isolation procedure. No radioactive substances other than actinomycins I, IV, and V were detected.

The high specific activity of the material should be of value in elucidating the mechanism of action, tissue distribution, and metabolism of this agent.

The authors would like to express their appreciation to Drs. Emil Frei, Sidney Udenfriend, and Bernhard Witkop for their help in the initiation of this study. Thanks are due to New England Nuclear Corporation for their cooperation in supplying the highly labeled isotope in sufficient quantities, and in discussion of the various technical details involved in using highly labeled tritiated methionine. This investigation was supported by research grant CA-06926 of the National Cancer Institute, National Institutes of Health.

REFERENCES

1. L. H. Goldberg, M. Rabinowitz and E. Reich, *Proc. Natl. Acad. Sci. U.S.* **48**, 2094 (1962).
2. E. Reich, *Science* **143**, 684 (1964).
3. S. Farber, G. D'Angio, A. Evans and A. Mitus, *Ann. N.Y. Acad. Sci.* **89**, 421 (1960).
4. G. T. Ross, L. Stolbach and R. J. Hertz, *Cancer Res.* **22**, 1015 (1962).
5. A. Sivak, M. Meloni, F. Nobili and E. Katz, *Biochim. Biophys. Acta* **57**, 283 (1962).
6. E. Katz and H. Weissbach, *Biochem. Biophys. Res. Commun.* **8**, 186 (1962).
7. E. Katz and H. Weissbach, *J.. Biol Chem.* **238**, 666 (1963).
8. G. Bray, *Anal. Biochem.* **1**, 279 (1960).
9. E. Katz, P. Pienta and A. Sivak, *Appl. Microbiol.* **6**, 236 (1958).
10. E. Katz and W. Goss, *Biochem. J.* **73**, 458 (1959).

Insensitivity to Valine of Streptomycin-Dependent *Escherichia coli* K12

W. J. POLGLASE

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

(Received May 28, 1965)

SUMMARY

A streptomycin dependent mutant was obtained from wild-type (valine-sensitive) *Escherichia coli* K12. This mutant (*E. coli* DK 12) grew exponentially in minimal medium containing valine (plus dihydrostreptomycin). The inhibition by valine of the acetohydroxy acid synthetase from both dependent and parent cells was quantitatively similar. However, the activity of the acetohydroxy acid synthetase of DK 12 cells was higher than that of the parent cells. It was concluded that the insensitivity to valine of *E. coli* DK 12 was the result of derepression of (acetohydroxy) acid synthetase in this mutant, the role of the antibiotic in dependent cells being that of a "derepressor."

Bonner (1) reported that the growth of a wild-type strain of *Escherichia coli* K12 was inhibited by L-valine and that this inhibition was overcome by L-isoleucine. Leavitt and Umbarger (2) proposed that inhibition of the growth of *E. coli* K12 by valine was a consequence of the relatively high sensitivity to inhibition by valine of the initial condensing enzyme (acetohydroxy acid synthetase) of the isoleucine-leucine-valine pathway. Consequently,

valine would inhibit the growth of *E. coli* K12 by preventing the formation of acetohydroxy butyrate (and thus of isoleucine) through inhibition of acetohydroxy acid synthetase. Recently (1965) Temple, Umbarger, and Magasanik (3) provided further experimental evidence to support the view that valine exerts its inhibitory effect on the growth of *E. coli* K12 solely by preventing the biosynthesis of isoleucine.

Bragg and Polglase (4) and Tirunarayanan *et al.* (5) reported (independently) that streptomycin-dependent strains of *E. coli* excrete the amino acid L-valine during growth on glucose-salts medium. Streptomycin-dependent *E. coli* K12 was one of the strains found to excrete valine (5). During the course of studies on enzyme activities of several strains of *E. coli*, it was observed that the acetohydroxy acid synthetase activity of streptomycin-dependent cells growing with adequate antibiotic was higher than that of the parent streptomycin-sensitive cells (6, 7) or of antibiotic-depleted, dependent cells (8). Studies on the relation between dihydrostreptomycin concentration and enzyme activities in streptomycin-dependent *E. coli* indicated that one of the effects of the antibiotic appeared to be to derepress acetohydroxy acid synthetase (7).

It was of interest therefore, to study the response to L-valine of streptomycin-dependent *E. coli* K12.

