteus vulgaris, and Leuconostoc mesenteroides, as well as crude liver homogenates, to high concentrations of purified plague endotoxin. A marked diminution of α-ketoglutaric acid oxidation resulted. As far as can be ascertained, this was the first use of bacterial preparations in a study of toxin mechanisms.

The present study indicates that whole cell preparations may be employed profitably in similar studies with an exotoxin and that streptolysin O preparations effect a marked increase in oxygen consumption by washed suspensions of Aerobacter aerogenes in the presence of certain Krebs cycle acids with and without added coenzyme I.

EXPERIMENTAL METHODS

Stock cultures of the departmental strain of A. aerogenes were maintained on Koser's citrate agar slants and were grown in Koser's citrate broth at 37 C for 24 hr prior to each experiment. Cells harvested by centrifugation were washed twice with phosphate buffer and resuspended to allow 50 per cent transmittance at 600 mμ in the spectronic 20 colorimeter. This turbidity was shown by dry weight determinations to represent a concentration of 3.5 mg of cells per ml. Manometric experiments were carried out in the conventional Warburg respirometer at 37 C. Citrate was added to the manometer vessels at a concentration of 20 μmoles per flask and coenzyme I at a concentration of 2 mg per flask.

Carlson and co-workers (1956) prepared various lots of streptolysin solutions equivalent to 10,000 to 55,000 hemolytic units per ml. For their manometric studies 200 units per flask (100 units per ml) were employed. Kellner and co-workers (1956) state that “15 hemolytic units represent not more and probably considerably less than 1 μg of streptolysin O.” Accordingly, for this investigation streptolysin O (Difco) was dissolved in water to a concentration of 100 μg per ml. The lysin was added to the Warburg vessels in 0.3-ml amounts, which are approximately equivalent to 450 units per flask (150 Units per ml). Although the comparative degrees of purity are not known, it was felt that this concentration would furnish streptolysin in the range described above.

Streptococcus pyogenes strain C203U (kindly furnished by Dr. Alan Bernheimer) was grown in the medium of Bernheimer et al. (1942) and from this strain streptolysin O was prepared according to the method of Kellner et al. (1956). This strain does not produce streptolysin S. The metabolic effects of the final concentration were compared on a weight basis with two different lots (nos. 494893 and 494214) of the commercial product.

RESULTS

The utilization of citrate by citrate-grown cells was enhanced approximately 2-fold by the addition of coenzyme I to the vessel system.
The addition of streptolysin O to a cell-citrate-coenzyme system again resulted in a 2-fold increase in oxygen uptake. This doubling effect was obtained in all subsequent experiments. Although Kellner et al. (1956) found that prior activation of streptolysin O by cysteine was necessary in their myocardial perfusion experiments, no effect of addition or deletion on lysin activity was observed in the A. aerogenes system when 0.05 per cent cysteine was pre-incubated with the lysin. A depressed enhancement of oxygen uptake resulted when one half the initial concentration of streptolysin O was used.

Since Kellner and co-workers further reported that cholesterol and anti-streptolysin O inhibited the myocardial contractile effect of streptolysin, these substances were tested in the bacterial system. Figure 1 also shows that no inhibition occurs when a 1:100 dilution of a saturated solution of cholesterol is incubated with the lysin for 20 min at room temperature prior to tipping. It also may be seen that prior incubation with homologous antibody (equal concentration, w/v, lot no. 433316) caused no diminution of streptolysin O activity. Incubation times varying from 20 min to 2½ hr were tested. After incubation at 55 C for 15 min the lysin did not cause a comparable increase in oxygen uptake. Oxygen uptake was decreased to an amount somewhat less than that obtained with the cell-citrate-coenzyme system in the absence of streptolysin. Streptolysin O significantly increased the oxygen uptake by the cells in the absence of coenzyme I. This increase, roughly comparable to that effected by the coenzyme alone, is less than that observed when the lysin is added to the system containing coenzyme.

