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Action of Streptomycin on *Vibrio cholerae*

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Arora & Krishna Murti (1955) showed that broad-spectrum antibiotics inhibit oxygen uptake by *Vibrio cholerae* cells in the presence of the tricarboxylic acid-cycle intermediates, but have no perceptible effect on the corresponding oxidations in animal-tissue homogenates. The availability of a *V. cholerae* strain sensitive to streptomycin and a resistant mutant derived from it has made it possible to extend this work, which was initiated originally with the object of elucidating the mechanism of action of antibiotics (Krishna Murti, 1959), to a more detailed evaluation of streptomycin inhibition. The results presented in this paper support our earlier findings and indicate that succinic dehydrogenase and fumarase are among the enzymes inhibited by streptomycin.

METHODS AND MATERIALS

Organisms. A strain of *V. cholerae* Inaba S^S (InSS), and a streptomycin-resistant mutant derived from it, Inaba S^R (InSR), and Ogawa 82/L were kindly supplied by Dr K. Bhaskaran of this Institute.

Medium. Nutrient agar made of Lab-Lemco (1%, w/v), Difco Peptone (1%, w/v), Marmite (0·5%), NaCl (0·5%) and Difco agar (2·5%) was used throughout for the maintenance and growth of the organisms. Before the addition of agar and before autoclaving, the broth was adjusted to pH 8·2 (glass electrode) with 2N-NaOH. Subculturing on this medium was carried out every fortnight.

Washed suspensions. Roux flasks containing 80 ml. of nutrient agar were seeded with an overnight growth of the organism in 10 ml. of nutrient broth. The cells were harvested with ice-cold 0·85% NaCl solution after 18 hr. growth at 37°. Since previous studies of Iyer, Dudani, Krishna Murti & Shrivastava (1953–54) had emphasized the importance of aqueous NaCl for washing and preparing cells of vibrios for enzymic studies, the organisms were washed three times with cold 0·85% NaCl solution on the centrifuge (1500 *g* for 20 min. at 5°) and finally suspended in 0·85% NaCl solution. For manometric studies the opacity of the suspension was adjusted to a Klett reading of 250 (red filter 660 mμ). Dry-weight determination of random samples of the suspensions showed that at this opacity value they contained 1 mg. bacterial dry wt./ml. Cell suspensions thus prepared showed a negligible endogenous O_2 uptake.

Cell-free extracts. These were prepared by sonic oscillation (at 25 kcyc./sec. with an output amperage of 2·5A) from freshly harvested cells suspended in cold 0·85% NaCl solution in a 500w Mullard Magneto strictor oscillator. The cell suspension was kept in a stainless-steel inducer flask which was cooled by continuous circulation of chilled water in an outer vessel. Cells subjected to impulses below 15 kcyc./sec. showed little lysis. A 15 min. exposure at 25 kcyc./sec., however, lysed the cells completely, as was evident by the clearing of turbidity and subsequent staining, which revealed less than 1% of Gram-negative bodies. The resulting material was centrifuged for 15 min. at 1500 *g* at 5° to remove unbroken cells and debris and the cell-free supernatant was stored in the frozen state at −10°.

An extract prepared as described was, however, devoid of succinic-dehydrogenase and fumarase activities. To obtain a cell-free preparation rich in these two enzymes, the following procedure was evolved. Cultures grown for 22 hr. on medium in 10 Roux flasks were harvested and washed as described before. The sedimented cells were maintained at −10° for 12 hr. The thawed mass was ground with acid-washed carborundum (200 mesh, Delhi Chemical Works, Delhi) in a chilled mortar and extracted with two 5 ml. portions of cold 0·85% NaCl solution. The extracts were freed from unbroken cells, cell debris and abrasive by centrifuging at 2000 *g* for 20 min. and precipitated at 5° by saturation with $(NH_4)_2SO_4$. The precipitated material was centrifuged off (800 *g* for 15 min. at 0°) dispersed in 5 ml. of cold 0·85% NaCl solution and dialysed against running cold distilled water for 18 hr. The volume of the suspension after dialysis was made up to 10 ml. with ice-cold glass-distilled water and clarified by centrifuging (800 *g* at 0° for 15 min.). The residue possessed appreciable succinic-dehydrogenase and fumarase activities and was used as such in inhibition studies.

Buffers. These were 0·2M-Na_2HPO_4–KH_2PO_4, pH 8·0, and 0·2M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl, pH 8·6.

Manometry. Air-filled Warburg manometers were used at 37°. Single-side-arm flasks contained buffer, cell suspension and drug in the main compartment and the substrate in the side arm. The total volume was maintained at 3 ml. The centre well always contained 0·2 ml. of 10% KOH with a 2 $cm.^2$ filter-paper roll.

