EFFECT OF DİHYDROSTREPTOMYCİN ON TETRAZOLİUM DYE REDUCTION IN ESCHERICHIA COLI

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Received for publication 8 November 1962

ABSTRACT

Bragg, P. D. (University of British Columbia, Vancouver, British Columbia, Canada) and W. J. Polglase. Effect of dihydrostreptomycin on tetrazolium dye reduction in Escherichia coli. J. Bacteriol. 85:795-800. 1963.—Sonic-disrupted extracts of Escherichia coli, grown without added antibiotic (sensitive and resistant), contained (in supernatant of fraction centrifuged at 100,000 \( \times g \)) a dihydrostreptomycin-inhibitable, succinate-triphenyltetrazolium chloride (TTC) reductase activity. The succinate-TTC reductase activities of extracts of \( E. \) coli grown in the presence of dihydrostreptomycin (resistant and dependent) were relatively low and were not inhibited by the antibiotic. At a moderate magnesium concentration, the degree of inhibition by dihydrostreptomycin of succinate-TTC reductase activity was sufficiently marked to indicate an important site of action of the antibiotic. Magnesium, putrescine, and spermidine antagonized the action of dihydrostreptomycin in the succinate-TTC reductase system.

The observation (Bragg and Polglase, 1962) that pyruvate, and certain amino acids derived from pyruvate, were formed after addition of dihydrostreptomycin to antibiotic-sensitive Escherichia coli growing exponentially on glucose-salts medium provided new evidence in support of the hypothesis that the antibiotic affects terminal respiration (Umbricht, 1949).

In this respect, dihydrostreptomycin was found to differ from neomycin and chloramphenicol to resemble cyanide, azide, and amytal (Bragg and Polglase, 1963). The inhibitory action of dihydrostreptomycin was antagonized by magnesium salts and by the polyamines, putrescine and spermidine (Mager, Benedict, and Artman, 1962; Bragg and Polglase, 1963). As a consequence of these observations and the work of others (Flaks et al., 1962a, 1962b) on the effects of streptomycin on protein synthesis, it was suggested (Bragg and Polglase, 1963) that streptomycin may exert an inhibitory action at more than one site.

The present report deals with observations on the effect of dihydrostreptomycin on the reduction of tetrazolium salts by cell extracts from \( E. \) coli mutants.

MATERIALS AND METHODS

The \( E. \) coli used in this work and the method of culture (in glucose-inorganic salts medium) have been described previously (Bragg and Polglase, 1962). Strains SB and RB are, respectively, streptomycin-sensitive and streptomycin-resistant strains, and DA is a streptomycin-dependent \( E. \) coli. The streptomycin-dependent culture was grown in medium containing a minimal amount (35 \( \mu \)g per ml) of dihydrostreptomycin. The resistant strain (RB) was grown both with 35 \( \mu \)g per ml of dihydrostreptomycin, in which case it was designated RB (+), and without added antibiotic, designated RB (−).

Cell-free extracts were prepared from cells harvested towards the end of the exponential growth phase. The cells, collected by centrifugation, were suspended in 0.01 M tris(hydroxymethyl)aminomethane (tris) buffer containing 0.01 M MgCl\(_2\) at pH 7.4. This suspension was treated for 15 min in a Raytheon 9-ke sonic oscillator and then centrifuged at 30,000 \( \times g \) for 30 min. The supernatant fluid (referred to as the cell-free extract) was fractionated further by centrifuging at 100,000 \( \times g \) for 2.5 hr. The upper half of the supernatant liquid was removed and is referred to in the following experiments as the supernatant fraction. The precipitate was resuspended in buffer and washed by centrifugation (100,000 \( \times g \) for 2 hr).
ments below, this fraction is referred to as the "100,000 × g precipitate."

