ELECTRON-TRANSPORT COMPONENTS OF STREPTOMYCIN-DEPENDENT *ESCHERICHIA COLI*

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ABSTRACT

BRAGG, P. D. (University of British Columbia, Vancouver, B. C., Canada) AND W. J. POLGLASE. Electron-transport components of streptomycin-dependent *Escherichia coli*. *J. Bacteriol.* **86**:544–547. 1963.—When a streptomycin-dependent strain of *Escherichia coli* was grown in antibiotic-free medium, the resulting streptomycin-depleted cells were found to contain relatively large amounts of vitamin K₂. Cytochromes *a*₁ and *a*₂ were absent from depleted cells, and these cells metabolized glucose with the accumulation of lactic acid. Depleted cells were normal in their content of coenzyme Q₈, total flavine, and cytochrome *b*. Growth of depleted cells on medium containing dihydrostreptomycin restored their ability to metabolize lactic acid concomitantly with a decrease in vitamin K₂ to a normal level, and with the formation of cytochromes *a*₁ and *a*₂. It is concluded that streptomycin (or dihydrostreptomycin) is required by dependent *E. coli* for maintenance of the integrity of the electron-transport system.

and Mager, Benedict, and Artman (1962), demonstrated inhibition of uptake of amino acids by ribosomes of streptomycin-sensitive *E. coli*, but streptomycin dependence in corresponding fractions from streptomycin-dependent *E. coli* could not be demonstrated (Flaks et al., 1962b).

Tirunarayanan, Vischer, and Renner (1962) and Bragg and Polglase (1962) observed that strains of streptomycin-dependent *E. coli* produced considerable quantities of L-valine. This observation, together with observations on other extracellular metabolites (Bragg and Polglase, 1962, 1963a), suggested an effect of streptomycin on respiration, as proposed earlier by Umbreit (1949, 1953) and others. Further studies (Bragg and Polglase, 1963b) showed that dihydrostreptomycin inhibited a reaction of the electron-transport chain in extracts of streptomycin-sensitive *E. coli*. These observations together with those of Engelberg and Artman (1961) led us to focus our attention on the electron-transport components of a strain of streptomycin-dependent *E. coli*.

MATERIALS AND METHODS

Miller and Bohnhoff (1947) first reported streptomycin-resistant microorganisms which required streptomycin for growth. Such "streptomycin-dependent" mutants have since been isolated from a great variety of microorganisms (see Paine and Finland, 1948). The biochemical basis of streptomycin dependence has not been explained but, presumably, in accordance with the hypothesis of Spotts and Stanier (1961), the reaction which requires streptomycin in a dependent mutant is closely related to the reaction which is inhibited by streptomycin in the parent, sensitive organism.

Polglase, Peretz, and Roote (1957) and Peretz and Polglase (1957) have shown that a strain of *Escherichia coli* which required streptomycin for growth also required the antibiotic for induced enzyme synthesis. Flaks, Cox, and White (1962a), as well as Speyer, Lengyel, and Basilio (1962)

All studies were performed with streptomycin-dependent *E. coli* strain DA, which had been used in previous work (Bragg and Polglase, 1962). The following medium, adjusted to pH 7.2, was used: 0.7% K₂HPO₄, 0.3% KH₂PO₄, 0.05% sodium citrate, 0.01% MgSO₄, 0.1% (NH₄)₂SO₄, 0.2% glucose, and 1000 units per ml of dihydrostreptomycin (when present) as the sulfate. Three different growth conditions (always with vigorous aeration) were studied. First, the culture was grown (at 37 C) at an optimal concentration of dihydrostreptomycin (1000 units per ml), to the end of the log phase (about 5 hr). Cells grown in this way were referred to as "normal DA." Second, normal DA cells were collected by centrifugation (8000 × *g*) and depleted of antibiotic by forced growth in fresh antibiotic-free medium for about 7 hr, followed by another centrifugation, resuspension, and incubation in fresh anti-

biotic-free medium for about 4 hr. During the depletion, the rate of growth was initially linear, and then approached zero. Morphologically, antibiotic-depleted, dependent cells are elongated rods. Cells obtained in this way were referred to as "depleted DA." Third, depleted DA cells were grown in fresh medium supplemented with dihydrostreptomycin (1000 units per ml) for periods up to 3 hr.