To study possible interactions of the supplements, various arrangements in flask and side arm incubation mixtures were made. All incubations were carried out for 20 min. Table 1 shows the usual increase in oxygen uptake as a result of lysin addition to the cell-citrate-coenzyme system. When streptolysin O and coenzyme I are incubated together prior to tipping, an increase in oxygen uptake over the base cell-citrate level is obtained, but the total uptake (94 and 99 μL) is less than that obtained when streptolysin O is added alone to the complete system (143 μL). No effect on total oxygen uptake resulted when the cells were incubated with streptolysin O before the citrate and coenzyme were added (66 vs. 68 μL). A decreased oxygen uptake (44 μL) was observed when the cells were allowed to incubate with the lysin and coenzyme before the substrate was added.

The effect of streptolysin O on oxygen uptake
Effect of various preincubation mixtures on the oxygen uptake of resting cells of Aerobacter aerogenes

<table>
<thead>
<tr>
<th>Vessel Contents: Cells +</th>
<th>Side Arm Supplements</th>
<th>Oxygen Uptake µL/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate + coenzyme</td>
<td>—</td>
<td>68</td>
</tr>
<tr>
<td>Citrate + coenzyme</td>
<td>Streptolysin</td>
<td>143</td>
</tr>
<tr>
<td>Citrate</td>
<td>Streptolysin + coenzyme</td>
<td>94, 99</td>
</tr>
<tr>
<td>Streptolysin</td>
<td>Citrate + coenzyme</td>
<td>66</td>
</tr>
<tr>
<td>Streptolysin + coenzyme</td>
<td>Citrate</td>
<td>44</td>
</tr>
</tbody>
</table>

Effect of streptolysin O on the oxygen uptake of resting cells of Aerobacter aerogenes in the presence of various Krebs cycle intermediates

<table>
<thead>
<tr>
<th>Vessel Contents: Cells +</th>
<th>Side Arm Supplement</th>
<th>Oxygen Uptake µL/hr</th>
<th>Increase %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate + coenzyme</td>
<td>—</td>
<td>86</td>
<td>95</td>
</tr>
<tr>
<td>Citrate + coenzyme</td>
<td>Streptolysin</td>
<td>108</td>
<td>115</td>
</tr>
<tr>
<td>Fumarate + coenzyme</td>
<td>—</td>
<td>81</td>
<td>95</td>
</tr>
<tr>
<td>Malate + coenzyme</td>
<td>Streptolysin</td>
<td>115</td>
<td>120</td>
</tr>
<tr>
<td>α-Ketoglutarate + coenzyme</td>
<td>—</td>
<td>95</td>
<td>129</td>
</tr>
<tr>
<td>α-Ketoglutarate + coenzyme</td>
<td>Streptolysin</td>
<td>120</td>
<td>143</td>
</tr>
</tbody>
</table>

by citrate-grown cells in the presence of certain other Krebs cycle intermediates was determined. Streptolysin O was tipped into vessels after the cells were incubated with citrate, fumarate, malate, or α-ketoglutarate. The data in table 2 show that citrate-grown cells, in the presence of coenzyme I, utilize equally well citrate, fumarate, and malate. Oxygen uptake in the presence of α-ketoglutarate exceeded that obtained in the presence of the other substrates. Significant increases also resulted when streptolysin O was added to the fumarate, malate, and α-ketoglutarate systems.

Because in the utilization of Krebs cycle intermediates, permeability often is a problem, some of the preceding determinations were repeated using a lysed cell preparation. After centrifugation and washing, the citrate-adapted cells were lysed by incubation at 37 C in distilled water for 1½ hr. The same w/v relationship was maintained as with the whole cell suspensions.

The lysed cell-citrate-coenzyme system was affected as before by the addition of streptolysin O; i.e., an approximate 100 per cent increase in oxygen uptake resulted. Comparable results were obtained in systems containing the other substrates. Heat-treated streptolysin O was again inactive, and antibody, again, had no inhibitory effect on the lysin's activity.