Enzyme-assay methods. Nitrate reduction and acetoin production were estimated according to Clarke & Cown (1952), succinic dehydrogenase by methylene-blue reduction (Bonner, 1955) or tetrazolium reduction (Srikantan & Krishna Murti, 1955), fumarase by the optical method devised by Racker (1950), β-galactosidase according to Lederberg (1950) as modified by Kuby & Lardy (1953) and nucleases according to Sagar, Krishna Murti & Shrivastava (1958) after measurements of extinction at 260 mμ of the reaction products.

Glyoxalate production was tested in evacuated Thunberg tubes. Cell-free extract, buffer and inhibitor were placed in the main tube and *iso*citrate in the hollow bent stopper. In a typical assay the constituents were 100 μmoles of tris, 40 μmoles of DL-*iso*-citrate and 0·5 ml. of cell-free extract in

a total volume of 2 ml. The tubes were evacuated, closed and the contents mixed. After a 2 hr. incubation period at 37° the glyoxalate formed was fixed as a dinitrophenylhydrazone and identified by paper chromatography (Saxena, Krishna Murti & Shrivastava, 1956) with butan-1-ol saturated with 0·4 N-NH_3 solution as the solvent for development.

Analytical methods. Protein concentration in cells and cell-free extracts was measured after precipitation with a 5% trichloroacetic acid solution according to Lowry, Rosebrough, Farr & Randall (1951). Nucleic acid concentration was estimated by measuring extinction at 260 mμ. Dihydrostreptomycin was estimated by the nitroprusside colour according to Monastero (1952).

Incorporation experiments. The medium for incorporation of labelled glycine contained Casamino acids (Difco) supplemented with L-tryptophan and L-cystine, purines, pyrimidines, buffered salt solutions (Gale, 1947), cells and labelled glycine diluted with carrier glycine (5 mg./ml.). This was made up by mixing 16 ml. of salts–buffer, pH 8·0, 7 ml. of amino acid solutions containing 40 mg. of Casamino acids and 20 mg. each of L-tryptophan and L-cystine/ml. (pH 7·5), 2·8 ml. of purine–pyrimidine solution (1 mg. each of adenine sulphate, hypoxanthine, xanthine, guanine–HCl, thymine, and uracil/ml.), 7 ml. of 10% glucose and 10 ml. of labelled glycine diluted with carrier. This medium (6 ml.) thus constituted is referred to as 'incorporation medium' and was mixed with 3 ml. of cell suspension and 1 ml. of water or drug as indicated. The concentration of cells in the medium was adjusted so as to give 10 mg. of cell mass/ml. This concentration of cell mass was found to be the minimum required for the subsequent isolation and plating of labelled protein. The mixture kept in 500 ml. Erlenmeyer flasks was shaken in a rotary shaker at 37°. At intervals 2 ml. portions were added to 1 ml. of cold 20% trichloroacetic acid and centrifuged at 1500 *g* for 10 min. The sedimented cells were extracted with 10 ml. of 5% trichloroacetic acid containing 10 mg. of unlabelled glycine/ml., by placing the tubes in boiling water for 10 min., and the protein was sedimented by centrifuging at 1500 *g* for 15 min. The trichloroacetic acid extraction was repeated, the protein pellet finally dispersed in 0·5 ml. of aqueous NH_3 (sp.gr. 0·92) and plated on planchets; a drop of an aqueous solution (1% w/v), of dodecylquaternarypyridinium (Imperial Chemical Industries Ltd.) was used to facilitate the plating. The protein films were dried under an infrared lamp and counted in a thin-window Geiger–Müller counter. Counts up to 10 000 were made in active samples and the total count for a 30 min. period was recorded for weaker samples. Since 'infinitely thin' films of radioactive material were used for counting, no correction for self-absorption was made.

Carrier-free [2-^{14}C]glycine solution (1 μc/ml.), made by dissolving in 100 ml. of glass-distilled water, 1·94 mg. of the radioactive powder (The Radiochemical Centre, Amersham, Bucks.) having a total activity of 0·1 mc, was kindly supplied by Dr S. C. Agarwala of this Institute.

Protein synthesis by cells. The medium was essentially similar to the one used in incorporation experiments but the proportion of the individual constituents was different. It was made of 5 ml. of salts–buffer, pH 8·0, 0·8 ml. of Casamino acids supplemented with L-tryptophan and L-cystine, 0·2 ml. of purine–pyrimidine mixture, 1·0 ml. of 1% glucose solution and 1·5 ml. of water or drug solution. To this mixture was added 5 ml. of cell suspension to give a final concentration of 1 mg. of cell mass/ml. Flasks containing cell suspensions were shaken in a rotary shaker at 37°. Samples (1 ml.) were withdrawn at the specified intervals and pipetted into 1 ml. of 10% trichloroacetic acid. The tubes containing the precipitated cells were kept in boiling water for 10 min. and centrifuged for 15 min. at 2500 *g*. The supernatants were discarded, the residue was dissolved in alkaline copper reagent and their protein content estimated. Bovine plasma albumin (Armour Laboratories, Chicago, Ill., U.S.A.) was used as standard.

