AN INVESTIGATION OF MODIFIED METABOLIC REGULATION IN STREPTOMYCIN-DEPENDENT ESCHERICHIA COLI

by

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ABSTRACT

The acetohydroxy acid synthetase levels in streptomycin-sensitive, -dependent and -resistant mutants have been studied in four different strains of Escherichia coli. The activity of the α-acetolactate-forming system was found to be greater both at pH 6.0 and at pH 8.0 in streptomycin-dependent mutants than in the corresponding streptomycin-sensitive cultures. In general, streptomycin-resistant mutants demonstrated enzyme activities within the range found for streptomycin-sensitive organisms regardless of whether they were grown in the presence or absence of antibiotic. The acetohydroxy acid synthetase activity of streptomycin-sensitive and -resistant revertants was observed to be lower than that of the dependent Escherichia coli culture from which they were derived by back-mutation. Mutation to streptomycin-resistance or -dependence had no effect on glucokinase and glutamic dehydrogenase activities. The addition of the coenzyme flavin adenine dinucleotide to the incubation mixtures markedly stimulated the activities of all the extracts. This enhancement of acetohydroxy acid synthetase activity had little or no effect on the ratio of activities of this enzyme in the dependent and sensitive Escherichia coli strains investigated. α-Acetohydroxybutyrate formation was found to be greater in extracts from the streptomycin-dependent

organism than in extracts prepared from the same strain of sensitive and resistant Escherichia coli. The degree of elevation of α-acetohydroxybutyrate paralleled that of α-acetolactate formation in the dependent mutant. It was concluded from these observations that excretion of L-valine by streptomycin-dependent Escherichia coli was a consequence of the elevated acetohydroxy acid synthetase activity of these mutants. In the dependent organism, it was postulated that streptomycin functioned as a "de-repressor" of aceto-hydroxy acid synthetase thus permitting the biosynthetic pathway leading to L-valine to serve as an important route of pyruvate dissimilation.

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A. INTRODUCTION

I. Metabolic Effects of Streptomycin in Microorganisms

The primary site of the antibacterial action of streptomycin has yet to be elucidated. However, several divergent hypotheses have been proposed: (1) inhibition of specific enzyme reactions (Umbreit, 1953; Rosanoff and Sevag, 1953; Barkulis, 1953); (2) altered permeability due to membrane damage (Anand, et al, 1960; Landman and Burchard, 1962); and (3) inhibition of protein synthesis (Erdos and Ullman, 1959; Mager, et al, 1962; Speyer, et al, 1962, Flax, et al, 1962a, 1962b; Cox, et al, 1964).

Two groups of investigators (Tirunarayanan, et al, 1962, and Bragg and Polglase, 1962) reported, independently, that streptomycin-dependent microorganisms grown on glucose-salts medium excreted relatively large amounts of L-valine and a lesser amount of L-leucine. Each group however, interpreted these results differently. Tirunarayanan and co-workers (1962) attributed the excretion of L-valine and L-leucine to a "partial blockage" of protein synthesis. Since the streptomycin-dependent strains were able to grow and multiply in spite of the partial block in protein synthesis, they suggested this phenomenon constituted only the preliminary fixation of streptomycin to the cell and that the antimicrobial effect was due to subsequent metabolic changes. Experiments carried out by Bragg and Polglase (1962) suggested that the excretion of L-valine resulted from an

alteration in the pathway of pyruvate dissimilation.

Extracellular metabolites are not normally detectable in the supernatant fluids of streptomycin-sensitive or -resistant organisms growing in the medium without antibiotic supplement. However, a streptomycin-resistant mutant in the presence of antibiotic excretes significant quantities of both lactate and pyruvate. These results suggest that the resistant mutant uses pathways of anaerobic metabolism in antibiotic containing medium but in the absence of the antibiotic employs a pathway of pyruvate dissimilation similar to that employed by sensitive organisms. The streptomycin-dependent mutant differs from both the sensitive and resistant organisms in its production of substantial amounts of L-valine. As much as 10% of the glucose carbon could be accounted for in this product. Subsequent work (Bragg and Polglase, 1964a) clearly indicated that depleted streptomycin-dependent cells, produced large quantities of lactate while the same cells supplemented with streptomycin, at a concentration in excess of that essential for growth, excreted only L-valine. When streptomycin-dependent cells were grown anaerobically, their metabolism resembled that of aerated, antibiotic depleted cells, (production of lactic acid) even in the presence of the optimal streptomycin concentration. For the streptomycin-dependent mutant, the relationship between aerobic metabolism and the requirement for the antibiotic is summarized diagrammatically in Fig. 1. The primary products of the aerobic catabolism

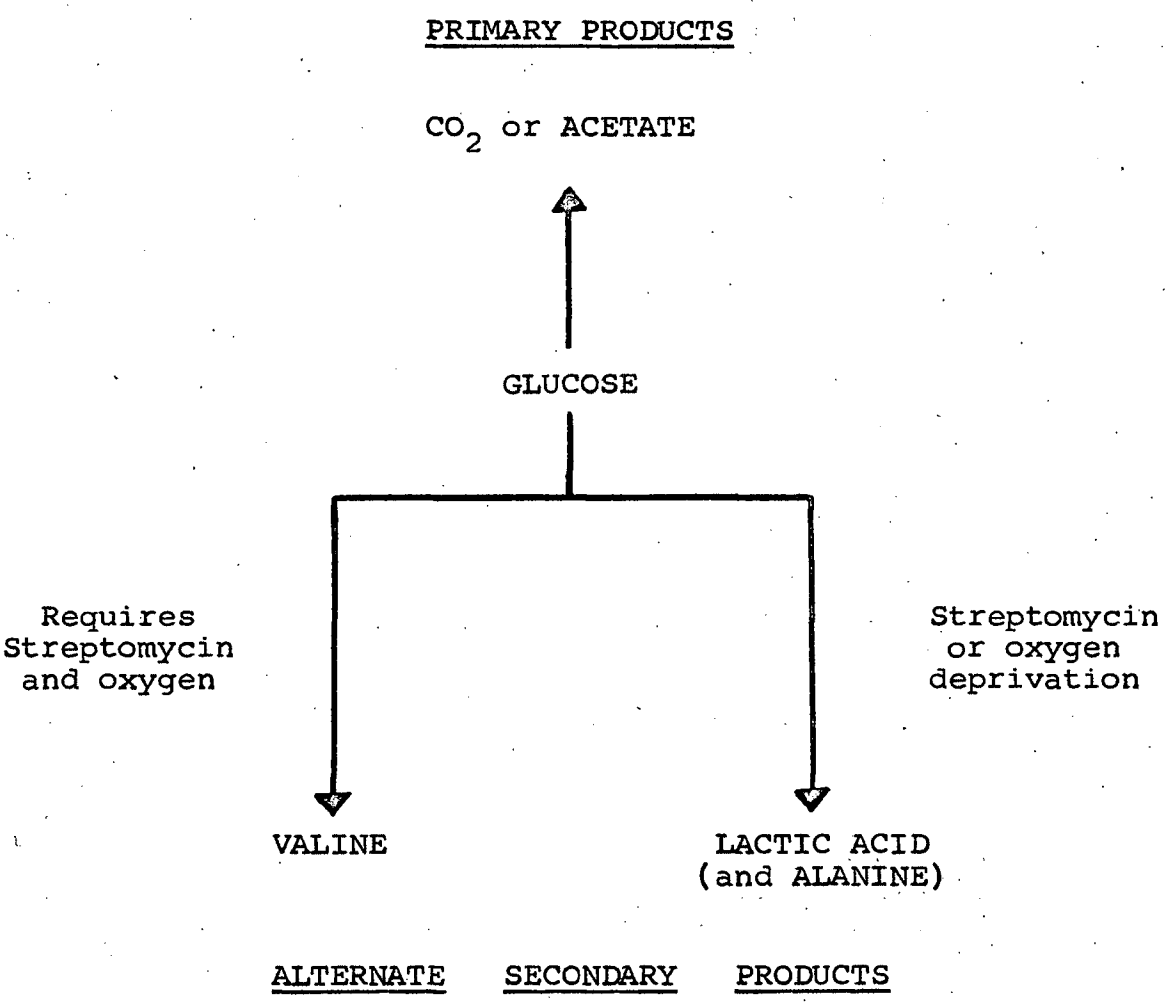

FIG. 1. Products of catabolism of glucose by streptomycin-dependent *Escherichia coli* (Taken from Bragg and Polglase, 1964a).

of glucose in streptomycin-dependent E. coli are carbon dioxide or acetate. The secondary products are L-valine (aerobic and supplemented with antibiotic) or lactate and L-alanine (either anaerobic or deprived of antibiotic). The small quantities of alanine probably arise from pyruvate by transamination. These workers proposed that the formation of L-valine appeared to be a secondary aerobic pathway of glucose metabolism existing in streptomycin-dependent mutants. Studies from the same laboratory (Bragg and Polglase, 1963b) on the effect of dihydrostreptomycin on electron transport in E. coli, suggested that L-valine may function as a neutral hydrogen acceptor in carbohydrate metabolism. If this were true, then, in streptomycin-dependent cells, the antibiotic might activate a mechanism enabling a biosynthetic pathway to function catabolically as a major route of pyruvate dissimilation.

