CATABOLITE REPRESSION IN
STREPTOMYCIN-DEPENDENT
ESCHERICHIA COLI

by

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ABSTRACT

Biosynthetic reactions in micro-organisms normally are under precise control, usually by a feed-back mechanism in which the end product inhibits the activity and/or represses the formation of the enzyme which initiates the biosynthetic pathway. Streptomycin (Sm)-dependent mutants of *Escherichia coli*, unlike the parent wild-type strains excrete the amino acid, L-valine, thereby indicating a loss of precise regulation in the biosynthesis of this amino acid (Tirunarayanan, Vischer and Renner, 1962; Bragg and Polglase, 1962). The initiating enzyme for valine biosynthesis, acetohydroxy acid (AHA) synthetase, is derepressed in Sm-dependent *E. coli* (Coukell and Polglase, 1965), thus providing an explanation for the excretion of valine by this mutant. However, it was apparent that the extent and cause of regulatory insufficiency in Sm-dependent *E. coli* required further investigation.

A study of the effect of the carbon source used for growth on AHA synthetase formation revealed that in wild-type *E. coli* B this enzyme was subject to catabolite repression. End-product inhibition of AHA synthetase by L-valine in *E. coli* B attained a maximum at 60-70% inhibition. These previously unreported properties of AHA synthetase (sensitivity to catabolite repression and incomplete end-product inhibition) are significant in the regulation of the biosynthetic pathway leading to the aliphatic amino acids and pantothenate.

In Sm-dependent *E. coli* B, growing with non-limiting antibiotic, catabolite repression of AHA synthetase was
relaxed. Additional evidence for relaxation of catabolite repression in Sm-dependent *E. coli* was provided by the observation that Sm-dependent mutants of *E. coli* (strains B and E) were inducible for β-galactosidase in the presence of glucose. Furthermore, several glucose-sensitive enzymes of wild-type *E. coli* B (citrate synthase, fumarase, aconitase and isocitrate dehydrogenase) were found to be insensitive to variation in the nature of the carbon source in a Sm-dependent mutant. Cell yield experiments revealed that aerobic glucose metabolism in the Sm-dependent mutant was one-third less efficient than in the Sm-sensitive strain, although the two strains were equally efficient under anaerobic conditions. Moreover, the rate of ATP synthesis in the Sm-dependent mutant was less than that of the wild-type parent organism. Therefore, relaxation of catabolite repression in Sm-dependent *E. coli* B appears to result from an impairment of aerobic energy metabolism in this mutant.

Catabolite repression in Sm-dependent *E. coli* B under conditions of antibiotic-limitation was investigated. The growth rate of Sm-dependent *E. coli* B on limiting concentrations of dihydrostreptomycin (DHSm) was evaluated by means of a constant (*K*<sub>DHSm</sub>) relating half-maximal growth rate to antibiotic concentration. *K*<sub>DHSm</sub> varied with the nature of the carbon source being highest with energy-rich compounds (e.g. gluconate) and lowest with energy-poor compounds (e.g. lactate). Glucose-sensitive enzymes of Sm-
dependent *E. coli* B were specifically repressed by antibiotic-limitation and exhibited specific activities lower than those observed for the same enzymes in glucose-grown extracts of wild-type *E. coli* B. Parallelism was observed between decreasing antibiotic concentration, decreasing growth rate, and increasing catabolite repression of certain glucose-sensitive enzymes (notably AHA synthetase and fumarase). The decreased efficiency of aerobic glucose metabolism of Sm-dependent *E. coli* B was not affected by variation in the concentration of antibiotic. Thus, it is improbable that carbohydrate metabolism is the antibiotic dependent site in the Sm-dependent mutant. The results are compatible with the hypothesis of Spotts and Stanier (1961) that the primary site of action of DHSm in the Sm-dependent organism is the ribosome (i.e., protein synthesis). However, the growth-limiting effect of antibiotic deprival appears to be augmented by catabolite repression. Additionally, the Sm-dependent mutant is deficient in energy metabolism which can explain the relaxation of control by catabolite repression when antibiotic is present in non-limiting concentration.
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ABBREVIATIONS

AHA acetohydroxy acid
ATP adenosine triphosphate
DHSm dihydrostreptomycin
DNA deoxyribonucleic acid
ilva isoleucine-leucine-valine
mRNA messenger ribonucleic acid
NAD$^+$ nicotinamide adenine dinucleotide
NADH reduced nicotinamide adenine dinucleotide
NADP$^+$ nicotinamide adenine dinucleotide phosphate
NADPH reduced nicotinamide adenine dinucleotide phosphate
RNA ribonucleic acid
Sm streptomycin
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INTRODUCTION

I. Genetics of Streptomycin Mutants

Antibiotics of the streptomycin family include streptomycin (Sm), mannosidostreptomycin and hydroxystreptomycin and their chemically reduced derivatives of which dihydrostreptomycin (DHSm) is the most important therapeutically. A microorganism selected for resistance to any one of these compounds is also resistant to other members of this family of antibiotics. The expression of resistance to the streptomycin antibiotics is complex. In addition to mutating to modest levels of resistance, various microorganisms give rise, in single steps, to highly resistant mutants and also to dependent mutants which require the antibiotic for growth. Since Sm-dependent mutants have been isolated from many Gram-positive and Gram-negative microbial species, there is good reason to believe that mutation to an absolute dependence on these compounds is a wide-spread genetic potentiality of bacteria. In Escherichia coli, genetic analyses have shown that sensitivity, dependence and single-step resistance (indifference) to a high concentration of Sm are determined by multiple alleles at a single locus, known as the "Sm" locus (Newcombe and Nyholm, 1950; Lennox, 1955; Hashimoto, 1960). This genetic fact implies that the three phenotypic states in question are all determined by alternative structural modifications of a single chemical substance within the cell. Low-level Sm-resistance (resistance to antibiotic concentrations up to approximately 25 μg./ml.),
on the other hand, is due to multiple loci at sites unlinked to the high-level Sm-resistance locus (Watanabe and Watanabe, 1959a; 1959b). In addition, low-level Sm-resistant mutants have been isolated which possess an episome for multiple drug resistance. In this case, the organism was found to be resistant to Sm because of a change in the permeability of the cell membrane to the antibiotic (Rosenkranz, 1964) or to the presence of an enzyme which inactivates the Sm molecule (Umezawa, et al., 1967).

II. Proposed Sites of Streptomycin Action

At neutral pH, both Sm and DHSm are cationic molecules and as such precipitate in vitro a wide variety of polyanionic macromolecules, presumably by cross-linking adjacent polymers and building up insoluble lattices (see Brock, 1964). Sm, however, is approximately ten times more effective than DHSm at precipitating most polyanions (Brock, 1964), despite the fact that both molecules possess the same net charge (+3) (Polglase, 1965). Since Sm and DHSm are equally active as antibacterial agents, the non-specific binding of these compounds to RNA, DNA, phospholipids, etc. (Rybak and Gros, 1947; Waksman, 1949) and their cationic effects on the activities of a number of unrelated enzymes (Zeller, et al., 1951; Darrow and Creveling, 1964) probably are not involved in the primary action of these antibiotics in the bacterial cell. This non-specific binding of Sm (and DHSm) to cellular components, however, may be responsible for many of the secon-
In 1961, Spotts and Stanier proposed a unitary hypothesis of Sm action which was developed in a logical manner from genetic, biochemical and physiological data obtained primarily from studies on Sm-dependent mutants. They suggested that Sm-sensitivity, dependence and high-level resistance were ultimately determined by structural modifications of a specific protein at a single intracellular site. It was proposed that this protein resided in the ribosome. The sensitive ribosome was pictured as possessing a structure that conferred on it a very high affinity for Sm. The resulting Sm-ribosome complex would prevent the attachment of mRNA, thus inhibiting protein synthesis. The corresponding structures of the resistant and dependent ribosomes were such that Sm had no effect on the former and its presence was obligatory for the latter to function normally.

This prediction that the ribosome was the Sm-sensitive site in the cell was supported by early studies in vitro on protein synthesis using various combinations of crude supernatant fluids and ribosomes from Sm-sensitive and Sm-resistant E. coli (Speyer et al., 1962; Flax et al., 1962). Similarly, using various combinations of 50S and 30S ribosomal subunits from Sm-sensitive and Sm-resistant E. coli, Davies (1964) and Cox et al. (1964) showed that the Sm-sensitive site resided on the 30S subunit of the 70S ribosome. Recently, Traub and Nomura (1968) reported that the alteration induced by the
Sm-resistance mutation is in a particular protein ("core") of the 30S subunit and is not in the RNA or other protein fractions ("split") of this particle. Davies (1964) demonstrated that Sm did not interfere with the binding of $^{14}$C-polyuridylic acid (poly U) to Sm-sensitive ribosomes (as suggested by Spotts and Stanier, 1961) even though poly U-directed polyphenylalanine synthesis was markedly inhibited by the antibiotic. Therefore, the Sm effect appeared to be localized somewhere between codon recognition and amino acid polymerization. Recent studies by Luzzatto, et al. (1968) indicated that Sm had no effect on Sm-sensitive ribosomes which were beyond the initiation step of the translation process. However, as these ribosomes completed the translation of one strand of messenger and attached themselves to a new mRNA molecule, Sm bound the ribosome to the initiation site. This ultimately resulted in the formation of the "70S initiation complexes" (70S ribosomes bound to the initiation site of mRNA molecules). Furthermore, they showed that a Sm-sensitive, cell-free protein-synthesizing system directed by messengers possessing the normal initiating codon, AUG (e.g., phage RNA or poly AUG) was considerably more sensitive to Sm than was the same system directed by a synthetic messenger such as poly U which functioned via an abnormal initiation mechanism (Nakamoto and Kolakofsky, 1966; Luzzatto et al., 1968).

Although poly U predominately stimulates the incorporation of phenylalanine into protein in cell-free extracts, the addition of Sm to a Sm-sensitive system can induce the incor-
poration of substantial quantities of leucine, isoleucine or other amino acids into protein (Davies et al., 1964; Van Knippenberg et al., 1965). This effect has been termed "miscoding" or "misreading" and can be induced non-specifically by a number of environmental factors (as well as Sm) including temperature, magnesium concentration, organic solvents and pH (Friedman et al., 1968). Miscoding stimulated by Sm was small or negligible in cell-free systems containing Sm-resistant ribosomes (Davies et al., 1964). The observation of Sm-induced translational errors in cell-free preparations from Sm-sensitive cells led to the inference that Sm may kill the sensitive organism by flooding the cell with non-functional protein (Davies et al., 1964). Subsequent studies, however, suggested that this was highly unlikely since cells of certain strains can survive and grow in the presence of Sm while making as much as 80% faulty protein (Gorini and Kataja, 1964). Thus, Sm can exhibit two distinct and experimentally separable effects on protein synthesis in Sm-sensitive cells; first, inhibition of peptide bond formation and second, stimulation of miscoding. Both of these effects are virtually absent in cell-free systems prepared from Sm-resistant organisms.

Until recently attempts were unsuccessful at demonstrating stimulation by Sm (or DHSm) of protein synthesis in vitro using ribosomes from Sm-dependent organisms. This was probably due to the fact that Sm, bound to Sm-dependent ribosomes, is not removed by standard washing procedures
(Kurland, 1966); thus further addition of antibiotic would have little or no effect on the activity of the Sm-dependent ribosomes. When Sm-dependent ribosomes were prepared from cells starved of antibiotic and were extensively washed with high concentrations of salt, Sm was shown to stimulate polypeptide synthesis (Likover and Kurland, 1967a). Furthermore, the Sm effect was shown to be a specific property of the 30S subunit. Thus, the Sm phenotype of an organism can be correlated with the phenotype in vitro of a particular component (30S subunit) of the ribosomes obtained from the corresponding Sm-sensitive, Sm-resistant or Sm-dependent cells. These results strongly support the hypothesis of Spotts and Stanier (1961) that the ribosome is a primary site of Sm action in bacteria. Studies on protein synthesis in Sm-sensitive and Sm-dependent E. coli suggest that the Sm phenotype of an organism probably results from the inhibitory effect or the stimulatory effect of Sm on the rate of protein synthesis rather than from the ability of this compound to induce or suppress miscoding (Likover and Kurland, 1967b).

In addition to the effects of Sm on the ribosome, early studies suggested that Sm also may act on the cell membrane. Roth et al. (1960) reported that the addition of Sm to Sm-sensitive cultures resulted in the excretion of 5'-ribonucleotides. Since the intracellular pool of nucleotides was not elevated under these conditions excretion must be due to membrane damage. This conclusion was verified by
the demonstration that the addition of Sm to Sm-sensitive cells caused increased excretion of amino acids (Anand and Davis, 1960), increased accessibility of β-galactosidase in a cryptic mutant (Brock and Brock, 1959) and an increase in K⁺ efflux (Dubin and Davis, 1961). Furthermore, since electron-transport components involved in terminal oxidative processes are believed to be located in the cytoplasmic membrane (see Smith, 1961), the effect of Sm on respiration (Umbreit, 1950) and on oxidative phosphorylation (Bragg and Polglase, 1963b) are consistent with the hypothesis that Sm acts on the cell membrane. Despite the extensive evidence that the membrane is a site of Sm action, however, the inability of these studies to pinpoint a single Sm-sensitive site coupled with the enormous success of related studies on the ribosome has led to a lessening of interest in this area of investigation.

III. Effect of Streptomycin on Pyruvate Metabolism

Two groups of investigators (Tirunarayanan et al., 1962; Bragg and Polglase, 1962) reported independently that Sm-dependent micro-organisms grown on glucose-salts medium excrete 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 these amino acids to a "partial blockage" of protein synthesis. Since the Sm-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 Sm 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 valine resulted from an alteration in the pathway of pyruvate dissimilation.

Extracellular metabolites were not normally detectable in the supernatant fluids of Sm-sensitive cultures or Sm-resistant cultures grown without antibiotic supplement. However, a Sm-resistant mutant, in the presence of antibiotic, excreted significant quantities of both lactate and pyruvate (Bragg and Polglase, 1962). These results suggested that the resistant mutant used pathways of anaerobic metabolism in a medium containing the antibiotic but in the absence of the antibiotic employed a pathway of pyruvate dissimilation similar to that employed by sensitive organisms. The Sm-dependent mutant differed from both the sensitive and resistant organisms in its production of substantial amounts of valine. As much as 10% of the glucose carbon could be accounted for in this product (Bragg and Polglase, 1962). Subsequent work (Bragg and Polglase, 1964a) indicated that antibiotic-depleted, Sm-dependent cells, produced large quantities of lactate but no valine while the same cells supplemented with non-rate-limiting concentrations of Sm, excreted only valine. When Sm-dependent cells were grown anaerobically their metabolism resembled that of aerated, antibiotic-depleted cells (production of lactate) even in the
presence of the optimal Sm concentration. Thus, the nature of the extracellular products of Sm-dependent organisms appears to be regulated by both the concentration of oxygen and of antibiotic in the growth medium. These workers proposed that the formation of valine was a secondary aerobic pathway of glucose metabolism existing in Sm-dependent mutants (Bragg and Polglase, 1964a). Studies from the same laboratory (Bragg and Polglase, 1963a) on the effect of DHSm on electron transport in \textit{E. coli}, suggested that valine may function as a neutral hydrogen acceptor in carbohydrate metabolism. If this were true, then, in Sm-dependent cells, the antibiotic might activate a mechanism enabling a biosynthetic pathway to function catabolically as a major route of pyruvate metabolism.

IV. **Biosynthesis of Aliphatic Amino Acids and Regulatory Mechanisms**

The biosynthetic pathways leading to \textit{L}-valine, \textit{L}-isoleucine, \textit{L}-leucine and pantothenate in \textit{E. coli} have been investigated through isotope studies on selected auxotrophic mutants. This work has been reviewed by Umbarger and Davis (1962) (Fig. 1). Valine and the precursors of leucine are synthesized from two molecules of pyruvate while the precursors for isoleucine result from the condensation of one molecule each of pyruvate and α-ketobutyrate. Umbarger and Brown (1958) reported that the enzymes catalyzing the last three reactions in the biosynthetic pathway leading to isoleucine also catalyze the corresponding reactions in valine synthesis. This
Fig. 1 The biosynthetic pathway to isoleucine, valine, leucine and pantothenate in Escherichia coli.

I Acetohydroxy Acid Synthetase
II Reductoisomerase
III Dihydroxy Dehydrase
IV Transaminase B
first became evident when it was found that auxotrophic mutants lacking an enzyme on the valine pathway generally lacked the corresponding enzyme on the isoleucine pathway. Thus "single-site" mutants occur which are multi-auxotrophic for valine, leucine and isoleucine. These findings have been supported by kinetic studies (Umbarger and Brown, 1958; Leavitt and Umbarger, 1961). It should be noted, however, that the enzyme which catalyzes the first step in the valine pathway, namely, the condensation of two molecules of pyruvate to one molecule of $\alpha$-acetolactate, also catalyzes the second step in the isoleucine pathway, the conversion of $\alpha$-ketobutyrate to $\alpha$-acetohydroxybutyrate (Leavitt and Umbarger, 1961). This enzyme complex (Fig. 2) has been designated the condensing enzyme or acetolactate-forming system (Umbarger and Brown, 1958) and more recently as acetohydroxy acid (AHA) synthetase (Bauerle et al., 1964). This enzyme is extremely labile and has not yet been purified from bacterial sources. An early investigation in crude extracts of *E. coli* of the mechanism of action of AHA synthetase suggested that two reactions were 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 $\alpha$-ketobutyrate. This reaction proceeds optimally only in the presence of a divalent cation such as $\text{Mg}^{++}$ or $\text{Mn}^{++}$. 
Fig. 2 The formation of \( \alpha \)-acetolactate and \( \alpha \)-aceto\( \alpha \)-hydroxybutyrate in *Escherichia coli*. 
Bauerle et al., (1964) reported that AHA-synthetase activity is greatly stimulated by the presence of the coenzyme flavin adenine dinucleotide (FAD). The function of this unexpected cofactor is as yet not understood.

It has been reported (Umbarger and Brown, 1958) that the AHA synthetase of E. coli, like initial enzymes of other biosynthetic pathways, is subject to end-product inhibition when assayed in the presence of L-valine. Regulation of the formation of AHA synthetase, however, has been the subject of a number of reports. Umbarger and Brown (1958) presented evidence that in E. coli the enzyme was repressed by growth in the presence of valine. However, in later work with "derepressed" mutants of Salmonella typhimurium and E. coli it was found that repression was multivalent (Freundlich et al., 1962) and required the presence of leucine and isoleucine in addition to valine. In wild-type organisms, however, multivalent repression of the enzyme was not observed (Armstrong et al., 1963). Freundlich and Umbarger (1963) reported that multivalent repression of AHA synthetetase was observed if pantothenate was supplied in addition to the three branched-chain aliphatic amino acids. More recent studies by Polglase (1966a) with several strains of E. coli, revealed that the repression of AHA synthetase was generally greater in the presence of the four end products (valine, leucine, isoleucine and pantothenate) than in the presence of valine alone; however, the degree of repression was not large
and there were differences between strains. The obvious conclusion that can be drawn from these reports is that the regulation of AHA-synthetase formation in these organisms is complex. This conclusion is not surprising in view of the intricate role played by this enzyme in the biosynthesis of four important products. If valine or any of the other end products alone could repress the formation of AHA synthetase, the production of the remaining products would be impaired. Theoretically, this problem should be eliminated by multivalent repression (Freundlich et al., 1962). However, the experimental evidence available suggests that this mechanism does not play a major role in the regulation of AHA-synthetase formation in wild-type micro-organisms.