Isolation of nucleic acids. Freshly harvested and washed cells were suspended in 30 ml. of arsenate–citrate–0·85% NaCl medium (0·05 M-sodium dihydrogen arsenate and 0·1 M-sodium citrate in 0·85% NaCl solution, pH 7·0) and mixed with 5 ml. of 10% sodium deoxycholate solution. The suspension became very viscous and the pH rose to 7·5. The gel was washed into a 500 ml. Pyrex bottle with 30 ml. of arsenate–citrate–NaCl and mixed with 20 ml. of $CHCl_3$ and 6 ml. of amyl alcohol. The bottle was shaken mechanically for 30 min. at 5° and the resulting suspension was centrifuged at 2500 *g* for 10 min. The process of deproteinization was repeated three times to yield an aqueous solution of nucleic acid fairly free from protein, the nucleic acids were precipitated with 20% cetylpyridinium bromide solution (Jones, 1953). The stringy nucleic acid complex could be taken up on a glass rod. The complex was dispersed in NaCl solution and shaken repeatedly with $CHCl_3$ to remove the cetylpyridinium bromide. Absorption spectra of NaCl solutions of this preparation gave curves similar to that of yeast ribonucleic acid and sperm deoxyribonucleic acid (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) with a characteristic absorption peak at 260 mμ and no hump at 280 mμ. The solution was used as such for preparing the dihydrostreptomycin complexes by adding dihydrostreptomycin solution (100 mg./ml.) dropwise. The precipitated material was washed with 0·85% NaCl solution to remove the drug. The precipitate obtained from 2 ml. of the original solution was homogenized in a Potter–Elvehjem homogenizer with 5 ml. of 0·2 M-sodium acetate, pH 7·5, and used as substrate for the action of crystalline ribonuclease and deoxyribonuclease (Sigma Chemical Co., St Louis, Mo., U.S.A.).

Drug sensitivity of the organism. Assay of drug sensitivity or resistance was carried by visually recording growth of InSS and InSR in nutrient broths (10 ml.) containing dihydrostreptomycin (0–2 mg./ml.). Growth of InSS was completely suppressed by concentration of dihydrostreptomycin as low as 2 μg./ml., whereas InSR grew very well in concentrations up to 2 mg./ml.

RESULTS

Effect of streptomycin on acetate oxidation. Volumes of oxygen used (μl./mg. dry wt./hr.; Q_{O_2} values) for acetate oxidation by fresh washed suspensions of InSS and InSR are given in Table 1. A perceptible inhibitory effect is produced even by the lowest concentration of dihydrostreptomycin used; the resistant strain is not inhibited by five times the concentration of the drug that causes 30% inhibition in InSS.

Effect of streptomycin on oxidation of Krebs-cycle intermediates. The Q_{O_2} values are given in Table 2. Dihydrostreptomycin considerably retarded the rate of oxidation of all the substrates by InSS excepting malate and lactate. Citrate was not included, because resting cells of *V. cholerae* are not permeable to citrate (Krishna Murti & Shrivastava, 1955*a*). The effect of various concentrations of dihydrostreptomycin and malate on the Q_{O_2} values are given in Tables 3 and 4. The consistent failure to observe inhibition with malate suggests that dihydrostreptomycin inhibits fumarase. Insensitivity of lactate oxidation to dihydrostreptomycin may be due to the conversion of lactate into malate by lactic dehydrogenase and 'malic enzyme'.

Effect of streptomycin on the oxidation of amino acids. The Q_{O_2} values are given in Table 5. The drug exerts an appreciable depressing action on the oxidation of the amino acids tested only by InSS.

Age of harvested cells and the degree of inhibition by streptomycin. Oxidation of acetate, succinate and fumarate was followed with freshly prepared cell suspensions of InSS and periodically thereafter with the same suspension stored at 8°. The Q_{O_2} values are given in Table 6. Attention is particularly drawn to the gradual diminution in the degree of inhibition of acetate oxidation by dihydrostreptomycin with aging of cells and the persistence of inhibition of fumarate and succinate oxidation even after 96 hr. of storage.

Effect of streptomycin on the rates of oxidation of various substrates. The rates of oxidation of a number of substrates by InSS and InSR grown under different conditions (cf. legends to Figs. 1–4) were compared in order to find out whether resistance leads to alterations in metabolic activity. Results of some typical experiments are presented graphically in Figs. 1–4. Dihydrostreptomycin always inhibited oxidation of acetate, succinate and fumarate by InSS. This inhibition is evident from the time the substrate is added to the reaction system. Oxygen uptake in the presence of glucose, mannose and maltose is, however, inhibited by the drug only after a lag period of 30–60 min. InSR grown with or without dihydrostreptomycin in the medium showed almost the same activity on all the substrates tested as strain InSS. Results presented in Fig. 4 show a lag period of about 30 min. before oxidation of histidine commences. Presumably the cells are adapted to use this substrate. Although dihydrostreptomycin inhibits oxygen uptake by InSS on this amino acid, the results do not conclusively indicate inhibition of adaptation.

