Respiration-dependent uptake of dihydrostreptomycin by *Escherichia coli*

Its irreversible nature and lack of evidence for a uniport process

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The transport of [^3H]dihydrostreptomycin into the cytoplasm of *Escherichia coli* was distinguished, by its respiration-dependent nature, from binding within the cell envelope. 1. Of the radiolabel in the cytoplasm, 70–90% was dissolved in, or quickly equilibrated with, the cytoplasmic aqueous phase because this proportion rapidly left cells treated with toluene or with butan-1-ol. 2. After a period of respiration-dependent uptake of [^3H]dihydrostreptomycin, cells were washed repeatedly by centrifugation and resuspension. Radiolabel did not leave the cells at any appreciable rate. 3. Uptake of dihydrostreptomycin (at an exogenous concentration of 1 mg of base/ml) was monitored for 2 h to an apparent equilibrium. Then the specific radioactivity of exogenous dihydrostreptomycin was raised without significantly altering its chemical concentration. There was no exchange of radiolabel between the exogenous pool and the cytoplasmic pool. 4. Dihydrostreptomycin was not taken up by respiring, cytoplasm-free membrane vesicles which accumulated L-proline in control experiments. These data support the view that respiration-dependent uptake of dihydrostreptomycin by *E. coli* is not simply a secondary translocation process such as uniport.

Dihydrostreptomycin exerts its antibiotic action at the level of the ribosome (Spotts & Stanier, 1961; Brock, 1966; Gale *et al.*, 1981). It is presumed therefore that it must cross the bacterial cytoplasmic membrane before the antibiotic effect can occur (Kogut & Carrier, 1980). The dihydrostreptomycin molecule is large (M_r of the 3^+ -charged base = 586) and polar (it carries two positively charged guanidino groups) so that it is unlikely to diffuse freely across the cytoplasmic membrane at any appreciable rate (Mitchell, 1958). Despite a large literature [see Hancock (1981*a,b*) for a comprehensive review] the identity and properties of the transport system(s) for dihydrostreptomycin have not been unequivocally defined. Moreover the nature of energy-coupling to the process is obscure.

When dihydrostreptomycin at concentrations between 5 and 30 μg of base/ml is added to an aerobically-growing culture of susceptible *Escherichia coli*, three consecutive phases occur. First

there is a rapid metabolism-independent binding of antibiotic to the cells (McQuillen, 1951; Anand *et al.*, 1960; Hurwitz & Rosano, 1962; Brock, 1966; Bryan & Van den Elzen, 1976). This is followed by a lag period of up to 30 min during which either no further net uptake of dihydrostreptomycin occurs (Anand *et al.*, 1960; Dubin *et al.*, 1963) or during which slow respiration-dependent uptake occurs (Bryan & Van den Elzen, 1976). The third phase follows, beginning with the onset of rapid respiration-dependent uptake of dihydrostreptomycin (Hurwitz & Rosano, 1962; Dubin *et al.*, 1963; Bryan & Van den Elzen, 1976). The end of the third phase has not been defined (Hancock, 1981*a*).

A current proposal for the mechanism of the respiration-dependent, third phase of uptake is that the transport system allows positively-charged dihydrostreptomycin to diffuse across the cytoplasmic membrane in response to the inwardly-directed transmembrane electric potential difference ($\Delta\psi$). This hypothesis was implied by Damber & Epstein (1981) and proposed by Bryan & Kwan (1983) in their general model for aminoglycoside uptake and killing in bacteria. Translocation across the cytoplasmic membrane as outlined

Abbreviations used: DHBP, 3,4-dihydroxybutyl-1-phosphonate; EDP-I and EDP-II, energy-dependent phases I and II.

above is defined as a uniport process (Mitchell, 1967, 1970; Bryan & Kwan, 1983; Young & Nichols, 1984). Here we report studies to examine whether the respiration-dependent uptake of dihydrostreptomycin by *E. coli* is indeed such a passive (i.e. the direction of flux being determined solely by the transmembrane difference in electrochemical potential of the substrate) process. In particular we investigated whether respiration-dependent uptake was reversible (it was not) and whether cytoplasmic membrane vesicles accumulated dihydrostreptomycin by a respiration-dependent process (they did not).

