EXTRACELLULAR METABOLITES OF STREPTOMYCIN MUTANTS OF *ESCHERICHIA COLI*

P. D. BRAGG AND W. J. POLGLASE

Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

Received for publication March 15, 1962

ABSTRACT

BRAGG, P. D. (University of British Columbia, Vancouver, Canada) AND W. J. POLGLASE. Extracellular metabolites of streptomycin mutants of *Escherichia coli*. J. Bacteriol. **84**:370–374. 1962.—A comparison of the extracellular products of glucose metabolism during aerobic exponential growth of *Escherichia coli* showed that a streptomycin-dependent strain produced large amounts of L-valine while only trace amounts of this amino acid were produced by streptomycin-sensitive strains. A further difference between sensitive and dependent mutants was the production by the latter of lactic acid when the gas phase was changed from air to nitrogen. Resistant cultures grown in antibiotic-free medium were similar to sensitive cultures, but when dihydrostreptomycin was added, the resistant organism produced lactic and pyruvic acids. Three strains of streptomycin-sensitive *E. coli* accumulated pyruvic acid from glucose oxidation in the presence of concentrations of dihydrostreptomycin which inhibited multiplication. Further evidence is thus provided to implicate reactions of pyruvate as being of significance in the mechanism of action of streptomycin.

Studies on the mechanism of action of streptomycin have resulted in a considerable number of divergent hypotheses. Thus, it has been proposed that the antibiotic effect of streptomycin may be due to (i) inhibition of specific enzyme reactions (*see* Lightbown, 1957), (ii) cell-membrane damage (Anand and Davis, 1960; Landman and Burchard, 1962), or (iii) inhibition of general or specific protein and enzyme synthesis (Erdös and Ullman, 1959; Roote and Polglase, 1955). Recently, Spotts and Stanier (1961) proposed that the primary site of action of streptomycin is the ribosome. According to their hypothesis, the action of streptomycin on the ribosome inhibits the synthesis of certain enzymes in sensitive organisms by preventing the binding of specific "messenger" ribonucleic acid. On the other hand, in streptomycin-dependent organisms, the functional consequences of a genetic impairment of ribosomal structure can be specifically overcome by combination of the ribosome with streptomycin. This hypothesis of Spotts and Stanier would seem to imply that streptomycin-resistant and -dependent mutants should exhibit metabolic pathways identical with the related sensitive strain when all three mutants are grown under optimal conditions.

The present investigation of extracellular substances was initiated as a result of an observation that streptomycin-dependent *Escherichia coli* produced, extracellularly, larger quantities of ninhydrin-positive material than did a streptomycin-sensitive mutant when both were grown under optimal conditions.

MATERIALS AND METHODS

Three strains of *E. coli*, sensitive to streptomycin, were used in this investigation: SB, SE, and SA. Strain SB is no. 482 of the culture collection of the National Research Council of Canada. Strain SE was obtained from the culture collection of Laval University, Montreal. Strain SA and a resistant mutant (RA) were obtained by back-mutation from a streptomycin-dependent *E. coli* (DA). The mutants SA, RA, and DA have been used in studies reported previously (Roote and Polglase, 1955; Polglase, Peretz, and Roote, 1957).

The following medium was used: K_2HPO_4 (0.7%), KH_2PO_4 (0.3%), sodium citrate (0.05%), $MgSO_4$ (0.01%), $(NH_4)_2SO_4$ (0.1%), glucose (0.2%). The dependent strain was found to grow at its maximal rate in this medium when 1,000 units of dihydrostreptomycin per ml were added. For each experiment, 500 ml of the medium was inoculated with 50 ml of a 16-hr culture of the

strain under investigation. After continuous vigorous aeration for 2 hr, the culture was divided into two equal parts in 500-ml flasks. One of these flasks was maintained under the original conditions as a control, while the other was varied, e.g., by the addition of antibiotic or by alteration of the gas phase. At hourly intervals during the course of the experiment, the optical density of the cultures was recorded and 2-ml samples were removed for the estimation of glucose by the method of Montgomery (1957). At the same time, samples of 20 ml were centrifuged at high speed immediately after removal from the growth vessels. The clear supernatant fluid was then concentrated to dryness under diminished pressure, and the residue was dissolved in water to give a volume of 3 ml. Samples of this solution were assayed for lactic acid (Barker and Summerson, 1941), total keto acid (Friedemann, 1957), and amino acids (Bode, 1955). The keto acid was shown to be pyruvic acid by chromatography of the 2,4-dinitrophenylhydrazone in *n*-butanol–ethanol–0.5 N ammonia (70:10:20; El Hawary and Thompson, 1953).