The lipoquinones were extracted by a modification of the method of Lester and Crane (1959). Sufficient packed cells were dried in vacuo at 80 C overnight to give 0.25 to 1.0 g of dried cells. The dried cells were extracted twice by shaking with 50-ml volumes of isooctane. The combined extracts were evaporated to a solid residue which was then taken up in about 5 ml of isooctane. The isooctane solution was added to the top of a column (5 by 15 mm) of Magnesol-Celite (5:1; McNeely, Binkley, and Wolfrom, 1945). For elution of vitamin K₂, 1% ethyl ether in isooctane was added to the column, and two or three 10-ml fractions were collected. Ethanol (1%) in isooctane was next added to elute coenzyme Q, and one or two 10-ml fractions were collected. Vitamin K₂ and coenzyme Q were estimated quantitatively as described by Bishop, Pandya, and King (1962). Chromatography by the method of Lester and Ramasarma (1959) established the benzoquinone to be coenzyme Q₈.

Difference spectra were measured in a Cary 15 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.). Cell suspensions examined in the spectrophotometer contained about 6 mg per ml of protein as estimated by the method of Lowry et al. (1951). The suspension in the top cell compartment was reduced with sodium dithionite, and that in the reference compartment was oxidized with 0.3% hydrogen peroxide.

Production of pyruvate and lactate by the cells was measured as previously described (Bragg and Polglase, 1962).

RESULTS AND DISCUSSION

The chromatographic adsorbent, Magnesol-Celite (McNeely et al., 1945), proved very effective in the separation of vitamin K₂ and coenzyme Q₈. These lipoquinones were firmly bound by the adsorbent in the presence of isooctane. Vitamin K₂ was eluted completely with a rela-

tively small volume of isooctane containing 1% ether, and all of the coenzyme Q₈ remained on the column. Coenzyme Q₈ was then (usually) completely eluted in 10 ml of isooctane containing 1% ethanol. The use of Magnesol-Celite as described appears to offer the advantage of requiring smaller quantities of cells for extraction and smaller volumes of solvent for column elution than does the use of Decalso as described by Lester and Crane (1959).

Streptomycin-dependent *E. coli* cells grown with an optimal concentration of dihydrostreptomycin (normal DA) were found to contain amounts of coenzyme Q₈ and vitamin K₂ comparable with those reported for *E. coli* by Bishop et al. (1962). Depleted DA cells showed a three-fold increase in vitamin K₂ over normal DA, and the coenzyme Q₈ content decreased slightly (Table 1). When the depleted cells were placed in medium supplemented with dihydrostreptomycin, the vitamin K₂ content decreased to one-half of the depleted value in 1 hr (Table 1). In several similar experiments, it was found that the vitamin K₂ content increased during depletion and decreased to normal values after growth on medium containing dihydrostreptomycin. An additional quantity of coenzyme Q₈ was extracted from the cells after saponification (Gale et al., 1963), but no additional vitamin K₂ was obtained (Table 1).

The difference spectrum of normal DA cells showed peaks at 560 and 530 mμ (cytochrome b), 590 mμ (cytochrome a₁), and 625 mμ (cytochrome a₂). (For nomenclature, see Smith, 1961.) In addition, a trough at 469 mμ was observed,

TABLE 1. Effect of dihydrostreptomycin depletion and supplementation on lipoquinone content of streptomycin-dependent *Escherichia coli* DA*

Cell type	Coenzyme Q ₈	Vitamin K ₂
Normal DA	0.70	0.044
Depleted DA	0.60 (+.22)†	0.134
Depleted DA after 1 hr of growth with dihydrostreptomy- cin	0.51 (+.23)†	0.067

* For growth conditions see Materials and Methods. Results are expressed as mg/g of dried cells.