Effect of dihydrostreptomycin on cell-free extracts

Production of acetoin. Streptomycin dependence and resistance in *Salmonella typhosa* are accompanied by the cell's gaining the ability to produce acetylmethylcarbinol which the parent sensitive strains were unable to do (Reitmann & Iverson, 1953–54). We therefore examined whether a similar change occurs in *V. cholerae*; acetylmethylcarbinol production was followed in a number of assay systems with both cell suspensions and extracts of InSS and InSR and no positive test was observed.

Nitrate reduction. Reduction of nitrate by cell-free extracts was followed for a period of 30 min. and no significant difference could be observed between the activities of InSS and InSR.

Production of glyoxalate. Suspensions were incubated in evacuated Thunberg tubes, and a dinitrophenylhydrazone was isolated which was identified by paper chromatography as that of glyoxalate. No difference could be established in the rate of production of this compound by extracts of InSS and InSR. This would suggest the presence of the glyoxalate shunt in both the strains. Since malate is formed in certain micro-organisms from acetate via glyoxalate (Kornberg & Madsen, 1958) the glyoxalate shunt may be involved in resistance.

Action of dihydrostreptomycin on fumarase and succinic dehydrogenase. For following the action of dihydrostreptomycin on succinic dehydrogenase

Table 1. *Oxidation of acetate by InSS and InSR*

The flasks contained 100 μmoles of phosphate, pH 8·0, 5 μmoles of sodium acetate, cells equivalent to 1 mg. cell mass and dihydrostreptomycin as indicated, in a total volume of 3 ml. The substrate was tipped in from the side arm after pre-incubating the cells with drug for 30 min.

	Concn. of dihydrostreptomycin (μg./ml.)					
	0	16·7	33·3	50·0	66·7	83·3
	Q_{O_2}					
InSS	62	44	31	39	34	34
InSR	77	78	78	79	77	78

Table 2. *Oxidation of tricarboxylic acid-cycle intermediates and lactate by InSS and InSR*

The conditions are as in Table 1 with 5 μmoles of the substrates as shown; dihydrostreptomycin (16·7 μg./ml.) present (+); absent (−). The values are given as Q_{O_2} values.

	*iso*Citrate		α-Oxo-glutarate		Succinate		Fumarate		Malate		Lactate	
	−	+	−	+	−	+	−	+	−	+	−	+
InSS	80	62	20	8	72	12	100	12	70	71	114	113
InSR	80	82	24	24	78	80	98	102	68	69	115	113

0·5 ml. of enzyme extract was mixed with 0·5 ml. of 0·2 M-potassium phosphate buffer, pH 8, and incubated at room temperature with 0·2 ml. of water (control) or 0·2 ml. of dihydrostreptomycin (1 mg./ml.) for 30 min.; 0·5 ml. of triphenyl tetrazolium chloride (0·05%, pH 7·5) and 0·2 ml. of 0·5 M-sodium succinate were added and incubated for a further period of 4 hr. at 37°. The formazan colour formed was extracted with a total volume of 6 ml. of toluene–acetic acid (1:1). A portion (1·5 ml.) of the coloured layer was diluted with 1·5 ml. of the same solvent mixture and the extinction was read in cuvettes (optical path 1 cm.) at 495 mμ in a Unicam spectrophotometer. The extinction values for InSS were 0·1 for control and 0·055 with dihydrostreptomycin. The extinction values in InSR were 0·15 and 0·145 respectively. A 45% inhibition of succinic dehydrogenase is brought about by dihydrostreptomycin on InSS without affecting the enzyme made from InSR.

To study the action of dihydrostreptomycin on fumarase, 1 ml. of enzyme extract was mixed with 1 ml. of 0·2 M-phosphate buffer, pH 8·0, and incubated with 0·2 ml. of water (control) or 0·2 ml. of dihydrostreptomycin (1 mg./ml.) at 30° for 30 min. A portion (1 ml.) of 0·1 M-sodium fumarate was added to the mixture and 1 ml. portions were pipetted into 1 ml. of 10% trichloroacetic acid immediately and 15 min. after the addition of fumarate. The proteins were centrifuged down and the extinction of clear supernatants (diluted 1:5) was read in silica cuvettes (1 cm. optical path) at 300 mμ. The decrease in extinction of the control

Table 3. *Effect of dihydrostreptomycin concentration on oxidation of malate by InSS and InSR*

The conditions are as in Table 1; the concentration was 5 μmoles of substrate/flask.