Experimental

Growth of the organism

E. coli K12 (N.C.T.C. 10538) was grown with shaking at 37°C in the liquid minimal medium described by Davis & Mingioli (1950) except that disodium succinate (10 g/l) was the carbon source in place of glucose. Cells were harvested in the exponential phase of growth (density ≤ 0.5 mg dry wt./ml; $A_{650}^{1\text{cm}} \leq 1.0$).

The minimum inhibitory concentration of dihydrostreptomycin for *E. coli* K12 was 0.8–1.0 µg of base/ml by the agar-incorporation method (Barry, 1980).

Preparation of membrane vesicles

Membrane vesicles were prepared by the method of Kaback (1971), the final vesicle pellet being resuspended in 0.1 M-potassium phosphate (38.7 mM-K₂HPO₄/61.3 mM-KH₂PO₄, pH 6.6) brought to 10 mM-EDTA using 0.5 M-EDTA (adjusted at room temperature to pH 7.0 with NaOH solution). The vesicles were frozen, and then stored, in liquid N₂. The protein concentrations of suspensions of membrane vesicles were measured by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Uptake of [³H]dihydrostreptomycin by whole cells

Cells were harvested at 4°C by centrifugation at 2300 g (r_{av} , 16 cm) for 15 min. They were then washed twice by resuspension in about the same volume as the growth medium of ice-cold 0.1 M-NaCl/10 mM-Hepes (pH 7.0) followed by recentrifugation as above. The final pellet was resuspended in ice-cold 50 mM-Hepes (pH 7.0) to 1–2 mg dry wt. of cells/ml and was stored in crushed ice.

[³H]Dihydrostreptomycin was obtained from Amersham International as 1 mCi/ml, 669 µg of base/ml. From this we prepared 10 ml volumes of working stock solutions which had approx. 305 µg of dihydrostreptomycin base/ml and 40 µCi/ml.

The 'reaction mixture' for the uptake experiments was held at 37°C in the well of a water-jacketed, Clark-type oxygen electrode. This allowed continuous monitoring of dissolved oxygen during each experiment. The vessel contents were either oxygenated or rendered anaerobic by bubbling with water-saturated O₂ or O₂-free N₂ respectively. The reaction mixture contained 50 mM-Hepes (pH 7.0), cells at about 0.3 mg dry wt./ml and 41 mM-succinate (pH 7.0). Uptake was started by the addition of [³H]dihydrostreptomycin.

At times indicated in the Figures, 100 µl volumes of reaction mixture were removed and diluted in 2.0 ml of 0.1 M-NaCl/10 mM-Hepes (pH 7.0) held at 37°C. The diluted mixture was immediately vacuum filtered (Oxoid 'Nufflow' 25 mm diameter filters, 0.45 µm pore size; the filters were pre-soaked in a solution of 100 µg of dihydrostreptomycin base/ml) and two further 2 ml portions of the diluent at 37°C were used in turn to wash the tube, filter funnel and filter. For determining specific radioactivity, five 25 µl samples of reaction mixture were removed and put directly onto quarter-size pieces of filter. Filters were dried, placed in plastic scintillation minivials and covered with scintillation fluid [5.0 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(5-diphenyloxazol-2-yl)benzene/litre of toluene]. A further vial containing scintillation fluid only was used to determine a background count rate. Samples were counted for 10 min on an Isocap/300 (Searle), or a 1218 Rackbeta (LKB), liquid-scintillation counter. Values of c.p.m. were corrected for the background count rate. The samples for measuring cell-associated dihydrostreptomycin were also corrected for radiolabel which bound to the filters (measured in a separate experiment).