V. Effect of Streptomycin on Enzyme Formation

Fitzgerald et al. (1948) reported that Sm inhibited the synthesis of an adaptive (inducible) benzoic acid-oxidizing enzyme in two Sm-sensitive species of Mycobacteria. This effect of Sm on inducible enzyme formation in Sm-sensitive organisms was confirmed by a series of studies on E. coli (Roote and Polglase, 1955; Polglase et al., 1956; Peretz and Polglase, 1957). In addition, these authors showed that the presence or absence of antibiotic had no effect on β-galactosidase induction in Sm-resistant (indifferent) mutants and that Sm was obligatory for significant β-galactosidase production in the Sm-dependent organism.
In an attempt to explain the excretion of valine by Sm-dependent mutants, the specific activity of AHA synthetase was determined in extracts of Sm-sensitive, Sm-resistant and Sm-dependent cells of 4 different strains of *E. coli* (Coukell and Polglase, 1965). The results of this study indicated that AHA-synthetase formation was derepressed in all Sm-dependent mutants grown in the presence of excess DHSm when compared to either the Sm-sensitive organism or the Sm-resistant mutant grown in the presence or the absence of antibiotic. The specific activities of two other enzymes, glucokinase and glutamic dehydrogenase, however, remained constant in all extracts. Subsequent work (Polglase, 1966a; 1966b) revealed that growth of Sm-dependent mutants on growth-rate-limiting concentrations of antibiotic repressed AHA synthetase to a level less than that observed in the corresponding wild-type organism. Upon the addition of antibiotic, however, AHA-synthetase formation was rapidly and specifically derepressed. Thus, DHSm has a positive regulatory effect on AHA-synthetase formation in Sm-dependent mutants. An examination of multivalent end-product repression of AHA synthetase in Sm-sensitive and in Sm-dependent organisms of several strains of *E. coli* indicated that although repression was slight, the AHA synthetase of the Sm-dependent mutants was just as responsive to this form of control as was the AHA synthetase from the respective parent Sm-sensitive organisms (Polglase, 1966a). However, during the course of this study,
it was noted that high concentrations of glucose in the growth medium consistently resulted in a diminished AHA-synthetase activity in extracts of wild-type E. coli B. Since it has long been known that the synthesis of β-galactosidase as well as many other inducible enzymes is repressed by growth on glucose (Monod, 1947), the observed effect of glucose on AHA synthetase suggested that the antibiotic-dependent derepression of enzymes in Sm-dependent mutants may be the result of interference of DHSm with general catabolite repression (Magasanik, 1961).

VI. Object of this Investigation

The object of the present investigation was to determine whether the derepressed formation of AHA synthetase and β-galactosidase by DHSm in Sm-dependent mutants was due to a direct effect of the antibiotic on transcription or translation or whether derepression was mediated through an effect of the antibiotic on some general regulatory process such as catabolite repression.

Since preliminary experiments indicated that the formation of AHA synthetase (like β-galactosidase) was inhibited by growth on glucose, it was necessary initially to determine whether or not the AHA synthetase of wild-type E. coli B was subject to catabolite repression. Subsequently, when it had been established that the formation of AHA synthetase was regulated by this mechanism, catabolite repression of other enzyme systems was investigated in Sm-dependent mutants grown on growth-rate-limiting and non-limiting concentrations
of antibiotic. As a result of these studies an attempt was made to correlate the concentration of DHSm on which a Sm-dependent mutant was grown with the growth rate of the organism and the degree of catabolite repression in the cell. In addition, it was hoped that this investigation would clarify the metabolic significance of valine excretion by Sm-dependent micro-organisms.
METHODS AND MATERIALS

I. Organisms

(i) Wild-type cultures

Two strains of *Escherichia coli* were used in this work; *E. coli* B (ATCC 11303) and *E. coli* E, the latter obtained originally from the Department of Bacteriology, University of Laval, Montreal. In this report, these two Sm-sensitive strains were designated SB and SE, respectively.

(ii) Streptomycin-dependent mutants

Spontaneous Sm-dependent mutants were isolated from each Sm-sensitive strain by the following procedure.

A volume of approximately 500 ml. of glucose (0.4%)-salts medium (see METHODS II,(i)) was inoculated with a Sm-sensitive culture which had been stored on a heart infusion agar slope at 5°. This culture was incubated without agitation for 20–24 hr. at 37°. The resulting growth was transferred aseptically to two 300 ml. sterile, glass bottles and centrifuged at 1,000 g for 1 hr. at 4°. The pellet was resuspended in 6.0 ml. of buffer (0.05 M-potassium phosphate, pH 7.4). Exactly 0.5 ml. of the resuspended cells (~10^8 cells) were pipetted on to the surface of several Petri plates containing heart infusion agar [heart infusion broth supplemented with 1.5% agar and 1 mg./ml. of dihydrostreptomycin (DHSm)]. The slurry of cells was evenly distributed over the agar surface with the aid of a glass spreader, and the plates
were incubated at 37° for 24 hr. The colonies which formed were subcultured into 5 ml. of sterile glucose-salts medium, either devoid of antibiotic or supplemented with 1 mg./ml. of DHSm, and incubated as described above. Sm-resistant mutants grew in both tubes, whereas, Sm-dependent mutants grew only in the presence of DHSm. To ensure that growth in the absence of antibiotic was in fact due to the Sm-resistant mutant and not to growth of a Sm-dependent culture resulting from a carry-over of DHSm from the plate, a loopful of this culture was further transferred both to antibiotic supplemented and unsupplemented medium in tubes. Sm-dependent mutants obtained in this way, were maintained both on heart infusion agar slopes at 5°, and as lyophilized preparations. All cultures stored on heart infusion agar slopes were subcultured monthly.

Sm-dependent mutants derived in this way from E. coli strain B and strain E were designated DB and DE, respectively.

II. Growth of Cultures and Preparation of Extracts

(i) Media

Throughout this work a minimal medium was used, which is based on that described by Davis and Mingioli (1950) with the omission of citrate. The composition is as follows: $K_2HPO_4$ (0.7%), $KH_2PO_4$ (0.3%), $MgSO_4 \cdot 7H_2O$ (0.02%) and $(NH_4)_2SO_4$ (0.1%). The carbon sources (glucose, glycerol, lactose, lactate or gluconate) were autoclaved separately in a concentrated solution and were added as indicated in each experi-
ment. In certain experiments, the minimal medium was supplemented with specific amino acids or calcium pantothenate as specified. Unless indicated otherwise, Sm-dependent cultures were routinely grown on minimal medium supplemented with 1 mg. of antibiotic/ml. (supplemented Sm-dependent cells). DHSm (sesquisulfate) (Merck, Sharp and Dohme, Montreal, Canada) was used in this work. For cultures which were to be grown overnight without agitation, the pH of this medium was adjusted to 7.6 by the addition of sodium hydroxide solution.

(ii) Estimation of growth and cell mass

Growth of bacterial cultures was determined turbidimetrically by measuring the change in absorbancy of the cultures at 420 m\(\mu\) (A\(420\)) in a Beckman B spectrophotometer (light path, 1.0 cm.). Turbidity was measured against a blank containing either distilled water, for cultures grown on minimal medium, or fresh medium when the cultures were grown on minimal medium supplemented with other constituents.

Standard curves relating total cellular protein or cell mass (dry wt.)/ml. of culture to A\(420\) were prepared for each organism. Total cellular protein was estimated (see METHODS VI,(i)) on aliquots of cells from exponential cultures. The cells were washed twice in distilled water and resuspended in water to give absorbancies ranging from 0.1 to 1.0. To determine cell mass, 20 ml. volumes of the washed, resuspended cells were added to dry, pre-weighed sample bottles. The
bottles were heated in an oven at 85° for 40 hr., cooled in a
dessicator and re-weighed. Cell mass is expressed as μg. of
dry cells/ml. Under the conditions employed for the growth
of cultures, absorbancy was found to be directly proportional
to total cellular protein and to cell mass up to an A_{420} of
1.0.

(iii) Procedures for growing and harvesting cultures

(a) Streptomycin-sensitive and supplemented
streptomycin-dependent cells

An inoculum (50-200 ml.) was grown aerobically for 18 hr.
at 37° under conditions (medium and antibiotic supplementation)
similar to those employed in the final growth experiment. The
culture was diluted in fresh, warm (37°) medium (200-1000
ml.) to an A_{420} of approximately 0.1, and a carbon source was
added to give the desired initial concentration. The culture
was incubated aerobically at 37° until the absorbancy reached
0.8-0.9. Growth was then terminated by chilling the culture.
The cells were harvested by centrifugation (16,300 g for 20
min.) washed once in 0.1 M-potassium phosphate buffer, pH
7.4 and stored as packed cells at 0°. All centrifugations
were performed at 4°. Normally the packed cells were stored
no longer than 24 hr. before they were disrupted and assayed
for enzymic activity.

(b) Antibiotic-limited, streptomycin-dependent cells

The Sm-dependent culture was transferred from a heart in-
fusion agar slope to approximately 50 ml. of minimal salts
medium containing 30 μg. of antibiotic/ml. The culture was incubated at 37° without agitation until heavy growth resulted (24-48 hr.). A portion (2-4 ml.) of this culture was transferred to another 50 ml. of the same medium and the procedure was repeated. After three or four such transfers, the entire culture (50 ml.) was added to 500 ml. of the same medium and the cells were grown aerobically for 18 hr. at 37°. The resulting cells were centrifuged at 16,300 g for 20 min. and resuspended in 6 l. of fresh, warm (37°) medium to give an $A_{\text{420}}$ of approximately 0.1. The culture was divided into six 1 liter portions, and supplemented with the required amount of DHSm usually in the range 3-25 μg./ml. A carbon source was added and the cultures were grown aerobically at 37° to an $A_{\text{420}}$ of 0.9-1.0. The cells were harvested and stored as described above. Cells grown in this way are referred to as "antibiotic-limited, Sm-dependent cells".

(c) Antibiotic-depleted, streptomycin-dependent cells

A culture (50 ml.) of Sm-dependent cells was grown aerobically for 18 hr. at 37° on minimal medium supplemented with 0.4% glucose and 100 μg. of DHSm/ml. The cells were centrifuged at 16,300 g for 20 min., washed once with fresh, warm (37°) antibiotic-free medium (glucose omitted) and resuspended in 1 liter of the same medium to give an $A_{\text{420}}$ of 0.10-0.15. Glucose (0.4%) was added and the culture was incubated at 37° with aeration. After 6 hr. the culture was centrifuged at 16,300 g for 20 min. and the cells were resuspended
in the same volume of antibiotic-free medium to an A_{420} of 0.35-0.40. The culture was incubated aerobically at 37° for a further 6 hr. If the culture exhibited arithmetic growth during the final 3 hr. the cells were harvested as previously described. If arithmetic growth did not occur during this period, the time of incubation was extended 1-2 hr. prior to harvesting. Cells starved for antibiotic in this way are referred to as "antibiotic-depleted, Sm-dependent cells".

(iv) Preparation of crude cell extracts and permeabilized cells

(a) Sonic treatment

To disrupt the cells by sonic oscillation, the wet, packed cells were resuspended homogeneously in ice-cold 0.1 M-potassium phosphate buffer, pH 8.0, at a concentration of 1 g. of cells (wet wt.)/15 ml. of buffer. Twelve-20 ml. of suspension were placed in a Bronwill 20-K.C. sonic oscillator and the cells were disrupted for 4 min.

(b) Disruption in a French pressure cell

Approximately 20 ml. of the cell suspension described above (see METHODS II,(iv),(a)) were added to a freshly greased, chilled (5°) French pressure cell (American Instrument Co., Silver Spring, Maryland, U.S.A.). The cells were disrupted by a single passage through the cell with a pressure differential of 15,000 p.s.i.

Enzymatic activities of extracts prepared by sonic
treatment or by treatment in a French pressure cell were determined either in the crude cell extracts or in the supernatant fluids following centrifugation at 12,000 g for 10 min. Crude extracts and supernatant fluids were stored at -20°.

(c) Disruption by grinding with alumina

To disrupt the cells by alumina grinding, approximately 1 g. (wet wt.) of packed cells was placed in a prechilled (5°) porcelain mortar and 1 g. of chilled alumina (Merck and Co. Ltd.) was added. The cells were ground vigorously to a thick paste (5 min.) and an additional 1 g. of alumina was added. Grinding was continued for a further 10 min. The paste was then suspended evenly in 15 ml. of 0.1 M-potassium phosphate buffer, pH 8.0, transferred to a centrifuge tube and centrifuged at 12,000 g for 10 min. The resulting supernatant fluid was either assayed immediately or stored at -20°.

(d) Toluene treatment

Permeabilized cells were prepared by treating the resuspended cells (see METHODS II,(iv),(a)) with toluene in the following manner. Distilled toluene was added to 3.0 ml. of cells to give a final concentration of either 5 or 10% v/v and the suspension was agitated vigorously on a Vortex mixer for 1 min. and then placed in a 37° water bath. After the desired incubation time (2 or 6 min.), the cells were transferred to ice and assayed immediately. Under these conditions less than 15% of the total cellular protein was released from the cell.
III. β-Galactosidase Induction Experiments

(i) Diauxie growth

The cells were grown in a Gilson (Omni) shaking water bath for 18 hr. at 37° in minimal medium supplemented with 0.2% of glucose. To remove the residual constituents of the medium, the cultures were harvested by centrifugation (12,000 g for 10 min.) and resuspended in one-half the original culture volume of 0.1 M-potassium phosphate buffer, pH 7.4 at 37°. The suspensions were again centrifuged and the washed cells were resuspended in fresh minimal medium to give an $A_{420}$ of approximately 0.1. After the addition of glucose (0.025%) and lactose (0.20%) the cultures were vigorously aerated by means of a sparger during agitation in the shaking water bath at 37°. The $A_{420}$ of the culture was followed during growth and, at intervals, 1.0 ml. samples of culture were removed for determination of β-galactosidase activity (see METHODS VII, (i)). Growth was generally expressed as a change in the μg. of total cellular protein/ml. of culture. Preliminary experiments established that under these conditions, all the glucose was consumed when the protein concentration reached 85-95 μg./ml. (see Figs. 8 and 9).

(ii) Determination of the differential rate of β-galactosidase synthesis

The inoculum was grown aerobically for 18 hr. at 37° in minimal medium supplemented with 0.2% of glycerol. The cells were washed once (see METHODS III, (i)) and resuspended in
fresh minimal medium to give an A$_{420}$ of 0.10-0.15. Glycerol (0.1%) and lactose (0.2%) were added and the culture was shaken vigorously at 37°. Samples (1.0 ml.) of culture were taken at frequent intervals after the initiation of growth and assayed for β-galactosidase activity (see METHODS VII, (i)). Units of enzyme activity/ml. of culture were plotted against the total protein concentration. The slope of the resulting line indicates the rate of β-galactosidase synthesis relative to the rate of total protein synthesis in the cell; i.e. the differential rate of β-galactosidase synthesis.

(iii) β-Galactosidase synthesis in pseudo-resting cells

Owing to the extreme sensitivity of β-galactosidase induction to catabolite repression, the effects of different carbon sources on β-galactosidase induction could not be evaluated reproducibly in cells starved for nitrogen. To overcome this problem a "pseudo-resting" cell system was devised in which both a carbon source and a nitrogen source were added to cells previously starved for both of these compounds.

Cultures grown aerobically on glycerol (0.2%) at 37° were harvested by centrifugation at an A$_{420}$ of 1.0 and were washed once in warm (37°) 0.1 M-potassium phosphate buffer, pH 7.4. The washed cells were resuspended in buffer of the same composition and were shaken vigorously at 37° for 30 min. At the end of this incubation period, the absorbancy of the culture was adjusted to 1.0 with buffer and the cells were transferred to a 30° water bath. After 5 min., a carbon
source (glycerol, 0.05%), a nitrogen source (ammonium sulfate, 0.1%) and the inducer, lactose (0.2%) were added and β-galactosidase formation was followed during a 2 hr. induction period. Cell suspensions treated in this manner, showed less than 15% increase in $A_{420}$ during the 2 hr. induction period and were referred to as "pseudo-resting cells".

IV. Cell Yield Experiments

(i) Definition

Cell yield on a minimal ammonium sulfate medium can be defined (Monod, 1942) by the formula:

$$Y = \frac{G}{C}$$

where $Y$ is the cell yield constant; $G$ is the total change in cell mass; and $C$ is the total amount of energy source consumed. In this study, $Y$ is equivalent to $\mu g.$ dry wt. of cells formed/$\mu g.$ dry wt. of carbon source consumed.

(ii) Aerobic cell yield from glucose

Cells growing exponentially at 37° on minimal medium (with or without antibiotic) supplemented with 0.4% glucose were harvested by centrifugation, washed once with 0.1 M-potassium phosphate buffer, pH 7.0, and resuspended in fresh, warm (37°) glucose-free medium to give an $A_{420}$ of approximately 0.1. The washed cells were incubated aerobically at 37° for 30 min. After this treatment, the absorbancy was readjusted to 0.1 with warm medium and a known amount of glucose (usually sufficient to give a final concentration of 400
μg./ml.) was added. A 2.0 ml. portion of culture was immediately taken for glucose determination (see METHODS VI(ii)) and the culture was shaken vigorously at 37°. The increase in absorbancy of the culture was followed closely until growth ceased. At this time another sample of culture was removed for glucose determination. To ensure that growth was complete, the A₄₂₀ of the culture was checked at 10 min. intervals for a further 40-60 min.

Some experiments concerned a study of the rate of uptake of glucose into the cells relative to the rate of formation of cell mass. In these experiments, samples for glucose estimation were taken at more frequent intervals during the growth of the culture.

Portions of culture to be assayed for glucose were immediately placed in 15 ml. thick-walled, glass centrifuge tubes previously chilled in an ice-salt bath. Under these conditions the temperature of the sample dropped from 37° to 8° in 1½ min. After 5-10 min. in the ice-salt bath, the cultures were centrifuged at 17,000 g for 12 min. and the supernatant was transferred to a clean, dry tube and stored at -20°. All samples were assayed for glucose (see METHODS VI,(ii)) within 7 days.

(iii) Anaerobic cell yield from glucose

To estimate the anaerobic cell yield from glucose washed, exponential cells starved for glucose were prepared
as described in METHODS IV,(ii). The washed cells were re-
suspended in fresh, warm (37°) minimal medium (150 ml.) to
give an \(A_{420}\) of 0.1. Glucose was added to give a final con-
centration of 900 \(\mu g./ml\). The culture was grown aerobically
and portions of the culture were removed for glucose deter-
mination as previously described (see METHODS IV,(ii)). When
the \(A_{420}\) of the culture reached approximately 0.4, the entire
culture was rapidly transferred to a 250 ml. graduated cylinder
partially immersed in a water bath at 37°. The cylinder was
quickly capped with a rubber stopper fitted with three pieces
of glass tubing and sealed around the rim with plasticine
and tape. Spectroscopic grade nitrogen (Canadian Liquid Air
Ltd., Vancouver, Canada) was bubbled vigorously beneath the
surface of the culture through one tube. After 5 min., the
tube supplying nitrogen was withdrawn from the culture and
elevated to a position approximately 4 cm. above the surface
of the liquid. The flow of nitrogen was decreased to a rate
which would maintain anaerobic conditions while causing mini-
mal evaporation. Samples of culture could be removed without
disturbing anaerobiosis by closing the nitrogen exhaust tube
causing the increased pressure to drive the culture up the
third tube into a collecting flask. The initial portion of
culture (5-10 ml.) from each sampling was discarded, while
subsequent samples were used to determine the cell mass and
glucose content of the culture. Glucose uptake was followed
closely until growth ceased. The anaerobic cell yield from
glucose was calculated from the increase in cell mass (dry wt.) and glucose consumption occurring after changing from aerobic to anaerobic conditions.