II. Biosynthesis of Aliphatic Amino Acids and Regulatory Mechanisms.

The biosynthetic pathways leading to L-isoleucine, L-valine, L-leucine and pantothenate in E. coli have been investigated through isotope studies on selected auxotrophic mutants. A review of this work has been given by Umbarger and Davis (1962) (see Fig. 2). L-valine and L-leucine are synthesized from two moles of pyruvate while the precursors for L-isoleucine result from the condensation of one mole each of pyruvate and α-ketobutyrate. Umbarger and Brown (1958b) reported that the enzymes catalysing the last three reactions in the biosynthetic

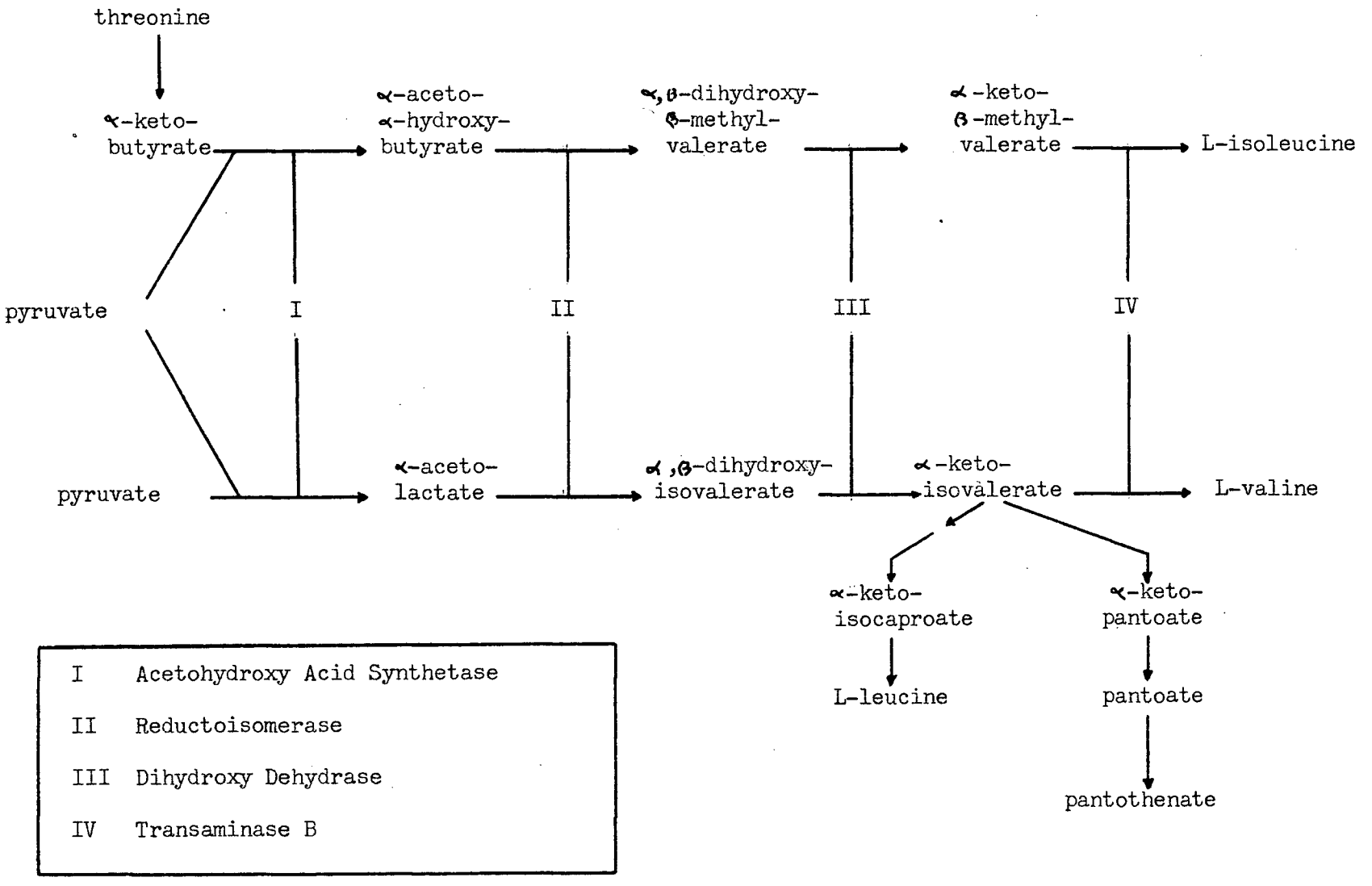

Fig. 2 The biosynthetic pathway to isoleucine, valine, leucine and pantothenate in Escherichia coli.

pathway leading to L-isoleucine also catalyse the corresponding reactions in L-valine synthesis. This first became evident when it was found that auxotrophic mutants lacking an enzyme on the L-valine pathway generally lacked the corresponding enzyme on the L-isoleucine pathway. Thus "single-step" mutants occur, multi-auxotrophic for L-valine, L-leucine and L-isoleucine. These findings have been supported by enzyme kinetic studies (Umbarger and Brown, 1958b; Leavitt and Umbarger, 1961). It should be noted however, that the enzyme which catalyses the first step in the L-valine pathway, viz. the condensation of two moles of pyruvate to one mole of ∝-acetolactate, also catalyses the second step in the L-isoleucine pathway, the acetylation of ∝-ketobutyrate to ∝-acetohydroxybutyrate (Leavitt and Umbarger, 1961). This enzyme complex (see Fig. 3) has been designated by various authors as the condensing enzyme, ∝-acetolactate-forming system (Umbarger and Brown, 1958b) and recently as acetohydroxy acid synthetase (Bauerle, et al, 1964). Although the enzyme complex has not been fractionated, early investigations (Umbarger and Brown, 1958b) suggest two reactions are involved. The first consists of the generation of an "active acetaldehyde", presumably as an acetal-diphosphothiamine (DPT) complex. The second reaction is the actual transfer of the acetal group to the acceptor molecule, either pyruvate or ∝-ketobutyrate. This reaction proceeds optimally only in the presence of a divalent cation such as Mg^{++} or Mn^{++}. Bauerle, et al (1964) recently reported that

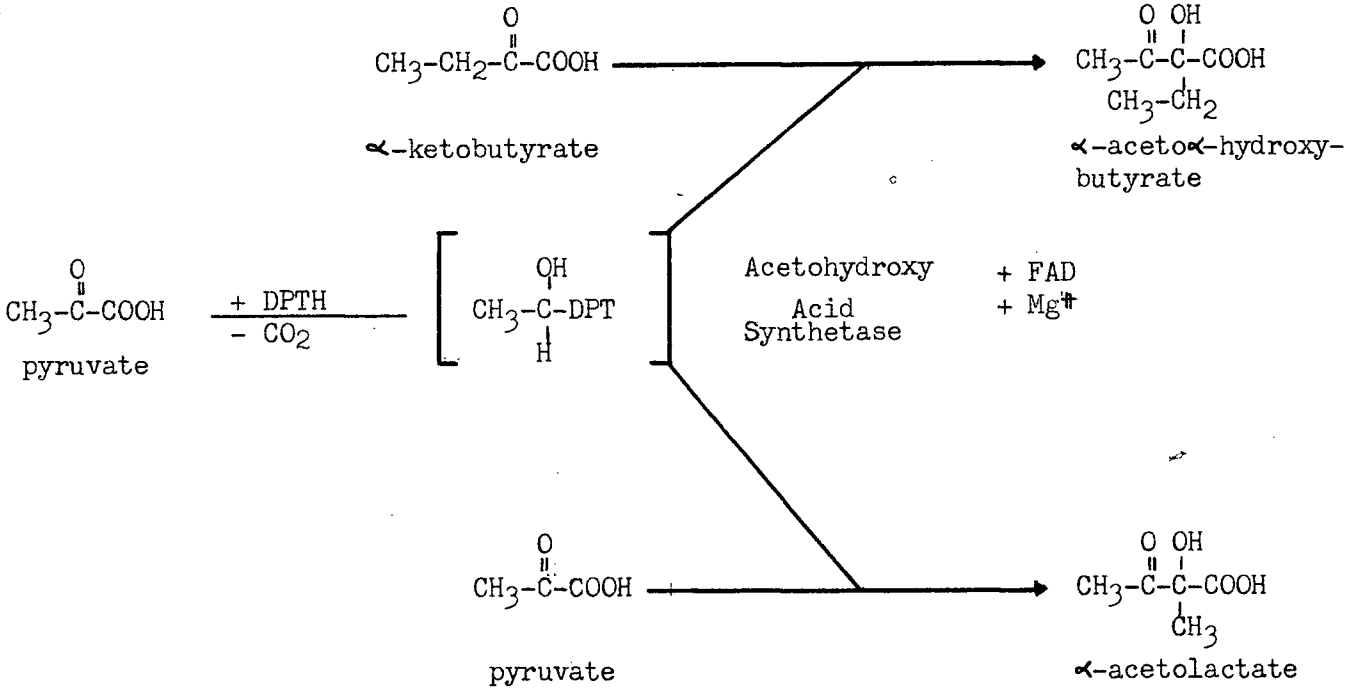

Fig. 3 The formation of ∝-acetolactate and ∝-aceto∝-hydroxy-butyrate in Escherichia coli.

acetohydroxy acid synthetase activity is greatly stimulated by the presence of the coenzyme flavin adenine dinucleotide (FAD). The function of this unexpected cofactor is as yet undefined. The pH optimum of the acetohydroxy acid synthetase system in E. coli has involved considerable re-investigation. Halpern and Umbarger (1959) clearly demonstrated the presence of two distinct acetolactate forming systems in A.aerogenes, one at pH 6.0 and the other at pH 8.0. However, they could find enzymic activity only at the higher pH in E. coli. A later paper (Radhakrishnan and Snell, 1960) described two pH optima in E. coli corresponding to the pH values reported for A. aerogenes. Recently, a thorough kinetic study on the acetohydroxy acid synthetase activity of a streptomycin-dependent E. coli mutant (Desai and Polglase, 1965) under optimal conditions (including supplementation with FAD), strongly supported the original prediction of a single pH 8.0 enzyme system in this organism.

It has been reported (Umbarger and Brown, 1958b) that the acetohydroxy acid synthetase complex, like initial enzymes of other biosynthetic pathways, is subject to end-product inhibition when assayed in the presence of L-valine. Repression studies, (Freundlich, et al, 1962) however, indicate that regulation of the enzymes associated with branched chain amino acid biosynthesis involves a control mechanism thus far unique in biological systems. Since L-valine and L-isoleucine are formed directly by a common sequence of enzymes, repression of the pathway

by the elevation of one product, could seriously affect the synthesis of the other product. In addition, L-leucine synthesis would also be impaired since the initial reaction leading to L-leucine biosynthesis involves an intermediate of the valine pathway. This problem is overcome in E. coli by the requirement that all three end-products (also possibly pantothenate) must be in excess for repression to occur. This has been termed "multivalent repression" (Freundlich, et al, 1962; Umbarger and Freundlich, 1965). Although the mechanism of this repression is not completely understood in regard to acetohydroxy acid synthetase, recent studies with streptomycin-dependent mutants of E. coli indicate that this enzyme is definitely repressible by the end-products (Polglase, in press). Therefore, it appears to be well established that the acetohydroxy acid synthetase system not only catalyses the initial step in the biosynthesis of L-valine and L-leucine from pyruvate but also controls the production of these amino acids. Since this enzyme complex is intimately involved with L-isoleucine biosynthesis, an alteration in acetohydroxy acid synthetase activity or a change in enzyme level would be expected to influence the synthesis of L-isoleucine as well as L-valine.