Removal of exogenous [³H]dihydrostreptomycin following a period of respiration-dependent uptake by intact cells

The uptake of [³H]dihydrostreptomycin at 15 µg of base/ml was monitored for 225 min as described above. Subsequent resuspensions (below) were in 0.1 M-NaCl/10 mM-Hepes (pH 7.0). Measurements of cell-associated [³H]dihydrostreptomycin were made by subjecting 10 µl, 50 µl or 100 µl samples to the filtration assay described above. Measurements of supernatant [³H]dihydrostreptomycin were made by placing 50 µl samples on pieces of filter, drying and counting as described above. After 225 min, 4.2 ml of the reaction mixture was added to 60 ml of buffer at 37°C. The suspension was stirred for 15 min at 37°C with sampling. It was then centrifuged at 10000 g (r_{av} , 7 cm) for 15 min at room temperature (20°C). The pellet was resuspended in 5.0 ml of buffer at 37°C

and cell-associated [^3H]dihydrostreptomycin was measured. A further 60 ml of buffer was then added and the suspension was again stirred for 15 min at 37°C with sampling. The cells were resedimented as above, the pellet was resuspended in 15 ml of buffer at 37°C and the cell-associated dihydrostreptomycin was measured. Three 5.0 ml portions were treated as follows. One was chilled in an ice/water slurry for 10 min followed by five 20 s bursts of sonication (MSE Soniprep 150 focused for maximum agitation, an amplitude of roughly 14 μm) with cooling at 0°C for 1 min between bursts. Cell-associated dihydrostreptomycin was measured (i) following chilling and (ii) following sonication. Toluene (0.1 ml, 2% v/v) was added to the second portion at room temperature and the mixture was stirred for 10 min. Cell-associated dihydrostreptomycin was then measured. Butanol (0.35 ml, 6.5% v/v) was added to the third portion which was stirred and sampled as in the toluene-treatment above.

Uptake of [^3H]dihydrostreptomycin by membrane vesicles

The reaction mixture was again placed in the well of the oxygen electrode vessel at 37°C. The reaction mixture (volume 1.9 ml) consisted of 10 mM-potassium phosphate (pH 6.6) and vesicles at a density of 1.0 mg of protein/ml. At zero time, 66 μl of the working stock [^3H]dihydrostreptomycin solution was added, either with or without simultaneous addition of 20 μl of unlabelled solution (100 mg of base/ml). At 8 or 11 min (as shown on the relevant Figure), 40 μl of 0.5 M-sodium succinate (pH 6.6) was added and oxygenation was started if necessary. At intervals, 50 μl samples were withdrawn and treated as described above for intact cells except that the diluent/wash solution was 0.1 M-NaCl and filters of 0.22 μm pore size were used.

Uptake of [^{14}C]proline by membrane vesicles

L-[^{14}C]Proline was obtained from Amersham International as a solution containing 0.176 mM-proline and 50 $\mu\text{Ci/ml}$. A working stock solution of final concentrations 1.0 mM and 5 $\mu\text{Ci/ml}$ was prepared. The uptake of [^{14}C]proline at a concentration of 15 μM was assayed as described for [^3H]dihydrostreptomycin except that the experiment was started by the addition of [^{14}C]proline solution and that the diluent in one case contained 1 mM-MgCl₂.

Chemicals

Dihydrostreptomycin sesquisulphate was from Sigma. Sucrose was castor sugar from Tate & Lyle, Croydon, Surrey, U.K.

Results

Respiration-independent uptake of dihydrostreptomycin

Under anaerobic conditions the plateau level of binding of dihydrostreptomycin (10.5 μg of base/ml) to cells was 248 (S.D. = 56, $n = 19$) ng/mg dry wt. Plateau levels of binding to cells inhibited by *N*-ethylmaleimide or by inhibitors of respiration were similar. The concentration-dependence of this respiration-independent binding followed a Langmuir adsorption isotherm (Oscik, 1982) with an apparent adsorption equilibrium constant of 2.7 ml/mg of base and a saturation level of binding of 11 μg of base/mg dry wt. of cells. There were 2.9×10^9 cells/mg dry wt., which gives about 4×10^6 adsorption sites/cell.

Respiration-dependent uptake of dihydrostreptomycin

Under conditions of aeration the uptake of [^3H]dihydrostreptomycin followed the time course shown in Fig. 1(a) (see also Nichols & Young, 1983; Young & Nichols, 1984). Despite several experiments we were unable to distinguish reproducibly between a plateau before the onset of rapid uptake (Anand *et al.*, 1960; Dubin *et al.*, 1963) and a slow, respiration-dependent rise in cell-associated dihydrostreptomycin as reported by Bryan & Van den Elzen (1976). We have analysed our results as if a plateau occurred. The level of this early plateau was estimated as shown by the lower broken lines in Figs. 1(a) and 1(b). The results of ten such estimations gave a mean value of 292 (S.D. = 78) ng of base/mg dry wt. of cells at a concentration of 10.5 μg of base/ml. This was not significantly different from the above amount of respiration-independent binding ($t = 1.75$ at 27 d.f., $P > 0.05$).