RESULTS AND DISCUSSION

Comparison of the mutants. The formation of various products during aerobic growth of *E. coli* SA, SB, SE, RA, and DA was followed by hourly sampling until all the glucose had been consumed and growth had ceased. The significant features of the results are recorded (Table 1) for 27% glucose consumption (exponential growth) and 82% glucose consumption (conclusion of exponential-growth phase). There were small differences among the strains SA, SB, and SE in the amounts of lactic acid and pyruvic acid produced.

TABLE 1. *Extracellular metabolites of mutants of Escherichia coli*

Culture	Glucose consumed	Pyruvic acid	Lactic acid	Valine
	μmoles	*μmoles*	*μmoles*	*μmoles*
SB	300	0.6	0	0
	900	0.6	0	0
SE	300	0	11.0	0
	900	0	3.9	0
SA	300	0	3.0	0
	900	8.5	13.5	0.9
RA	300	0	11.0	0
	900	0	0	0
RA*	300	21.0	26.5	1.0
	900	4.0	53.0	3.0
DA*	300	0.3	0	41.0
	900	2.0	0	102

* The medium contained dihydrostreptomycin (1,000 units/ml).

The resistant strain (RA) was not unlike the sensitive strains SA, SB, and SA when grown in antibiotic-free medium, but when grown in medium containing 1,000 units per ml of dihydrostreptomycin, strain RA produced elevated quantities of both lactic and pyruvic acids (Table 1). These results suggest that the resistant strain uses pathways of anaerobic metabolism in antibiotic-containing medium. Somewhat similar results were recorded by Rosanoff and Sevag (1953), who observed that a resistant strain produced more lactic acid from pyruvate and glucose than did the parent sensitive strain. These workers did not, however, observe an increase in lactic acid after addition of antibiotic. This difference between the observations of Rosanoff and Sevag (who worked with nonproliferating cells) and the present work with cells growing exponentially may be due to the differences in experimental techniques.

It appears that the resistant strain RA, when grown in the absence of antibiotic, may have certain metabolic pathways for pyruvate utilization in common with the sensitive strains. In the presence of dihydrostreptomycin, however, the resistant strain is able to utilize other metabolic pathways.

The dependent strain, DA, differed from the other strains in the production of substantial amounts of L-valine. As much as 10% of the glucose carbon could be accounted for in this product (Table 1). In one experiment, 200 mg of this amino acid was isolated as the crystalline hydrochloride from 11 liters of medium. The product was positively identified as L-valine hydrochloride by chromatography, elementary analysis, optical rotation, and infrared-absorption spectrum. That the amino acid was formed *de novo* was established by the observation that, during the period of formation of valine, there was no concomitant decrease in the amino-acid content of the trichloroacetic acid-soluble frac-

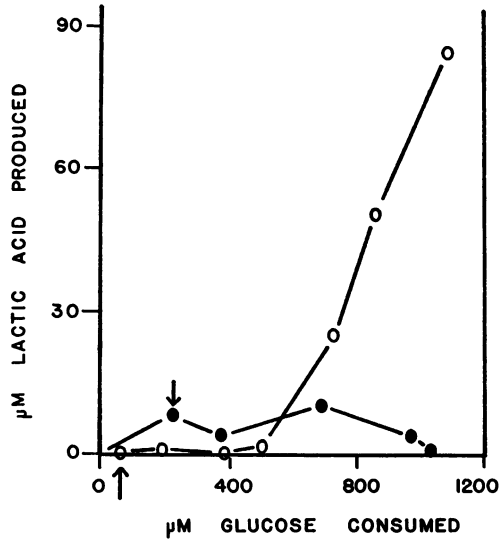

FIG. 1. *Production of lactic acid from glucose by sensitive (●) and dependent (○) Escherichia coli. Gas phase was changed from air to nitrogen at arrows.*

tion of the cell. Only trace amounts of other amino acids (alanine, leucine, or isoleucine) were produced by strain DA, so that this phenomenon of production of large amounts of valine differs from previous observations (Dagley and Johnson, 1956) on the production of small amounts of several amino acids (histidine, alanine, aspartic and glutamic acids) by *E. coli.*

Further differences were observed between the dependent and sensitive *E. coli* when the gas phase was changed from air to nitrogen after 2 hr of growth (Fig. 1). Although both strains produced about the same amount of pyruvic acid, DA produced far more lactic acid than did SA, suggesting that DA may be more limited in the number of pathways for the dissimilation of pyruvate than is SA.

The foregoing results show that a streptomycin-dependent culture, grown at a concentration of dihydrostreptomycin adequate to permit a maximal growth rate, is not identical in its metabolic reactions with sensitive *E. coli* in antibiotic-free medium. This might seem to contradict the unitary hypothesis of Spotts and Stanier (1961) which would lead one to expect similar metabolic reactions from parent and mutant strains under conditions of optimal growth.