(iv) Aerobic cell yield from gluconate

The efficiency of gluconate utilization for cell synthesis was determined in washed, gluconate-grown cells prepared as described in METHODS IV,(ii). The cells were suspended in fresh, warm (37°) minimal medium to give an A_{420} of 0.1, and were supplemented with a known amount of gluconate (usually 400 μg./ml.). The culture was grown aerobically at 37° until the increase in absorbancy of the culture ceased. The change in cell mass of the culture was obtained from a standard curve relating A_{420} to μg. dry cells/ml.

V. Estimation of the Rate of ATP Formation and the ATP Pool

The rate of ATP formation and the concentration of the ATP pool in exponential cells were studied in cultures treated in two different ways.

(i) Method I

Cells growing exponentially on glucose (0.4%)-salts medium at 37° were diluted in fresh, warm medium of the same composition to give an A_{420} of approximately 0.25. The diluted culture was placed on a shaker at 37° and agitated vigorously. Samples (2.0 ml.) of culture were removed for ATP determination (see METHODS VI,(iii)) at A_{420} increments of 0.07-0.10 from 0.3 to 1.0.
Aliquots of culture to be assayed for ATP were immediately added to 0.2 ml. portions of ice-cold 30% (w/v) perchloric acid in centrifuge tubes. After 10 min. on ice, the extracts were shaken well and then neutralized with 0.8 ml. of 2M-KOH (the final pH was 7.0-7.8). The precipitate was removed by centrifugation and the supernatant fluids were either assayed immediately or stored at -20°. All extracts were assayed within 48 hr.

(ii) Method II

In some experiments the initial concentration of the ATP pool of the cells was decreased below the level normally observed in exponential cultures by treating the cells in the following manner. Exponential cultures growing aerobically on glucose (0.1%)-salts medium at 37° were rapidly harvested by centrifugation (12,000 g for 10 min.) at 4°. The cells were washed once with cold (0°) 0.1 M-potassium phosphate buffer, pH 7.4 and resuspended in warm (37°) glucose-free minimal medium to give an A_420 of 0.20-0.25. The flask was stoppered tightly and incubated as a stationary culture at 37° for 30 min. It was found that this treatment resulted in a 50-75% reduction in the initial concentration of the ATP pool. At the end of this period, glucose (0.4%) was added and the culture was grown aerobically to an A_420 of 1.0. Portions of culture were removed and treated with perchloric acid as described in the previous section.
VI. Chemical Analysis

(i) Protein

Protein was estimated by the Folin-Phenol method as described by Lowry et al. (1951). Bovine γ-globulin (Calbiochem, Los Angeles, Calif., U.S.A.) was used as a standard. Protein samples were generally diluted (with distilled water) to contain 25 to 250 µg. of protein/ml.

(ii) Glucose

Glucose was determined by the commercial preparation, Glucostat (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) as follows: to 0.5 ml. aliquots containing 0-300 µg. of glucose/ml. were added 2.5 ml. of 0.1 M-potassium phosphate buffer, pH 7.0 and 2.0 ml. of the Glucostat reagent (see below). This mixture was incubated at 37° until the reaction had reached completion (30 min.). Two drops of 6 N HCl were added to each tube, followed by 5.0 ml. of distilled water. The contents of the tube were mixed vigorously on a Vortex mixer and left at room temperature for 3-5 min. The A_{420} of the resulting solutions was measured and converted by means of a standard curve into µg. of glucose. After the solution is acidified (with 6 N HCl) the color is stable at room temperature for several hr.

The Glucostat reagent was prepared by dissolving one vial of lyophilized enzyme preparation and one vial of chromogen in 50 ml. of distilled water. The pH was adjusted, if neces-
sary, to 7.0 and the solution was stored at 5° in the dark. As the reconstituted Glucostat reagent is extremely unstable, it was prepared immediately before use.

(iii) ATP

ATP was determined enzymatically by a luciferase assay system based on a method described by Cole et al. (1967).

(a) Reconstitution of the firefly lantern extract

A lyophilized preparation of firefly lanterns (250 mg.) (Sigma FLE-250) was dissolved in 25 ml. of distilled water. According to the supplier (Sigma), after reconstitution, the suspension contained 0.05 M-potassium arsenate and 0.02 M-magnesium sulfate at pH 7.4. The suspension was stored at 0° for 24 hr., clarified by centrifugation at 5,000 g for 10 min. and then stored in 1.0 ml. aliquots at -20°.

(b) Assay system

The following components were added to a clean, dry scintillation vial: 0.2 ml. of 0.2 M-glucylglycine buffer, pH 7.4; 0.7 ml. of distilled water; 0.05 ml. of reconstituted firefly lantern extract and 0.02 ml. of a solution containing ATP (standard or sample). The vial was immediately shaken, capped and placed in a Nuclear-Chicago Mark I scintillation counter which previously had been programmed to the most sensitive setting. Following a standard delay time of 45 sec. after the addition of the ATP solution, five 12 sec. counts
were taken at 15 sec. intervals. Under these conditions, the luminescence decays exponentially. Therefore, it was possible to estimate the maximum luminescence by extrapolating back to zero time. The background count, under these conditions, was 8-12 counts/12 sec., whereas, sample counts generally ranged from $10^3$ to $10^6$ over the same time period.

A stock solution of 1 mM ATP was prepared in distilled water and frozen at -20°. Each day fresh dilute ATP standards containing 2-25 μmole/0.02 ml. were prepared from the stock solution.

The ATP content of the perchloric acid-extracted cells is expressed as μmole of ATP/ml. of culture. The ATP pool is defined as μmole of ATP/mg. dry wt. of bacterial cells.

(iv) Valine

The valine content of culture supernatant fluids was estimated chromatographically by the method of Bode (1955). Samples (50 ml.) of supernatant fluids from cultures centrifuged at 16,000 g for 20 min. were concentrated 10 or 20-fold by evaporation to dryness under diminished pressure at 50° and resuspending the residue in distilled water. Exactly 0.10 ml. of each concentrated sample and a series of valine standards (0.1 to 1.0 mM) were spotted on a sheet of Whatman No. 1 chromatography paper. The chromatogram was run by descending chromatography for 16 hr. in a solvent composed of butanol-acetic acid-water (4:1:1 v/v). The dried chromatogram was developed by immersing it in a ninhydrin solution
(ninhydrin, 500 mg.; acetone, 90 ml.; water, 5.0 ml. and acetic acid, 5.0 ml.) followed by heating at 90° for 15 min. The resulting spots were fixed by treating the chromatogram with a copper nitrate solution (saturated aqueous CuNO₃, 1.0 ml; concentrated nitric acid, 0.02 ml. and acetone, 99 ml.). To measure valine quantitatively, the spots were excised from the chromatogram, shredded, and eluted with 1.0 ml. of methanol. The absorbancy of the eluant was determined at 530 μm. The μmoles of valine present was determined from a standard curve prepared in an identical manner.

VII. Enzyme Assays

All spectrophotometric determinations were made with a Cary 15 recording spectrophotometer at 25°. Specific activities were calculated as units of enzyme/mg. of protein.

(i) β-Galactosidase

β-Galactosidase was determined in samples (1.0 ml.) of culture by the method of Pardee et al. (1959). Toluene (1 drop) and β-mercaptoethanol (final concentration 15 mM) were added to the cell suspensions. The cells were lysed by agitation on a Vortex mixer for 30 sec. followed by incubation at 37° for 30 min. The contents of the tubes were then brought to 28°; 0.2 ml. of a solution of 0.02 M o-nitrophenyl β-D galactopyranoside in 0.25 M-sodium phosphate buffer, pH 7.0, was added, and the tubes were incubated a measured period of time, until the desired intensity of color had developed. The
reaction was stopped by the addition of 0.5 ml. of 1 M-sodium carbonate, and the absorbancy was determined at 420 μm. A correction for turbidity of the cells could be made by multiplying the $A_{550}$ of the solution by 1.60 and subtracting this value from the $A_{420}$ of the same solution. Under the above conditions, 1 μmole of o-nitrophenol/ml. has an $A_{420}$ of 0.0075 (Pardee et al., 1959). One unit of enzyme is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmole of o-nitrophenyl β-D-galactopyranoside/min. at 28° and pH 7.0.

(ii) Acetohydroxy acid synthetase

Although AHA synthetase is stable for several days in stored, whole packed cells at 0°, it is extremely labile in cell extracts (Desai and Polglase, 1965). Therefore, AHA-synthetase activity was determined only on freshly prepared crude cell extracts.

Each assay tube contained, in a total volume of 1.0 ml.: potassium phosphate buffer, pH 8.0, 100 μmoles; sodium pyruvate, 0.25 m-mole; MgCl$_2$, 0.5 μmole; flavin adenine dinucleotide (FAD), 10 μmoles; thiamine pyrophosphate (TPP), 0.3 μmole and 0.5 ml. of crude extract (2.5-3.0 mg. of protein). After incubation for 15 min. at 37° the reaction was stopped by the addition of 0.1 ml. of 40% (w/v) trichloroacetic acid. This was followed by incubation for 15 min. at 60° to convert the α-acetolactate into acetoin. The reaction mixture was diluted 100-fold and a portion of the resulting solution was
analyzed for acetoin by the method of Westerfeld (1945). To a 1.0 ml. aliquot of the diluted reaction mixture was added 1.0 ml. of 0.5% creatine in water and 1.0 ml. of freshly prepared 5% α-naphthol in 2.5 M-sodium hydroxide. The solution was mixed vigorously and the color allowed to develop for 1 hr. in the dark. The resulting color was read at 540 nm and converted to μmoles of acetoin (or α-acetolactate) by means of a standard curve. One unit of activity is equivalent to the formation of 1 μmole of acetoin/hr. at 37°.

(iii) Threonine dehydratase

The assay system consisted of the following in a total volume of 1.0 ml.: potassium phosphate buffer, pH 8.0, 100 μmoles; L-threonine, 80 μmoles; pyridoxal phosphate, 100 μμmoles and 0.5 ml. of crude cell extract (2-3 mg. of protein). The mixture was incubated at 37° for 10 min. and the reaction stopped by the addition of 0.1 ml. of 40% (w/v) trichloroacetic acid. Following a 10-fold dilution with distilled water the precipitate was removed by centrifugation at 2,000 g for 5 min. and the supernatant was analyzed for α-ketobutyric acid by the method of Friedemann (1957). To 1.0 ml. of the supernatant solution was added 2.0 ml of distilled water and 1.0 ml. of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl. After 5 min. at room temperature, 3.0 ml. of distilled benzene and 1.0 ml. of 95% ethanol were added and the aqueous phase was extracted for 3 min. by agitating the contents
of the tube with a stream of air. The two layers were separated by low speed centrifugation and the aqueous layer was removed and discarded. The organic phase was extracted again (3 min.) with 6.0 ml. of 10% sodium carbonate. Exactly 2.0 ml. of the aqueous carbonate layer were removed and added to 2.0 ml. of 1.5 N sodium hydroxide. Color was allowed to develop for 5 min. at room temperature. The absorbancy of the solution was read at 435 m\textmu and converted by means of a standard curve into \textmu moles of \(\alpha\)-ketobutyric acid. One unit of threonine dehydratase activity is equivalent to the amount of enzyme required to form 1 \textmu mole of \(\alpha\)-ketobutyrate/hr. at 37\textdegree.

(iv) Citrate synthase

The condensing enzyme, citrate synthase, was determined spectrophotometrically by measuring the rate of change in A\textsubscript{233} (Srere and Kosicki, 1961). Each assay contained in a volume of 1.0 ml.: tris-HCl buffer, pH 8.0, 100 \textmu moles; oxaloacetic acid, 0.40 \textmu mole and acetyl-CoA, 0.16 \textmu mole. The reaction was initiated by the addition of crude extract (25-35 \textmu g. of protein). The change in A\textsubscript{233} was recorded continuously against a blank containing all the ingredients except extract. The decrease in A\textsubscript{233} is due both to cleavage of the thiol ester bond and to the utilization of oxaloacetic acid. The combined molar extinction coefficient of 5.4 \times 10^3 determined by Srere and Kosicki (1961) was used in this work.
A unit of enzyme activity is defined as the formation of 1 mMmole of citrate/min.

(v) Fumarase

Fumarase was determined spectrophotometrically at 240 μm as described by Hanson and Cox (1967). Each cuvette contained in a total volume of 1.0 ml.: potassium phosphate buffer, pH 7.2, 50 μmoles; malic acid, 17.5 μmoles and extract (80-120 μg. of protein). The rate of increase in absorbancy due to the formation of fumaric acid was followed continuously against a blank containing distilled water and extract. One unit of fumarase activity is defined as an $A_{240}$ change of 0.001/min.

(vi) Aconitase

Aconitase activity was determined by measuring at 240 μm the rate of formation of cis-aconitic acid from isocitric acid as described by Hanson and Cox (1967). Each assay mixture contained in a volume of 1.0 ml.: potassium phosphate buffer, pH 7.2, 50 μmoles; DL-isocitric acid, 15 μmoles and cell extract (80-120 μg. of protein). A unit of aconitase activity is equivalent to the amount of enzyme which produces an $A_{240}$ change of 0.001/min.

(vii) Isocitric dehydrogenase

Isocitric dehydrogenase was determined by measuring at 340 μm the rate of reduction of nicotinamide adenine dinucleotide phosphate (NADP$^+$). Each reaction mixture contained
in a volume of 1.0 ml.: tris-HCl buffer, pH 7.5, 50 μmoles; MgCl₂, 10 μmoles; sodium DL-isocitrate, 10 μmoles; NADP⁺, 0.3 μmole and extract (50-70 μg. of protein). A unit of activity is equivalent to the formation of 1 μmole of reduced NADP⁺/min.

(viii) Glucokinase

Glucokinase activity was determined spectrophotometrically by measuring the rate of reduction of NADP⁺ in a reaction system coupled to glucose 6-phosphate dehydrogenase. The reaction system contained the following in a volume of 1.0 ml.: tris-HCl buffer, pH 7.0, 100 μmoles; glucose, 4 μmoles; ATP, 2 μmoles; MgSO₄, 4.5 μmoles; NADP⁺, 100 μmole; glucose-6-phosphate dehydrogenase (C.F. Boehringer and Soehne, Mannheim, Germany), 1 unit, and crude extract (0.25-0.35 mg. of protein). One glucokinase unit is defined as that amount of enzyme that forms 1 μmole of NADPH/min.

(ix) Glucose 6-phosphate dehydrogenase

The reaction mixture used for the determination of glucose 6-phosphate dehydrogenase contained in 1.0 ml.: glucose 6-phosphate, 5 μmoles; MgCl₂, 10 μmoles; NADP⁺, 0.14 μmole; glycylglycine buffer, pH 7.5, 50 μmoles and cell extract (0.25-0.35 mg. of protein). The change in A₃₄₀ was recorded as a function of time. A unit of activity is defined as the formation of 1 μmole of NADPH/min.

In the absence of exogenous substrates, NADP⁺ was not reduced by the crude cell extracts under the assay conditions described.
PART A: Repression by Glucose of Acetohydroxy Acid Synthetase in Escherichia coli B

RESULTS

I. Effect of Carbon Source on Acetohydroxy Acid Synthetase Formation

When *E. coli* B was grown on glucose-salts medium, the specific activity of AHA synthetase decreased with increasing glucose concentration. The results of a typical experiment are presented in Table I. Although the specific activities of AHA synthetase fluctuated slightly from one experiment to the next, a decrease of 35-40% in specific activity during a 20-fold increase in glucose concentration invariably was observed. Evidence that a general repression by glucose of all enzymes in the extract did not occur under these conditions was provided by the constancy of the specific activities of two other enzymes, glucokinase and threonine dehydratase (Table I).

It has long been known that the formation of a number of catabolic enzymes in certain micro-organisms is repressed by growth on an energy-rich carbon source such as glucose but not by growth on poor carbon sources such as glycerol or lactate. This phenomenon, which was originally termed the "glucose effect" (Epps and Gale, 1942) has been shown to be a property not exclusive to glucose metabolism and as a result is now generally referred to as "catabolite..."
Table I. Effect of increasing glucose concentration on enzyme specific activities in *Escherichia coli* B

<table>
<thead>
<tr>
<th>Initial concn. of glucose in the medium (%)</th>
<th>Specific activity</th>
<th>Threonine dehydratase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AHA synthetase*</td>
<td>Glucokinase†</td>
</tr>
<tr>
<td>0.05</td>
<td>7.45</td>
<td>38.5</td>
</tr>
<tr>
<td>0.10</td>
<td>6.50</td>
<td>39.7</td>
</tr>
<tr>
<td>0.20</td>
<td>5.55</td>
<td>38.4</td>
</tr>
<tr>
<td>0.40</td>
<td>5.40</td>
<td>37.5</td>
</tr>
<tr>
<td>0.60</td>
<td>4.90</td>
<td>37.5</td>
</tr>
<tr>
<td>1.00</td>
<td>4.55</td>
<td>38.5</td>
</tr>
</tbody>
</table>

The cultures were grown aerobically from an A$_{260}$ of 0.1 to 0.8, harvested, disrupted by sonic treatment and assayed for enzymic activities as described in the METHODS.

*μmoles of product formed/mg. of protein/hr. at 37°
†μmole of NADP$^+$ reduced/mg. of protein/min. at 25°
repression" (Magasanik, 1961). We decided, therefore, to investigate the effect of glycerol as carbon source on AHA-synthetase formation. The results shown in Fig. 3, indicate that increasing concentrations of glycerol have no significant effect on AHA-synthetase formation. In other experiments slight repression was occasionally observed at the highest glycerol concentrations employed (1%).

Further evidence that the formation of AHA synthetase in \textit{E. coli} B is subject to catabolite repression is provided in PARTS B and C of this thesis.

II. Evidence for a Single Acetolactate-Forming System in \textit{Escherichia coli} B

In \textit{E. coli} K-12 the presence of two acetolactate-forming systems has been reported (Radhakrishnan and Snell, 1960): a biosynthetic, pH 8 enzyme which is inhibited by the ultimate product of the pathway (L-valine) but is resistant to repression by glucose, and a catabolic pH 6 enzyme, which is resistant to inhibition by valine but is repressed by growth on glucose. Therefore, it was essential to establish whether a single or multiple acetolactate-forming system existed in \textit{E. coli} B under the conditions used for growing these cultures. Owing to the extreme lability of acetolactate-forming activity in cell lysates, it was necessary to limit this investigation to an examination of crude cell extracts. In order to distinguish between catabolic (pH 6) and anabolic
Fig. 3. Effect of increasing (initial) concentrations of carbon source on the formation of AHA synthetase in *Escherichia coli* B. The basal-salts medium was supplemented with either glucose (●) or glycerol (○) at various initial concentrations as indicated. Cultures were harvested at an *A*$_{420}$ of 0.8 and sonic extracts were prepared as described in the METHODS. Specific activity is defined as µmoles of acetoin formed/mg. of protein/hr. at 37°.
(pH 8) acetolactate-forming activities we took advantage of the distinctive regulatory properties reported for each system, i.e. sensitivity to repression by glucose and sensitivity to inhibition by valine, respectively.