Fig. 1 shows initial experiments designed to assess the reversibility of respiration-dependent dihydrostreptomycin uptake. Removal of the driving force for dihydrostreptomycin uptake by making the cells anaerobic did not result in efflux of dihydrostreptomycin taken up (Fig. 1b; Young & Nichols, 1984). Addition of unlabelled dihydrostreptomycin to a concentration 100-fold greater than that of the radiolabelled dihydrostreptomycin during respiration-dependent uptake caused a slight loss of cell-associated radiolabel (Fig. 1a). A steady rate of uptake was then re-established. Extrapolating back, as shown with the upper broken line in Fig. 1(a), allowed an estimation of an amount of dihydrostreptomycin apparently lost from the cells (the loss was apparent, because the calculations used the specific radioactivity present at the start of the experiment rather than the actual specific radioactivity obtained after the addition of

Fig. 1. Time courses of uptake of [^3H]dihydrostreptomycin by respiring *Escherichia coli*: the effects of adding unlabelled dihydrostreptomycin (a) during respiration-dependent uptake and (b) when respiration-dependent uptake had been halted by making the suspension anaerobic

The lower broken line in (a) and the broken line in (b) show how initial apparent plateau levels of uptake were estimated. When unlabelled dihydrostreptomycin was added the subsequent data points were calculated as if the specific radioactivity remained unchanged. (a) ○, 15 μg of dihydrostreptomycin base/ml; ●, 10.5 μg of dihydrostreptomycin base/ml. At 80 min (arrow) unlabelled dihydrostreptomycin was added to 1.23 mg of base/ml. (b) ○, 10.0 μg of dihydrostreptomycin base/ml. At 80 min (arrow) the O_2 line was withdrawn and the suspension was bubbled with water-saturated N_2 . ●, As for (○) except that at 100 min unlabelled dihydrostreptomycin was added to 0.99 mg of base/ml; △, uptake under anaerobic conditions for comparison (10.0 μg of base/ml).

unlabelled dihydrostreptomycin). For a narrow range of dihydrostreptomycin concentrations (10.0 or 10.5 μg of base/ml added at zero time followed by the addition of unlabelled dihydrostreptomycin to a final concentration of 0.95 or 0.99 mg of base/ml) the apparent losses of dihydrostreptomycin from the cells under respiring conditions were as follows. Before the onset of rapid uptake it was 54–148 ng of base/mg dry wt. and during rapid uptake (e.g. Fig. 1a) it was 87–120 ng of base/mg dry wt. Under anaerobic conditions a similar reversibility was observed (although then the rapid uptake phase was absent) and the apparent loss of dihydrostreptomycin from the cells was 63–104 ng of base/mg dry wt. The apparent losses from cells inhibited from time zero by *N*-ethylmaleimide or azide were 112 and 47 ng of base/mg dry wt., respectively. Fig. 1(b) shows an experiment in which an excess of unlabelled dihydrostreptomycin was added to cells after respiration-dependent uptake had been halted by making the suspension anaerobic. The mean apparent loss of dihydrostreptomycin from the cells in two such experi-

ments was 100 ng of base/mg dry wt. Although respiration-dependent uptake of dihydrostreptomycin was halted by *N*-ethylmaleimide, *N*-ethylmaleimide did not prevent the type of exchange shown at 80 min in Fig. 1(a) and at 100 min in Fig. 1(b) (Nichols & Young, 1983). The mean apparent loss of dihydrostreptomycin from the cells measured in two experiments where *N*-ethylmaleimide was added followed by unlabelled dihydrostreptomycin was 50 ng of base/mg dry wt.