TABLE 2. *Effect of addition of dihydrostreptomycin to exponentially growing sensitive Escherichia coli SE*

Time	OD at 420 mμ	Glucose consumed	Pyruvic acid	Leucine or isoleucine	Valine	Alanine
hr		*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*
0	0.96	0	0	0	0	0
1	1.49	397	1.6	0	0	0
1*	1.12	140	0.3	1.6	4.2	6.3
2	1.82	719	0	0	0	0
2*	1.07	200	7.1	2.6	7.3	13.5
3	1.85	763	0	0	0	0
3*	1.02	249	25	3.1	6.3	20.0

* Dihydrostreptomycin (1,000 units/ml) was added at zero time.

TABLE 3. *Effect of addition of dihydrostreptomycin to exponentially growing sensitive Escherichia coli SA*

Time	OD at 420 mμ	Glucose consumed	Pyruvic acid	Lactic acid	Valine	Alanine
hr		*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*
0	0.60	0	0	0	0	0
1	0.96	264	2.5	4.4	0	0.6
1*	0.97	185	0.9	2.2	3.3	1.3
2	1.40	792	8.8	13.9	1.3	1.3
2*	1.35	426	17.2	9.9	10.3	2.0
3	1.59	892	1.0	17.2	4.6	0.6
3*	1.66	845	64.3	58.9	39.4	3.9
4	1.61	898	0.6	15.7	8.3	0
4*	1.79	890	1.2	63.6	71.2	7.1

* Dihydrostreptomycin (1,000 units/ml) was added at zero time.

Effect of dihydrostreptomycin on the sensitive strains. Strain SA differed from strains SB and SE in antibiotic sensitivity. Upon addition of 1,000 units per ml of dihydrostreptomycin to a growing culture of SA, the optical density continued to increase. Viable-cell counts and microscopy showed that a significant part of the increase in optical density was due to an increase in cell length, while the rate of cell division was substantially decreased. Strains SB and SE behaved similarly at a concentration of 10 to 20 units per ml of dihydrostreptomycin. At higher concentrations (10,000 units per ml for SA; 100 units per ml for SB and SE), the antibiotic also inhibited cell elongation. Addition of dihydrostreptomycin to sensitive strains after 2 hr of growth in antibiotic-free medium produced

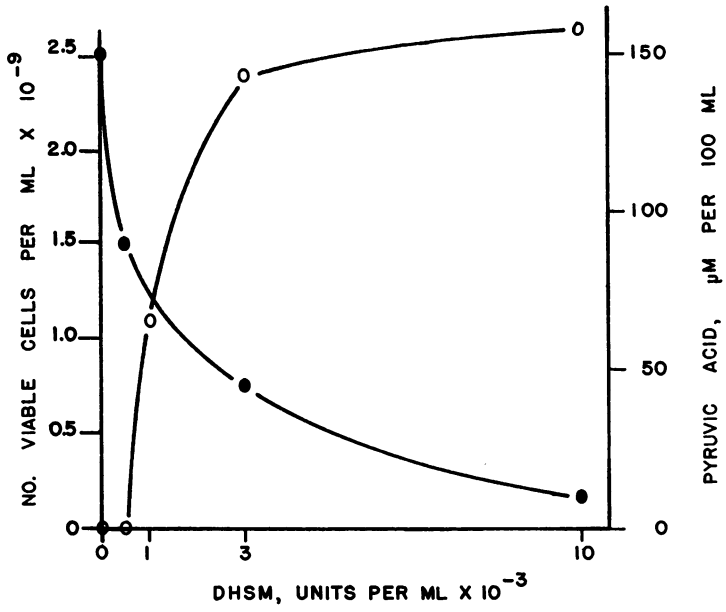

FIG. 2. *Effect of dihydrostreptomycin (DHSM) on viable-cell count (●) and on pyruvic-acid production (○) in culture medium (strain SA).*

marked changes in composition of the medium. First, all sensitive strains accumulated large amounts of pyruvic acid. Second, there was an increase in the production of valine and alanine, and, in the case of strain SA, an increase in lactic acid also (Tables 2 and 3). The formation of these metabolites can be related to the increased amount of pyruvic acid, since alanine and lactic acid would be formed by amination and reduction of pyruvic acid, respectively, and valine would be formed through acetolactic acid from pyruvic acid (Radhakrishnan and Snell, 1960).

In strain SA, the pyruvic acid, produced after the addition of dihydrostreptomycin, decreased towards the end of exponential growth. It was possible to account, almost quantitatively, for the decrease in pyruvic acid (63.1 μmoles; 189 μmoles of carbon) by the formation of valine (31.8 μmoles), alanine (3.2 μmoles), and lactic acid (4.7 μmoles; total: 173 μmoles of carbon). The major ninhydrin-positive substances produced by all three sensitive strains, after the addition of dihydrostreptomycin, were valine and alanine. In addition, strains SB and SE produced significant amounts of substances which appeared to be peptides.