The reduction of triphenyltetrazolium chloride (TTC) was measured in Thunberg tubes using the following systems: 0.06 M tris containing 0.03 M MgCl₂, 1 ml (pH 8.0); 10⁻³ M TTC, 0.3 ml; cell extract, 0.5 ml; inhibitor solution (or water), 0.3 ml. The stopper contained 0.5 ml of sodium succinate (5 μmoles), or 0.5 ml of reduced nicotine adenine dinucleotide (NADH; 0.5 μmoles). After evacuation, the tube was preincubated (37°C) prior to the addition of substrate. At the end of the incubation period (5 min to 1 hr), the reaction was stopped by adding 4 ml of acetone. The precipitate which formed was removed by centrifugation, and the optical density of the supernatant was measured at 485 mμ (Bril, 1954). Results were expressed in one of two ways: as activity units where 1 unit was defined as a change in optical density of 0.01 per hr per mg of protein (Tables 1 to 5), or as a percentage of the optical density obtained in the absence of inhibitor (Fig. 1 to 3). The use of the terms NADH-TTC (or succinate-TTC) reductase activity does not connote individual enzyme systems but refers only to the over-all reaction under observation.

For experiments on heat-treated preparations, the cell extract was heated for 90 sec at 100°C. After cooling, the precipitate which formed was redispersed with a glass homogenizer.

Pyruvic acid was determined as total keto-
acid (Friedemann, 1957), protein by the method of Lowry et al. (1951), and total pyridine nucleotides by the cyanide method (Ciotti and Kaplan, 1957). Difference spectra of cytochromes, with and without added sodium dithionite, were measured using the Cary double-beam spectrophotometer.

Results and Discussion

Tetrazolium reductase activity of cell fractions.

With NADH as substrate, the TTC reductase activity of the supernatant fractions from the various strains of E. coli was greater than that of the corresponding "100,000 × g precipitates" (Table 1). The TTC reductase activities of the "100,000 × g precipitates" from all strains were decreased slightly by relatively high concentrations of dihydrostreptomycin (Table 1). The TTC reductase activities of strains SB and RB (-) were not affected by dihydrostreptomycin. On the other hand, there was a stimulation of

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Inhibitor</th>
<th>Dihydrostreptomycin (μg/ml)</th>
<th>Q-N-oxide†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>385</td>
<td>1,150</td>
</tr>
<tr>
<td>Supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB</td>
<td></td>
<td>235</td>
<td>254</td>
</tr>
<tr>
<td>RB (-)</td>
<td></td>
<td>295</td>
<td>288</td>
</tr>
<tr>
<td>RB (+)</td>
<td></td>
<td>88</td>
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</tr>
<tr>
<td>DA</td>
<td></td>
<td>135</td>
<td>147</td>
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<tr>
<td>&quot;100,000 × g precipitate&quot;</td>
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</tr>
<tr>
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<td></td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>RB (-)</td>
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<td>DA</td>
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<td>3.1</td>
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* Activities given in units defined in Materials and Methods.
† 2-Heptyl-4-hydroxy quinoline-N-oxide (1.4 × 10⁻³ M).

TTC reductase activity by dihydrostreptomycin in DA and RB (+).

When the supernatant fraction and the "100,000 × g precipitate" of SB were combined, there was no observable inhibition of TTC reductase by dihydrostreptomycin if NADH was the substrate; but when succinate was used as substrate, partial inhibition by dihydrostreptomycin was evident at relatively high concentrations of the antibiotic (Table 2). Since the "100,000 × g precipitate" of E. coli SB was found to be devoid of succinate-TTC reductase activity, attention was focused on the supernatant fraction. The succinate-TTC reductase activities of SB and DA supernatant fractions are given in Table 3. This reductase activity appears to be dependent upon the presence of thiol groups, since preparations from DA showed no activity until cysteine was added (1 μmole per mg of protein). Furthermore, although the maximal activity (about 15 units) was obtained in freshly prepared SB supernatant fractions, aged preparations required the addition of cysteine (1 μmole per mg of protein) for maximal activity. Dihydrostreptomycin, at relatively high concentrations, inhibited the succinate-

TTC reductase of sensitive (SB) E. coli SB but the dependent strain DA was not affected (Table
TABLE 2. NADH and succinate-TTC reductase activities* in combined supernatant and "100,000 × g precipitate" from Escherichia coli SB

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dihydrostreptomycin (µg/ml)</th>
<th>Amytal (2.1 × 10⁻³ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>323</td>
<td>970</td>
</tr>
<tr>
<td>Succinate</td>
<td>7.2</td>
<td>5.9</td>
</tr>
<tr>
<td>NADH</td>
<td>90</td>
<td>87</td>
</tr>
</tbody>
</table>

* Activities given in units defined in Materials and Methods.