III. Objective of the Investigation of Streptomycin Mutants

It was suggested (Bragg and Polglase, 1962) that in streptomycin-dependent E. coli mutants, the antibiotic may evoke

an alteration in aerobic carbohydrate metabolism resulting in amino acid excretion. This may be further interpreted as an alteration in the control of branched chain amino acid biosynthesis. Preliminary studies (Bragg and Polglase, 1964a) on acetohydroxy acid synthetase activities in depleted and supplemented streptomycin-dependent E. coli, indicated that maximal α-acetolactate formation occurred only in the presence of antibiotic. Streptomycin appeared to de-repress the level of this enzyme in supplemented dependent mutants regardless of whether or not the cells were grown in the presence of the end-products. Since this suggests that streptomycin functions at the genetic level, it is essential to establish whether this is a general phenomenon or merely the characteristic response of a particular streptomycin-dependent mutant.

The objective of this investigation was therefore to determine through a study of several strains of E. coli whether consistent differences exist in streptomycin mutants in the level of acetohydroxy acid synthetase (the regulatory enzyme for L-valine biosynthesis). It was assumed at the outset that the generality of the phenomenon of L-valine excretion by streptomycin-dependent mutants had been established by previous work (Tirunarayanan, et al, 1962; Bragg and Polglase, 1962).

In its initial phase, the investigation required the isolation of several new E. coli mutants. The second phase of the study involved assays for enzymatic activities of bacterial

extracts. Subsequently, when adequate evidence had been adduced to establish that streptomycin-dependent mutants of E. coli do indeed differ quantitatively to sensitive and resistant strains in enzyme content, an attempt was made to explain the advantage to the streptomycin-dependent organism of this difference.

B. METHODS AND MATERIALS

I. Original Cultures

Four strains of E. coli were used in this work. E. coli "A" as previously described by Roote and Polglase (1955) was originally obtained as the streptomycin-dependent culture. This mutant has been designated DA. A streptomycin-sensitive (SA), and streptomycin-resistant (RA) mutant was derived by "back-mutation" from the dependent culture. The strain designated E. coli "C" was obtained from the Laboratory of Hygiene, Ottawa, Ontario and strain E. coli "E" was isolated at the Department of Bacteriology, University of Laval, Montreal. These were supplied to us as streptomycin-sensitive strains. An additional sensitive strain was obtained from the American Type Culture Collection (ATCC 12407). This strain is a spontaneous radiation-resistant mutant of E. coli "B" and will be referred to, hereinafter, as E. coli B/r (Witkin, 1947).

II. Isolation of Streptomycin Mutants

(i) From streptomycin-sensitive parent cultures.

A volume of approximately 500 ml. of glucose-salts medium (composition described under "Medium") was innoculated with one or two loopfuls of streptomycin-sensitive culture stored in heart infusion broth (25 gm. of heart infusion broth per liter) at 5°C. This culture was incubated without agitation for 20 - 24 hours at 37°C. The resulting growth was transferred

aseptically to two 300 ml. sterile bottles and centrifuged at 2000 RPM for 1 hour at 4°C (International Refrigerated Centrifuge). The pellet was resuspended in 6.0 ml. of buffer (0.05M potassium phosphate, pH 7.4), called the "Standard Inoculum" (SI). Exactly 0.5 ml. of the SI ($\sim 10^8$ cells) were pipetted on to the surface of several Petri plates containing heart infusion agar (heart infusion broth fortified with 1.5% agar) and 1000 units per ml. (1 unit is equivalent to 1 µg of free streptomycin base) of either dihydrostreptomycin (DHSM) or streptomycin (SM). The slurry of cells was evenly distributed over the agar surface and the plates were incubated at 37°C for 24 hours. The colonies which formed were subcultured into 5 ml. of sterile glucose-salts medium, either devoid of antibiotic or supplemented with 1000 µg per ml. and incubated as described above. Dihydrostreptomycin-resistant mutants would grow in both tubes, whereas dependent mutants would grow only in the presence of dihydrostreptomycin. To ensure that growth in the absence of antibiotic was in fact due to the resistant mutant and not to growth of a dependent culture resulting from a carry-over of dihydrostreptomycin from the plate, a loopful of this culture was further transferred both to antibiotic supplemented and to unsupplemented medium in tubes. Pure resistant and dependent cultures of each strain were stored at 5°C on both heart infusion agar slopes and in heart infusion broth. All cultures were subcultured monthly.

(ii) Isolation of revertants from a streptomycin-dependent culture.

To 100 ml. of glucose-salts medium supplemented with 100 µg of dihydrostreptomycin per ml. was added 10 ml. of the E. coli dependent strain grown overnight at the same antibiotic concentration. The culture was incubated at 37°C for 20 - 24 hours and centrifuged aseptically at 2000 RPM for 1 hour. The pellet was washed twice in 0.05M potassium phosphate buffer, pH 7.4, added to 1000 ml. of heart infusion broth (antibiotic-free) and incubated a further 48 hours at 37°C. The cells were harvested as previously described. The pellet was suspended in 10 ml. of buffer. One loopful of this cell suspension was streaked on glucose-salts agar medium (glucose-salts broth containing 1.5% agar) in such a manner as to produce isolated colonies. Colonies were subcultured to liquid glucose-salts medium to yield pure sensitive (growth only in the absence of DHSM) and resistant (indifferent to the presence of DHSM) cultures. All cultures were stored as indicated.

III. Growth of Cultures and Preparation of Extracts

(i) Medium

The basal medium was of the composition previously described by Davis and Mingioli (1950) and consisted of the following: K_2HPO_4(0.7%), KH_2PO_4(0.3%) sodium citrate (0.05%), $MgSO_4$(0.02%), $(NH_4)_2SO_4$(0.1%). Glucose was autoclaved separately and added to the basal medium to give a final concentration of 0.4%.

Rather than streptomycin, the more stable analog, dihydrostreptomycin (sesqui sulfate) (Merck, Sharp and Dohme, Montreal, Canada) was generally used in this work. When streptomycin sulfate was used, it was sterilized by passage through millipore filters. In all cases the medium was adjusted to a final pH of 7.0.

(ii) Procedure for growing and harvesting cultures.

Prior to growth, the mutants involved were transferred at least three times on glucose-salts medium, under conditions (temperature and antibiotic concentration) similar to those employed in the final growth experiment.

The glucose-salts medium (900 ml.) was inoculated with 100 ml. of culture previously grown as a stationary culture overnight at 37°C. The two-liter flask was incubated at the same temperature in a water bath with moderate aeration provided as follows. Air was supplied by glass tubing fitted through a cork stopper, running below the surface of the medium and connected by rubber tubing to an air line. Rate of growth in the flask was observed by recording optical densities hourly at 420 mμ. The cultures were generally grown for 5 to 6 hours and were harvested during the latter half of the exponential growth phase (usually at an $O.D._{420\ m\mu}$ of approximately 1.4). Streptomycin-sensitive organisms were grown in antibiotic-free medium, while streptomycin-dependent cultures were routinely grown on the same medium fortified with 1,000 units per ml. of dihydrostreptomycin.

The resistant mutants were grown on both supplemented (R^+ cells) and unsupplemented (R^- cells) medium.

The cells were harvested immediately by centrifugation at 6,000 x g for 20 minutes in a refrigerated centrifuge (4°C) and were then washed by centrifugation with 0.05M potassium phosphate buffer, pH 7.0.

(iii) Bacterial extracts.

The pellet was resuspended in the same buffer in a ratio of 1 gm. of cells to 15 ml. of buffer. The suspensions were then treated in a Bronwill 20-kc sonic oscillator for 3 minutes followed by centrifugation at 10,000 x g for 15 minutes. The supernatant solutions were either assayed immediately or stored at -20°C and assayed within 20 - 24 hours.

IV. Enzyme Assays

(i) Measurement of ∝-acetolactate-forming activity.

Early studies on this enzyme complex (Radhakrishnan and Snell, 1960) suggested activity maxima at both pH 6.0 and 8.0. Therefore, initial assays were carried out at both pH values. Later experiments (Desai and Polglase, 1965) established the existence of only one optimal pH (pH 8.0) for this enzyme system and thereafter pH 6.0 assays were discontinued. During the course of this work, two assay methods were employed.

(a) Method I

The earlier method was modified from the procedure of Umbarger and Brown, (1958b). Each tube contained in 2.6 ml:

potassium phosphate, pH 8.0 and pH 6.0, 100 μmoles; sodium pyruvate, 50 μmoles; $MgCl_2$, 5 μmoles; thiamine pyrophosphate, 0.3μmoles; 0.5 ml. of E. coli extract (protein; 2-4 mg. per ml.) and when indicated flavin adenine dinucleotide 10 mμmoles. Assay tubes were generally incubated at 37°C for 30 minutes (longer periods were used for extracts of very low activity or shorter periods for high activity). The reaction was stopped by the addition of 0.5 ml. of 10% trichloroacetic acid and the tubes were centrifuged to clarify the reaction mixtures. To 1.0 ml. of the supernatant solution was added 0.05 ml. of 36 N sulphuric acid, and the sample was autoclaved at 10 p.s.i. for 10 minutes to convert the α-acetolactate to acetoin. The solution was then diluted to 10 ml. To a 5 ml. aliquot of this solution was added 1.0 ml. of 0.5% creatine in water and 1.0 ml. of freshly prepared 5% α-naphthol in 2.5 N sodium hydroxide. The solution was mixed vigorously and the color allowed to develop for one hour in the dark. The optical density at 540 mμ was read on a Beckman B spectrophotometer. Since the enzyme acetolactate decarboxylase is not present in extracts of E. coli (Juni, 1952; Umbarger and Brown, 1958b; Radhakrishnan and Snell, 1960) acetoin production can be attributed entirely to chemical decarboxylation of α-acetolactate and provides a measure of acetohydroxy acid synthetase activity. Determination of the acetoin content of untreated extracts of E. coli (not acidified and heated) was accomplished by stopping the reaction mixtures with 0.1 ml. of 10% zinc sulphate

and 0.1 ml. of 1 N sodium hydroxide as described by Umbarger and Brown (1958b). Acetoin was determined immediately in one aliquot and a second aliquot was heated following addition of acid and treated as usual (See Table II).

(b) Method II

The second procedure used to assay acetohydroxy acid synthetase activity was described by Desai and Polglase, (1965). It differs from the former method primarily in the concentration of certain components of the incubation mixture and in the regular supplementation with FAD.