In Fig. 4 are pH curves for the formation of acetolactate in crude extracts of cells grown on minimal medium supplemented with glucose or glycerol. Although the specific activity of the glycerol-grown cells was nearly double that of the glucose-grown cells the pH optima of both extracts was between 7.8 and 8.0. Addition of valine to the assay system containing the glycerol-grown extract strongly inhibited acetolactate formation (lower curve), although the position of the maximum of the pH curve was not significantly altered. These results suggest that the pH 6 enzyme is absent, since under these conditions (grown on glycerol and assayed in the presence of valine) the activity profile of a pH 6 enzyme should be prominent.

During an investigation of feedback inhibition of acetolactate-forming activity in crude extracts of glycerol-grown cells, it was observed that inhibition by valine did not exceed 60-70%, regardless of the concentration of substrate and valine employed. In order to investigate the problem of incomplete inhibition three alternate hypotheses were considered: (1) complete inhibition may require the concerted action of more than one inhibitor, (2) incomplete inhibition by valine may be an artifact of the method by
Fig. 4. Effect of pH on the acetolactate-forming system from cultures of *Escherichia coli* B grown on different carbon sources. Crude extracts were prepared from cells grown on either 0.4% glucose (-----) or on 0.4% glycerol (-----). Assay mixtures were prepared in either 0.1 M-Tris-HCl (o) or potassium phosphate (o). The composition of the assay mixtures was as described in the METHODS with the exception of L-valine (bottom curve) which was added at a concentration of 1 mM. The cells were disrupted by sonic treatment in distilled water and assayed immediately. Specific activity is expressed as μmoles of acetoin formed/mg. of protein/hr. at 37°.
which the crude cell extracts were prepared, and (3) two pH 8 enzymes may be present under these conditions, one which is responsible for 60-70% of the total activity and is 100% inhibited by valine and a second enzyme which contributes only 30-40% of the activity but is valine insensitive. To examine the first possibility, acetyl-CoA-forming activity was determined in extracts prepared from glycerol-grown cells in an assay system containing 25 mM substrate and in the same system supplemented with valine or with the four known end products of the isoleucine-valine ("ilva") pathway (valine, leucine, isoleucine and pantothenate). The results are presented in Table II. The addition of four end products to the assay system did not enhance (or relieve) the inhibition which resulted from the presence of valine alone. The following compounds also failed to inhibit (or stimulate) acetyl-CoA-forming activity when added to the reaction mixture at a final concentration of 1 mM: citrate, acetate, lactate, phosphoenolpyruvate, 2-phosphoglycerate, NAD\(^+\), NADH, NADP\(^+\), NADPH and ATP. Thus, while concerted feedback control of this activity cannot be ruled out, it seems highly unlikely that this mechanism is responsible for the limited inhibition observed. To ascertain whether incomplete inhibition by valine is an artifact resulting in vitro from ultra-sonic treatment of the cells, valine inhibition of acetyl-CoA-forming activity was compared in crude extracts and supernatant fluids of glycerol-grown cells disrupted in various ways. The results of this
Table II. *Effect of the four end products of the valine-isoleucine pathway on the acetolactate-forming activity of* Escherichia coli B

<table>
<thead>
<tr>
<th>Additions</th>
<th>μmoles of acetoin formed/mg. of protein/hr.</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.71</td>
<td>-</td>
</tr>
<tr>
<td>Valine</td>
<td>1.76</td>
<td>62.6</td>
</tr>
<tr>
<td>Valine, leucine, isoleucine, and pantothenate</td>
<td>1.76</td>
<td>62.6</td>
</tr>
</tbody>
</table>

The cells were grown on glycerol (0.4%)–salts medium, harvested and crude sonic extracts were prepared as described in the METHODS. The cell extract was assayed for AHA-synthetase activity in a reaction system containing 25 mM substrate. End products when present, were added to give a final concentration of 0.5 mM each.
experiment are summarized in Table III. The degree of inhibition by valine of acetolactate-forming activity remained constant (59-67%) regardless of whether the cells were disrupted by sonic oscillation, alumina grinding or passage through a French pressure cell. Evidence that incomplete inhibition also occurs in permeabilized whole cells is provided by the results of the experiments with toluene-treated cells (Table III). It was found that when cell suspensions were briefly exposed at 37° to dilute solutions of toluene, the permeability of the cell membrane increased without cell lysis, permitting small molecules such as pyruvate and acetolactate to enter and leave the cell rapidly. Permeabilized cells assayed for acetolactate formation in the presence and absence of valine exhibited high activity and incomplete valine inhibition comparable to that observed in cell extracts (65%) (Table III). Prolonged exposure to, or higher concentrations of toluene resulted in decreased enzymic activity and caused cell lysis. Even under these conditions, however, the degree of inhibition by valine remained constant (Table III). The possibility that extracts of glycerol-grown cells contain two pH 8 acetolactate-forming enzymes possessing different valine sensitivities (0 or 100%) was investigated by measuring acetolactate formation in extracts from glucose and glycerol-grown cells in the presence of increasing concentrations of valine. Although the specific activities of the acetolactate-forming systems of the glycerol and glucose-
Table III. Valine inhibition of acetolactate-forming activity in cells of Escherichia coli
B disrupted or permeabilized by various methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Crude extract</th>
<th>Supernatant</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (mg./ml.)</td>
<td>µmoles of acetoin /mg. of protein/hr.</td>
<td>Inhibition (%)</td>
<td>Protein (mg./ml.)</td>
<td>µmoles of acetoin /mg. of protein/hr.</td>
<td>Inhibition (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Valine</td>
<td>+Valine</td>
<td></td>
<td>-Valine</td>
<td>+Valine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole cells</td>
<td>5.75</td>
<td>0†</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sonic oscillation</td>
<td>5.80</td>
<td>5.11</td>
<td>2.06</td>
<td>60</td>
<td>4.93</td>
<td>5.23</td>
<td>1.87</td>
<td>64</td>
</tr>
<tr>
<td>Alumina grinding</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.96</td>
<td>1.17</td>
<td>0.48</td>
<td>59</td>
</tr>
<tr>
<td>French pressure cell</td>
<td>5.88</td>
<td>2.74</td>
<td>0.94</td>
<td>66</td>
<td>4.40</td>
<td>3.00</td>
<td>0.98</td>
<td>67</td>
</tr>
<tr>
<td>Toluene*</td>
<td>5.56</td>
<td>6.21</td>
<td>2.19</td>
<td>65</td>
<td>0.16</td>
<td>0†</td>
<td>0†</td>
<td>0</td>
</tr>
<tr>
<td>Toluene†</td>
<td>5.32</td>
<td>3.55</td>
<td>1.21</td>
<td>66</td>
<td>0.80</td>
<td>0†</td>
<td>0†</td>
<td>0</td>
</tr>
</tbody>
</table>

The cells were grown aerobically on glycerol (0.4%)-salts medium, harvested by centrifugation and washed with 0.1 M-potassium phosphate buffer, pH 7.4. The washed cells were treated as described in the METHODS. AHA-synthetase activity was determined in the crude extracts and in the 12,000 g (10 min.) supernatant fluids. The assay system was as described in the METHODS with the exception of the concentration of pyruvate which was reduced to 25 mM. Valine, when present, was added to give a final concentration of 1 mM.

*5% (v/v) toluene followed by incubation at 37° for 2 min.
†10% (v/v) toluene followed by incubation at 37° for 6 min.
‡no activity was detected.
Fig. 5. Valine inhibition of acetolactate-forming activity in extracts of *Escherichia coli* B grown on different carbon sources. Crude sonic extracts were prepared from cells grown on either 0.4% glucose (●) or on 0.4% glycerol (○). Acetolactate formation was determined in the assay system described in the METHODS except for the concentration of substrate which was reduced to 25 mM. Valine was added at the concentrations indicated. The specific acetolactate-forming activities on glucose and on glycerol were 4.6 and 8.1, respectively.
grown cells were different (8.1 and 4.6, respectively), the percentage end-product inhibition at each valine concentration was the same (Fig. 5). Results such as these would not be expected if the increased acetolactate-forming activity of extracts from glycerol-grown cells had been due to the derepression of a second enzyme (sensitive to glucose repression and resistant to inhibition by valine). Therefore, the increased pH 8 acetolactate-forming activity of glycerol-grown cells (Fig. 4) appeared to result from the derepression of a single enzyme which is partially sensitive to valine.

Radhakrishnan and Snell (1960) reported that in *E. coli* K-12, the pH 6 enzyme could be readily distinguished from the pH 8 enzyme by the greater stability of the former activity during storage and purification. Since the pH 6 enzyme was also resistant to valine inhibition (Radhakrishnan and Snell, 1960), an experiment was designed to determine if a change in valine sensitivity occurred during storage of a crude extract of *E. coli* B. The results of this experiment are given in Table IV. The concentrations of pyruvate and valine employed in this study (see later) resulted in a 62.5% inhibition of acetolactate formation in the fresh extracts from glycerol-grown cells. Following storage for 29 hr. at 0°, the extract lost approximately 70% of its acetolactate-forming activity. Valine sensitivity, however, remained relatively constant during storage, indicating the presence of a single acetolactate-forming system which, after release from
Table IV. *Lability of acetolactate-forming activity during storage*

<table>
<thead>
<tr>
<th>Time at 0° (hr.)</th>
<th>µmoles of acetoin formed /mg. of protein/hr.</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Valine +Valine</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.93 2.23</td>
<td>62.5</td>
</tr>
<tr>
<td>6</td>
<td>3.78 1.25</td>
<td>66.9</td>
</tr>
<tr>
<td>12</td>
<td>3.17 1.00</td>
<td>68.5</td>
</tr>
<tr>
<td>22</td>
<td>2.47 0.89</td>
<td>64.0</td>
</tr>
<tr>
<td>29</td>
<td>1.92 0.72</td>
<td>62.5</td>
</tr>
</tbody>
</table>

Cells were grown on minimal medium supplemented with 0.4% glycerol. The crude sonic extract was stored in an ice bath at 0°. At the times indicated, portions (0.5 ml.) were assayed for enzymic activity. The composition of the reaction mixture was identical to the system described in the METHODS except for the concentration of pyruvate which was decreased to 25 mM. Valine, when present, was added to give a final concentration of 1 mM.
Table V. *Desensitization of AHA synthetase to valine inhibition*

<table>
<thead>
<tr>
<th>Time at 25° (hr.)</th>
<th>μmoles of acetoin formed /mg. of protein/hr.</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Valine</td>
<td>+Valine</td>
</tr>
<tr>
<td>0</td>
<td>4.02</td>
<td>1.46</td>
</tr>
<tr>
<td>1</td>
<td>2.45</td>
<td>0.89</td>
</tr>
<tr>
<td>2</td>
<td>1.88</td>
<td>0.76</td>
</tr>
<tr>
<td>3</td>
<td>1.65</td>
<td>0.84</td>
</tr>
<tr>
<td>5</td>
<td>1.49</td>
<td>0.90</td>
</tr>
<tr>
<td>7</td>
<td>1.30</td>
<td>0.95</td>
</tr>
</tbody>
</table>

The culture was grown on glycerol (0.4%)-salts medium, harvested and a crude sonic extract was prepared as described in the METHODS. The extract was immediately placed in a water bath at 25° and portions (0.5 ml) were removed at the times indicated for enzyme determination. AHA synthetase was assayed in a reaction system containing 25 mM pyruvate and when present, 0.5 mM L-valine.
the cell by ultra-sonic treatment, showed a parallel loss both in catalytic activity and in sensitivity to feedback inhibition.

When crude cell extracts were incubated at 25° for 2 hr. or longer, AHA synthetase was partially desensitized to valine inhibition (Table V). Although the decrease in valine sensitivity caused by warming the extract was accompanied by a decrease in enzymic activity, it should be noted that the latter loss was disproportionate with the former. Thus pyruvate and valine probably bind to the enzyme at separate sites.

III. Kinetic of Valine Inhibition of Acetohydroxy Acid Synthetase of *Escherichia coli* B

As a result of the observation (Fig. 5) that valine, even at high concentrations could only partially (60-70%) inhibit AHA synthetase activity of *E. coli* B, a preliminary investigation was undertaken in crude extracts of the inhibition kinetics of this system. Examination of a plot of enzyme velocity versus substrate concentration in the presence or in the absence of valine (Fig. 6A) suggested that valine inhibition was non-competitive. However, a double reciprocal plot of the same data (Fig. 6B) indicated that the type of inhibition involved here was neither true non-competitive nor true competitive inhibition. At valine concentrations less than that required to produce maximum inhibition (<0.5 mM) (see Fig. 5), inhibition at low substrate concentrations appeared non-competitive (Fig. 6B) but at very high substrate
Fig. 6. Valine inhibition of AHA synthetase in *Escherichia coli* B. AHA-synthetase activity was determined in a crude sonic extract prepared from cells grown aerobically at 37° on glycerol (0.4%)-salts medium. Valine was added to give a final concentration of 0.25 mM. S, initial concentration of substrate (pyruvate). V, μmoles of product formed/mg. of protein/hr. at 37°.

A. A plot of substrate concentration (S) versus reaction velocity (V).
B. A double reciprocal plot of substrate concentration (1/S) versus reaction velocity (1/V).
concentrations inhibition became slightly competitive (Fig. 6B). When the valine concentration exceeded 0.5 mM, changes in substrate concentration had little or no effect on the degree of inhibition (non-competitive). For example, at a valine concentration of 0.5 mM a 10-fold decrease in pyruvate concentration (250 to 25 mM) increased inhibition by less than 10%. Therefore, in valine inhibition experiments the pyruvate concentration used was 25 mM ensuring that valine inhibition was maximal.

IV. Relationship between Glucose Repression and End-Product Repression of Acetohydroxy Acid Synthetase of Escherichia coli B

The results of an investigation of the relationship between glucose repression and end-product repression of AHA synthetase in E. coli B are shown in Table VI. Previous reports have presented evidence that in E. coli, AHA synthetase is repressed by growth in a minimal medium supplemented with valine (Umbarger and Brown, 1958) or a mixture of valine, leucine, isoleucine and pantothenate (Freundlich and Umbarger, 1963). The effect of these four end products or valine alone was examined in the presence of glycerol or glucose or both. Of the total repression observed in the presence of glucose and end products, the greatest proportion, in all experiments was due to glucose (or glucose and glycerol). When glycerol was the only carbon source present, the specific activities in the presence of end products (glycerol and end products)
Table VI. Effect of biosynthetic end products and carbon source on the formation of AHA synthetase in Escherichia coli B

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Additions to basal medium</th>
<th>Specific activity (µmoles of acetoin formed/mg. of protein/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>5.30</td>
</tr>
<tr>
<td></td>
<td>Glucose + L-valine</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>9.30</td>
</tr>
<tr>
<td></td>
<td>Glycerol + L-valine</td>
<td>10.30</td>
</tr>
<tr>
<td>2</td>
<td>Glycerol</td>
<td>8.73</td>
</tr>
<tr>
<td></td>
<td>Glycerol + glucose</td>
<td>5.27</td>
</tr>
<tr>
<td></td>
<td>Glycerol + end products</td>
<td>8.40*, 8.83†</td>
</tr>
<tr>
<td></td>
<td>Glycerol + glucose + end products</td>
<td>5.64</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>6.16</td>
</tr>
<tr>
<td></td>
<td>Glucose + end products</td>
<td>5.28*, 5.68†</td>
</tr>
</tbody>
</table>

The inoculum was grown for 16 hr. in a shaking water bath on basal medium supplemented with 0.4% glycerol. After harvesting, the cells (stationary phase cells) were washed once in phosphate buffer and resuspended in the same medium plus glucose (0.4%), glycerol (0.4%), L-valine (0.5 mM) or end products as indicated. End products when present were added to give the following concentrations: L-valine, L-leucine, L-isoleucine, 0.5 mM each; and calcium pantothenate, 0.1 mM* or 0.001 mM†. The cultures were grown, harvested, disrupted by sonic treatment and assayed for enzymic activity as described in the METHODS.
were as high as or higher than in the control (glycerol) (Table VI, expt. 2). The presence of valine alone (Table VI, expt. 1) consistently resulted in a slight derepression of AHA-synthetase activity whether the cells were grown on glucose or on glycerol.
The results reported here indicate that in *Escherichia coli* strain B, grown aerobically on minimal salts medium, a single acetolactate-forming system exists which is subject to glucose repression. Derepressed formation of this enzyme system (AHA synthetase) occurs when this strain is grown either on a very low concentration of glucose or on any concentration of a poor carbon source such as glycerol. Although this enzyme is sensitive to catabolite repression, its pH optimum (7.8-8.0) and its sensitivity to feedback inhibition by valine, are in accord with the previously established properties of the biosynthetic AHA synthetase.

Valine inhibition of AHA synthetase activity has been reported to be competitive with respect to substrate in *E. coli* K-12 (Umbarger and Brown, 1958) but non-competitive for the equivalent activities in *A. aerogenes* (Halpern and Umbarger, 1959) and *S. typhimurium* (Bauerle et al., 1964). In the present study, AHA synthetase of *E. coli* B is incompletely inhibited by valine (60-70%), even at very high concentrations, and exhibits inhibition kinetics which are neither purely competitive nor non-competitive with substrate (Fig. 6). Several reports of incomplete feedback inhibition have appeared in the literature. Magee and de Robichon-Szulmajster (1968) found that the pH 7.2, AHA-synthetase activity in crude lysates of *Saccharomyces cerevisiae* was inhibited to a maximum of 60% by the addition of excess L-valine. Subsequent
experiments revealed that, in this case, incomplete inhibition resulted from the selective desensitization of the valine feedback inhibition site during the preparation of cell extracts. When the enzyme was assayed in benzene-treated, permeabilized, intact cells, valine inhibition approached 100%. The investigation reported herein of the effect of various methods of cellular disruption on the valine sensitivity of AHA synthetase of *E. coli* B indicated that incomplete feedback inhibition in this strain was not an artifact of cell lysis since this enzyme demonstrated the same partial response to valine in the intact cell (Table III). Studies on the control of aspartokinase activity in various micro-organisms (Datta and Gest, 1964; Patte et al., 1965) uncovered another example of incomplete feedback inhibition. Aspartokinase is rather unusual in that this enzyme regulates the flow of metabolites into a branched, biosynthetic pathway. The presence of high concentrations of a single end product only partially reduces enzymic activity. Complete inhibition of aspartokinase activity in *Rhodopseudomonas capsulatus* is achieved only by the concerted action of two or more products of this complex pathway. The obvious similarity between the role of aspartokinase and that of AHA synthetase in regulating a multi-product biosynthetic system suggested that 100% inhibition of the latter enzyme might be achieved by the cooperative action of more than one end product. Results of such an experiment obtained in this investigation with AHA synthetase of *E. coli* B
indicated that the degree of partial inhibition of this enzyme by valine was neither enhanced nor lessened by the presence of the other known end products (leucine, isoleucine and pantothenate) of the "ilva" pathway.

In many micro-organisms, biosynthetic pathways leading to the formation of more than one end product are controlled by several enzymes which catalyze the same initial reaction, but which are regulated by different end products. *E. coli* K-12, for example, possesses three aspartokinases each of which is specifically inhibited by either lysine, threonine or homoserine (Stadtman, 1963). Since these enzymes differ somewhat in primary structure their presence can generally be detected by changes in the inhibition kinetics of the crude activity during storage, heat or acid treatment or preliminary fractionation. Valine inhibition and enzyme stability studies performed on crude extracts of *E. coli* B in the present investigation suggest that a single, pH 8, AHA synthetase is present in this organism.