TABLE 3. Effect of cysteine and inhibitors on succinate-TTC reductase activities* of supernatant fractions of Escherichia coli SB and DA

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Inhibitor</th>
<th>Dihydrostreptomycin (µg/ml)</th>
<th>Q-N-oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>385</td>
<td>1,150</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3,850</td>
</tr>
<tr>
<td>SB</td>
<td>15.4</td>
<td>10.2</td>
<td>9.2</td>
</tr>
<tr>
<td>DA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SB*</td>
<td>15.2</td>
<td>11.0</td>
<td>—</td>
</tr>
<tr>
<td>DA*</td>
<td>11.2</td>
<td>11.2</td>
<td>11.2</td>
</tr>
</tbody>
</table>

* Activities given in units defined in Materials and Methods.
† 2-Heptyl-4-hydroxyquinoline-N-oxide (1.4 × 10⁻⁴ M).
‡ Cysteine (2 µmoles) added.

3. Krishna Murti (1960), using a similar technique, reported 45% inhibition by dihydrostreptomycin of succinic dehydrogenase in a cell-free extract from antibiotic sensitive Vibrio cholerae. The enzyme system from the streptomycin-resistant V. cholerae was not inhibited.

Supernatant fractions from E. coli RB (−) extracts also had a dihydrostreptomycin-inhibitable succinate-TTC reductase activity (Table 4). The high reductase activity in these preparations appeared to be due partly to endogenous substrate, since some dye reduction commenced before the addition of succinate. The succinate-TTC reductase activity of RB (−) was relatively low and was not inhibited by dihydrostreptomycin (Table 4). Thus, E. coli cells grown in the absence of antibiotic [SB and RB (−)] have a dihydrostreptomycin-inhibitable succinate-TTC reductase system, whereas cells grown in the presence of dihydrostreptomycin [DA and RB (+] have lower succinate-TTC reductase activities which are not inhibited by the antibiotic.

Antagonism of dihydrostreptomycin by cations in the succinate-TTC reductase system. Relatively high concentrations of dihydrostreptomycin were necessary to demonstrate inhibition of succinate-TTC reductase activity. However, in the preparation of the "100,000 × g supernatant fractions," a high (1 × 10⁻² M) magnesium concentration had been used to provide a cleaner separation of the particulate fraction. When a lower concentration of magnesium (1.67 × 10⁻³ M) was used, the degree of inhibition by dihydrostreptomycin was increased and occurred at a lower concentration of antibiotic (Fig. 1). Both putrescine (Fig. 2) and spermidine (Fig. 3) were antagonistic towards dihydrostreptomycin at the lower magnesium level.

A detailed quantitative study is not warranted until a purified system is obtained, but the magnitude of the effect of dihydrostreptomycin on succinate-TTC reductase activity is sufficient to indicate that there is an important site of action of the antibiotic in the electron-transport system of E. coli.

Site of TTC reduction. Measurements of difference spectra of the supernatant fractions of SB (−), RB (−), RB (+), and DA indicated that cytochromes were present in trace amounts only (about 0.01 µmoles per mg of protein). Addition of the cytochrome-containing "100,000 × g precipitate" had no effect on succinate-TTC reductase activity. It appears, therefore, that TTC must receive electrons directly from the succinic dehydrogenase flavoprotein (Ling,
FIG. 1. Antagonism by Mg of inhibition by dihydrostreptomycin (DHSM) of succinate-TTC reductase activity in "100,000 X g supernatant fraction" from sensitive Escherichia coli SB. Curve A: 1.1 X 10^{-2} M; curve B: 1.67 X 10^{-3} M Mg.