In an attempt to correlate commercial streptolysin O with that prepared by the authors, duplicate systems were set up. Six tenths ml of a solution of streptolysin O calculated to contain 50 µg of the streptococcal protein precipitate was tipped into the complete system. This concentration of the lysin resulted in an approximate 40 per cent increase in oxygen uptake over that elicited by the commercial product (30 µg per flask).

Discussion

A specific effect of streptolysin O preparations on respiration of cells has been demonstrated. In every case solutions of streptolysin O enhanced oxygen uptake by resting and lysed cells of A. aerogenes in the presence of citrate and other Krebs cycle intermediates. This stimulation is observed in the absence of coenzyme I although the effect is much greater when the cells are furnished with an exogenous supply of the coenzyme. Since cysteine was shown to have no real effect on the lysin and since cholesterol did not inhibit its action, it might be inferred that this metabolic enhancement effect is, perhaps, distinct from the previously determined roles of streptolysin: the hemolytic, the leukotoxic and the inhibitory effect on muscle contractility. Although the commercial product contains cysteine, the lysin prepared in our laboratory had a greater effect than did the commercial product, although no reducing compound was added. The commercial product in the oxidized state gave results similar to those obtained when it was tested in the reduced state.

Although the effect of the lysin on myocardial contractility was reduced in the presence of specific antibody, the antibody had no compa-
rable effect in this study. Sevag (1951) has pointed out that in some instances the chemical groups responsible for antigenicity and for the enzyme activity may be located at different sites on the molecule. It would seem that the effect observed in this study represents a new role for the toxin—the accentuation of some oxidative mechanism related to normal tri-carboxylic acid metabolism. The work of Pillemer and Muntz (cited in Pillemer and Robbins, 1949) showed that an accentuated metabolic rate occurred in animals after tetanal exotoxin injection and that of Frei and Witchard (1955) indicated that a cell preparation of a β-hemolytic streptococcus increased the dehydrogenase rate of guinea pig liver mitochondria, rendering this view possible.

The cells must be actively utilizing substrate in the presence of coenzyme before the full effect of streptolysin occurs, for the stimulation resulting from tipping the lysin and the coenzyme concurrently into the cell-substrate system was approximately only 50 per cent in contrast to the usual 100 per cent stimulation resulting from tipping streptolysin into the complete system. There was no effect on the respiration of the cells when they were preincubated with the lysin prior to the addition of the substrate and coenzyme. This result seems to pinpoint the effect to the metabolism of, in this case, citric acid. One further point must be made. There was a 50 per cent reduction in oxygen uptake when the cells were incubated with the lysin and the coenzyme before the addition of the substrate. No clear explanation can be offered at this time for this inhibition, but this result does emphasize the fact that the cells must be utilizing substrate before enhancement due to streptolysin O occurs, and does indicate a possible complementary action of the lysin and the coenzyme.

The utilization of the other intermediates was also enhanced by the addition of streptolysin O although to a lesser extent than that of citrate. Since it was necessary to adapt the cells to a rapid utilization of citrate, it is probable that adaptation to these other substrates might show comparable results. A preliminary experiment employing nonadapted cells showed slight uptake in the presence of citrate and none in the presence of the other intermediates.

Finally these same procedures were employed with cell-free preparations. The results duplicated, in every case, those obtained with the washed, resting cell preparations. Since purified toxin is unavailable, it is possible that the effects reported may not be a property of the extracellular lysin itself, but may result from the action of streptococcal diphosphopyridine nucleotidase or of some associated, heat-labile impurity.

**SUMMARY**

Streptolysin O of *Streptococcus pyogenes* stimulates the aerobic oxidation of citric acid and other Krebs cycle intermediates by resting cells and lysed preparations of *Aerobacter aerogenes* in the presence of coenzyme I and, to a lesser degree, in the absence of coenzyme I. Heat inactivation studies suggest this stimulation to be enzymatic.

**REFERENCES**