In *E. coli* K-12 (Radhakrishnan and Snell, 1960) and in *A. aerogenes* (Halpern and Umbarger, 1959) two acetolactate-forming systems have been reported: (1) a catabolic system functioning at an optimum of pH 6 and (2) a pH 8 system involved in the biosynthesis of the aliphatic amino acids and of pantothenate. The formation of the pH 6 enzyme in *A. aerogenes* can occur only in an acidic environment (<pH 6) (Halpern and Umbarger, 1959). Since the conditions employed by Radhakrishnan
and Snell to grow *E. coli* K-12 can also result in an acidic medium (unpublished observation) perhaps the pH 6 activity observed in the study with strain K-12 but not in the present study with strain B can be attributed to differences in growth conditions of the cultures. An earlier study in this laboratory (Desai and Polglase, 1965) on the properties of the acetolactate-forming system from a streptomycin-dependent mutant of *E. coli*, also failed to detect activity at pH 6. Hence, although the occurrence of a pH 6 enzyme under certain conditions cannot be excluded in *E. coli* B, the results presented here indicate that this enzyme is not responsible for the augmented AHA synthetase activity observed in glycerol-grown cultures of this organism.

Many properties of the feedback system observed for the AHA synthetase of *E. coli* B indicate that this enzyme belongs to the class of allosteric proteins described by Monod et al. (1963). First, the sensitivity of the enzyme to valine is markedly affected by changes in pH (Fig. 4), as is the case in the inhibition of threonine dehydratase by isoleucine (Changeux, 1961) and phosphoribosyl-ATP pyrophosphorylase by histidine (Martin, 1962). Second, the selective removal of sensitivity to valine can be effected by heat treatment (Table V). Similar results have been reported for threonine dehydratase (Changeux, 1961), aspartate transcarbamylase (Gerhart and Pardee, 1962) and homoserine dehydrogenase (Patte *et al.*, 1962).
1963). In the present study, however, desensitization of AHA synthetase, even by mild heat treatment (25°), results in a rapid loss of activity. Nevertheless, the assumption that the enzyme possesses a separate site for valine binding (inhibitor site), the integrity of which is independently destroyed by heat treatment, seems valid due to the disproportionality between the loss of valine sensitivity and the loss of catalytic activity under certain conditions (Table V). Finally, the complex inhibition kinetics of this enzyme (Fig. 6) are consistent with the idea of a distinct inhibitor site (Monod et al., 1963). In addition to properties of the feedback system, the allosteric nature of AHA synthetase of E. coli B is suggested by the extreme lability of this enzyme in cell extracts. A common property of allosteric enzymes is the rapid loss of catalytic activity following release from the cell (Monod et al., 1965). Allosteric properties have been reported for the AHA synthetase of E. coli K-12 (Umbarger and Brown, 1958) and of S. typhimurium (Bauerle et al., 1964).

The fact that threonine dehydratase, the enzyme that initiates the biosynthesis of isoleucine (Changeux, 1961), does not show a glucose effect (Table I) indicates that repressibility by catabolite repression is not a general property of the regulatory enzymes involved in the biosynthesis of the branched, aliphatic amino acids. In E. coli K-12 the structural gene governing AHA synthetase (condensing enzyme)
is controlled by an operator locus (opr. B) entirely independent of that of the other enzymes of the pathway (Ramakrishnan and Adelberg, 1965). Threonine dehydratase, however, along with transaminase B and the dihydroxy acid dehydrase, is under the control of a second operator locus designated opr. A.

It has been reported that the AHA synthetase in valine-isoleucine auxotrophs of E. coli is subject to "multi-valent repression" by the four end products, valine, isoleucine, leucine and pantothenate (Freundlich and Umbarger, 1963; Freundlich, 1967). Results of the present study (Table VI) indicate that, in the wild-type organism, whether in the presence or absence of added end products, the AHA synthetase is repressed to a level determined by the carbon source on which the cells were grown. Hence, growth on an energy-rich carbon source such as glucose can repress further the level of AHA synthetase in cells previously grown on a poor carbon source plus four end products (Table VI). These data suggest that this enzyme may be under the control of two distinct types of co-repressor molecules; a catabolite co-repressor which is derived from the carbon source and a biosynthetic end-product co-repressor which in this case may be derived from a number of end products.

Similarity exists between the regulatory mechanism described here for AHA synthetase and the regulation of the Krebs-cycle in various micro-organisms. Hanson and Cox (1967) reported that while glucose alone could partially
(40-60\%) repress many of the Krebs-cycle enzymes in *Bacillus subtilis*, *Bacillus licheniformis* and *E. coli*, complete repression was achieved only when the cells were grown in the presence of a rapidly metabolizable energy source and certain biosynthetic end products of the cycle, particularly glutamate. Growth on end products plus a poor carbon source (e.g. acetate) failed to repress enzyme levels. The requirement for dual control of this cycle was attributed to its amphibolic role in intermediary metabolism. For AHA synthetase, the observation of dual control suggests that this enzyme also may play an amphibolic role. The requirement of this enzyme for FAD supports this suggestion.

Alternatively, the sensitivity of AHA synthetase to catabolite repression along with incomplete feedback inhibition by valine and the high Km of this enzyme for substrate may be properties of a regulatory system possessed by this organism to control the function of the branched "ilva" pathway. AHA synthetase is involved in the synthesis of leucine, isoleucine and pantothenate in addition to valine, therefore complete inhibition of AHA synthetase activity by valine would impair the synthesis of the other products of the pathway. In *E. coli* B this problem appears to be alleviated by the presence of an AHA synthetase which is only partially sensitive to feedback inhibition (Fig. 5 and Table II). Additional control of this pathway may be provided by catabolite
repression which regulates the concentration of AHA synthetase in the cell. For example, when the wild-type organism is growing on an energy-rich carbon source such as glucose, pyruvate and other metabolic intermediates are rapidly formed and accumulate (Magasanik, 1961). Catabolite repression is strong under these conditions and the synthesis of AHA synthetase is repressed (Fig. 3). However, since growth on glucose results in the elevation of the intracellular concentration of pyruvate, the increased activity of the remaining enzyme will be sufficient to satisfy the biosynthetic requirements of the cell. When glucose is replaced by a carbon source which is metabolized more slowly (e.g. glycerol), the concentration of the pyruvate pool decreases. Due to the high Km of this enzyme for substrate (∼20 mM) (Fig. 6), this decrease in pyruvate concentration in the cell, reduces AHA-synthetase activity and subsequently the activity of the entire "ilva" pathway. However, since catabolite repression is also relaxed during growth on a poor energy source, AHA synthetase is derepressed (Fig. 3). Thus, the cell compensates for the decrease in the concentration of the pyruvate pool by increasing the concentration of AHA synthetase in the cell. Control of AHA synthetase activity by a combination of catabolite repression and partial feedback inhibition enables the organism to satisfy its leucine, isoleucine and pantothenate requirements under various growth conditions, without synthesizing excessive quantities of valine. Further evidence in
support of this hypothesis will be presented in PART B of this thesis.

Although it appears anomalous that AHA synthetase, an enzyme involved in the regulation of a biosynthetic pathway, should be subject to catabolite repression, this case is not unique. Gorini and Gundersen (1961) have reported that the ornithine transcarbamylase of *E. coli* B (but not of several other strains of *E. coli* examined) was subject to a glucose effect. Repression of ornithine transcarbamylase by the carbon source appears to play a major role in the regulation of the arginine pathway in this strain (B), since ornithine transcarbamylase is not regulated by end-product repression. Thus, although regulation of the valine-isoleucine and of the arginine pathways appears to differ in many respects in *E. coli* B (Gorini and Gundersen, 1961), the involvement of catabolite repression in the control of these two anabolic pathways represents an interesting deviation from the general hypotheses concerning regulation of biosynthetic systems. Gorini (1963) suggested that the sensitivity to catabolite repression of ornithine transcarbamylase in *E. coli* B may represent a vestigial control mechanism from a period in the past when the arginine pathway of this organism functioned catabolically as well as anabolically. The present-day strain B then may have lost the degradative use of the pathway while retaining the "catabolite repression" type of control. Although this hypothesis may explain the origin of
this unexpected control mechanism in the "ilva" pathway of 
*E. coli*. B, it is difficult to understand how this mechanism 
could withstand natural selection unless it can bestow on a 
cell a greater survival value than that provided by the more 
common end-product repression observed in other anabolic 
pathways.
PART B: Relaxation of Catabolite Repression in Streptomycin-Dependent *Escherichia coli*

RESULTS

I. Relaxation of the Glucose Effect on Acetohydroxy Acid Synthetase in Streptomycin-Dependent *Escherichia coli*

In PART A, evidence was presented (Table I and Fig. 3) which indicated that the biosynthetic acetolactate-forming system (AHA synthetase) of wild-type *E. coli* B, is repressed by growth on a rapidly metabolizable carbon source such as glucose. The results of a similar experiment using a Sm-dependent mutant of *E. coli* B are presented in Fig. 7. After a slight decrease in specific activity at concentrations of glucose or glycerol greater than 0.05% the activity remained constant during a 10-fold increase in carbon-source concentration. Unlike the AHA synthetase of the wild-type organism (Fig. 3), the specific activity of this enzyme in the Sm-dependent mutant was not significantly altered by changing the carbon source from glucose to glycerol (Fig. 7). These results suggest that in the Sm-dependent mutant, in contrast with the wild-type organism, there is at least partial relaxation of repression by the carbon source of AHA synthetase.

II. Modification of Diauxie Growth in Streptomycin-Dependent Mutants

For a more detailed investigation of the mechanism of
Fig. 7. Effect of increasing (initial) concentrations of carbon source on the formation of AHA synthetase in streptomycin-dependent *Escherichia coli* B. The minimal salts medium was supplemented with either glucose (○) or glycerol (○). The results of an identical experiment (----) with the wild-type parent of this mutant, grown on glucose, were taken from Fig. 3. Specific activity is expressed as μmoles of acetoin formed/mg. of protein/hr. at 37°.
relaxation of catabolite repression in Sm-dependent mutants, the \( \beta \)-galactosidase system was chosen as the experimental tool (Jacob and Monod, 1961).

The classical experiment that demonstrates the "glucose effect" (Epps and Gale, 1942) is the occurrence of a biphasic growth curve in a bacterial culture growing on a mixture of glucose and lactose. This biphasic growth pattern was named the "diauxie effect" by Monod (1947). Wild-type \( E. \text{coli} \) (Fig. 8A) exhibits a classical diauxie growth curve resulting from the complete repression of \( \beta \)-galactosidase during growth on glucose. The corresponding Sm-dependent culture (DB), however, showed only a slight diauxie effect (Fig. 8B). The partial elimination of diauxie growth in the Sm-dependent mutant appeared to result from initiation of induction of \( \beta \)-galactosidase at least 1½ hr. before the exhaustion of glucose (3½ hr.). Varying the lactose:glucose ratio did not prevent early induction nor restore typical diauxie growth.

To confirm the differences in the diauxie response of Sm-sensitive and Sm-dependent organisms, another strain of \( E. \text{coli} \) was examined. The growth curves are given in Fig. 9 for wild-type \( E. \text{coli} \) E (strain SE) and the Sm-dependent derivative of this strain (DE). Strain SE, like strain SB, exhibited diauxie growth (Fig. 9A), whereas the Sm-dependent mutant DE (Fig. 9B), did not show even the slight diauxie effect obtained with mutant DB. Thus growth on glucose completely failed to repress \( \beta \)-galactosidase induction in
Fig. 8. Glucose-lactose diauxie growth of wild-type *Escherichia coli* B (SB) and a streptomycin-dependent mutant (DB) of this organism. Growth (○) and β-galactosidase formation (©) were followed in cultures growing aerobically at 37° on minimal medium supplemented with 0.025% glucose and 0.2% lactose. See METHODS for further experimental details. A, Strain SB; B, strain DB.
Fig. 9. Glucose-lactose diauxie growth of wild-type *Escherichia coli* E (SE) and a streptomycin-dependent mutant (DE) of this organism. Growth (o) and β-galactosidase formation (△) were followed in cultures growing aerobically at 37° on minimal medium supplemented with 0.025% glucose and 0.2% lactose. See METHODS for further experimental details. A, Strain SE; B, strain DE.
strain DE. This was confirmed by an examination of the
induction curve (Fig. 9B).

III. Differences between Streptomycin-Sensitive and
Streptomycin-Dependent Escherichia coli in the
Glucose Effect on β-Galactosidase Synthesis

A more direct approach to the measurement of catabolite
repression is to study the effect of various carbon sources
on the differential rate of enzyme synthesis (Cohn and Horibata,
1959). Fig. 10A shows the lactose-induced formation of β-
galactosidase in cultures of strain SB growing exponentially
on glycerol, a carbon source that exerts little catabolite
repression owing to its relatively low rate of metabolism
(Magasanik et al., 1959). The concentration of enzyme/ml.
is plotted as a function of total cellular protein (Fig.
10A). The addition of glucose (final concentration, 0.05%)
resulted in a rapid decrease in the differential rate of β-
galactosidase synthesis. In this strain, the rate of enzyme
synthesis on glucose was approximately 70% less than the rate
on the glycerol control.

The results of an identical experiment with strain DB
are given in Fig. 10B. In this case, the addition of glu-
cose evoked a sharp but short-lived repression of β-galac-
tosidase synthesis, which rapidly gave way to a differential
rate only 15% less than that of the control.

Experiments identical with those described for strains
Fig. 10. Effect of the addition of glucose on the differential rate of β-galactosidase induction in cultures of wild-type Escherichia coli B (SB) and a streptomycin-dependent mutant (DB) of this organism. Cells were induced with lactose (0.2%) while growing exponentially on glycerol (0.1%)-salts medium (o). Glucose (0.05%, final concentration) was added as indicated by the arrows (>). Further experimental details are given in the METHODS. A, Strain SB; B, strain DB.
Fig. 11. Effect of the addition of glucose on the differential rate of β-galactosidase induction in cultures of wild-type Escherichia coli E (SE) and a streptomycin-dependent mutant (DE) of this organism. Cells were induced with lactose (0.2%) while growing exponentially on glycerol (0.1%)-salts medium (o). Glucose (0.05%, final concentration) was added as indicated by the arrows (©). Further experimental details are given in the METHODS. A, Strain SE; B, strain DE.
SB and DB were carried out on wild-type strain E and its Sm-dependent derivative, DE. Addition of glucose to cultures of strain SE growing exponentially on glycerol caused an initial sharp repression of enzyme synthesis that was transient and gave way to a steady-state rate about 40% less than the rate observed on glycerol alone (Fig. 11A). In contrast, addition of glucose to strain DE had no effect on the rate of β-galactosidase synthesis (Fig. 11B).

Both of the Sm-dependent strains thus showed resistance toward catabolite repression when compared to the parent Sm-sensitive strains. However, there were quantitative differences between the two dependent strains, both in diauxie growth (Figs. 8B and 9B, respectively) and in β-galactosidase induction (Figs. 10B and 11B, respectively). Thus strain DE appeared completely resistant to catabolite repression, but strain DB was somewhat less resistant to this effect.

IV. β-Galactosidase Induction in Psuedo-Resting Cells

The question now may be asked: do Sm-dependent mutants lack the mechanism for the formation of the catabolite co-repressor, as suggested by results with strain DE, or is the co-repressor synthesized at a rate insufficient to cause repression, the situation indicated by results with strain DB? To answer this question, β-galactosidase induction was examined in "pseudo-resting" cell suspensions of strains SE and DE prepared as described in the METHODS section. These experimental
conditions resulted in a limitation of anabolism without limiting to the same degree the corresponding catabolic processes, thus permitting an accumulation of catabolite co-repressor molecules (Mandelstam, 1961). Results of a typical experiment are presented in Fig. 12. When glucose at a final concentration of 0.075% was added to cultures of strain SE or strain DE after 90 min. of lactose-induced synthesis of β-galactosidase, enzyme synthesis was rapidly inhibited in both cultures. Hence, the mechanism for formation of catabolite co-repressor was operative in Sm-dependent mutants, but under the conditions of exponential growth, apparently the rate of formation of co-repressor in strain DE was insufficient to cause a "glucose effect". Only when conditions were modified so as to favour the accumulation of high concentrations of catabolite co-repressor was catabolite repression demonstrable in this Sm-dependent organism.

In addition to lactose, the gratuitous inducers, methyl β-D-thiogalactopyranoside (TMG) and isopropyl β-D-thiogalactopyranoside (IPTG) were used as inducers of β-galactosidase in many of these studies. Results with TMG generally agreed with those obtained with lactose. IPTG, however, was unsatisfactory in this study; β-galactosidase induction by this powerful inducer (Jacob and Monod, 1961; Moses and Prevost, 1966) was completely resistant to repression by glucose under the conditions used.
Fig. 12. Effect of the addition of glucose on β-galactosidase induction in "pseudo-resting" cultures of wild-type *Escherichia coli* E (SE) and a streptomycin-dependent mutant (DE) of this organism. Experimental conditions are described in the METHODS. Glucose (0.075%, final concentration) was added to one flask as indicated by the arrows. Glycerol control (o); glucose added (o). A, Strain SE; B, strain DE.
V. Relaxation of Catabolite Repression in Pathways of Carbohydrate Metabolism

If the derepression of AHA synthetase in the Sm-dependent mutant (Fig. 7) was the result of a general decrease in the concentration of catabolite co-repressor molecules in the cells, then other enzymes subject to repression by carbon source should also reflect this loss of control. Many of the enzymes of intermediary metabolism are known to be sensitive to catabolite repression (Magasanik, 1961; Gorini and Gundersen, 1961; Hanson and Cox, 1967). For example, the Krebs-cycle enzymes are lower in micro-organisms grown on a salts medium containing glucose than in cells grown on a poor carbon source such as glycerol or lactate (Gray et al., 1966; Hanson and Cox, 1967). To determine whether or not the apparent decrease in concentration of catabolite co-repressor in Sm-dependent mutants influences the control of other enzymes, the specific activities of several Krebs-cycle enzymes involved in carbohydrate metabolism were determined in crude extracts of strains SB and DB grown on glucose and glycerol. The results are summarized in Table VII. Examination of the results for the wild-type organism (strain SB) indicates that the four Krebs-cycle enzymes investigated as well as AHA synthetase exhibit a "glucose effect" as demonstrated by glycerol/glucose specific activity ratios greater than unity (Table VII, column b/a): this effect was particularly marked in the case of fumarase and aconitase (Table VII). In the corres-
Table VII. Specific activities of enzymes from streptomycin-sensitive (SB) and streptomycin-dependent (DB) strains of Escherichia coli B grown on glucose or glycerol

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>SB Glucose</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>DB Glucose</th>
<th>Glucose</th>
<th>Glycerol</th>
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<th>DB Glucose</th>
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<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>b/a</td>
<td>(c)</td>
<td>(d)</td>
<td>d/c</td>
<td>c/a</td>
<td>d/b</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AHA synthetase</td>
<td>4.42</td>
<td>9.32</td>
<td>2.11</td>
<td>9.64</td>
<td>9.52</td>
<td>0.99</td>
<td>2.18</td>
<td>1.02</td>
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<tr>
<td>Citrate synthase</td>
<td>172</td>
<td>440</td>
<td>2.56</td>
<td>493</td>
<td>349</td>
<td>0.71</td>
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<tr>
<td>Fumarase</td>
<td>396</td>
<td>2070</td>
<td>5.23</td>
<td>2545</td>
<td>1885</td>
<td>0.74</td>
<td>6.42</td>
<td>0.91</td>
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<tr>
<td>Aconitase</td>
<td>111</td>
<td>460</td>
<td>4.14</td>
<td>789</td>
<td>1317</td>
<td>1.67</td>
<td>7.10</td>
<td>2.86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocitric dehydrogenase</td>
<td>365</td>
<td>629</td>
<td>1.73</td>
<td>880</td>
<td>943</td>
<td>1.07</td>
<td>2.41</td>
<td>1.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>130</td>
<td>109</td>
<td>0.85</td>
<td>137</td>
<td>137</td>
<td>1.00</td>
<td>1.05</td>
<td>1.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucokinase</td>
<td>55.4</td>
<td>68.9</td>
<td>1.22</td>
<td>67.0</td>
<td>64.2</td>
<td>0.96</td>
<td>1.20</td>
<td>0.93</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Glucose and glycerol were added to the minimal medium to give an initial concentration of 0.4%. Growth of the cultures, preparation of extracts and enzyme assays are described in the METHODS. Specific activities (Units/mg. of protein) reported are mean values of activities obtained in two or more, separate experiments.
ponding Sm-dependent mutant, the repression of glucose-sensitive enzymes observed in the wild-type organism was absent (Table VII), permitting many of the enzymes to reach specific activities equal to or even exceeding levels obtained by growth of the wild-type parent strain on glycerol. The relationship between carbon source and Sm phenotype with respect to catabolite repression is summarized by the ratios of the glucose sensitive enzymes in strain DB to those in strain SB (Table VII). After growth of cells on glucose, the ratios for all five glucose-sensitive enzymes exceeded 2 and for fumarase and aconitase DB/SB ratios greater than 6 were observed (Table VII, column c/a). In extracts of cells grown on glycerol, the DB/SB ratios of most enzymes approached unity (Table VII, column d/b) except the ratio for aconitase, which was relatively high.