Organisms and media. A streptomycin-dependent culture of *E. coli* K12 was isolated by selection of colonies which appeared after 48 hours' incubation of a nutrient agar plate (containing 1000 μg per milliliter of dihydrostreptomycin) which had been spread with 2 g of cell paste derived from growth of wild-type K12. The medium employed for growth and enzyme studies was that of Davis and Mingioli (9) with 0.4% glucose. For growth of the streptomycin-dependent K12 culture, this medium was supplemented with dihydrostreptomycin (1000 μg/ml). The procedure for growth of cells and for determination of acetohydroxy acid synthetase was as follows. Of an 18-hr stationary culture, 100 ml was added to 1 liter of medium (containing the appropriate additions) in a 2-liter Erlenmeyer flask. Cultures were grown with vigorous aeration for 4–5 hr, then chilled by immersion of the flasks in crushed ice. Cells were harvested by centrifugation (6000 *g*) and washed once with 0.1 M phosphate buffer, pH 8.0. The packed cells were then suspended in buffer (0.1 M phosphate, pH 8.0) at a concentration of 1 g of cells to 15 ml of buffer. This suspension was subjected for 4 min to sonic disruption in a 20 kc sonic oscillator (Biosonik, Bronwill Scientific Co., Rochester, New York). The resulting extracts, which contained 4.5–5.0 mg per milliliter of protein (10) were used directly for enzyme assays.

Determination of acetohydroxy acid synthetase. The method of analysis was developed as a result of observations on enzyme stability and cofactor requirements (11, 12). Each tube contained the following in a total volume of 1.0 ml of 0.1 M phosphate buffer, pH 8: thiamine pyrophosphate, 45 μg; flavin adenine dinucleotide, sodium salt, 100 μg; magnesium chloride, 0.1 ml of 0.05 M; sodium pyruvate, 250 μmoles; bacterial extract, 0.1 ml. For each determination, three tubes were incubated at 37° for 5, 10, and 15 min, respectively, whereupon the reaction was stopped by the addition to each tube of 0.1 ml of 50% trichloroacetic acid. Tubes were then heated at 60° for 15 min to convert acetolactate to acetoin, which was then determined by the method of Westerfeld (13). Specific activities were calculated from zero order plots and are expressed as micromoles of acetolactate per hour per milligram of protein.

Wild-type (streptomycin-sensitive) *E. coli* K12 (SK-12) grew exponentially on minimal medium, but growth was linear on the same medium containing L-valine (58 mg/l) (Fig. 1). Streptomycin-dependent *E. coli* K12 (DK-12) grew exponentially on minimal medium plus dihydrostreptomycin (1000 μg/ml), and the addition of L-valine (58 mg/l) to this medium had a negligible effect on growth of this mutant (Fig. 1).

In Table 1 are recorded the results of determinations of acetohydroxy acid synthetase in all four preparations. The activity of this enzyme was elevated in streptomycin-dependent *E. coli* K12 (DK-12) above that of the wild-type, or streptomycin-sensitive, K12 (SK-12). No acetohydroxy acid synthetase was detectable in SK-12 cells which had been grown in medium containing L-valine, whereas there was only a slight decrease in this enzyme in DK-12 cells after growth in the presence of L-valine (Table 1).

The acetohydroxy acid synthetase of

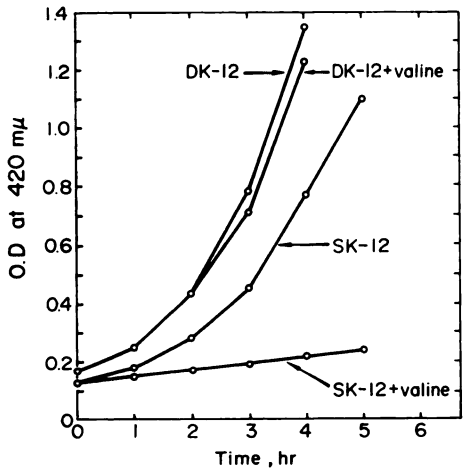

FIG. 1. *Growth of wild-type, streptomycin-sensitive E. coli K12 (SK-12) and a streptomycin-dependent mutant (DK-12) on minimal medium plus valine.*

At zero time, 58 mg valine was added per milliliter of medium, as indicated. For growth of the dependent organism (DK-12), dihydrostreptomycin (1.0 mg per ml) was present in the medium.

TABLE 1

Acetohydroxy acid synthetase activity of Escherichia coli K12

Culture	Additions to basal medium	μmoles of acetolactate per mg protein per hr
SK-12 (wild-type) streptomycin-sensitive	None	4.9
SK-12	Valine (58 mg/l)	Nil
DK-12 (streptomycin-dependent	None	8.0
DK-12	Valine (58 mg/l)	7.3

streptomycin-dependent *E. coli* was found to be valine sensitive (Table 2). The enzyme activities in Table 2 are recorded as optical density units at 540 mμ. The final column of Table 2 shows that the ratio of acetohydroxy acid synthetase activities in dependent versus sensitive cells remained constant at about 1.39 as the valine concentration was increased, indicating that the valine sensitivity of the enzyme was unaffected by mutation from streptomycin sensitivity to dependence.

TABLE 2

Inhibition by valine of acetohydroxy acid synthetase of streptomycin-sensitive (SK-12) and dependent (DK-12) Escherichia coli K12

Conditions: Each tube contained in a total volume of 2.0 ml of phosphate buffer (0.1 M pH 8.0): thiamine pyrophosphate, 45 μg; flavin adenine dinucleotide, sodium salt, 100 μg; magnesium chloride, 0.1 ml of 0.05 M; sodium pyruvate, 25 μmoles; bacterial extract 0.1 ml. Reaction time: 10 min at 37°. Acetolactate was determined after conversion to acetoin (Westerfeld, 13).