Each tube contained in 1.0 ml: potassium phosphate, pH 8.0, 100 μmoles; sodium pyruvate, 0.5 mmoles; $MgCl_2$, 0.5 μmole; thiamine pyrophosphate, 45 mμmoles; flavin adenine dinucleotide, 10 mμmoles; 0.5 ml. of E. coli extract (prepared in pH 8.0, 0.5 M potassium phosphate buffer). After incubation for 15 minutes at 37°C the reaction was stopped by the addition of 0.1 ml. of 50% trichloroacetic acid. This was followed by incubation for 15 minutes at 60°C to convert the α-acetolactate to acetoin. An aliquot of the resulting solution was analysed for acetoin by the method of Westerfeld (1945). Protein was determined by the method of Lowry et al, (1951). Results by both methods were expressed in micromoles of α-acetolactate formed per mgm. of protein per hour.

(ii) Measurement of α-acetohydroxybutyrate-forming activity.

α-Acetohydroxybutyrate was determined in a microbiological assay based on the fact that growth inhibition of strain K-12 of E. coli by L-valine is reversed by L-isoleucine (Tatum, 1946) or any six-carbon precursor of L-isoleucine (Umbarger, 1958). Since α-acetohydroxybutyrate is decarboxylated by boiling for 5 minutes, the assay was rendered specific for this compound by testing the extracts before and after heat treatment.

(a) Reversal of L-valine inhibition by L-isoleucine in E. coli K-12.

A series of tubes (12 x 200 mm.) were prepared containing, in glucose-salts medium, L-valine, 0.42 µmoles per ml. (50 µgm), and L-isoleucine in serial dilutions ranging from 0.38 to 0.012 µmoles (50 to 1.5 µgm) per ml., in a final volume of 5.0 ml. A control tube containing only L-valine (0.42 µmoles per ml.) was also prepared. All tubes were inoculated with two loopfuls of E. coli K-12 previously grown overnight in amino acid-free basal medium at 37°C. The inoculated tubes were then incubated at 37°C for three days. Relative turbidities were recorded at various intervals during this period (See Table VII).

(b) Preparation of assay plates.

The method employed was modified from the procedure originally described by Leavitt and Umbarger (1960). The assay organism (E. coli K-12) was grown overnight on glucose-salts medium. The culture was diluted aseptically with liquid medium

to an optical density (O.D. 420 mμ) of approximately 1.0 and stored at 5°C. This culture could be maintained at that temperature for upto 5 days with consistent results. The solid medium (L-valine agar) contained 1.5% agar and was supplemented with 0.42 μmoles (50 μgm) of L-valine per ml.

To seed the agar 0.1 ml. of the culture was mixed with 10 ml. of the melted valine agar previously cooled to 45°C. The seeded agar was poured into a plastic Petri dish (20 x 60 mm.), rotated to distribute the organisms, and allowed to solidify. A second layer of seeded agar identically prepared was then poured on top of the first layer and a porcelain assay cylinder (Penicylinder, 8 x 10 mm., Fisher Scientific Co. Ltd., Edmonton, Canada) was dropped into the upper liquid layer. After the second layer had solidified 0.1 ml. of the test sample was placed in the cup. The assay plates were incubated at 37°C for 16 hours. When a reaction mixture was assayed the cylinders were modified by inserting into the bottom of each a disk of Whatman #3 filter paper slightly larger in diameter than the cylinder. These disks serve to localize any precipitated protein or bacterial contamination introduced along with the reaction mixture.

(c) Enzymatic formation of α-acetohydroxybutyrate.

Bacterial extracts were prepared as previously described. Each incubation tube contained in a final volume of 1.0 ml: sodium pyruvate, 10 μmoles; α-ketobutyrate, 5 μmoles; $MgCl_2$, 10 μmoles; thiamine pyrophosphate, 175 mμmoles; flavin adenine

dinucleotide, 10 mμmoles; potassium phosphate 100 μmoles (pH 8.0) and 0.5 ml. of extract protein (prepared in pH 8.0 buffer). After 15 minutes incubation at 37°C, the reaction was stopped by the addition of 0.1 ml. each of 10% $ZnSO_4$ and N NaOH. The precipitated protein was removed by low speed centrifugation and the supernatant solution (or an aliquot thereof) was placed in the cup of the assay plate. Incubation mixtures were generally assayed in duplicate or in triplicate.

(d) Estimation of bacterial growth.

At the end of the incubation period zones of growth surrounded those cups which had contained α-acetohydroxybutyrate. The cylinders were removed and the agar which contained growth was cut out with a cork bore of a diameter slightly larger than the zones of growth. The agar plug was dropped into a 12 ml. tapered centrifuge tube containing 0.5 ml. of distilled water. The contents of the tube were heated above 96°C for 90 seconds and then treated with 0.7 ml. of 0.5 N hydrochloric acid. The tube was then heated for an additional 60 seconds to hydrolyse the agar. Before cooling, the tubes were centrifuged (Servall) at 3400 x g for 8 minutes. The supernatant was discarded and the precipitate was washed in 2.0 ml. of distilled water. The washed protein was dissolved in 0.5 ml. of 5% sodium carbonate in 0.1 N sodium hydroxide and estimated by the method of Lowry, et al, (1951).

(e) Preparation of a standard curve.

L-Isoleucine was used as a standard rather than the intermediate, α-acetohydroxybutyrate. Points on the standard curve were derived by averaging values obtained from several independent experiments (See Table IV).

(iii) Reference enzymes.

(a) Glucokinase

Glucokinase activity was determined spectrophotometrically at room temperature by observing the change in optical density at 340 mμ (Cary 15 spectrophotometer, Applied Physics Corporation, Monrovia, California). The system contained the following in a volume of 1.0 ml: glucose, 4 μmoles; adenosine triphosphate, 2 μmoles; $MgCl_2$, 4.5 μmoles; nicotinamide adenine dinucleotide phosphate ($NADP^+$) 100 μmoles; glucose-6-phosphate dehydrogenase (C.F. Boehringer and Soehne, Mannheim, Germany), 1 unit; tris (hydroxymethyl) aminomethane pH 7.0, 100 μmoles. The reaction was initiated by the addition of 0.1 ml. of cell extract (prepared as described above, pH 7.0). Activities are expressed as millimicromoles of coenzyme reduced per milligram of protein per minute.

(b) Glutamic dehydrogenase

For the determination of glutamic dehydrogenase the following solution was prepared: tris (hydroxymethyl) aminomethane, 50 μmoles (pH 7.0); α-ketoglutarate, 3 μmoles; ammonium sulfate, 40 μmoles; reduced nicotinamide adenine dinucleotide phosphate (NADPH) 150 mμmoles. To 1.0 ml. of this solution in

a cuvette was added 0.1 ml. of bacterial cell extract and the decrease in optical density at 340 mμ was recorded at 25°C in the Cary 15 spectrophotometer. Specific activities are expressed as millimicromoles of coenzyme oxidized per milligram of protein per minute.

C. RESULTS

I. Streptomycin Sensitivity of the Cultures

The sensitivity of streptomycin-sensitive (S) strains to the antibiotic is shown in Table I. The revertant obtained from the dependent parent (DA) is slightly less sensitive than wild type strains.

II. Specificity of the Method for α-Acetolactate Determination

Since α-acetolactate formation by cell free bacterial extracts is determined colorimetrically by measuring the concentration of the decarboxylated product, acetoin, it should be established that in E. coli, acetoin is not a normal end-product. Table II gives the results of α-acetolactate determination on the streptomycin-dependent mutant DE when the incubation mixtures are stopped under different conditions. It should be noted that acetoin produced after acidification and heating appears to represent the total α-acetolactate formed during the enzyme reaction (See Discussion). This supports data published on other strains of E. coli (Juni, 1952; Radhakrishnan and Snell, 1960; Umbarger and Brown, 1958b). For this reason, all α-acetolactate

TABLE I

Relative Sensitivities of Escherichia Coli Sensitive Strains to Dihydrostreptomycin.

Strain	Growth+ in [DHSM] μgm per ml.					
	1.5	3.0	6.0	12.5	25	50
SA*	++++	++	tr	0	0	0
SC	++++	0	0	0	0	0
SE	++++	0	0	0	0	0
SB/r	++++	+	0	0	0	0

* Derived from the dependent mutant (DA) by back-mutation.

\+ Each tube contained 5 ml. of glucose-salts medium and a constant inoculum. Growth was estimated after incubation at 37°C for 24 hours. Relative growth is represented by the scale 0 to ++++ with "tr" indicating a trace of growth.

TABLE II

Comparison of Methods for Determining α-Acetolactate in Extracts of *Escherichia coli*.

Reaction stopped with	μmoles of acetoin per mg. protein per hour	
	pH 6.0	pH 8.0
TCA*, not heated	0.319	0.677
TCA*, heated+	0.207	0.582
$ZnSO_4$, NaOH‡ not heated	0.075	0.056
$ZnSO_4$, NaOH, acidification and heated	0.235	0.620

* Trichloroacetic acid, final concentration 1.6%.

\+ Heated for 10 minutes in an autoclave at 10 p.s.i. following acidification with 0.05 ml. of 36 N H_2SO_4.

‡ As described in "Materials and Methods".

assays described in this study employ a total acetoin determination on reaction mixtures following acidification and heat treatment.

III. Activity of the α-Acetolactate-forming System of the Mutants

Relative acetohydroxy acid synthetase activities for streptomycin-sensitive (S) and streptomycin-dependent (D) mutants of E. coli are given in Table III. It can be seen that at both pH 6.0 and 8.0 the D/S ratio is greater than unity. In contrast to this are the results in Table IV for streptomycin-resistant mutants grown in the absence of antibiotic (R^- cells) or in a medium supplemented with 1000 units per ml. of dihydrostreptomycin (R^+ cells). In this case, the R^+/R^- ratio both at pH 6.0 and 8.0 deviates only slightly from unity.

The effect of flavin adenine dinucleotide (FAD) on acetohydroxy acid synthetase activity at pH 8.0 is given in Table V. It was observed that although a substantial (six fold) increase in enzyme activity had occurred in extracts containing FAD, the D/S ratios of these mutants remained constant. As a result of the elevated activity in the presence of FAD, all further acetohydroxy acid synthetase determinations included this coenzyme.

IV. Activity of the α-Acetohydroxybutyrate-forming System of the Mutants.

Inhibition of the growth of E. coli strain K-12 by L-valine and reversal of this inhibition by L-isoleucine is

TABLE III

Acetohydroxy acid Synthetase Activities in Extracts of Streptomysin-Sensitive (S) and -Dependent (D) Escherichia coli.