The specific activities of two other enzymes, glucose 6-phosphate dehydrogenase and glucokinase were essentially the same in the Sm-sensitive and Sm-dependent organisms and were not affected by changing the carbon source (glucose or glycerol) (Table VII). These results indicate that only those enzymes that respond to changes in the concentration of catabolite co-repressor molecules are elevated in Sm-dependent E. coli.
VI. Cell Yield from Glucose of Streptomycin-Sensitive and Streptomycin-Dependent Strains of *Escherichia coli* B

To determine if control by catabolite repression was relaxed in Sm-dependent mutants because of impaired carbohydrate metabolism, the efficiency of glucose utilization for cell mass synthesis was determined in Sm-sensitive and Sm-dependent cultures of *E. coli* B. Preliminary experiments indicated that in *E. coli* B, glucose is completely oxidized as soon as it is taken up by the cell, since growth (determined by the increase in cell mass) ceases almost immediately after the disappearance of glucose from the medium. Therefore, in cultures of *E. coli* B, the rate at which glucose is removed from the medium is a measure of the rate of glucose utilization by the cell. In Fig. 13, the dry wt. of Sm-sensitive and Sm-dependent cells obtained during aerobic growth on limiting glucose was plotted as a function of the decrease in glucose concentration of the medium. The yield of Sm-dependent cells was approximately 37% lower than for the wild-type organism (Fig. 13).

To determine whether the decrease in efficiency of carbohydrate metabolism in the Sm-dependent mutant occurred anaerobically as well as aerobically, the cell yield from glucose of each organism was estimated in the presence and in the absence of oxygen. The effect of a rapid shift from aerobic to anaerobic growth conditions on the rate of glucose consumption relative to cell mass formation is shown in Fig. 14.
Fig. 13. Yield of cells from glucose during aerobic growth of streptomycin-sensitive (SB) and streptomycin-dependent (DB) strains of *Escherichia coli* B. Exponential cells were washed and resuspended in minimal salts medium containing 400 µg. of glucose/ml. During aerobic growth, portions of culture were removed, rapidly cooled, centrifuged (16,000 g for 10 min.) and the supernatant solutions were assayed for glucose by the Glucostat method. Strain SB (o); strain DB (o).
Fig. 14. Effect of a change from aerobic to anaerobic growth on the yield of cells from glucose of streptomycin-sensitive (SB) and streptomycin-dependent (DB) strains of Escherichia coli B. Portions of aerobic and anaerobic culture were removed for glucose determination as described in the legend of Fig. 13. The arrows indicate the points at which the growth conditions were made anaerobic. Other experimental details are given in the METHODS. Strain SB, (○); strain DB, (○).
Table VIII. Cell yields from glucose of streptomycin-sensitive and streptomycin-dependent strains of Escherichia coli B during aerobic and anaerobic growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm-sensitive</td>
<td>0.423</td>
<td>0.161</td>
</tr>
<tr>
<td>Sm-dependent</td>
<td>0.261</td>
<td>0.164</td>
</tr>
</tbody>
</table>

*Y is the cell yield constant defined as the number of μg. dry wt. of cells formed/μg. of glucose consumed. Values of Y were calculated from the data of Fig. 14.
Under aerobic conditions, the Sm-dependent mutant (DB), exhibited the same decreased efficiency of glucose utilization described previously (Fig. 13). When growth conditions were made anaerobic, however (at the arrows), both the Sm-sensitive and the Sm-dependent cultures showed a diminished and similar efficiency of glucose metabolism (Fig. 14). This observation was supported by the cell yield constants (Y) calculated for each organism during aerobic and anaerobic growth (Table VIII). Although the aerobic cell yield constant for the Sm-dependent mutant was 38% lower than for the wild-type organism under the same conditions, anaerobically, the cell yield constants for each organism were the same (Table VIII). The decreased efficiency of carbohydrate metabolism observed in the Sm-dependent mutant thus appears to be the result of a specific impairment of oxidative processes in this organism.

VII. **ATP Synthesis in Streptomycin-Sensitive and Streptomycin-Dependent Strains of *Escherichia coli* B**

The existence of a direct relationship between the degree of catabolite repression and the energy content of an organism or between catabolite repression and the growth rate of an organism on a variety of energy sources is well documented in the literature (Magasanik, 1961; Mandelstam, 1961; Tustanoff and Bartley, 1964; Okinaka and Dobrogosz, 1967). In fact, results from indirect studies have led to the proposal that ATP (Mandelstam, 1961; Moses and Prevost, 1966)
or compounds metabolically related to ATP (Dobrogosz, 1968; Cox and Hanson, 1968) may be the catabolite co-repressor. The observations that cell yield (aerobic) from glucose (Figs. 13 and 14) and growth rates on glucose, glycerol or lactate (Table IX) of Sm-dependent mutants are lower than for the corresponding wild-type parent strains suggest that the loss of control by catabolite repression in these mutants may result from an impairment of general energy metabolism. To investigate this possibility, the rate of ATP synthesis and the concentration of the ATP pool were measured in exponential cultures of strains SB and DB growing aerobically on glucose salts medium. Results of initial experiments indicated that under these conditions the ATP pools of both the Sm-sensitive and the Sm-dependent organisms oscillated during growth. Therefore, a direct comparison of the ATP concentration in the two organisms at a particular cell density was meaningless. As a result of further experimentation, it was found that if the initial ATP pool was decreased by harvesting and pre-incubating the cells in the absence of an energy source (see METHODS), the subsequent rate of ATP synthesis was exponential during a 3-fold increase in cell mass. The results of a typical experiment are shown in Fig. 15. The doubling times for growth and for ATP formation in the Sm-sensitive culture were 52 and 36 min., respectively (Fig. 15A), and in the Sm-dependent culture were both approximately 70 min. (Fig. 15B). Thus, ATP formation paralleled growth rate in the
Table IX. *Growth rates of Escherichia coli strains*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Doubling time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB (Sm-sensitive)</td>
<td>Glucose</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>80</td>
</tr>
<tr>
<td>DB (Sm-dependent)</td>
<td>Glucose</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>124</td>
</tr>
<tr>
<td>SE (Sm-sensitive)</td>
<td>Glucose</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>66</td>
</tr>
<tr>
<td>DE (Sm-dependent)</td>
<td>Glucose</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>110</td>
</tr>
</tbody>
</table>

All cultures were grown aerobically on the basal salts medium supplemented with a carbon source at an initial concentration of 0.4%.
Fig. 15. Rate of ATP formation in cultures of streptomycin-sensitive (SB) and streptomycin-dependent (DB) *Escherichia coli* B growing aerobically on glucose-salts medium. Growth (o) and ATP concentration (o) were determined on portions of culture removed at the times indicated. The inoculum was prepared by method II (see METHODS). A, Strain SB; B, strain DB. The doubling times of cells for SB and DB were 52 min. and 71 min., respectively and of ATP formation were 36 min. and 70 min., respectively.
Fig. 16. The ATP pool of streptomycin-sensitive (SB) and streptomycin-dependent (DB) strains of *Escherichia coli* B during aerobic growth on glucose-salts medium. The pool was calculated from the data of Fig. 15. Strain SB (o); strain DB (o).
Sm-dependent mutant whereas in the Sm-sensitive organism the rate of energy production exceeded the rate of growth. This observation was supported by a plot of the ATP pool versus cell mass during the growth of these cultures (Fig. 16). The average concentration of the ATP pool was not significantly different in the Sm-sensitive and the Sm-dependent cultures. However, the pool concentration remained relatively constant in the Sm-dependent mutant during growth while it increased slowly in the wild-type parent strain. Therefore, although the rate of ATP synthesis is decreased in the Sm-dependent organism, the actual concentration of the ATP pool in this mutant does not differ greatly from that of the Sm-sensitive strain.

VIII. Effect of Carbon Source on Valine Excretion by Streptomycin-Sensitive and Streptomycin-Dependent Cultures of Escherichia coli B

AHA synthetase is derepressed to the same extent in strain SB grown on glycerol as in strain DB grown on glucose or on glycerol (Table VII). Sm-dependent mutants growing aerobically on glucose-salts medium are known to excrete L-valine (Bragg and Polglase, 1962, Tirunarayanan et al., 1962). Therefore, if derepression of AHA synthetase alone is sufficient to cause valine excretion, valine should be present in supernatant fluids of cultures of strains SB and DB grown on minimal medium supplemented with glycerol. The results of an experiment
Table X. Valine excretion by streptomycin-sensitive and streptomycin-dependent strains of Escherichia coli B

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Strain</th>
<th>Carbon source</th>
<th>Valine (µmoles/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SB (Sm-sensitive)</td>
<td>Glucose</td>
<td>4.2</td>
</tr>
<tr>
<td>2</td>
<td>DB (Sm-dependent)</td>
<td>Glucose</td>
<td>8.0</td>
</tr>
<tr>
<td>3</td>
<td>SB</td>
<td>Glycerol</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>DB</td>
<td>Glycerol</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>SB</td>
<td>Glycerol + Glucose</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Cultures of strains SB and DB were grown for 16 hr. on a shaker at 37° in minimal medium supplemented with 0.4% of either glucose or glycerol. The cells were diluted with fresh medium to give an $A_{420}$ of 0.1. Flasks nos. 1-4 received either glucose or glycerol (final concn., 0.4%) as indicated and the cultures were grown with vigorous aeration to an $A_{420}$ of 1.6. The cells were removed by centrifugation and portions of the supernatant fluid were assayed for valine (see METHODS). In flask no. 5, diluted, glycerol-grown cells of strain SB were supplemented with 0.4% glycerol and grown aerobically to an $A_{420}$ of 0.8. Glucose (0.4%) was then added and growth continued to an $A_{420}$ of 1.6.
designed to investigate the role of the carbon source in valine excretion by Sm-sensitive and Sm-dependent cultures of E. coli B are presented in Table X. Wild-type B (strain SB) unlike most Sm-sensitive strains (Bragg and Polglase, 1962; Tirunarayanan et al., 1962) excreted a significant amount of valine during aerobic growth on glucose even though AHA synthetase was repressed under these conditions (Fig. 3 and Table I). The corresponding Sm-dependent mutant (strain DB), however, produced approximately twice as much valine under the same conditions (Table X). When glycerol replaced glucose as the carbon and energy source, valine was not detected in the supernatant fluids of either the Sm-sensitive or the Sm-dependent cultures. However, when glucose was added to a culture of strain SB derepressed for AHA synthetase by growth on glycerol, a rapid excretion of valine resulted (Table X, line 5).
DISCUSSION

The results of experiments presented in PART B indicate that metabolic control by catabolite repression is relaxed in Sm-dependent mutants of *Escherichia coli* growing exponentially on a glucose-salts medium. This decrease in catabolite repression appears to be responsible for the derepressed formation of the AHA synthetase of Sm-dependent mutants of *E. coli* (Coukell and Polglase, 1965; Table VII). Studies on catabolite repression of the inducible β-galactosidase system in *E. coli* B and in an additional strain *E. coli* E indicate that control of this enzyme by the carbon source also is relaxed in Sm-dependent mutants (Figs. 8-11). Furthermore, certain enzymes of the Krebs-cycle which are sensitive to catabolite repression in wild-type strain B are derepressed in the Sm-dependent mutant (Table VII). In particular, the activities of aconitase, fumarase, citrate synthase and isocitrate dehydrogenase are higher in the Sm-dependent mutant than in the parent strain of *E. coli* B. Thus, relaxation of catabolite repression appears to be a general phenomenon in these mutants since all of the glucose-sensitive enzymes investigated exhibited this loss of control.

An obvious question to ask at this point is whether a single compound is responsible for the repression of all glucose-sensitive enzymes in *E. coli* or whether each enzyme responds only to its own specific co-repressor molecule.
During a series of studies by Neidhardt and Magasanik (1957) on the mechanism of catabolite repression of histidase induction in *Aerobacter aerogenes*, it was found that certain growth conditions which permitted the synthesis of histidine, still repressed the formation of other glucose-sensitive enzymes. They concluded that more than one co-repressor molecule must be involved in the regulation of glucose-sensitive enzymes. For example, a single nitrogen-containing co-repressor may be responsible for the repression of all enzymes acting on nitrogenous compounds, while another co-repressor may regulate the synthesis of enzymes which degrade sugars and related molecules (Magasanik, 1961). Recently, Moses and Prevost (1966) postulated a mechanism for general control by catabolite repression which requires only a single co-repressor molecule. According to this hypothesis, the various glucose-sensitive enzymes in the cell exhibit different sensitivities to the same catabolite co-repressor. Furthermore, the intracellular concentration of this co-repressor fluctuates in response to changes in the rates of the various catabolic and anabolic processes. Thus, when the co-repressor concentration in the cell is low, enzymes which are very sensitive to the co-repressor remain repressed, while enzymes which are less sensitive to this molecule are derepressed to varying degrees. Although the identity of the co-repressor molecule remains obscure, a number of independent studies suggest that ATP (Mandelstam, 1961; Moses and Prevost, 1966)
or compounds metabolically related to ATP (Dobrogosz, 1968; Cox and Hanson, 1968) may be the elusive co-repressor. Since the general relaxation of control by catabolite repression in strain DB correlates with a decreased rate of ATP synthesis in this mutant (Fig. 15), the single catabolite co-repressor hypothesis initially appears the most attractive. However, it is possible that a number of different co-repressor molecules are involved, all of which depend upon ATP for their synthesis.

Recent studies, primarily by Tyler et al. (1967) indicate that there is more than one type of glucose effect on β-galactosidase induction. In addition to the effect of glucose on the transport of inducer across the cell membrane, two types of catabolite repression have been described which can be distinguished from one another as being transient or permanent. Transient repression is a short-lived repression (usually less than one generation) which occurs when glucose or an analog of glucose is added to a culture growing exponentially on a carbon source such as glycerol, lactate or succinate. Since only glucose and glucose analogues can evoke transient repression, it is thought that the co-repressor is either glucose itself or a closely related metabolite (glucose 6-phosphate, NADPH, etc.) (Paigen, 1966; Hsie and Rickenberg, 1967; Prevost and Moses; 1967). Permanent catabolite repression, however, can be exerted by any carbon source provided that the rate of energy metabolism in the cell exceeds
the biosynthetic requirement of the cell (Magasanik, 1961; Mandelstam, 1961). Furthermore, permanent repression is elicited by catabolites which are metabolically distant from glucose and the degree of repression remains relatively constant in exponential cultures as long as the carbon source is present (Moses and Prevost; 1966).

The conditions employed in this work to study the effect of glucose on the differential rate of β-galactosidase synthesis (addition of glucose to cells growing exponentially on glycerol) would be expected to result in transient as well as permanent catabolite repression (Figs. 10 and 11). Glucose does not block the uptake of inducer into these cells since an increase in lactose concentration accompanied by a decrease in the concentration of glucose had no effect on diauxie growth or β-galactosidase induction (see Loomis and Magasanik, 1967). Transient repression is probably responsible for the biphasic induction kinetics which occurred following the addition of glucose to exponential cultures of strain SE (Fig. 11A). In strain SB (Fig. 10A), the severe permanent repression produced by glucose must "mask" a similar transient effect since transient repression is observed in the Sm-dependent mutant of this strain (DB) (Fig. 10B). The failure to detect a residual transient effect in strain DE (Fig. 11B) may be due to the limited sensitivity of this experimental system to slight changes in catabolite repression in strains which normally exhibit weak transient and permanent repression. These data
suggest that the decreased glucose effect observed in Sm-dependent mutants results from an impairment of permanent and not of transient catabolite repression. Further support for this conclusion is provided by the specific activities of enzymes involved in the initial steps of glucose metabolism. The specific activities of glucokinase and glucose 6-phosphate dehydrogenase (Table VII) of strain DB do not differ significantly from those of the wild-type parent strain (SB). Since the occurrence of transient catabolite repression is dependent upon a rapid conversion of glucose to the level of the sugar-phosphates (Prevost and Moses, 1967; Tyler et al., 1967), equivalent glucokinase and glucose 6-phosphate dehydrogenase activities in the two organisms tend to rule out differences in this form of catabolite repression.

Studies on the regulation of AHA synthetase in Sm-dependent E. coli B (DB) and of the inducible β-galactosidase of strain DB and of an additional Sm-dependent strain (DE) indicate that although control by catabolite repression is relaxed in these mutants it is not completely absent. For example, β-galactosidase induction in Sm-dependent strain DE was extremely sensitive to glucose repression under conditions where the anabolic rate of this mutant was limited (Fig. 12). Thus, the mechanism concerned with catabolite co-repressor synthesis is operative in Sm-dependent mutants but under conditions of exponential growth, the concentration of co-repressor in the cell apparently is not maintained at a high
enough level to regulate enzyme synthesis.

Neidhardt (1960) investigated carbohydrate metabolism in a mutant of \textit{A. aerogenes} lacking glucose repression. As a result of this study he concluded that any mutant that has lost its sensitivity to glucose for a number of glucose-sensitive enzymes should have either (1) an impaired glucose dissimilation or (2) an improved overall anabolic capacity. Since the growth rate of Sm-dependent \textit{E. coli} B on glucose-salts medium is lower than the growth rate of the wild-type strain (Table IX), the decrease in catabolite repression observed in this mutant must result from an impairment of glucose metabolism because a stimulation of anabolism (which is the rate-limiting process under these conditions) would increase the growth rate of the dependent mutant. This deduction was confirmed by the discovery that the efficiency of aerobic glucose utilization in Sm-dependent \textit{E. coli} B was only two-thirds that of the parent strain (Fig. 13). Further support for the hypothesis that the decrease in catabolite repression in the Sm-dependent mutant results from an effect on catabolic rather than on anabolic processes was obtained from an investigation of ATP formation in this organism. The results indicate that although the ATP pool in the Sm-dependent mutant was approximately equal to that of the sensitive organism (Fig. 16), the rate of ATP synthesis in the mutant was diminished and paralleled the growth rate of the Sm-dependent culture (Fig. 15). This suggests that the growth rate of the Sm-dependent
mutant (unlike that of the wild-type parent strain) is limited by the rate of energy production in the cell. Furthermore, this investigation of ATP formation suggests that in exponential cultures of Sm-sensitive and Sm-dependent organisms, catabolite repression may be related more closely to the rate of energy production than to the concentration of energy in the cell.