	Concn. of dihydrostreptomycin (μg./ml.)					
	0	33·3	66·6	100	133·3	166·7
	Q_{O_2}					
InSS	70	72	78	70	68	72
InSR	68	69	68	69	70	68

Table 4. *Effect of substrate concentration upon oxidation of malate by InSS and InSR*

The conditions are as in Table 1.

	Concn. of malate (μmoles/flask)				
	5	10	20	40	50
	Q_{O_2}				
InSS	80	123	128	147	170
InSR	84	122	126	148	172

Table 5. *Oxidation of amino acids by InSS and InSR*

Conditions were as given in Table 1. Substrate: 20 μmoles/flask. +, Dihydrostreptomycin present (100 μg./ml.); −, dihydrostreptomycin absent. Results are given as uptake of oxygen.

		L-Aspartate			Glycine			DL-Serine			L-Cysteine			L-Glutamate			DL-Threonine		
Strain	Time (min.)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)
InSS	60	63	33	48	26	26	0	35	22	37	60	59	0	15	15	0	28	22	22
	90	90	40	55	38	30	22	50	32	36	84	69	18	22	17	29	41	30	27
	120	118	49	58	51	33	36	72	41	43	94	68	29	33	17	50	58	37	36
	180	172	78	56	74	31	60	100	52	48	160	68	58	42	18	70	82	42	49
InSR	60	138	130	0	5	65	0	146	151	0	42	45	0	—	—	—	—	—	—
	90	200	202	0	84	88	0	168	170	0	64	65	0	—	—	—	—	—	—
	120	228	226	0	92	96	0	182	188	0	84	88	0	—	—	—	—	—	—
	180	248	250	0	112	120	0	212	220	0	108	112	0	—	—	—	—	—	—

for InSS was 0·1, whereas the dihydrostreptomycin-treated system showed no decrease in extinction during the period, indicating complete inhibition of fumarase activity. In contrast the fall in extinction was 0·15 in both the control and dihydrostreptomycin-treated system in InSR, indicating that the drug has no action on the fumarase of InSR.

Susceptibility of dihydrostreptomycin–nucleic acid complexes to the hydrolytic action of ribonuclease and deoxyribonuclease

0·2 M-Acetate homogenates (1 ml.) of dihydrostreptomycin–nucleic acid complexes prepared as described in the Methods and Materials section was mixed with 300 μg. of deoxyribonuclease or ribonuclease for 60 min. at 37°. The uncombined nucleic acids were precipitated with 1 ml. of MacFadyan's (1934) reagent and the extinctions of the supernatants measured at 260 mμ after appropriate dilution. Extinction values of the supernatants of InSS were 0·60 and 0·27 respectively for ribonuclease and deoxyribonuclease. The corresponding values for InSR were 1·60 and 1·40. From this it would appear that nucleic acid–dihydrostreptomycin complexes derived from InSS and InSR are both susceptible to the action of the enzymes. More material absorbing at 260 mμ is solubilized by the enzymes from the dihydrostreptomycin complex of InSR than from the corresponding complex of InSS.

Table 6. *Oxidation of acids in InSS as affected by aging of cells*

Conditions are as given in Table 1. Substrate: 10 μmoles/flask, +, Dihydrostreptomycin present (16·6 μg./ml.); −, dihydrostreptomycin absent. The age of cells represents the period stored at 8° after harvesting. Results are given as Q_{O_2} values.

Age of cells (hr.)	Acetate		Fumarate		Succinate	
	−	+	−	+	−	+
Fresh	126	98	118	14	120	12
24	109	90	116	14	116	23
48	74	68	98	32	98	36
96	72	72	54	30	52	30

Action of dihydrostreptomycin on protein synthesis and incorporation of ^{14}C

V. cholerae does not ordinarily ferment lactose (Wilson & Miles, 1955) and attempts to induce formation of β-galactosidase in InSS and InSR under a variety of conditions were unsuccessful. The Ogawa 82/L strain grew very poorly on nutrient agar even after 48 hr. incubation. Notwithstanding this sparse growth, freshly harvested cells of this strain were used in exploratory induction experiments and dihydrostreptomycin was found to inhibit enzyme formation. Since the results