Fig. 2 shows what occurred when, after a period of rapid respiration-dependent dihydrostreptomycin uptake, the exogenous dihydrostreptomycin was reduced first to a low, and then to a negligible, concentration. Sonication of the second resuspension resulted in a loss of about half of the cytoplasmic dihydrostreptomycin, from 1200 to 690 ng of base/mg dry wt. of cells (Fig. 2f). Adding toluene (2%, v/v) to the cells resuspended the second time resulted in a reduction in cell-associated dihydrostreptomycin of 86%, from 1230 to 172 ng of base/mg dry wt. (Fig. 2g). Exposure of the cells to butan-1-ol (6.5%, v/v) resulted in a

Fig. 2. Dihydrostreptomycin in the cytoplasm did not flow out of cells of *Escherichia coli* when exogenous dihydrostreptomycin was removed.

(a) Initial respiration-dependent uptake. The early plateau of respiration-independent binding (380 ng of base/mg dry wt. of cells) has been subtracted from each data point. (b) A 15-fold dilution of the reaction mixture from (a). The exogenous concentration of dihydrostreptomycin was 0.92 μg of base/ml. (c) The cells were sedimented and then resuspended in 5 ml of buffer. (d) The suspension was diluted a further 12-fold. The exogenous concentration of dihydrostreptomycin was 0.023 μg of base/ml. (e) The cells were sedimented and then resuspended in 15 ml of buffer. (f) The effect of sonication: (i) a sample of the suspension from (e) was chilled; (ii) the chilled suspension was sonicated for a total of 100s. (g) and (h) Addition of toluene and butan-1-ol respectively to samples of the suspension from (e).

similar reduction in cell-associated dihydrostreptomycin, from 1230 to 148 ng of base/mg dry wt. (Fig. 2h). Two other similar experiments were carried out and the results were qualitatively the same.

With dihydrostreptomycin at the concentrations used in the experiments shown in Figs. 1 and 2 (10–15 μg of base/ml), it would be necessary to monitor uptake for more than 4 h in order to observe a plateau level of intracellular dihydrostreptomycin (Fig. 2a). Increasing the dihydrostreptomycin concentration to 1 mg of base/ml resulted in attainment of a plateau of respiration-dependent uptake in about 2 h (Figs. 3 and 4). Fig. 3 shows that the addition of toluene to 2.5% (v/v) or of butan-1-ol to 8.7% (v/v) caused a loss of about 70% of cell-associated dihydrostreptomycin under such apparent equilibrium uptake-level conditions. The

amount of dihydrostreptomycin associated with the cells after butan-1-ol or toluene treatment was similar to that observed in the first 40 min when cells were anaerobic from the start of the experiment (Fig. 3).

Fig. 4 demonstrates that after a plateau level of uptake had been attained, intracellular dihydrostreptomycin did not exchange with extracellular dihydrostreptomycin. This is shown in two ways. Firstly, when the dihydrostreptomycin of high specific radioactivity was added after attainment of the plateau of uptake, the cell-associated radiolabel did not increase to the value observed in the control experiment. Secondly the experiment represented by the solid symbols in Fig. 4 may be analysed as follows to calculate the sizes of exchangeable and non-exchangeable pools of radio-

Fig. 3. Effects of adding toluene or butan-1-ol to cells of *Escherichia coli* which had carried out respiration-dependent uptake of dihydrostreptomycin to a plateau

The concentration of dihydrostreptomycin was 1.05 mg of base/ml. ○, Control; △, ●, butan-1-ol (△) or toluene (●) were added to the concentrations (v/v) 8.7% or 2.5% respectively at 160 min (arrow); □, uptake under anaerobic conditions for comparison.

Fig. 4. Exchange of cell-associated dihydrostreptomycin with exogenous dihydrostreptomycin after attainment of a plateau level of respiration-dependent uptake

(●) At zero time the experiment was started by the addition of [^3H]dihydrostreptomycin of low specific radioactivity (30 mg of base/ml, 8 $\mu\text{Ci/ml}$) to a concentration of 1.0 mg of base/ml. At 120 min (arrow) high specific radioactivity [^3H]dihydrostreptomycin (322 μg of base/ml, 42 $\mu\text{Ci/ml}$) was added to an extra concentration of 10.9 μg of base/ml. (○) Control experiment. The two solutions of [^3H]dihydrostreptomycin were added together at zero time to give the same final proportions of each.