μmoles of α-acetolactate+ formed per mg. protein per hour.

	pH 6.0			pH 8.0		
Strain	Sensitive	Dependent	D/S	Sensitive	Dependent	D/S
B/r	0.286	0.407	1.4	0.256	0.714	2.8
E	0.125	0.207	1.6	0.104	0.582	5.6
C	0.065	0.477	7.3	0.239	0.542	2.3
A	0.355	0.477	1.3	0.334	0.688	2.1

* D/S is the ratio of activities in dependent (D) and sensitive (S) extracts.

\+ Determined by Method I.

TABLE IV

Acetohydroxy Acid Synthetase Activities in Extracts of Streptomycin-Resistant Escherichia coli.

μmoles of α-acetolactate* formed per mg. protein per hour

	pH 6.0			pH 8.0		
Strain	R^-	R^+	R^+/R^-	R^-	R^+	R^+/R^-
B/r	0.251	0.139	0.6	0.321	0.282	0.9
E	0.167	0.183	1.1	0.192	0.141	0.7
C	0.179	0.140	0.8	0.152	0.108	0.7
A	0.117	0.118	1.0	0.152	0.218	1.4

R^- indicates extracts from cells grown without added antibiotic.

R^+ indicates extracts from cells grown in medium containing 1,000 units per ml. of dihydrostreptomycin.

* Determined by Method I.

TABLE V

Effect of Flavin Adenine Dinucleotide on Acetohydroxy acid Synthetase Activities in Sensitive (S) and Dependent (D) Extracts of Escherichia coli.

	μmoles ∝-acetolactate[+] formed per mg. protein per hour.			
Mutant	- FAD	D/S	+ FAD[*]	D/S
SA	0.438	1.8	2.601	1.7
DA	0.793		4.500	
SC	0.188	2.4	1.165	2.5
DC	0.456		2.960	

* Flavin adenine dinucleotide (FAD) was added to give a final concentration of 2 μgm. per ml.

\+ Determined by Method I.

shown on Table VI. The extreme sensitivity of this inhibition to specific reversal, and only by L-isoleucine or any six-carbon precursor of this amino acid, permits the quantitative determination of α-acetohydroxybutyrate by estimating relative bacterial growth. Table VII shows that growth of E. coli strain K-12 is due entirely to the presence of α-acetohydroxybutyrate and not to the straight chain five-carbon decarboxylation product (in confirmation of the observations of Leavitt and Umbarger, 1960). A standard curve of total protein versus L-isoleucine concentration as determined by the microbiological assay method previously described is given in Fig. 4. Due to the poor reproducibility of this system each point was derived by averaging values of several experiments. The resulting curve compared favorably with the L-isoleucine standard curve of Umbarger and Leavitt (1960).

A comparison of α-acetolactate and α-acetohydroxybutyrate formation by cell-free extracts prepared from streptomycin-sensitive, -dependent and -resistant mutants of E. coli is given in Table VIII. It is interesting to note that there was formed approximately 35% less α-acetohydroxybutyrate than α-acetolactate in all mutants.

V. Activity of the Reference Enzymes

In Table IX, glucokinase and glutamic dehydrogenase activities are shown for mutants of E. coli A. The activities of both enzymes appeared to remain constant in all mutants.

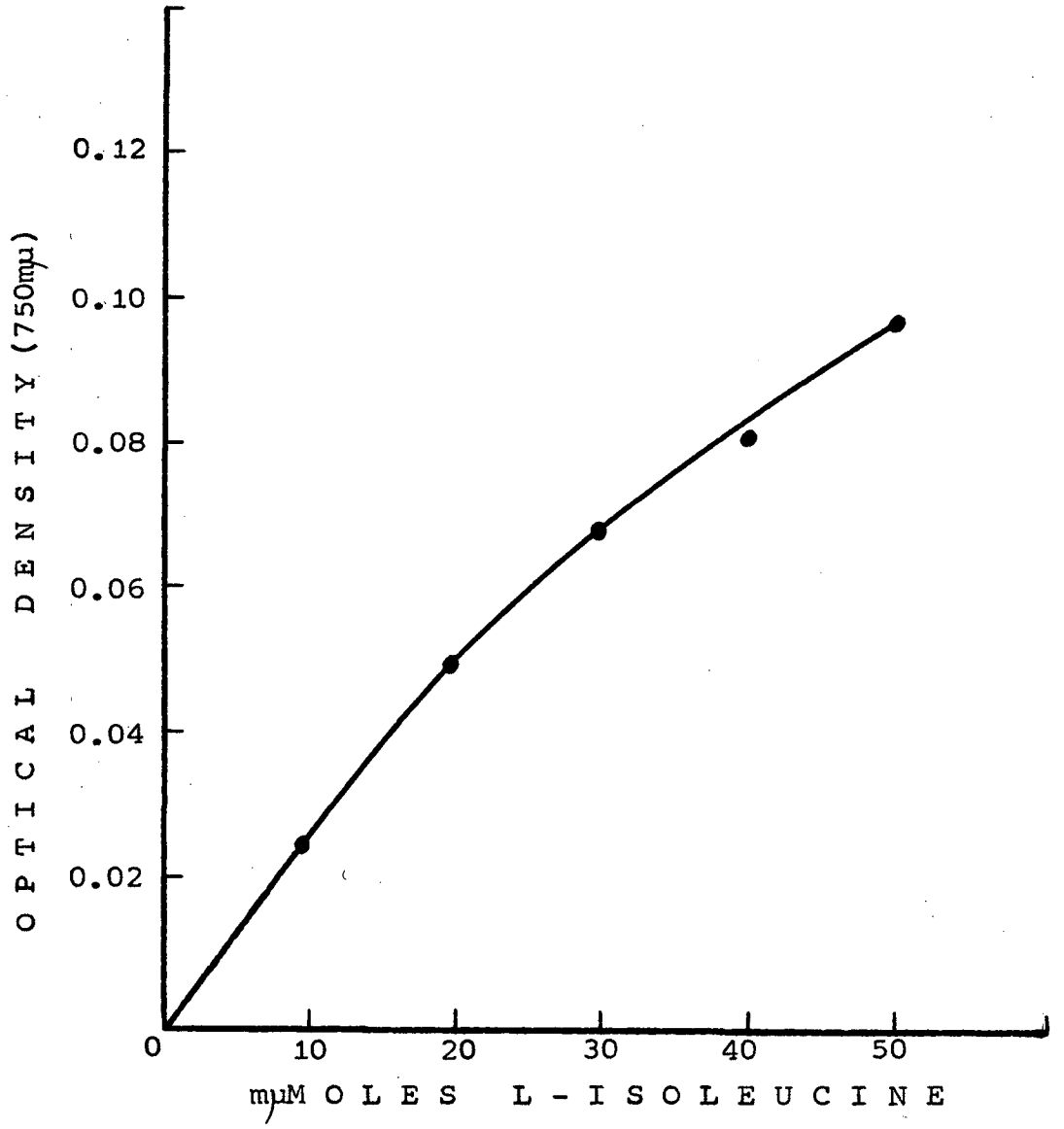

FIG. 4. Growth response of Escherichia coli K-12 to increasing concentrations of L-isoleucine. Each point was derived by averaging the results of five separate experiments.

TABLE VI

Sensitivity of Escherichia coli K-12 to L-Valine and Reversal of Inhibition by L-Isoleucine.

Concentration of L-Isoleucine	Relative Turbidity*	
µmoles per ml. of medium	20 hours	72 hours
0.380	++++	++++
0.190	+++	++++
0.095	+++	++++
0.048	+++	+++
0.024	++	+++
0.012	++	++
0.000	0	tr

All tubes contained L-valine (0.42 µmoles per ml.)

* Growth was estimated after incubation at 37°C for the period of time indicated. Relative growth is represented by the scale 0 to ++++ with "tr" indicating a trace of growth.

TABLE VII

Specificity of the α-Acetohydroxybutyrate Assay System for the Six-Carbon Intermediate.

Reaction stopped with	μmoles of α-Acetohydroxybutyrate* formed per mg. protein per hour
$ZnSO_4$, NaOH+	2.150
TCA, Heated ‡	0.050

* Calculated from the L-isoleucine standard curve Fig. 4. Extracts assayed at pH 8.0 only.

\+ As described in "Materials and Methods".

‡ The reaction was stopped by the addition of 0.2 ml. of trichloroacetic acid followed by boiling for 5 minutes.

TABLE VIII

Formation of α-Acetolactate and α-Acetohydroxybutyrate by Sensitive (S), Resistant (R) and Dependent (D) Extracts of Escherichia coli.

Culture	α-Acetolactate*	Ratio	α-Acetohydroxybutyrate+	Ratio
	μmoles of acetoin formed per mg. protein per hour	D/X‡	μmoles of isoleucine formed per mg. protein per hour	D/X
DA	5.50		3.62	
SA	3.74	1.5	2.36	1.5
RA^-	2.67	2.1	1.85	2.0
RA^+	3.64	1.5	2.48	1.5
DE	5.63		3.52	
SE	2.73	2.1	1.52	2.3

* Determined by Method II (pH 8.0 only).

\+ Calculated from the L-isoleucine standard curve. Fig. 4.

‡ X refers to cultures other than streptomycin-dependent mutants.

RA^- represents resistant cells grown in the absence of antibiotic, while RA^+ represents the same resistant culture grown in a medium supplemented with 1000 units of dihydrostreptomycin per ml.

TABLE IX

Reference Enzyme Activities of Streptomycin Mutants of *Escherichia coli* A.

Extract	Glucokinase*	Glutamic Dehydrogenase*
Sensitive (SA)	51	120
Dependent (DA)	50	120
Resistant (RA^-)†	58	112
Resistant (RA^+)†	67	122

---

* Activities are expressed as millimicromoles of coenzyme ($NADP^+$ or NADPH, respectively) changed per minute per milligram of protein.

† RA^- refers to resistant cells grown in antibiotic-free medium, while RA^+ cells were grown with 1,000 units per ml. of dihydrostreptomycin.

D. DISCUSSION

I. Genetic Characteristics of the Streptomycin Mutants

Plating methods employed in this work readily yielded spontaneous mutants exhibiting complete indifference to streptomycin or dihydrostreptomycin although the parent wild types were sensitive to low concentrations (above 3.0 μg per ml.) of either antibiotic (Table III). While individual mutation rates were not calculated, it became apparent during this work that streptomycin indifferent colonies were occurring at a relatively constant frequency from the various sensitive strains. Values ranging from 10^{-10} (Newcombe and Hawirko, 1949) to 10^{-8} (Demerec, 1951) have been suggested for rates of mutation to resistance. These values include the formation of both streptomycin resistant and dependent organisms.