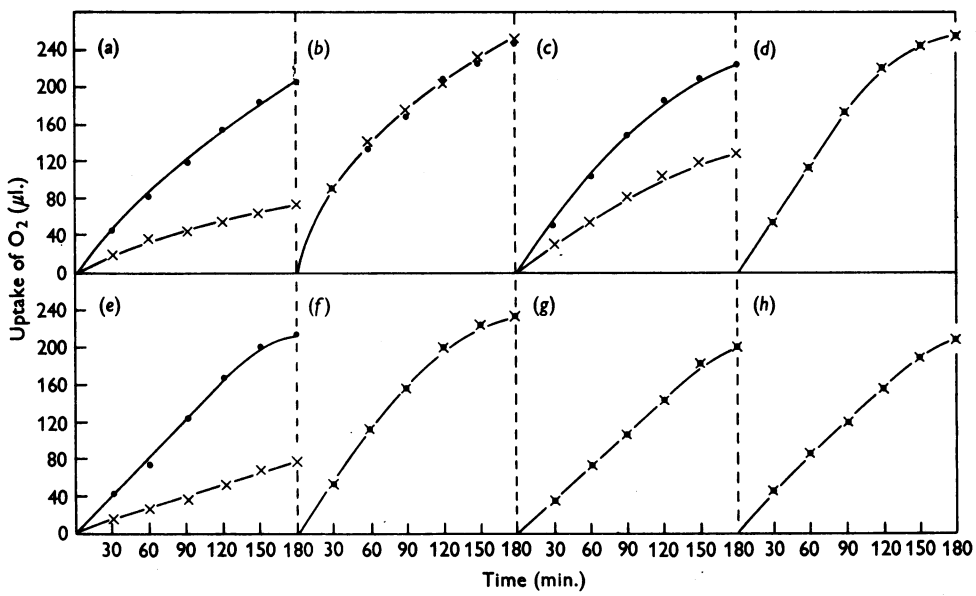

Fig. 1. Uptake of O_2 by InSS and InSR. The flasks contained: cells (1 mg. dry wt.), 20 μmoles of substrate, 33 g. of dihydrostreptomycin (present, ×; absent, ●), 100 μmoles of phosphate buffer, pH 8·0. The substrate was tipped in from the side arm after 30 min. pre-incubation. (*a*) InSS + succinate; (*b*) InSR + succinate; (*c*) InSS + acetate; (*d*) InSR + acetate; (*e*) InSS + fumarate; (*f*) InSR + fumarate; (*g*) InSS + malate; (*h*) InSR + malate.

were not conclusive, the effect of dihydrostreptomycin was studied on protein synthesis measured by increase in cellular nitrogen. The results of a typical experiment are summarized in Table 7. Protein synthesis is inhibited by dihydrostreptomycin in InSS whereas in InSR it proceeds even in the presence of the drug. It is also apparent that the net synthesis of protein/ml. of medium is greater in InSR than in InSS.

The growth rates of the two strains in nutrient broth under identical conditions were, however, comparable and the protein and nucleic acid contents of cells on dry-wt. basis at 18–24 hr. growth did not show any quantitative differences between InSS and InSR. Analysis of cell suspensions of InSS and InSR of equal opacity value had a protein content of 500–530 μg./mg. dry wt. and extinction values at 260 mμ of trichloroacetic acid extracts of cells of InSS and InSR were comparable.

Action of dihydrostreptomycin on incorporation of [^{14}C]glycine into the trichloroacetic acid-precipitable fraction of cells

Incorporation of labelled amino acid was followed for a period of 3 hr. and the results have been presented in Fig. 5. The ^{14}C-incorporation data run almost parallel to results on protein synthesis. Here again rate of incorporation of ^{14}C/ml. of suspension was greater in InSR than in InSS. A comparison of the data for protein synthesis given in Table 7 and the data for incorporation shown in Fig. 5 reveals that whereas chloramphenicol stops protein synthesis completely, incorporation of labelled glycine is inhibited only 50%.

DISCUSSION

The results presented in this paper establish clearly that streptomycin exerts its primary cyto-

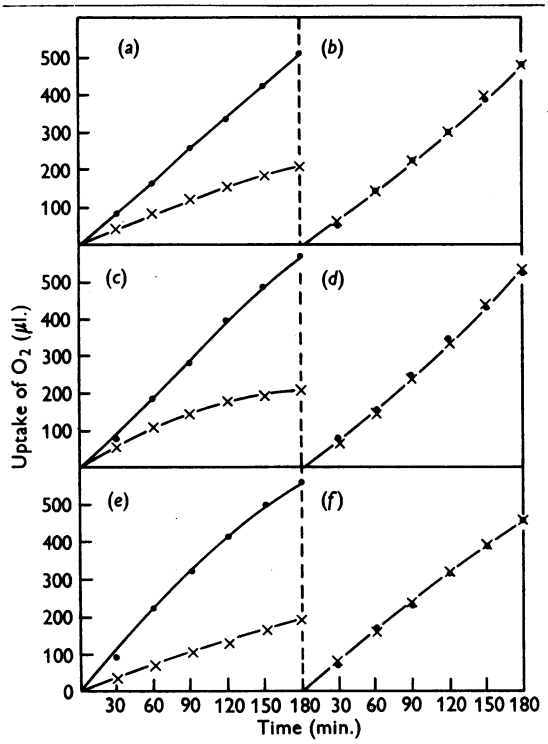

Fig. 2. Uptake of O_2 by InSS and InSR. InSR were grown on nutrient agar containing 500 μg. of dihydrostreptomycin/ml. The conditions are as for Fig. 1. (*a*) InSS + acetate; (*b*) InSR + acetate; (*c*) InSS + fumarate; (*d*) InSR + fumarate; (*e*) InSS + succinate; (*f*) InSR + succinate.