label. Let M be the amount of dihydrostreptomycin taken up which exchanged with the exogenous pool (reversible uptake). Let N be the amount of dihydrostreptomycin taken up which did not exchange with the exogenous pool (irreversible uptake). Let A_1 be the specific radioactivity

(116 c.p.m./ μg of base) of the dihydrostreptomycin added at zero time. Let A_2 be the specific radioactivity (681 c.p.m./ μg of base) of exogenous dihydrostreptomycin after the addition of the second solution. Let C_1 be the radioactivity taken up at the first plateau (8233 c.p.m./mg dry wt. of cells), i.e. before the addition of the second solution at 120 min. Let C_2 be the radioactivity taken up at the second plateau (17070 c.p.m./mg dry wt. of cells). The first plateau represented irreversible plus reversible uptake at the first specific radioactivity:

$$C_1 = A_1(N + M).$$

The cell-associated radiolabel at the second plateau was due to the irreversible uptake at the first specific radioactivity and the reversible uptake at the second specific radioactivity so that:

$$C_2 = A_1N + A_2M.$$

M remained unchanged because the change in exogenous dihydrostreptomycin concentration was negligible. Combining these two equations yields:

$$M = (C_2 - C_1)/(A_2 - A_1).$$

Using this resulting equation we obtained a value for M of 15.6 μg of base/mg dry wt. of cells. The amount of exchangeable cell-associated dihydrostreptomycin was thus similar to the amount adsorbed under anaerobic conditions (8–15 μg of base/mg dry wt. of cells). The above value of M was put into the first equation and N , the non-exchangeable fraction, was calculated to be 55.1 μg of base/mg dry wt. This was similar to the 35 $\mu\text{g}/\text{mg}$ dry wt. lost from the cells after treatment with toluene or butan-1-ol (Fig. 3). In a second identical experiment the amount of exchangeable dihydrostreptomycin associated with the cells was 11.8 μg of base/mg dry wt. and the non-exchangeable fraction was 53.2 μg of base/mg dry wt.

Membrane vesicles

Cytoplasmic membrane vesicles from *E. coli* showed active uptake of [^{14}C]proline which was dependent on the addition of succinate (Fig. 5a). In other words they behaved as normal 'active transport' membrane vesicles (Kaback, 1972). Figs. 5(b) and 5(c) show that these vesicles did not take up [^3H]dihydrostreptomycin under conditions under which they did take up proline. Succinate-driven dihydrostreptomycin uptake into membrane vesicles was absent whether dihydrostreptomycin was present at a concentration of 10 μg of base/ml (Fig. 5b) or 1 mg of base/ml (Fig. 5c). These findings are similar to those of Bryan & Kwan (1983) who noted a lack of respiration-driven uptake of [^3H]gentamicin into membrane vesicles prepared from *E. coli* by the method of Kaback (1971).

Fig. 5. Lack of respiration-dependent uptake of dihydrostreptomycin by membrane vesicles from *Escherichia coli*

The arrows show the addition of succinate. (a) Proline uptake (control). The solution used for diluting and washing the samples was 0.1 M-NaCl/1 mM-MgCl₂ (▲) or 0.1 M-NaCl (△). (b) Dihydrostreptomycin (10.3 μg of base/ml) uptake under anaerobic (●) or aerobic (○) conditions. (c) Uptake under aerobic conditions at 1 mg of dihydrostreptomycin base/ml (□).

Discussion

We propose that the initial (i.e. within 10–40 min) uptake of dihydrostreptomycin to a plateau under aerobic conditions (Fig. 1) was due to the antibiotic binding to cells outside the cytoplasmic membrane by a metabolism-independent process. This is the same conclusion as was reached by Anand *et al.* (1960), Dubin *et al.* (1963) and Bryan