The occurrence of single step dependent mutants, unlike indifferent mutants, appeared to fluctuate with the strain of *E. coli* and form of antibiotic employed. *E. coli* strain B (obtained from the National Research Council of Canada), for example, refused to produce dependent mutants on medium containing either streptomycin or the dihydro derivative despite repeated attempts. The same strain, however, readily formed resistant mutants on either form of the antibiotic. It was generally found that the reduced form of the antibiotic (dihydrostreptomycin) was consistently more effective in isolating dependent mutants than was streptomycin itself. Once obtained, the mutants responded

equally well to either form of the antibiotic.

It was clearly shown by Scott (1949) that both streptomycin and dihydrostreptomycin are non-mutagenic. Resistant and dependent forms will appear with a characteristic frequency in susceptible populations irrespective of the presence of the antibiotic. At the present time with the limited understanding of the mechanism of action of streptomycin it is difficult to explain the enhanced ability of the dihydro form to select dependent mutants.

A single step back-mutation (reversion) from high level streptomycin-dependence to streptomycin-sensitivity can be demonstrated in many strains of dependent E. coli. Hashimoto (1960) has shown that mutation from dependence to sensitivity is in fact not a true reversion but is mediated by a suppressor mutation. This suppressor maps close to the locus governing antibiotic dependence and high level resistance and is capable of modifying the expression of either allele. It is obvious from Table I that the revertant (SA) is not quite as sensitive to dihydrostreptomycin as are the three wild type sensitive cultures. This suggests that suppression of the dependent locus is incomplete and the resulting sensitive progeny acquire a low level resistant phenotype as demonstrated by their ability to grow on slightly elevated streptomycin concentrations. Characteristics of the corresponding resistant mutant (RA) will be discussed later. The rate of back-mutation has been studied by Bertani(1951)

and his results indicate that for a given strain the mutation rate is constant and ranges as high as 10^{-8} per bacterium per division. Various strains, however, differ markedly in their ability to revert.

In addition to reversion from dependence to sensitivity, certain strains of dependent E. coli apparently are capable of converting to high level resistance if stored under unfavorable conditions in the presence of antibiotic. This alteration in phenotype is spontaneous and probably is induced by a suppressor mutation (Hashimoto, 1960) or a modifier mutation (Matney, et al, 1960). Due to the instability of many dependent mutants all strains were subcultured monthly and the state of each was determined prior to experimentation.

In E. coli genetic analysis has shown that sensitivity, dependence and single-step high level resistance are determined by multiple alleles at a single locus known as the "Sm locus" (Newcombe and Nyholm, 1950; Hashimoto, 1960). Consideration of this genetic fact, along with numerous physiological and biochemical observations, led Spotts and Stanier (1961) to propose a unitary hypothesis of streptomycin action. They suggested that the three phenotypes were ultimately determined by structural modification of a "specific" protein at a "single" intracellular site. Streptomycin would bind reversibly with this protein and depending on the structure of the receptor site may or may not influence the function of the cell. The ribosomes were

proposed as the streptomycin binding sites. The sensitive ribosome was pictured as possessing a structure that conferred on it a very high affinity for streptomycin. The resulting combination prevented the attachment of m-RNA, thus inhibiting protein synthesis. The corresponding structures of the resistant and dependent ribosomes were such that streptomycin had no effect on the former and its presence was obligatory for the latter to function normally. This hypothesis emphasized the fact that only a single site, the ribosome, was altered during mutation and that the streptomycin dependent cell in the presence of a supra-critical level of antibiotic (> 250 units per ml.) did not differ significantly from the corresponding sensitive strain grown under optimal conditions (Spotts, 1962). The discovery of ninhydrin-positive material present in culture supernatants of streptomycin-dependent mutants but absent from the corresponding fluids of -sensitive and -resistant cultures led Bragg and Polglase (1962) to an investigation of metabolic differences in the three pheno-types. Their results (Bragg and Polglase, 1964a; 1964b) indicated that streptomycin-dependent mutants, unlike the -sensitive and -resistant forms, underwent significant changes at the level of pyruvate metabolism in response to alterations in streptomycin concentrations and aerobic conditions. This suggests an impair-ment in respiration or streptomycin action at a site other than the ribosome. Recently, (Cox, et al, 1964; Davies, 1964) it has been shown by means of sucrose gradient centrifugation that

streptomycin does not prevent the attachment of m-RNA to the sensitive ribosome. Data presented in this thesis, as well as more current work from this laboratory lend further support to the steadily growing pool of evidence that the unitary hypothesis of Spotts and Stanier is less than adequate to explain the physiological and biochemical responses to streptomycin.

II. Formation of α-Acetolactate by Streptomycin Mutants

The synthesis of α-acetolactate by cell-free bacterial preparations was determined colorimetrically by estimating the decarboxylated product, acetoin, by the method of Westerfeld (1945). The original color reaction was shown by Voges and Proskauer (1898) to be due to the reaction between diacetyl or acetoin and a guanidino group in the presence of alkali. Attempts to increase the sensitivity of this reaction led to the addition of creatine and α-naphthol. The standard curve was linear for concentrations as high as 10 µgm. per ml. but deviated slightly from this relationship at greater concentrations.

The Westerfeld method is nearly specific for acetoin and diacetyl. Related 5-carbon ketols give a somewhat similar color, while the 6-carbon ketols give a light olive-green color within the time limit employed. The sensitivity of these analogs, however, is 10 to 100 fold lower than either diacetyl or acetoin (Green, et al, 1942). Color contributed by diacetyl can be distinguished from acetoin by its rate of formation.

Color development due to diacetyl is generally complete within 10-15 minutes, while the color complex due to acetoin reaches maximum intensity only after one hour. It should be noted that under the conditions required to decarboxylate α-acetolactate (acid and heat) it is possible to oxidize small quantities of acetoin to diacetyl (Westerfeld, 1945), hence early color formation does not necessarily indicate endogenous diacetyl. With the exception of assay mixtures containing high concentrations of α-acetolactate early color development was not observed in this work. Therefore, it seems safe to conclude that the total color derived from these reaction mixtures is due to α-acetolactate production.

Although the enzyme acetolactate decarboxylase is not present in extracts of E. coli (Juni, 1952), enzyme reaction mixtures still showed a low but significant acetoin level, even when the reactions were stopped under conditions chosen to prevent chemical decarboxylation (Table II, line 3). It is probable that this acetoin arose from a spontaneous decomposition of α-acetolactate caused by incubation at 37°C since α-acetolactate is known to be relatively heat labile even at this temperature (Umbarger and Brown, 1958b). Consequently, values obtained under these conditions were not subtracted from values for total acetoin.

Reaction mixtures treated with both acid and heat (Method I) generally resulted in acetoin values 10 - 15% lower than mixtures treated with acid alone (Table II, lines 1 and 2)

and 5 - 10% lower than mixtures treated with heat in the presence of zinc sulphate and sodium hydroxide (line 4). This strongly suggests that the severe conditions (autoclaved for 10 minutes at 10 p.s.i. at an acidic pH) employed for complete decarboxylation also destroyed a small quantity of the color complexing material in the reaction mixture. This observation led to the use of milder decarboxylation conditions as described under Method II.

It is evident from the data of Tables III and IV, that at pH 8.0 the sensitive and resistant mutants (grown with or without dihydrostreptomycin supplementation) of all strains exhibited similar acetohydroxy acid synthetase activities whereas, the corresponding dependent mutants possess activities two to five times greater. At pH 6.0 this relationship was less dramatic but the D/S ratio exceeded the R^+/R^- ratio in all strains. The degree of de-repression of acetohydroxy acid synthetase in the various dependent strains was not identical and this difference may be explained solely by strain variability in its response to streptomycin.

The enzyme activities of the sensitive and dependent mutants of E. coli strain A require particular note (Table III, line 4, and Table V, lines 1 and 2) since the sensitive strain (SA) was derived as a revertant of the dependent strain. In this case back-mutation from streptomycin-dependence to -sensitivity was accompanied by a decrease in acetohydroxy acid synthetase

activity at pH 6.0 and at pH 8.0. This repression of enzyme activity resulting from reversion appears to be incomplete and leaves the sensitive revertant with partial streptomycin-dependent properties as indicated by the slightly elevated acetohydroxy acid synthetase activity and the low D/S ratios (Table III and Table V). It was previously pointed out that the growth of this mutant (SA) in the presence of low antibiotic concentrations (Table I) suggested it might also possess streptomycin-resistant tendencies. The fact that the acetohydroxy acid synthetase level is de-repressed upon mutation from streptomycin sensitivity to dependence and reversibly re-repressed during back-mutation to the sensitive state, strongly suggests that genetic control of this enzyme is linked to the locus of streptomycin dependence.

Umbarger and Brown (1958b) reported a peculiar lack of linear response in the formation of α-acetolactate as a junction of extract concentration. After the completion of the present work, a cofactor, the addition of which corrected this anomalous behavior (Leavitt, 1964) was isolated and identified as flavin adenine dinucleotide (Bauerle, et al, 1964). Further experiments were therefore immediately carried out to determine whether or not the addition of FAD to the reaction system altered the enzyme activity and/or the relationship between the sensitive and dependent strains. The data of Table V clearly indicates that although α-acetolactate-formation is stimulated six to seven fold in the presence of this cofactor, the D/S ratio in

the presence or absence of FAD remained unchanged. The role which FAD plays in this reaction sequence is unknown. However, recently Hogg, et al, (1965) have reported finding iron (Fe^{++}) associated with this enzyme complex and suggest that its function may involve the coenzyme.

In order to eliminate the possibility that the elevated level of acetohydroxy acid synthetase was due to a general stimulation of enzyme formation in the dependent mutant, other enzymes were studied in the three mutants of strain A. It is obvious from the data of Table IX that mutation from antibiotic dependence to sensitivity or dependence to resistance has no effect on glucokinase or glutamic dehydrogenase activity. Hence, while the involvement of other enzymes is not excluded, the increased acetohydroxy acid synthetase activity cannot be attributed to a general de-repression of enzymic activity in the dependent mutant. In this connection, it should also be noted that calculation of specific activities per mgm. of protein excludes the possibility that de-repression of acetohydroxy acid synthetase is a non-specific event.