Gorini and Kataja (1964a) described a class of Sm-resistant, arginine auxotrophs of *E. coli* which would grow only if arginine or Sm were added to the minimal medium. These mutants were termed conditionally streptomycin-dependent (CSD) because of their dependence on antibiotic for growth in the absence of the required amino acid. Subsequent studies revealed that one of these CSD-mutants possessed a defective structural gene for the enzyme ornithine transcarbamylase (OTCase) and this mutation was phenotypically corrected by Sm at the level of translation when this mutant was grown in the presence of antibiotic. Examination of the specific activities of a number of enzymes in these "Sm-suppressed" mutants indicated that while the concentration of OTCase in CSD-mutants was elevated sufficiently to permit growth, the formation of certain other enzymes was impaired (Gorini and Kataja, 1964b). This "over-suppression" by Sm led to the formation of non-functional protein in the cell and a reduction in the growth rates of these mutants (Gorini and Kataja, 1964b). In the present study, many of the properties observed for the Sm-
dependent mutant of *E. coli* B (DB) are analogous to properties described for the CSD-mutants. For example, both mutants require antibiotic for growth but grow more slowly than their respective wild-type parent strains; both mutants exhibit irregularities in the concentrations of a number of metabolically unrelated enzymes and both mutants appear less efficient at cell synthesis than the corresponding antibiotic independent organisms. Despite these similarities, however, results of experiments described in this section indicate that the Sm-dependent mutant of *E. coli* B and the CSD-mutants described by Gorini are unrelated. First, the derepressed formation of certain enzymes in strain DB does not occur randomly, as would be expected if Sm were merely altering the fidelity of translation. Only glucose-sensitive enzymes appear to be affected, since glucokinase and glucose 6-phosphate dehydrogenase which are resistant to catabolite repression exhibit equivalent specific activities in the Sm-sensitive and in the Sm-dependent organisms. Furthermore, while the concentrations of a number of enzymes are elevated in the Sm-dependent mutant (DB), no enzymes have been detected which are synthesized at a decreased concentration. Second, while aerobic cultures of strain DB exhibit a decreased efficiency of glucose utilization for cell synthesis, anaerobically, the Sm-dependent mutant is equally as efficient as the wild-type parent strain (SB) (Table VIII). A change, such as this, in oxygen tension would not be expected to alter the coding properties of CSD-
mutant ribosomes. Therefore, most of the abnormal properties of strain DB appear to result from impaired energy metabolism rather than from a direct effect of DHSm on protein synthesis.

In PART A, it was suggested that the capacity of an organism to control AHA-synthetase formation by catabolite repression may assist it in regulating the activity of the complex, branched "ilva" pathway. This hypothesis is supported by the investigation (Table X) of the effect of the nature of the carbon source on valine excretion by cultures of wild-type E. coli B and a Sm-dependent derivative (strain DB) of this organism. When strain SB or DB is grown on a minimal medium supplemented with a slowly metabolizable carbon source such as glycerol, no extracellular valine is produced (Table X) despite the fact that AHA synthetase is derepressed under these conditions (Table VII). This result is consistent with the report that during exponential growth on glycerol, intermediates such as pyruvate do not accumulate significantly since they are synthesized slowly and metabolized rapidly (Magasanik et al., 1959). The important role played by the carbon source during valine excretion is further emphasized by an examination of the supernatant fluids of glucose-grown cultures. Strain SB, for example, excreted a small but significant quantity of valine (Table X) even though AHA synthetase was repressed by growth on glucose (Table VII). The enhanced amount of extracellular valine by the Sm-dependent
mutant (DB), under the same growth conditions, correlates with the derepressed level of AHA synthetase in this organism (Table VII). The most dramatic production of extracellular valine from glucose, however, resulted when glucose was added to a Sm-sensitive culture growing exponentially on glycerol. Addition of glucose caused a rapid increase in the pyruvate concentration in the cell (Magasanik et al., 1959). Since the AHA synthetase of these cells was already derepressed as a result of growth on glycerol, valine could be rapidly synthesized and excreted before the enzyme could be repressed (Table X). Thus, in *E. coli* B, while a slight excretion of valine can result simply from growth on a rapidly metabolizable carbon source such as glucose, maximum valine production requires both a high concentration of intracellular pyruvate and a derepressed level of AHA synthetase. These conditions are present in the Sm-dependent mutant of *E. coli* B (DB) growing exponentially on glucose.

It has been reported that valine is excreted when Sm (or DHSm) is added to growing cultures of Sm-sensitive but not high-level Sm-resistant micro-organisms (Tirunarayanan et al., 1962; Bragg and Polglase, 1962). In view of the relationship between pyruvate accumulation and valine excretion observed in *E. coli* B, it is now possible to speculate on the probable mechanism of this phenomenon. When Sm is added to a susceptible culture, protein synthesis is inhibited (Erdös and Ullmann, 1959). This impairment of anabolic activity will result in a rapid and
dramatic increase in the concentration of metabolic intermediates such as pyruvate. If feedback inhibition of AHA synthetase by valine is incomplete, as it is in *E. coli* B (Fig. 5), valine synthesis will not be shut off as the concentration of the amino acid increases in the cell. This will result in an overproduction and eventual excretion of valine. Protein synthesis in high-level Sm-resistant mutants is not inhibited by Sm (Erdős and Ullmann, 1959), thus the pyruvate concentration in the cell will not increase significantly upon the addition of antibiotic. Since AHA synthetase is also repressed under these conditions (Coukell and Polglase, 1965) valine will not be produced in excess. Support for this hypothesis comes from a study by Bragg and Polglase (1962) on the formation of extracellular metabolites by Sm-mutants of *E. coli*. They found that the addition of DHSm to sensitive cultures resulted in a progressive change in the composition of the growth medium. Approximately 2 hr. after the addition of antibiotic, pyruvate was detected. The amount of pyruvate produced by the cells increased as a function of the concentration of DHSm added to the culture and was related to the viable-cell count of the DHSm-treated culture. This was followed during the next 1-2 hr. by a gradual decrease in the concentration of extracellular pyruvate and an increase in the concentration of lactate, alanine and particularly of valine. It was possible to account, almost quantitatively, for the decrease in extracellular pyruvate by the formation of the secondary products, all of
which are synthesized from pyruvate. Growth of the corresponding Sm-resistant mutant on a high concentration of DHSm (1 mg./ml.) resulted in the excretion of a small quantity of pyruvate and some lactate but only a trace of valine.
PART C: Enhancement of Catabolite Repression by Dihydrostreptomycin-Limitation of Streptomycin-Dependent Escherichia coli B

RESULTS

I. Antibiotic-Requirement for Growth of Streptomycin-Dependent Escherichia coli B on Various Carbon Sources

Preliminary experiments indicated that the Sm-dependent mutant (DB) which was isolated on medium containing 1 mg./ml. of DHSm, had comparable growth rates at either 1 mg./ml. or 100 μg./ml. of antibiotic. As the concentration of DHSm in the medium was decreased below 50 μg./ml., however, the growth rate of this mutant was markedly diminished. Growth curves for cultures of strain DB on glucose-salts medium containing rate-limiting concentrations of DHSm are shown in Fig. 17. When antibiotic was not added to the culture, a slight increase in absorbancy was observed during the period of incubation. This increase was accompanied by the formation of filamentous forms from cells in the original inoculum, a characteristic response of Sm-dependent organisms to antibiotic starvation (Delaporte, 1949). The cultures which were supplemented with 5 to 100 μg./ml. of DHSm exhibited exponential growth but the growth rates decreased as the antibiotic concentration in the medium decreased. When the growth rates of these cultures were plotted against the concentration of antibiotic on which they were grown, a hyperbolic, saturation curve was obtained.
Fig. 17. Growth of a streptomycin-dependent mutant of \textit{Escherichia coli} B on rate-limiting concentrations of dihydrostreptomycin. The inoculum (600 ml.) was grown aerobically for 18 hr. on glucose (0.4\%) - salts medium supplemented with 30 \( \mu g./mL \) of dihydrostreptomycin. The resulting cells were harvested by centrifugation and resuspended in 6 l. of the same medium to give an \( A_{420} \) of 0.1. The culture was divided into six 1-l. volumes and supplemented with antibiotic to give the final concentrations (\( \mu g./mL \)) indicated. The increase in \( A_{420} \) of each culture was followed during aerobic growth at 37\°C.
Fig. 18. Relationship between the growth rate of *Escherichia coli* strain DB and the dihydrostreptomycin concentration of the medium. The data were obtained from the experiment shown in Fig. 17. Growth rate is defined as the reciprocal of the doubling time in hr.

A. A plot of growth rate versus dihydrostreptomycin concentration.

B. A plot of the reciprocal of the growth rate versus the reciprocal of the dihydrostreptomycin concentration.
The corresponding double reciprocal plot of these data is shown in Fig. 18B. From this graph, two constants may be calculated, $K_{DHSm}$ and $V_{max}$, relating the concentration of DHSm in the medium to the growth rate. These constants may be interpreted in the conventional manner so that $K_{DHSm}$ is equivalent to the concentration of DHSm which will permit one-half the maximum growth rate, and $V_{max}$ is the growth rate at a non-limiting concentration of DHSm. For this experiment with glucose as the carbon source the values for $K_{DHSm}$ and $V_{max}$ were 5.3 μg./ml. and 0.645 respectively (growth rate is expressed as the reciprocal of the doubling time in hr.).

Experiments were carried out to investigate the effect of a change in both the nature and the initial concentration of the carbon source in the medium on the values of $K_{DHSm}$ and $V_{max}$. A 10-fold increase in initial glucose concentration had no significant effect on the value of $K_{DHSm}$ but did increase the maximum growth rate of the culture as indicated by the 40% increase in $V_{max}$ (Table XI). The effect of a change in the nature of the carbon source on the DHSm-requirement of this mutant was investigated in additional experiments similar to the one described in Figs. 17 and 18 but employing different carbon sources. The $K_{DHSm}$ and $V_{max}$ values obtained for each carbon source are listed in Table XII. Average $K_{DHSm}$ values ranged from a high of 12.6 when gluconate was the carbon source to a low of 1.5 when lactate was the carbon source. Thus, in order to attain one-half maximal growth rates, Sm-dependent cultures
Table XI. **Effect of glucose concentration on the dihydrostreptomycin requirement for the growth of streptomycin-dependent**

*Escherichia coli B*

<table>
<thead>
<tr>
<th>Initial glucose concn. (%)</th>
<th>$V_{\text{max}}^*$</th>
<th>$K_{\text{DHSm}}$ $^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.527</td>
<td>5.40</td>
</tr>
<tr>
<td>0.4</td>
<td>0.661 $^\dagger$</td>
<td>5.30</td>
</tr>
<tr>
<td>1.0</td>
<td>0.741</td>
<td>5.00</td>
</tr>
</tbody>
</table>

$V_{\text{max}}$ and $K_{\text{DHSm}}$ values at each glucose concentration were obtained from double reciprocal plots of growth rates versus dihydrostreptomycin concentration. Details of the experimental procedure are given in the legend of Fig. 17 and in the METHODS.

*$V_{\text{max}}$* is the growth rate at the optimal concentration of antibiotic. Growth rate is defined as the reciprocal of the doubling time in hr.

$^+$ $K_{\text{DHSm}}$ is the dihydrostreptomycin concentration required to permit one-half the maximal growth rate.

$^\dagger$ Average value of three separate experiments.
Table XII. Relationship between the carbon source and the dihydrostreptomycin requirement for the growth of streptomycin-dependent Escherichia coli B

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>$V_{\text{max}}^*$</th>
<th>$K_{\text{DHSm}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconate</td>
<td>0.914</td>
<td>12.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.661</td>
<td>5.3</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.558</td>
<td>2.0</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.485</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Growth experiments were performed as described in the legend of Fig. 17. The carbon source was added to give an initial concentration of 0.4%. $V_{\text{max}}$ and $K_{\text{DHSm}}$ values are averages of at least two separate experiments.

$V_{\text{max}}^*$ is the growth rate at the optimal concentration of dihydrostreptomycin.

$K_{\text{DHSm}}$ is the dihydrostreptomycin concentration required to permit one-half the maximal growth rate.
of strain DB required 8.4 times the concentration of DHSm when gluconate was the carbon source as when lactate was used.

The order of increasing growth rate was found to be lactate < glycerol < glucose < gluconate. $K_{\text{DHSm}}$ values on these carbon sources followed the same order (Table XII). The values obtained for $K_{\text{DHSm}}$ and $V_{\text{max}}$ were found to be highly reproducible. Cultures grown on the energy-rich, rapidly metabolizable carbon sources, gluconate and glucose (see DISCUSSION) exhibited a relatively high DHSm-requirement while cultures grown on a poor energy source such as glycerol or lactate required less antibiotic.

II. Effect of Growth with Limiting-Dihydrostreptomycin on the Formation of Enzymes of Carbohydrate Metabolism

Previous studies with Sm-dependent mutants of *E. coli* have shown that certain enzymes are specifically repressed by antibiotic-deprivation. For example, constitutive $\beta$-galactosidase (Spotts, 1962), isocitrate dehydrogenase (Bragg and Polglase, 1964b) and AHA synthetase (Polglase, 1966a; 1966b) were repressed at least 80% in antibiotic-starved cells compared to the specific activities observed in the corresponding antibiotic-supplemented, Sm-dependent organisms. These enzymes have also been shown to be subject to catabolite repression (Table VII) (for constitutive $\beta$-galactosidase see Mandelstam, 1962). Therefore, it was anticipated that an investigation of catabolite repression in antibiotic-limited
cultures of strain DB might help to explain the diverse DHSm-requirements of this mutant when grown on different carbon sources. Catabolite repression as a function of antibiotic concentration was studied by determining the specific activities of several catabolite repression-sensitive enzymes of carbohydrate metabolism (Table VII) during growth on various carbon sources in the presence of growth-rate-limiting or non-limiting concentrations of DHSm. As a control, the specific activities of two glucose-insensitive enzymes, glucokinase and glucose 6-phosphate dehydrogenase (Table VII) were determined in the same extracts. The results of these experiments are summarized in Table XIII. In general, the specific activities of the catabolite repression-sensitive enzymes (AHA synthetase, isocitrate dehydrogenase, aconitase, fumarase and citrate synthase) from cells grown in the presence of 100 μg./ml. of DHSm, were lowest when the $K_{\text{DHSm}}$ values for the various carbon sources were highest. Moreover, when the antibiotic concentration of the medium was severely limiting (3 μg./ml.) this inverse relationship between enzyme activity and $K_{\text{DHSm}}$ was dramatically expanded for all glucose-sensitive enzymes except isocitrate dehydrogenase (see DISCUSSION). For any particular carbon source except glucose, the percentage repression of the glucose-sensitive enzymes (isocitrate dehydrogenase excluded) from cells grown on 3 μg./ml. of DHSm compared with cells grown on 100 μg./ml. of antibiotic was greater the higher the value of $K_{\text{DHSm}}$. Gluconate exhibited a
Table XIII. **Specific activities of enzymes from streptomycin-dependent Escherichia coli B grown on various carbon sources and either a growth-rate-limiting or non-limiting concentration of dihydrostreptomycin**

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Gluconate</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K&lt;sub&gt;DHSm&lt;/sub&gt; (µg./ml.)</strong></td>
<td>12.6</td>
<td>5.3</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>AHA synthetase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity</td>
<td>100</td>
<td>6.0</td>
<td>11.4</td>
<td>11.0</td>
</tr>
<tr>
<td>% Repression</td>
<td>75</td>
<td>78</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td><strong>Isocitric dehydrogenase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity</td>
<td>100</td>
<td>564</td>
<td>547</td>
<td>800</td>
</tr>
<tr>
<td>% Repression</td>
<td>-8</td>
<td>16</td>
<td>-11</td>
<td>0</td>
</tr>
<tr>
<td><strong>Aconitase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity</td>
<td>100</td>
<td>270</td>
<td>216</td>
<td>478</td>
</tr>
<tr>
<td>% Repression</td>
<td>77</td>
<td>41</td>
<td>-11</td>
<td>-47</td>
</tr>
<tr>
<td><strong>Fumarase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity</td>
<td>100</td>
<td>552</td>
<td>1942</td>
<td>2260</td>
</tr>
<tr>
<td>% Repression</td>
<td>76</td>
<td>65</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td><strong>Citrate synthase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity</td>
<td>100</td>
<td>197</td>
<td>320</td>
<td>523</td>
</tr>
<tr>
<td>% Repression</td>
<td>57</td>
<td>72</td>
<td>19</td>
<td>21</td>
</tr>
</tbody>
</table>

....continued on following page
TABLE XIII. (Continued from preceding page)

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Gluconate</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K&lt;sub&gt;DHSm&lt;/sub&gt; (µg./ml.)</strong></td>
<td>12.6</td>
<td>5.3</td>
<td>2.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Glucose 6-phosphate dehydrogenase

| Specific activity | 100 | 125 | 109 | 120 | 79 |
| % Repression | 3 | 118 | 109 | 122 | 70 |

Glucokinase

| Specific activity | 100 | 79 | 115 | 81 | 75 |
| % Repression | 3 | 77 | 92 | 79 | 76 |

Growth experiments were performed as described in the legend of Fig. 17. The carbon source was added to give an initial concentration of 0.4%. Harvesting of cultures, preparation of sonic extracts and enzyme assays were carried out as described in the METHODS. K<sub>DHSm</sub> is the concentration of dihydrostreptomycin required for the half maximal growth rate (see Table XII). Specific activities are expressed as units of enzyme/mg. bacterial protein.
relatively high level of repression even when the DHSm concentration was non-limiting (100 μg./ml.) (Table XIII).

Glucokinase and glucose 6-phosphate dehydrogenase formation were relatively unaffected by DHSm-limitation in strain DB. Although the specific activities of both enzymes varied slightly on the different carbon sources, these differences showed no correlation with either the antibiotic requirements or the growth rates of the Sm-dependent cultures (Table XIII).

Under certain conditions, growth of strain DB on a rate-limiting concentration of antibiotic led to an increase in the formation of a number of the glucose-sensitive enzymes. Aconitase was repressed when Sm-dependent cells were grown on glucose or gluconate at a concentration of 3 μg./ml. of DHSm but was derepressed when cells were grown on glycerol or lactate at the same antibiotic concentration (Table XIII). When the DHSm concentration of Sm-dependent cultures growing on lactate or glycerol was decreased from 100 μg./ml. to 15-25 μg./ml. the formation of AHA synthetase was also significantly derepressed. Further reduction in the antibiotic concentration, however, resulted in a sharp repression of AHA synthetase (Fig. 19). When the same organism was grown on glucose or on gluconate, derepression of AHA synthetase was not observed at any rate-limiting concentration of antibiotic (Fig. 19).