FIG. 2. Antagonism by putrescine of inhibition by dihydrostreptomycin (DHSM) of succinate-TTC reductase activity of "100,000 X g supernatant fraction" from sensitive Escherichia coli SB. Mg = 1.67 X 10^{-3} M. Curve A: 1.86 X 10^{-2} M; curve B: 4.83 X 10^{-3} M putrescine; curve C: no putrescine.

FIG. 3. Antagonism by spermidine of inhibition by dihydrostreptomycin (DHSM) of succinate-TTC reductase activity of "100,000 X g supernatant fraction" from sensitive Escherichia coli SB. Mg = 1.67 X 10^{-3} M. Curve A: 1.86 X 10^{-2} M; curve B: 4.83 X 10^{-3} M spermidine; curve C: no spermidine.

Su, and Tung, 1957) or from some unknown intermediate electron acceptors(s) between the flavoprotein and the dye.

The streptomycin antagonist, 2-heptyl-4-hydroxy quinoline-N-oxide (Lightbown, 1954; Cornforth and James, 1956) was found to inhibit succinate-TTC reductase activity by about 50% (Tables 3 and 4). The work of Lightbown and Jackson (1956) has suggested that in E. coli quinoline-N-oxides act at a site preceding the cytochrome level. It is noteworthy that both dihydrostreptomycin and quinoline-N-oxide show an effect on electron transport in the same general area. If it is presumed that NADH-TTC reduction is a direct transfer of electrons from the pyridine nucleotide to the dye (Lester and Smith, 1961), whereas succinate-TTC reduction proceeds via additional electron acceptors between the succinic dehydrogenase flavoprotein and the tetrazolium dye, then the inhibition of the latter system and not of the former by dihydrostreptomycin and quinoline-N-oxide can be understood.

Effect of nicotinamide adenine dinucleotide (NAD) on succinate-TTC reductase activity. Table 5 shows that NAD stimulated the succinate-TTC reductase activity. Stimulation of succinate-TTC reductase activity by NAD in mammalian tissue homogenates has been re-
ported previously (Bril, 1954; Sprints and Waldschmidt-Leitz, 1953). According to Bril (1954), full activity of the (particulate) enzyme was dependent on the presence of a soluble fraction. In the present work, it was found that the succinate-TTC reductase activity of E. coli (SB) supernatant fraction was stimulated by the addition of heat-treated supernatant fraction from SB (Table 5). That this stimulation may have been due to the presence of pyridine nucleotides (0.075 μmole per mg of protein) in the supernatant fraction was indicated by the following. Addition of heat-treated supernatant fraction containing 0.23 μmole of pyridine nucleotides gave a 1.6-fold stimulation (sevenfold per μmole) in TTC reduction, whereas 0.9 μmole of NAD gave a 4.3-fold stimulation (fivefold per μmole). These results are considered to agree within experimental error. In what manner NAD stimulates succinate-TTC reductase activity is not known, but since the percentage inhibition by dihydrostreptomycin is the same in the presence or absence of NAD or heated supernatant fraction (Table 5) it appears that NAD normally participates in the succinate-TTC reduction. However, the reduction of TTC in the presence of succinate cannot pass through NADH since, if this were the case, succinate-TTC reductase activity would not be inhibited by dihydrostreptomycin.

The foregoing results have indicated that dihydrostreptomycin will inhibit a pathway of electron transport in the supernatants of SB (−) and RB (−) but not in RB (+) and DA (+). As with other tetrazolium reductases (Oda and Goldman, 1962), the significance of succinic-TTC reductase to the organism is not known. The work of Flaks et al. (1962a, b) and Mager et al. (1962) demonstrated inhibition by streptomycin of amino acid incorporation into ribosomes of E. coli. Whether or not there is a direct relationship between these observations and the observations herein recorded of the effect of the antibiotic on electron transport cannot be decided on the basis of present data. Experimental work is now being directed towards purifying the succinate-TTC reductase system and towards determining whether or not the system was liberated from the ribosomes as a result of sonic disruption.

**Acknowledgments**

The 2-heptyl-4-hydroxy quinoline-N-oxide was obtained from J. W. Lightbown, Medical Research Council, Mill Hill, London, England. This work was supported by a grant (MT 750) from the Medical Research Council of Canada.

**LITERATURE CITED**


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