Concurrent work from this laboratory suggests that the enzymes reductoisomerase (Lau, 1966) and Transaminase B (Unpublished Observation) which catalyse subsequent steps in the biosynthetic pathway are not de-repressed in the dependent organism. The dihydroxy acid dehydrase has not yet been studied. Therefore, the evidence accumulated thus far infers that the

excretion of L-valine (and L-leucine) from streptomycin-dependent mutants of E. coli results solely from a de-repression of the control enzyme, acetohydroxy acid synthetase and not of the subsequent biosynthetic enzymes. The fact that only trace quantities of L-leucine are detected in the supernatant fluids (Bragg and Polglase, 1962; Tirunarayanan, et al, 1962) suggests some feedback mechanism controls the drain of α-ketoisovalerate to the 6-carbon product (Freundlich, et al, 1962).

III. Formation of α-Acetohydroxybutyrate by Streptomycin Mutants

As indicated in Fig. 2 and Fig. 3 the enzyme aceto-hydroxy acid synthetase is involved not only with the synthesis of L-valine and L-leucine but also with the synthesis of L-isoleucine via the intermediate α-acetohydroxybutyrate. Since there is no evidence for the excretion of L-isoleucine by dependent mutants, it was important to determine whether an elevated α-acetohydroxybutyrate level is formed in response to the de-repression of acetohydroxy acid synthetase. If aceto-hydroxy acid synthetase stimulation is due to an increased rate of enzyme formation rather than to an effect on the kinetics of the enzyme, then the D/S ratio for α-acetolactate production should correspond to the D/S ratio for synthesis of α-aceto-hydroxybutyrate.

As mentioned in the "Materials and Methods", the only quantitative assay method sensitive enough to measure the level

of α-acetohydroxybutyrate formed in this system was the microbiological assay described by Leavitt and Umbarger (1960). This procedure measures the degree to which inhibition of E. coli strain K-12 by L-valine can be reversed by L-isoleucine or precursors of this compound. Umbarger and Brown (1955) noted that L-isoleucine was a non-competitive antagonist of L-valine, as would be expected if L-isoleucine served to restore a deficiency created by interference with its biosynthesis. Furthermore, while the 6-carbon precursors of L-isoleucine, α-keto β-methylvalerate and α, β dihydroxy β-methylvalerate reversed inhibition as well as did L-isoleucine itself, a 4-carbon precursor, α-ketobutyrate was ineffective. This suggested that the pathway was blocked at the acetohydroxy acid synthetase step. Since the acetohydroxy acid synthetase of strain K-12 is extremely sensitive to feedback by L-valine (Umbarger and Brown, 1958a; Leavitt and Umbarger, 1962) the addition of L-valine in the absence of L-isoleucine would simultaneously inhibit the synthesis of both α-acetolactate and α-acetohydroxybutyrate and ultimately would prevent the growth of the organism as a consequence of starvation for L-isoleucine.

To establish the sensitivity of the E. coli K-12 culture to L-valine and the reversibility of this inhibition by L-isoleucine, a growth experiment was carried out, the results of which are given in Table VI. In the absence of L-isoleucine growth was negligible even after 72 hours. However, the addition

of L-isoleucine at concentrations considerably lower (35 fold) than the inhibitor, L-valine, permitted significant growth.

Technical problems associated with a microbiological assay of this type on occasion gave erratic results particularly during the determination of the standard curve. For this reason, the standard curve employed in this work was a composite plot of values obtained in several separate experiments each carried out in duplicate. The standard curve is shown in Fig. 4. It was reported by Leavitt and Umbarger (1960) that at low concentrations (upto 0.02 μmoles) α-acetohydroxybutyrate and L-isoleucine stimulate the growth of inhibited E. coli to the same extent. Therefore, the end-product rather than the intermediate was used as a standard and the reaction mixtures were diluted to give concentrations of α-acetohydroxybutyrate which would fall on the linear portion of the standard curve. In addition, extracts were routinely assayed in triplicate (initially in duplicate) in order to reduce systematic error.

Since α-acetohydroxybutyrate is decarboxylated by boiling for 5 minutes, the assay was rendered specific for this compound by testing each sample before and after heat treatment. It can be seen from Table VII that the growth stimulated by α-acetohydroxybutyrate is 50-fold greater than that stimulated by the remainder of the intermediates and by L-isoleucine itself. These data are supported by the work of Umbarger, et al (1960). They found that even with crude extracts which were capable of

converting α-acetohydroxybutyrate to later intermediates in the L-isoleucine pathway, no other L-isoleucine precursors were detected as products unless NADPH had also been added to the reaction system.

Table VIII clearly indicates that the D/X ratios of acetohydroxy acid synthetase are constant, regardless of whether the activity is determined by the formation of α-acetolactate or α-acetohydroxybutyrate.

It is interesting to note that the resistant mutant of strain A in the presence of dihydrostreptomycin consistently exhibited a higher enzyme level than did the same mutant in the absence of antibiotic (see Tables IV and VIII). Although this phenomenon, which has already been mentioned in regard to the sensitive (SA) revertant, cannot be explained entirely, it probably arises as a consequence of "incomplete suppression" of the dependent locus during reversion (Hashimoto, 1960).

Enzyme activities as determined by the formation of α-acetohydroxybutyrate were lower by 30-36% for strain A and 37-44% for strain E than enzyme activities determined by the formation of the shorter-chained intermediate, α-acetolactate. Umbarger and Brown (1958b) reported similar diminished enzyme activity for α-acetohydroxybutyrate formation in extracts of E. coli strain K-12. It is difficult to evaluate the significance of these figures with respect to relative substrate specificity, due to the unique substrate requirements of this enzyme complex.

Since α-acetolactate formation involves the condensation of two moles of pyruvate and α-acetohydroxybutyrate formation requires one mole each of α-ketobutyrate and pyruvate, it is not possible even in the presence of a high α-ketobutyrate concentration to estimate the proportion of pyruvate entering each product. Wagner, et al, (1965) have studied the *in vitro* synthesis of L-valine and L-isoleucine by particulate fractions of *Neurospora crassa*. Their results indicate that pyruvate and α-ketobutyrate definitely compete for the catalytic site on the condensing enzyme. In addition, L-isoleucine formation increases linearly with increasing α-ketobutyrate concentration but never exceeds 70% of the L-valine forming capacity of the preparation. They interpret this as meaning that the "active acetaldehyde" (from pyruvate) is synthesized and reacts with the two keto-acids at a common active site on the enzyme. Hence, supra-critical concentrations of α-ketobutyrate would inhibit the formation of the active acetaldehyde and eventually the synthesis of both L-valine and L-isoleucine. For this reason they suggest there must be a regulatory mechanism *in vivo* controlling the α-ketobutyrate level. The evidence accumulated thus far suggests the enzyme *threonine dehydratase* (Umbarger and Brown, 1958a; Changeux, 1961).

Therefore, it seems relatively safe to conclude that although acetohydroxy acid synthetase is de-repressed with respect to α-acetohydroxybutyrate formation in the dependent mutant, L-isoleucine does not accumulate *in vivo* due to the control of

the α-ketobutyrate level at a second point. If enzyme de-repression is not accompanied by increased amounts of α-keto-butyrate the elevated pyruvate level (Bragg and Polglase, 1962; 1964a) will rapidly be converted to α-acetolactate and ultimately to L-valine.

IV. Significance of Acetohydroxy Acid Synthetase De-repression in Dependent Mutants of Escherichia coli.

Previous studies in this laboratory (Bragg and Polglase, 1962; 1963a; 1963b; 1963c) concerning the involvement of streptomycin with the metabolism of streptomycin mutants, have implicated this antibiotic with two phenomena: (1) the excretion of extracellular metabolites; and (2) the impairment of certain oxidative processes. It is interesting therefore to consider the results of the present work in terms of a possible explanation for the abnormal metabolic behavior exhibited by the various streptomycin mutants.

In general, streptomycin sensitive and resistant strains of E. coli demonstrate no metabolic irregularities when grown aerobically on glucose in the absence of antibiotic. The addition of streptomycin to exponentially growing sensitive cultures however, is usually accompanied by an immediate cessation of exponential growth followed by the liberation of extracellular metabolites. The major excretion product is pyruvate with lesser quantities of L-alanine and L-valine (Bragg and Polglase, 1962).

The formation of the amino acids can be directly related to the increased amounts of pyruvate, and L-alanine by direct amination and L-valine via α-acetolactate and reduction. Streptomycin also reportedly inhibits oxidative phosphorylation in sensitive organisms (Bragg and Polglase, 1963d).

The resistant mutant, when grown in the presence of antibiotic (1000 units per ml.), produced elevated levels of both pyruvate and lactate (Bragg and Polglase, 1962). This observation supported earlier observations by Rosanoff and Sevag (1953). This suggests that the resistant organism utilizes pathways of anaerobic metabolism when grown in the presence of antibiotic. However, no inhibition of oxidative phosphorylation could be demonstrated in the resistant extracts. As previously pointed out, neither the sensitive nor resistant organisms exhibited elevated acetohydroxy acid synthetase activities whether in the presence or absence of streptomycin.

The streptomycin dependent mutant accumulates and excretes L-valine during oxidation of glucose if two environmental requirements are satisfied. First, streptomycin or dihydrostreptomycin must be present and second aeration is essential. Growth under oxygen or antibiotic deprivation results in the production of lactate from glucose instead of L-valine (Bragg and Polglase, 1964a). Subsequent work showed that the addition of streptomycin to depleted cells initiated a rapid formation of L-valine with a concurrent decrease in pyruvate and lactate accumulation. It has

recently been shown that the level of acetohydroxy acid synthetase in streptomycin-depleted-dependent cells is repressed but increases linearly upon the addition of antibiotic to a level several fold greater than that of the corresponding sensitive and resistant strains (Polglase, in press). Therefore, in this mutant, streptomycin appears to stimulate the de-repression of acetohydroxy acid synthetase in order to alleviate the accumulation of metabolites which arise as a result of an alteration in aerobic metabolism. Although the actual site of the metabolic impairment remains unknown, Bragg and Polglase (1963c; 1963d) have presented data which advocates the electron-transport chain as a site of action of streptomycin. In the dependent mutant, streptomycin (or dihydrostreptomycin) may act to maintain the integrity of an "alternate electron-transport system" which utilizes lactate, under conditions of antibiotic or oxygen starvation or L-valine, under aerobic, antibiotic-supplemented conditions as terminal hydrogen acceptors. In such a scheme de-repression of acetohydroxy acid synthetase and hence stimulation of L-valine synthesis could serve two functions. First, pyruvate which may accumulate due to modifications in terminal oxidation can be efficiently removed (two moles of pyruvate per mole of α-acetolactate) and converted to a near neutral end-product. Second, since the formation of L-valine requires NADPH, the stimulation of this pathway will help to maintain the level of oxidized coenzyme essential for oxidative

metabolism. It has been reported (Bragg and Polglase, 1964b) that the isocitric dehydrogenase (NADPH enzyme) level in supplemented-dependent cells was several fold greater than in the depleted cells. At the present stage of experimental investigation, it is difficult to ascertain the primary function of this stimulated pathway, but it is known that the intracellular accumulation of "catabolites" of glucose metabolism can impair the normal control of certain inducible enzymes (Monod, 1947; Neidhardt and Magasanik, 1956; Mandelstam, 1961; 1962), a situation which may become deleterious to the organism.