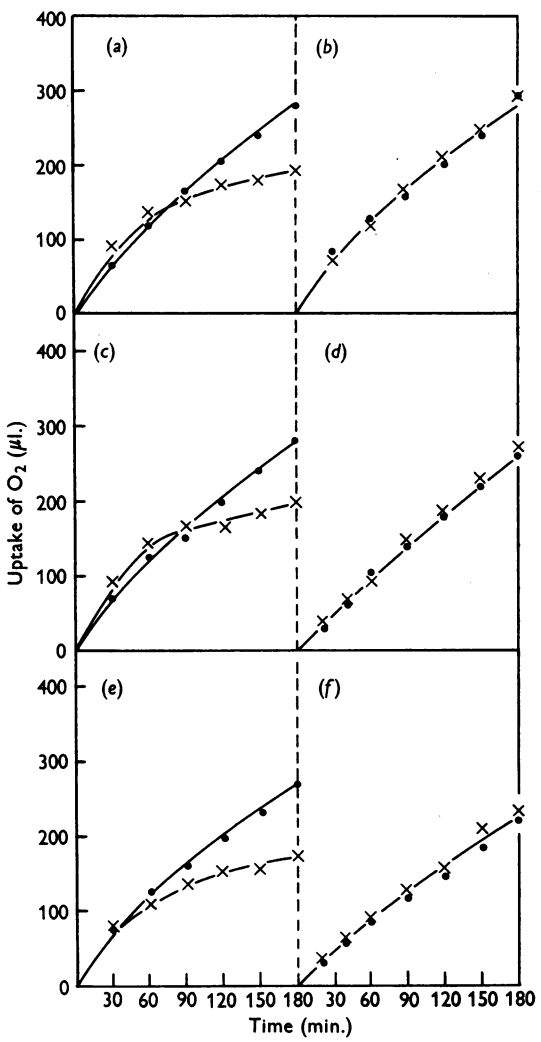

Fig. 3. Uptake of O_2 by InSS and InSR. InSR were grown on nutrient agar containing 500 μg. of dihydrostreptomycin/ml. The conditions were as for Fig. 1. (*a*) InSS + glucose; (*b*) InSR + glucose; (*c*) InSS + maltose; (*d*) InSR + maltose; (*e*) InSS + mannose; (*f*) InSR + mannose.

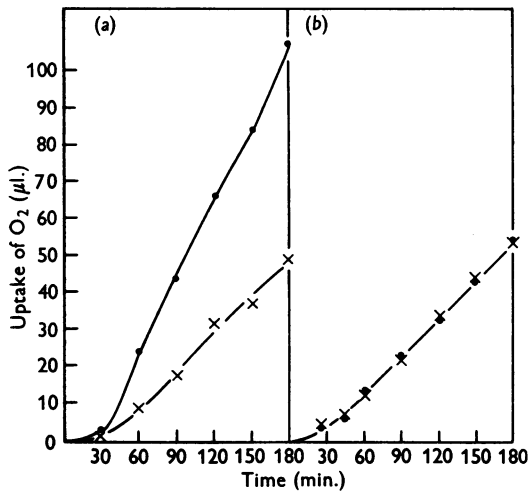

Fig. 4. Action of dihydrostreptomycin on oxidation of histidine. InSR were grown on nutrient agar containing 500 µg. of dihydrostreptomycin/ml. The conditions were as for Fig. 1. (*a*) InSS; (*b*) InSR.

Table 7. *Protein synthesis by InSS and InSR*

Values are given as µg./ml. of medium. Dihydrostreptomycin or chloramphenicol was present at concentrations of 111 µg./ml.

Culture	Time (min.)	Control	Dihydro-streptomycin	Chloram-phenicol
InSS	30	21	10	0
	60	41	25	0
	90	61	40	0
	120	96	50	0
	180	136	50	0
InSR	30	60	80	0
	60	150	205	0
	90	270	280	0
	120	240	320	0
	180	380	390	0

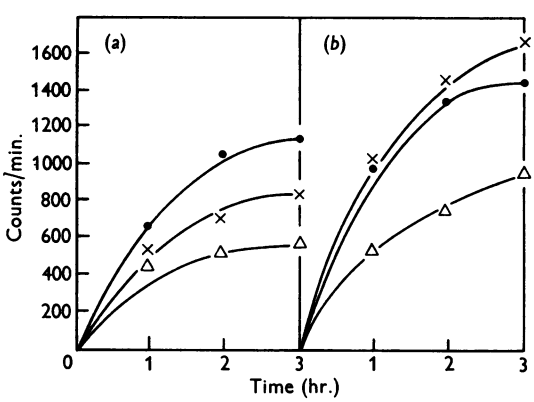

Fig. 5. Action of dihydrostreptomycin on incorporation of ^{14}C into trichloroacetic acid-precipitable fraction. (*a*) InSS; (*b*) InSR. ●, Control; ×, dihydrostreptomycin (100 µg./ml.); △, Chloramphenicol (100 µg./ml.).