The production of pyruvic acid as a function of dihydrostreptomycin concentration reached a constant value at approximately the level which inhibited cell division. For strain SB, the viable-cell count was minimal (1.4×10^8 per ml) and the pyruvic acid maximal (68 μmoles per 100 ml of medium) at 30 units of dihydrostreptomycin per ml. With the less-sensitive strain, SA, approximately 100 times as much antibiotic produced a similar effect (Fig. 2).

The block in the utilization of pyruvate in the sensitive strains may be a specific site of action of dihydrostreptomycin, or the site of action may be more remote along the pathway of pyruvate dissimilation. Umbreit (1953) claimed that streptomycin inhibited a condensation between pyruvic and oxaloacetic acids; other workers (Barkulis, 1953; Rosanoff and Sevag, 1953) postulated an inhibition of the phosphoroclastic cleavage of pyruvic acid to acetyl phosphate and formic acid.

Either of these hypotheses would account for the accumulation of pyruvic acid, although both hypotheses have been criticized in view of certain observed inconsistencies (Lightbown, 1957).

ACKNOWLEDGMENTS

Dihydrostreptomycin sulfate was a gift of Merck & Co., Montreal.

This work was supported by a grant (MT 750) from the Medical Research Council of Canada.

LITERATURE CITED

ANAND, N., AND B. D. DAVIS. 1960. Damage by streptomycin to the cell membrane of *Escherichia coli*. Nature **185**:22–24.

BARKER, S. B., AND W. H. SUMMERSON. 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. **138**:535–554.

BARKULIS, I. L. 1953. Inhibition of the anaerobic pyruvate metabolism of *Escherichia coli* by dihydrostreptomycin. J. Bacteriol. **65**:337–343.

BODE, F. 1955. Eine Vereinfachung und Verbesserung der Methode zur Quantitativen Bestimmung von Aminosäuren und Peptiden mittels des Ninhydrin-Kupferkomplexes. Biochem. Z. **326**:433–435.

DAGLEY, S., AND A. R. JOHNSON. 1956. Appearance of amino-acids and peptides in culture filtrates of microorganisms growing in mineral salt media. Biochim. et Biophys. Acta **21**:270–276.

EL HAWARY, M. F. S., AND R. H. S. THOMPSON. 1953. Separation and estimation of blood keto acids by paper chromatography. Biochem. J. **53**:340–347.

ERDÖS, T., AND A. ULLMANN. 1959. Effect of streptomycin on the incorporation of amino-acids labelled with carbon-14 into ribonucleic acid and protein in a cell-free system of a *Mycobacterium*. Nature **183**:618–619.

FRIEDEMANN, T. E. 1957. Determination of α-keto acids, p. 414–418. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, v. 3. Academic Press Inc., New York.

LANDMAN, O. E., AND W. BURCHARD. 1962. The mechanism of action of streptomycin as revealed by normal and abnormal division in streptomycin-dependent salmonellae. Proc. Natl. Acad. Sci. U.S. **48**:219–228.

LIGHTBOWN, J. W. 1957. Metabolic processes underlying streptomycin resistance. Giorn. ital. chemioterap. **4**:22–32.

MONTGOMERY, R. 1957. Determination of glycogen. Arch. Biochem. Biophys. **67**:378–386.

POLGLASE, W. J., S. PERETZ, AND S. M. ROOTE. 1957. Adaptive enzyme formation by dihydrostreptomycin-dependent *Escherichia coli*. Can. J. Biochem. Physiol. **34**:558–562.

RADHAKRISHNAN, A. N., AND E. E. SNELL. 1960. Biosynthesis of valine and isoleucine. II. Formation of α-acetolactate and α-aceto-α-hydroxybutyrate in *Neurospora crassa* and *Escherichia coli*. J. Biol. Chem. **235**:2316–2321.

ROOTE, S. M., AND W. J. POLGLASE. 1955. The effect of dihydrostreptomycin on the formation of adaptive enzymes by a strain of *Escherichia coli*. Can. J. Biochem. Physiol. **33**:792–796.

ROSANOFF, E. I., AND M. G. SEVAG. 1953. Alternate metabolic pathways in streptomycin sensitive and resistant strains of *Escherichia coli*. Antibiotics & Chemotherapy **3**:495–504.

SPOTTS, C. R., AND R. Y. STANIER. 1961. Mechanism of streptomycin action: a unitary hypothesis. Nature **192**:633–637.

UMBREIT, W. W. 1953. The action of streptomycin. VI. A new metabolic intermediate. J. Bacteriol. **66**:74–81.