Related studies (Hepner, 1966) on streptomycin-sensitive, -resistant and -dependent mutants of Aerobacter aerogenes, have demonstrated a corresponding de-repression of the acetohydroxy acid synthetase in dependent organisms of this species. However, since this organism possesses the enzyme acetolactate decarboxylase (June, 1952) the terminal products are acetoin and 2,3 butylene glycol rather than L-valine.

Rosenkranz (1963) has reported de-repression of alkaline phosphatase in streptomycin-dependent Escherichia coli. Although his interpretation of this phenomenon differs somewhat from the one presented here, it is consistent with the idea that one role of the antibiotic in the dependent organism is that of an enzyme "de-repressor" (Jacob and Monod, 1961).

The regions of the bacterial cell concerned with carbohydrate metabolism are not the only areas presently being studied

in an attempt to elucidate the primary site of streptomycin action. Evidence (Flax, et al, 1962a; Speyer, et al, 1962; Davies, 1964; Pestka, et al, 1965) accumulated over the past five years partially supports the "unitary hypothesis" of Spotts and Stanier (1962) that the ribosome is the region of the sensitive cell principally affected by streptomycin. The addition of low levels of antibiotic to an in vitro protein synthesizing system containing sensitive ribosomes generally leads to a misincorporation of C^{14}-amino acids with the resulting formation of "nonsense protein" (Davies, et al, 1964). It was suggested that in the streptomycin-sensitive organism this protein floods the cell and irreversibly inhibits cell division. Streptomycin has no effect on the resistant ribosome under the same conditions (Flax, et al, 1962b). It is important to note that ribosomes prepared from streptomycin-dependent cells have never been shown to demonstrate a functional dependence on streptomycin. In fact, amino acid incorporation and streptomycin binding studies indicate that the 30S subunit of the resistant and dependent ribosomes are identical (Flax, et al, 1962b; Cox, et al, 1964).

At the present time it is difficult to derive a single hypothesis capable of explaining the numerous genetic, physiological and biochemical observations which have been reported on streptomycin mutants. Since a number of chemical lesions at divergent sites of the bacterial genome can give rise to various streptomycin-resistant phenotypes and at least one dependent

phenotype and since many workers inadequately describe the characteristics of their mutants and the conditions under which they were obtained, it is difficult to ascertain whether equivalent mutants have been studied. It is highly probable that not only do the phenotype of the resistant mutants differ but also the biochemical mechanisms of resistance controlled by these mutants (Watanabe and Watanabe, 1959a; 1959b; Brock, 1964). Hence, if progress is to be made towards the understanding of streptomycin action by comparing the properties of mutants, it is essential that the genetic and physiological characteristics of the organisms under investigation be clearly defined.

E. REFERENCES

Anand, N., Davis, B.D., and Armitage, A.K. 1960 Nature 185, 22.

Barkulis, I.L. 1953 J. Bact 65, 337.

Bauerle, R.H., Freundlich, M., Størmer, F.C. and Umbarger, H.E. 1964 Biochim. Biophys. Acta 92, 142.

Bertani, G. 1951 Genetics 36, 598.

Bragg, P.D. and Polglase, W.J. 1962 J. Bact. 84, 370.

Bragg, P.D. and Polglase, W.J. 1963a J. Bact. 85, 590.

Bragg, P.D. and Polglase, W.J. 1963b J. Bact. 85, 795.

Bragg, P.D. and Polglase, W.J. 1963c J. Bact. 86, 544.

Bragg, P.D. and Polglase, W.J. 1963d J. Bact. 86, 1236.

Bragg, P.D. and Polglase, W.J. 1964a J. Bact. 88, 1006.

Bragg, P.D. and Polglase, W.J. 1964b J. Bact. 88, 1399.

Brock, T.D. 1964 Federation Proc. 23, 965.

Changeux, J.P. 1961 Cold Spring Harb. Symp. Quant. Biol. 26, 313.

Cox, E.C., White, J.R. and Flax, J.G. 1964 Proc. Natl. Acad. Sci. U.S. 51, 703.

Davies, J.E. 1964 Proc. Natl. Acad. Sci. U.S. 51, 659.

Davies, J.E., Gilbert, W. and Gorini, L. 1964 Proc. Natl. Acad. Sci. U.S. 51, 883.

Davis, B.D. and Mingioli, E.S. 1950 J. Bact. 60, 17.

Demerec, M. 1951 Genetics 36, 585.

Desai, I.D. and Polglase, W.J. 1965 Biochim. Biophys. Acta. 110, 181.

Erdos, T. and Ullman, A. 1959 Nature 192, 633.

Flax, J.G., Cox, E.C. and White, J.R. 1962a Biochem. Biophys. Res. Commun. 7, 385.

Flax, J.G., Cox, E.C., Witting, M.L. and White, J.R. 1962b Biochem. Biophys. Res. Commun. 7, 390.

Freundlich, M., Burns, R.O. and Umbarger, H.E. 1962 Proc. Natl. Acad. Sci. U.S. 48, 1804.

Green, D.E., Westerfeld, W.W., Vennesland, B. and Knox, W.E. 1942 J. Biol. Chem. 145, 69.

Halpern, Y.S. and Umbarger, H.E. 1959 J. Biol. Chem. 234, 3067.

Hashimoto, K. 1960 Genetics 45, 49.

Heptner, A.S. 1966 B. Sc. Thesis (Honours), University of British Columbia, Vancouver, Canada.

Hogg, R.W., Chitra, A.B. and Broquist, H.P. 1965 J. Bact. 90, 1265.

Jacob, F. and Monod, J. 1961 J. Mol. Biol. 3, 318.

Juni, E. 1952 J. Biol. Chem. 195, 715.

Landman, O.E. and Burchard, W. 1962 Proc. Natl. Acad. Sci. U.S. 48, 219.

Lau, D. 1966 M. Sc. Thesis, University of British Columbia, Vancouver, Canada.

Leavitt, R., and Umbarger, H.E. 1960 J. Bact. 80, 18.

Leavitt, R., and Umbarger, H.E. 1961 J. Biol. Chem. 236, 2486.

Leavitt, R., and Umbarger, H.E. 1962 J. Bact. 83, 624.

Leavitt, R., 1964 J. Bact. 88, 172.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951 J. Biol. Chem. 192, 265.

Mager, J., Benedict, M. and Artman, M. 1962 Biochim. Biophys. Acta. 62, 202.

Mandelstam, J. 1961 Biochem. J. 79, 489.

Mandelstam, J. 1962 Biochem. J. 82, 489.

Matney, T., Goldschmidt, E.P. and Bausum, H.T. 1960 Bacteriol. Proc., p. 186.

Monod, J. 1947 Growth 11, 223.

Neidhardt, F.C. and Magasanik, B. 1956 Biochim. Biophys. Acta. 21, 324.

Newcombe, H.B. and Hawirko, R. 1949 J. Bact. 57, 565.

Newcombe, H.B. and Nyholm, M.H. 1950 Genetics 35, 603.

Pestka, S., Marshall, R. and Nirenberg, M. 1965 Proc. Natl. Acad. Sci. U.S. 53, 639.

Polglase, W.J. Can. J. Biochem. in press.

Radhakrishnan, A.N. and Snell, E.E. 1960 J. Biol. Chem. 235, 2316.

Roote, S.M. and Polglase, W.J. 1955 Can. J. Biochem. Physiol. 33, 792.

Rosanoff, E.I. and Sevag, M.G. 1953 Antibiot. Chemotherapy 3, 495.

Rosenkranz, H.S. 1963 Biochem. 2, 122.

Scott, G.W. 1949 British J. Expt. Path. 30, 501.

Speyer, J.F. Lengyel, P. and Basilio, C. 1962 Proc. Natl. Acad. Sci. U.S. 48, 684.

Spotts, C.R. and Stanier, R.Y. 1961 Nature 192, 633.

Spotts, C.R. 1962 J. Gen. Microbiol. 28, 347.

Tatum, E.L. 1946 Cold Spring Harb. Symp. Quant. Biol. 2, 278.

Tirunarayanan, M.O., Vischer, W.A. and Renner, U. 1962 Antibiot. Chemotherapy 12, 117.

Umbarger, H.E. and Brown, B. 1955 J. Bact. 70, 241.

Umbarger, H.E. 1958 Bacteriol. Proc. 1958, 111.

Umbarger, H.E. and Brown, B. 1958a J. Biol. Chem. 233, 415.

Umbarger, H.E. and Brown, B. 1958b J. Biol. Chem. 233, 1156.

Umbarger, H.E., Brown, B. and Eyring, E.J. 1960 J. Biol. Chem. 235, 1425.

Umbarger, H.E. and Davis, B.D. 1962 In The Bacteria Vol. III, 167. Edited by I.C. Gunsalus and R.Y. Stanier, New York: Academic Press, Inc.

Umbarger, H.E. and Freundlich, M. 1965 Biochem. Biophys. Res. Commun. 18, 889.

Umbreit, W.W. 1953 J. Bact. 66, 74.

Voges, O. and Proskauer, B. 1898 Hyg. u. Infektiouskr 28, 20.

Wagner, R.P., Bergquist, A. and Baree, T. 1965 Biochim. Biophys. Acta. 100, 444.

Watanabe, T. and Watanabe, M. 1959a J. Gen. Microbiol. 21, 16.

Watanabe, T. and Watanabe, M. 1959b J. Gen. Microbiol. 21, 30.

Westerfeld, W.W. 1945 J. Biol. Chem. 161, 495.

Witkin, E.M. 1947 Genetics 32, 221.