& Van den Elzen (1976). The second phase (see the introduction for an explanation of the three phases of dihydrostreptomycin uptake) was one during which little further uptake of dihydrostreptomycin occurred before the onset of rapid, respiration-dependent transport (Fig. 1). Some authors concluded that no uptake occurred during this phase (Anand *et al.*, 1960; Dubin *et al.*, 1963) and others that slow, respiration-dependent uptake occurred (Bryan & Van den Elzen, 1976, 1977). When the second phase of dihydrostreptomycin uptake was assumed to be a plateau, estimating this plateau for ten experiments gave a value indistinguishable from the plateau observed under anaerobic conditions in 19 experiments (see the Results section). Thus in our experiments significant uptake did not occur during this second phase. The third phase of dihydrostreptomycin uptake was rapid, respiration-dependent uptake (Andry & Bockrath, 1974; Bryan & Van den Elzen, 1976; Hancock, 1981a; Fig. 1b). This phase has been called 'EDP-II' by Bryan & Van den Elzen (1977). Previous workers have concluded that during this phase, dihydrostreptomycin crosses the cytoplasmic membrane to enter the cytoplasm (Andry & Bockrath, 1974; Bryan & Van den Elzen, 1976; Holtje, 1978). It was this process which our experiments were intended to characterize further.

Our principal observation was that respiration-dependent uptake was irreversible. Dihydrostreptomycin did not flow out of the cells when the driving force for uptake was removed by making the suspension anaerobic (Young & Nichols, 1984; Fig. 1b), by inhibiting respiration (Andry & Bockrath, 1974; Bryan & Van den Elzen, 1976) or by adding the uncoupler dinitrophenol (Andry & Bockrath, 1974). In contrast, when either the electron transport inhibitor azide or the uncoupler dinitrophenol were added to *E. coli* containing the growth inhibitor DHBP in radiolabelled form, about 70% of the radiolabel flowed out of the cells in 5 min (Leifer *et al.*, 1977). Secondly, repeated dilution, centrifugation and resuspension of cells following a period of respiration-dependent uptake of [³H]dihydrostreptomycin did not wash out radiolabel from the cytoplasm (Fig. 2). Again, in contrast, greater than 90% of accumulated [³H]DHBP was lost from *E. coli* on washing with distilled water (Leifer *et al.*, 1977). Thirdly, the irreversible nature of respiration-dependent dihydrostreptomycin uptake was also evident from the lack of exchange diffusion between exogenous and cytoplasmic pools of the antibiotic (Fig. 4). Attempts to detect a decrease in cytoplasmic radiolabel following the addition of excess unlabelled dihydrostreptomycin to the medium during respiration-dependent uptake were more difficult to interpret. However neither was there in those

experiments any evidence of exchange between cytoplasmic and exogenous dihydrostreptomycin (Figs. 1a and 1b; Nichols & Young, 1983; Young & Nichols, 1984). Once more in contrast, the DHBP transport system of *E. coli* does catalyse exchange diffusion between exogenous and cytoplasmic DHBP (Leifer *et al.*, 1977).

There are several possible explanations for respiration-dependent uptake of dihydrostreptomycin being irreversible. Three are discussed briefly below. Firstly dihydrostreptomycin could be bound irreversibly to ribosomes in the cytoplasm as suggested by Kogut & Carrier (1980). This is unlikely because dihydrostreptomycin binding to ribosomes *in vitro* is rapidly reversible (Chang & Flaks, 1972). Moreover, when the cytoplasmic membrane was made permeable to small molecules by treatment with toluene (Jackson & DeMoss, 1965; Andry & Bockrath, 1974) or butan-1-ol (Tabor & Tabor, 1966) radiolabel rapidly left the cells (Figs. 2 and 3). Secondly translocation across the membrane might be a rapidly reversible, passive process such as uniport (Bryan & Kwan, 1983) with irreversibility being imposed by covalent modification of dihydrostreptomycin making it a poor substrate for the porter in the in $\rightarrow$ out direction. However a rapidly equilibrating porter would be expected to mediate respiration-dependent transport of dihydrostreptomycin into cytoplasm-free membrane vesicles. This did not occur (Fig. 5). Thirdly, respiration-dependent uptake could be coupled to a chemical reaction, not necessarily involving dihydrostreptomycin. Such a process would be termed primary translocation (Mitchell, 1967) and examples are the binding protein-dependent transport systems reviewed by Dills *et al.* (1980). This would also explain why no uptake occurred in cytoplasmic membrane vesicles, but we have not tested the suggestion further.

Gale *et al.* (1981) have pointed out that the irreversible step in the bactericidal action of dihydrostreptomycin is unknown. They speculated that transport into the cytoplasm might be the irreversible step. Our results are consistent with this view.

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