Valine (moles/liter)	Spectrophotometer reading at 540 mμ (DK-12)	(SK-12)	Ratio DK-12:SK-12
3.1×10^{-5}	0.812	0.590	1.38
6.2×10^{-5}	0.710	0.503	1.41
1.25×10^{-4}	0.590	0.430	1.37

Interest in the inhibition by L-valine of the growth of *E. coli* K12 stems from the importance of this phenomenon to an understanding of regulation of metabolic pathways. In a recent study of regulation of the isoleucine-leucine-valine enzymes in *Escherichia coli* K12, Umbarger and Freundlich (14) concluded that not only is the acetohydroxy acid synthetase of this strain more sensitive to valine inhibition than that of other strains of *E. coli*, but this enzyme is "rather uniquely not derepressed when isoleucine is limiting." These investigators (14) concluded that resistance to valine in *E. coli* K12 could be achieved by one of two types of mutation: (a) by mutation leading to the formation of a resistant enzyme (acetohydroxy acid synthetase) or (b) by a mutation that would result in derepressed formation of the isoleucine-leucine-valine enzymes. Resistance to valine by process (b) above would "*include derepression of the sensitive enzyme, acetolactate synthetase.*"

In the present work, it was observed that the acetohydroxy acid (acetolactate) synthetase of streptomycin-dependent *E. coli* K12 and that of the streptomycin-sensitive parent organism were equally

sensitive to valine (Table 2). Thus, resistance to valine by mutation leading to a resistant enzyme (see (a) above) is excluded as the explanation of valine resistance in streptomycin-dependent *E. coli* K12. Mutation resulting in derepressed enzyme formation (see (b) above) appears to explain the observed results satisfactorily. Only the first of the isoleucine-leucine-valine enzymes was determined in this investigation, and its activity in the streptomycin-dependent mutant was higher than that of the streptomycin-sensitive parent culture (Table 1). Presumably, the enzyme was sufficiently derepressed in streptomycin-dependent K12 to permit the observed exponential growth in the presence of an amount of valine sufficient to prevent exponential growth of the parent organism. These results appear to provide further evidence in support of the mechanism of valine inhibition of K12 proposed by Temple *et al.* (3) and by Umbarger and Freundlich (14).

Our interpretation of the significance of valine excretion to the phenomenon of streptomycin dependence has been reported elsewhere (4, 15). The apparent derepression of acetohydroxy acid synthetase in a number of strains of streptomycin-dependent *E. coli* and the relation between enzyme derepression and antibiotic concentration have been described recently (7). These results taken together with earlier observations on the requirement for streptomycin for enzyme induction in dependent *E. coli* (16, 17) suggest an action of the antibiotic at the level of expression of regulatory genes.

Bacterial cultures were grown by J. Withaar. Streptomycin and dihydrostreptomycin were gifts of Merck, Sharp & Dohme, Montreal, Canada. This work received financial support through grant No. MT-750 from the Medical Research Council of Canada.

REFERENCES

1. D. Bonner, *J. Biol. Chem.* **166**, 545 (1946).
2. R. I. Leavitt and H. E. Umbarger, *J. Bacteriol.* **83**, 624 (1962).
3. R. J. Temple, H. E. Umbarger, and B. Magasanik, *J. Biol. Chem.* **240**, 1219 (1965).
4. P. D. Bragg and W. J. Polglase, *J. Bacteriol.* **84**, 370 (1962).
5. M. O. Tirunarayanan, W. A. Vischer, and U. Renner, *Antibiot. Chemotherapy* **12**, 117 (1962).
6. M. B. Coukell and W. J. Polglase, *Can. J. Microbiol.*, in press.
7. W. J. Polglase, *Federation Proc.* **24**, 416 (1965).
8. P. D. Bragg and W. J. Polglase, *J. Bacteriol.* **89**, 1158 (1965).
9. B. D. Davis and E. S. Mingioli, *J. Bacteriol.* **60**, 17 (1950).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
11. F. C. Störmer and H. E. Umbarger, *Biochem. Biophys. Res. Commun.* **17**, 587 (1964).
12. I. D. Desai and W. J. Polglase, *Biochim. Biophys. Acta* (in press).
13. W. W. Westerfeld, *J. Biol. Chem.* **161**, 495 (1945).
14. H. E. Umbarger and M. Freundlich, *Biochem. Biophys. Res. Commun.* **18**, 889 (1965).
15. P. D. Bragg and W. J. Polglase, *J. Bacteriol.* **88**, 1006 (1964).
16. W. J. Polglase, S. Peretz, and S. M. Roote, *Can. J. Biochem. Physiol.* **34**, 558 (1957).
17. S. Peretz and W. J. Polglase, *Antibiot. Ann.* **1956–1957**, 533 (1957).