† After saponification.

corresponding to reduced flavine. In depleted DA cells, the cytochrome *b* peaks and the flavine trough were essentially unchanged, but the cytochrome *a*₁ and *a*₂ peaks were absent (Fig. 1). Then, 3 hr after the addition of fresh medium containing dihydrostreptomycin to depleted DA cells, cytochrome *a*₁ and *a*₂ were observed in the difference spectrum (Fig. 1).

The disappearance of glucose from the medium containing depleted DA cells was accompanied by the formation of considerable amounts of lactic acid (Table 2). In contrast, depleted DA cells in dihydrostreptomycin-containing medium metabolized glucose without formation of lactic acid (Table 2). These observations indicate that, functionally, the depleted DA cells were more anaerobic than were normal DA cells. The accumulation of lactic acid by the depleted DA cells correlated with the absence of cytochromes *a*₁ and *a*₂. Both of these abnormalities were corrected when depleted DA cells were grown in medium containing dihydrostreptomycin; i.e., cytochromes *a*₁ and *a*₂ reappeared and lactic acid disappeared (Table 2). According to Castor and Chance (1959), cytochromes *a*₂ and *o* are the terminal oxidases of *E. coli*. Engelberg and Artman (1961) found that a strain of streptomycin-dependent *E. coli* grown in a minimal concentration of antibiotic lacked cytochrome *o*, i.e., the carbon monoxide-sensitive oxidase. However, cytochromes *a*₁ and *a*₂ could not be detected by these workers in a dependent *E. coli* grown with either minimal or optimal antibiotic con-

TABLE 2. *Effect of dihydrostreptomycin depletion and supplementation on acid production by streptomycin-dependent Escherichia coli DA**

Cell type	Time	Glucose consumed	Pyruvic acid	Lactic acid
	hr	μmoles	μmoles	μmoles
Depleted DA	1	320	3.0	151
	2	630	14.0	140
	3	1034	2.0	300
Depleted DA + dihydrostreptomycin	1	320	2.0	0
	2	540	10.8	0
	3	1634	11.8	0

* For growth conditions see Materials and Methods.

centration. Although we have not determined the cytochrome *o* content of our dependent strain, it seems likely that the terminal oxidase systems of the strain of dependent *E. coli* used by Engelberg and Artman (1961) and the strain used by us are different.

Neither coenzyme Q₈ nor cytochrome *b* changed appreciably in total amount during antibiotic deprivation or supplementation (Table 1, Fig. 1). On the other hand, the increase in vitamin K₂ was accompanied by a decrease in cytochromes *a*₁ and *a*₂, and this may imply a functional relationship between vitamin K₂ and these cytochromes.

In previous work with an extract from streptomycin-sensitive *E. coli* (Bragg and Polglase, 1963b), we observed an inhibition by dihydrostreptomycin of the reduction of triphenyltetrazolium chloride (TTC) in the presence of succinate. Although the significance to the organism of succinate-TTC reductase activity is not known, the action of dihydrostreptomycin on this reaction implicates the electron-transport chain as a site of action of the antibiotic in sensitive *E. coli*. Thus, both in streptomycin-sensitive and in streptomycin-dependent *E. coli*, effects of the antibiotic on the electron-transport system have been observed. Since the electron-transport components of bacteria are believed to be located in the cytoplasmic membrane (see Smith, 1961), the effects of dihydrostreptomycin in the dependent strain may be related to the damage by streptomycin to the cell membrane of sensitive *E. coli* reported by Anand and Davis (1960). We observed that depleted DA cells are relatively fragile and more easily ruptured by sonic or mechanical treatment than are normal DA cells.

FIG. 1. *Difference spectra (reduced/oxidized) of antibiotic-depleted streptomycin-dependent Escherichia coli (DA). (a) After growth for 3 hr in medium containing dihydrostreptomycin. (b) No additions.*

It can thus be concluded that the integrity of the electron-transport chain and thus of the bacterial cell membrane is antibiotic-dependent in *E. coli* requiring streptomycin for growth. However, the question of the primary site of action of streptomycin is not yet answered, since the effects herein recorded may be secondary to such phenomena as protein synthesis or coupled phosphorylation.

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