toxic effect on *V. cholerae* by inhibiting oxygen uptake in the terminal respiration sequence in a manner somewhat similar to its action on *Escherichia coli* (Umbreit, 1949; Oginsky, Smith & Umbreit, 1949; Umbreit, Smith & Oginsky, 1951; Oginsky, 1953; Umbreit, 1953). Oxidation of the intermediates of the tricarboxylic acid cycle (excepting citrate, to which the cells are not permeable, and malate, the oxidation of which is not affected at all) is inhibited by the drug from the moment the substrate and enzyme are brought together. Experiments with intact cells indicate a multiplicity of steps in streptomycin inhibition; unequivocal evidence with cell-free extracts could, however, be provided only for the inactivation of succinic dehydrogenase and fumarase.

The inhibition data on oxidations by intact cells of *V. cholerae* fit in with observations on other bacteria, particularly acetate accumulation in sensitive organisms and the reversal of streptomycin inhibition by organic acids such as fumaric acid and pyruvic acid (see Pratt & Dufrenoy, 1949). The insensitivity of malate oxidation to streptomycin is a unique characteristic of *V. cholerae*. In an independent study of the metabolism of dihydrostreptomycin-sensitive, dihydrostreptomycin-resistant and dihydrostreptomycin-dependent strains of *Vibrio comma*, Farkas-Himsley (1957) found striking differences among the three strains in the rate of reduction of neo-tetrazolium in the presence of various substrates, except with malate.

The interaction of streptomycin and nucleic acids first demonstrated by Cohen (1946) has been suggested by Donovick, Bayan, Canales & Pansy (1948) as a possible mechanism of action of the drug. Peretz & Polglase (1956–57) showed that dihydrostreptomycin–nucleic acid complexes made from sonic extracts of drug-sensitive *E. coli* are not attacked by nucleases, in contrast with the ready susceptibility to enzyme action of similar complexes of dihydrostreptomycin with yeast ribonucleic acid and with thymus deoxyribonucleic acid. The non-availability of nucleotides or nucleic acid fragments implicated to function as the 'incorporation factors' (Gale, 1958) by the neutralization of nucleic acids by dihydrostreptomycin could lead to cessation of incorporation and possibly also of protein synthesis. That such a situation does not occur in *V. cholerae* is clear from the fact that dihydrostreptomycin–nucleic acid complexes of both InSS and InSR are equally attacked by nucleases. Thus although dihydrostreptomycin precipitates the nucleic acids of InSS, the latter are presumably still available for protein synthesis.

Protein synthesis and incorporation of labelled amino acid in InSS are both inhibited by dihydrostreptomycin although not to the same extent as by

chloramphenicol. It may be that this inhibition in *V. cholerae* by dihydrostreptomycin is secondary to a primary block at some other site. An aerobic organism such as *V. cholerae* derives its energy for synthetic processes, such as protein formation, by the terminal oxidation of carbon compounds. On the basis of the inhibition of two or more enzymes participating in the tricarboxylic acid cycle, it does not appear to be unreasonable to conclude that oxidative metabolism is the process primarily attacked by the drug in *V. cholerae*. A more rigorous proof of this could be provided only with isolated enzymes in reconstituted systems where the trapping of energy derived by oxidative breakdown of primary substrates could be demonstrated. This has not been possible in the present study owing to the presence in cell extracts of a highly active nucleotidase (Krishna Murti & Shrivastava, 1955*b*) insensitive to fluoride ions.

SUMMARY

1. Washed suspensions and extracts of a streptomycin-sensitive Inaba strain (InSS) of *Vibrio cholerae* and a streptomycin-resistant mutant (InSR) derived from it were used in manometric enzymic studies with a view to locating the site of action of dihydrostreptomycin.

2. The oxidation of acetate, succinate, fumarate, L-aspartate, DL-serine, L-cysteine, glycine, DL-threonine, DL-histidine, glucose, mannose and maltose were inhibited appreciably by concentrations of dihydrostreptomycin that prevented the growth of InSS. Malate oxidation by InSS was, however, not affected by the drug.

3. Oxygen uptake by InSR on these substrates was unaffected by dihydrostreptomycin even at a concentration 100 times that which inhibited InSS.

4. Dihydrostreptomycin also inhibited partially purified preparations of succinic dehydrogenase and fumarase made from InSS but not the corresponding enzymes derived from InSR.

5. Dihydrostreptomycin interfered with protein synthesis and incorporation of ^{14}C from [2-^{14}C]-glycine into the trichloroacetic acid-precipitable fraction of InSS and not into that of InSR.

6. Dihydrostreptomycin–nucleic acid complexes isolated from InSS and InSR are equally susceptible to the action of added nucleases.

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