The strong repression of glucose-sensitive enzymes in extracts of antibiotic-supplemented cells grown on gluconate was unexpected since previous studies (Table VII) established that,
Fig. 19. Effect of antibiotic-limitation on the formation of AHA synthetase in cultures of streptomycin-dependent Escherichia coli B (DB) grown on various carbon sources. Cells were grown as described in the legend of Fig. 17 on minimal medium supplemented with the following carbon sources at an initial concentration of 0.4%: gluconate, (●); glucose (○); glycerol, (■) or lactate, (▲). Preparation of sonic extracts and determination of AHA-synthetase activities were performed as described in the METHODS. Specific activity is expressed as μmoles of acetoin formed/mg. of protein/hr. at 37°.
in this mutant, these enzymes were relatively resistant to repression when grown on glucose (a very good source of catabolite co-repressor). To determine whether the enhanced catabolite repression resulting from growth on gluconate was a general characteristic of this strain (B), or a specific property of the Sm-dependent mutant (DB), the specific activities of AHA synthetase were determined in extracts of Sm-sensitive (SB) and Sm-dependent cultures of *E. coli* B grown on glucose and on gluconate (Table XIV). As shown previously (Fig. 7 and Table VII), the AHA synthetase of this Sm-dependent mutant was derepressed in extracts of glucose-grown cells, when compared with the wild-type organism. In extracts of the Sm-dependent mutant grown on gluconate, however, AHA synthetase was repressed almost as strongly as it was in the wild-type organism (SB) grown on either glucose or gluconate. Furthermore, the doubling time of the Sm-dependent culture was considerably less on gluconate than on glucose, while the Sm-sensitive culture grew at the same rate on either carbon source (Table IX).

It was shown previously (Figs. 13 and 14), that aerobic metabolism is 35-40% less efficient in the Sm-dependent mutant of *E. coli* B than in the parent strain. Since the results of Table IX suggested that gluconate metabolism by this mutant may be more efficient than glucose metabolism, a cell yield experiment was carried out to estimate the efficiency of utilization of each energy source. Table XV, gives the dry wt. of
Table XIV. Repression of AHA synthetase by glucose and gluconate in cultures of streptomycin-sensitive and streptomycin-dependent Escherichia coli B

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon Source</th>
<th>Doubling time (min.)</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB (Sm-sensitive)</td>
<td>Glucose</td>
<td>54</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>Gluconate</td>
<td>54</td>
<td>5.90</td>
</tr>
<tr>
<td>DB (Sm-dependent)</td>
<td>Glucose</td>
<td>91</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>Gluconate</td>
<td>74</td>
<td>7.85</td>
</tr>
</tbody>
</table>

Inocula were grown 18 hr. on minimal medium containing glucose or gluconate. The cultures were grown aerobically from an A420 of 0.1 to 0.8, harvested, disrupted by sonication and assayed for AHA-synthetase activity as described in the METHODS. Specific activity is expressed as μmoles of acetoin formed/mg. of protein/hr. at 37°.
Table XV. Efficiency of glucose and gluconate utilization by cultures of streptomycin-sensitive and streptomycin-dependent Escherichia coli B

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon Source</th>
<th>μg. dry cells formed/μmole of carbon source consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB (Sm-sensitive)</td>
<td>Glucose</td>
<td>94.2</td>
</tr>
<tr>
<td></td>
<td>Gluconate</td>
<td>93.7</td>
</tr>
<tr>
<td>DB (Sm-dependent)</td>
<td>Glucose</td>
<td>59.7</td>
</tr>
<tr>
<td></td>
<td>Gluconate</td>
<td>59.3</td>
</tr>
</tbody>
</table>

Cultures of strains SB and DB were grown 18 hr. on minimal salts medium supplemented with either glucose or gluconate. The cells were washed once by centrifugation and the yield of cells from each carbon source was determined as described in the METHODS. Cell mass was obtained from a standard curve relating $A_{420}$ to μg. dry cells/ml.
Sm-sensitive and Sm-dependent cells formed from equal molar quantities of glucose and gluconate. The results indicate that gluconate metabolism like glucose metabolism, is less efficient (by 38%) in strain DB than in the wild-type organism. Therefore, the enhanced growth rate and the increased catabolite repression observed in the Sm-dependent mutant growing on gluconate does not result from an increased yield of energy from this carbon source.

III. Response of Catabolite Repression-Sensitive Enzymes of Antibiotic-Depleted Cells to Dihydrostreptomycin Supplementation

Following a lengthy period (10-12 hr.) of growth in medium containing glucose but no antibiotic, the Sm-dependent cells exhibited the filamentous growth which is characteristic of antibiotic-depletion. The addition of DHSm (1 mg./ml.) to antibiotic-starved cultures resulted in a progressive breakdown of the elongated cells which was usually complete within 3 hr. The effect of DHSm-supplementation of antibiotic-depleted cells on the formation of certain glucose-sensitive enzymes in strain DB is shown in Table XVI. The difference in specific activities of these enzymes in extracts of antibiotic-depleted and antibiotic-supplemented cells after 3 hr. indicates that the synthesis of these enzymes undergoes a rapid and dramatic derepression during this period. The glucose-insensitive enzymes, glucokinase and glucose 6-phosphate dehydrogenase were
Table XVI. Specific activities of various enzymes in dihydrostreptomycin-depleted and supplemented extracts of streptomycin-dependent Escherichia coli B

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (Units/mg. of protein)</th>
<th>Supplemented/Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Depleted</td>
<td>Supplemented</td>
</tr>
<tr>
<td>AHA synthetase</td>
<td>1.68</td>
<td>11.7</td>
</tr>
<tr>
<td>Fumarase</td>
<td>200</td>
<td>1622</td>
</tr>
<tr>
<td>Aconitase</td>
<td>34.4</td>
<td>182</td>
</tr>
<tr>
<td>Isocitric dehydrogenase</td>
<td>183</td>
<td>490</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>118</td>
<td>426</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>75.2</td>
<td>81.4</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>64.3</td>
<td>91.6</td>
</tr>
</tbody>
</table>

Preparation of antibiotic-depleted cells and enzyme assays are described in the METHODS. Supplemented cells received 1 mg./ml. of dihydrostreptomycin 3 hr. prior to harvesting.
relatively unaffected by DHSm-depletion or by the subsequent antibiotic supplementation (Table XVI).

IV. Relationship between Growth Rate and Enzyme Synthesis in Streptomycin-Dependent Cells Grown on Limiting-Antibiotic

Examination of the growth rates and the specific enzyme activities in a number of growth experiments in which DHSm was limiting indicated that a direct relationship existed between the diminished growth rate in the presence of limiting-antibiotic and the degree of repression of certain glucose-sensitive enzymes. This relationship is illustrated in Fig. 20 for AHA synthetase and fumarase activities in extracts of glucose and gluconate-grown cells. In general, both growth rate and enzyme formation were lower on gluconate than on glucose at the same antibiotic concentration. Not all glucose-sensitive enzymes exhibited this relationship, however. While the degree of repression of citrate synthase under these conditions often correlated with the growth rate of the culture, aconitase and isocitric dehydrogenase rarely displayed this parallelism. Except for fumarase, this relationship was seldom observed in cultures grown on poor carbon sources (glycerol and lactate).

V. Effect of Antibiotic Concentration on the Yield of Streptomycin-Dependent Cells from Glucose

Previous results showed that relaxation of catabolite
Fig. 20. Relationship between the decrease in growth rate and the repression of AHA synthetase and fumarase in dihydrostreptomycin-limited cultures of streptomycin-dependent Escherichia coli B. Growth experiments were carried out as described in the legend of Fig. 17. Glucose (o) or gluconate (O) were added to give an initial concentration of 0.4% (w/v). Growth rates and specific activities are expressed as percentages of the values obtained with cultures grown on 100 µg./ml. of dihydrostreptomycin. The antibiotic concentrations employed were 25, 15, 10, 5 and 3 µg./ml., respectively. A, AHA synthetase; B, fumarase.
repression in the Sm-dependent mutant growing on glucose with non-limiting antibiotic correlated with a lowered efficiency of carbohydrate metabolism (Fig. 13). Cell yield studies with gluconate as the carbon source gave similar results to those obtained with glucose, i.e. approximately one-third less than the wild-type organism (SB) (Table XV). Therefore, if the impairment of carbohydrate metabolism in the DHSm-supplemented, Sm-dependent cells growing on a non-limiting concentration of antibiotic were due to a direct interaction of DHSm with catabolic processes of these cells, then decreasing the antibiotic concentration would be expected to result in the restoration of efficient carbohydrate metabolism and enhanced catabolite repression. To determine whether the efficiency of carbohydrate metabolism of strain DB is dependent on antibiotic concentration, the yield of Sm-dependent cells from glucose was estimated in cultures growing exponentially on 10, 100 and 1000 µg./ml. of antibiotic. The results are presented in Table XVII. Although the growth rate of strain DB was significantly lower on 10 µg./ml., the efficiency of glucose utilization at this antibiotic concentration was identical to the efficiencies calculated for cultures growing on supra-critical concentrations of antibiotic (100 or 1000 µg./ml.). Therefore, the inefficiency of carbohydrate metabolism of the Sm-dependent mutant appears to be independent of DHSm-concentration.
Table XVII. *Effect of antibiotic concentration on the cell yield from glucose of streptomycin-dependent Escherichia coli B*

<table>
<thead>
<tr>
<th>Antibiotic concn. (µg./ml.)</th>
<th>Doubling time</th>
<th>Y*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>93</td>
<td>0.341</td>
</tr>
<tr>
<td>100</td>
<td>87</td>
<td>0.344</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>0.344</td>
</tr>
</tbody>
</table>

The inoculum was grown aerobically for 16 hr. on glucose-salts medium supplemented with 10 µg./ml. of dihydrostreptomycin. The cells were washed once by centrifugation and the cell yield from glucose was determined as described in the METHODS in media containing 10, 100 or 1000 µg./ml. of antibiotic. Cell mass was obtained from a standard curve relating A_{420} to dry wt. of cells/ml. of culture. *Y is the cell yield constant defined as the number of µg. of dry cells formed/µg. of glucose consumed.*
VI. Effect of Antibiotic-Limitation on the ATP Pool of Streptomycin-Dependent Cells

Results of the experiment shown in Fig. 15 suggested that the growth rate of DHSm-supplemented (1 mg./ml.) cultures of strain DB' was limited by the rate of ATP synthesis in this organism. Since growth of this mutant on low concentration of antibiotic (<100 µg./ml.) resulted in a further reduction of growth rate (Fig. 17), DHSm-starvation could conceivably be interfering with ATP synthesis by removing DHSm from some antibiotic-dependent energetic process. To examine this possibility, the ATP pool of Sm-dependent cells was determined following the addition of 1 mg./ml. of DHSm to a culture growing exponentially on a glucose-salts medium containing only 10 µg./ml. of DHSm (Fig. 21). Although the experimental conditions (see METHODS) caused the ATP pools of both the antibiotic-supplemented and the antibiotic-limited cells to oscillate during growth, the results indicated that the addition of a high concentration of DHSm to antibiotic-limited cells did not enhance the concentration of the ATP pool of the Sm-dependent mutant. In fact, the ATP pool of the antibiotic-limited cells (10 µg./ml.) was always greater than the ATP pool of the antibiotic-supplemented cells (1 mg./ml.) during a 4-fold increase in cell mass. The doubling times of antibiotic-limited and antibiotic-supplemented cultures, however, were 100 and 78 min., respectively.
Fig. 21. Effect of dihydrostreptomycin concentration on the ATP pool of streptomycin-dependent *Escherichia coli* B. The inoculum was grown aerobically for 16 hr. on glucose-salts medium supplemented with 10 μg./ml. of dihydrostreptomycin. The cultures to be assayed for ATP were prepared by method I (see METHODS) and contained either 10 (o) or 1000 (©) μg./ml. of antibiotic. The doubling times of the cultures were 100 and 78 min., respectively.
**DISCUSSION**

The Sm-dependent mutant of *E. coli* B (strain DB) can grow on glucose-salts medium in the presence of DHSm concentrations as low as 3 µg./ml. Cultures of this mutant grow exponentially on rate-limiting concentrations of antibiotic (<100 µg./ml.) and the growth rates of these cultures are directly related to the concentration of antibiotic in the medium (Figs. 17 and 18). The growth rate as a function of DHSm concentration can be evaluated reproducibly by determining a constant ($K_{DHSm}$) which is equivalent to the concentration of DHSm permitting one-half the maximal growth rate (Fig. 18). The value obtained for $K_{DHSm}$ depends on the nature of the carbon source, being highest for energy-rich, rapidly metabolizable compounds such as gluconate and glucose (Table XII). The value of $K_{DHSm}$ is independent of variations in the initial concentration of the carbon source in the medium (Table XI). In a related study by Spotts (1962) on Sm-dependent mutants of *E. coli* K-12, the growth rates of these mutants on glycerol were a direct function of the Sm concentration up to approximately 100 µg./ml. at which value ("critical concentration") Sm was no longer the rate-limiting factor. When glucose was the carbon source, Sm remained rate-limiting up to about 250 µg./ml. At rate-limiting concentrations of antibiotic, however, growth rates were independent of the nature of the carbon source (Spotts, 1962). Thus, while the critical
antibiotic concentration of the medium was dependent on the nature of the carbon source, the growth rates of the cultures on sub-critical concentrations of antibiotic were independent of carbon source. These results obtained by Spotts (1962) were not confirmed in the present work where it was found that the nature of the carbon source had a marked effect on growth rates when the concentration of DHSm was limiting.

Growth of the Sm-dependent mutant (DB) in the presence of limiting concentrations of antibiotic resulted in an enzyme imbalance in these cells (Tables XIII and XVI). Glucose-sensitive enzymes were specifically repressed indicating a substantial increase in catabolite repression. Furthermore, DHSm supplementation of antibiotic-starved cells resulted in a rapid and specific derepression of the catabolite repression-sensitive enzymes (Table XVI). Recently, Goodman and Spotts (1967) reported that the synthesis of both inducible and constitutive β-galactosidase and of constitutive alkaline phosphatase were inhibited during the growth of Sm-dependent strains of *E. coli* transferred to medium containing no added antibiotic. They attributed some, but not all, of this inhibition to an increase in catabolite repression in the antibiotic-deprived cells. They suggested that in addition to inhibition of enzyme synthesis by catabolite repression, inhibition of enzyme synthesis could occur randomly due to the misreading of certain codons by the abnormal Sm-deficient, dependent ribosomes. However, they were unable to detect the presence of
cross-reacting material by serological methods or to find any other evidence for infidelity in translation \textit{in vivo}, either in the presence or absence of Sm. Thus, whereas these authors attributed the enzyme imbalance in antibiotic-deprived Sm-dependent cells to a random inhibition of enzyme synthesis affecting all classes of enzymes, the results of the present investigation suggest that the inhibition is not random but, in fact, specifically affects enzymes which are subject to catabolite repression. The susceptibility of many of these enzymes to catabolite repression may not be readily apparent in the wild-type organism or in the antibiotic-supplemented, Sm-dependent mutant because of the moderate variation in concentration of catabolite co-repressor normally occurring in these cells and the relative insensitivity of these enzymes to catabolite repression. For example, the synthesis of isocitric dehydrogenase by wild-type \textit{E. coli} B was only slightly sensitive to catabolite repression (Tables VII and XIII) but in antibiotic-depleted cells of strain DB this enzyme was significantly repressed (63\%) (Table XVI).

The formation of several of the glucose-sensitive enzymes of strain DB (AHA synthetase, aconitase and isocitrate dehydrogenase) was derepressed when this mutant was grown on a poor carbon source (e.g. glycerol or lactate) in the presence of certain rate-limiting concentrations of antibiotic (Table XIII and Fig. 19). However, when catabolite repression was further increased in the cell, (by growth on a
better carbon source or by a reduction in the intracellular concentration of antibiotic), these enzymes were strongly repressed (Table XVI and Fig. 19). These results suggest that the synthesis of these glucose-sensitive enzymes in the Sm-dependent mutant growing on rate-limiting concentrations of DHSm is regulated by more than one factor. Gray et al. (1966) studied the effects of aerobiosis, anaerobiosis and nutrition on the formation of the Krebs-cycle enzymes in *E. coli*. As a result of this investigation, they concluded that the Krebs-cycle can be divided into at least three sectors each of which is under independent control. These are enzymes metabolizing (a), the tricarboxylic acids; (b), the 5-carbon dicarboxylic acids and (c), the 4-carbon dicarboxylic acids. During aerobic growth on a minimal salts medium, the Group (a) enzymes (which include aconitase and isocitric dehydrogenase) constitute an amphibolic pathway which is regulated by both catabolite repression and end-product repression. Under the same growth conditions, the Group (c) enzymes (which include fumarase) are principally catabolic in function. These enzymes are sensitive to catabolite repression but are relatively resistant to end-product repression (Gray *et al.*, 1966). Results of the present study with the Sm-dependent mutant of *E. coli* B indicate that fumarase formation is not derepressed under growth conditions which derepress aconitase and isocitrate dehydrogenase (see Table XIII). Similar results have been reported for the regulation of the Krebs-cycle enzymes in
B. subtilis and in B. lichenformis (Hanson and Cox, 1967). AHA-synthetase formation in E. coli also appears to be regulated by more than a single mechanism. In addition to control by catabolite repression which is described in PART A of this thesis for E. coli B, multivalent end-product repression has been repotted for this enzyme (Freundlich et al., 1962).

Thus, the derepression of certain glucose-sensitive enzymes during growth on limiting-antibiotic appears to be the result of interference by increasing catabolite repression with end-product control (or some other mechanism) of enzyme synthesis. Strong catabolite repression which occurs when this mutant is severely starved of antibiotic completely abolishes derepression (Table XVI).

The enhanced production of extracellular valine by antibiotic-supplemented, Sm-dependent mutants when compared to the corresponding wild-type parent strains was attributed to the derepressed level of AHA synthetase in these organisms (see PART B, DISCUSSION). Previous studies by Bragg and Polglase (1964a) indicated that valine was not a major excretion product of Sm-dependent mutants when they were grown in antibiotic-free medium. An explanation of this observation is suggested by examination of the AHA-synthetase activities of antibiotic-depleted and supplemented cultures of strain DB (Table XVI). The AHA-synthetase activity of the DHSm-starved cells is 86% lower than in the cells supplemented with antibiotic. Furthermore, the specific AHA-synthetase activity of the depleted,
Sm-dependent cells (1.67) is 63% lower than the lowest activity observed for the corresponding Sm-sensitive organism (4.5) grown on glucose. Sm-dependent cells with AHA synthetase repressed this strongly would not be expected to excrete significant quantities of valine, even if the pyruvate concentration in the Sm-dependent cells was elevated during growth in antibiotic-free medium.

Glucose generally is considered to be the most effective source of catabolite co-repressor (Magasanik, 1961). Therefore, it was surprising to find that growth of the Sm-dependent mutant (but not of the wild-type parent strain) on gluconate resulted in a higher rate of growth and stronger catabolite repression than observed when the same organism was grown on glucose (Table XIV). This response was not due to a higher yield of energy from gluconate since the yield of Sm-dependent cells was the same from both carbon sources (Table XV). Gluconate, therefore, must be metabolized more rapidly than glucose by this mutant. Eisenberg and Dobrogosz (1967) have recently reported that in *E. coli*, gluconate is metabolized via the inducible Entner-Doudoroff pathway rather than by the hexose monophosphate pathway, the major aerobic route of glucose dissimilation. Since pyruvate is an early product of the Entner-Doudoroff pathway, the rate of aerobic pyruvate-coupled energy formation may be higher in the antibiotic-dependent mutant growing on gluconate than in the same mutant growing on glucose. An increase such as this in the
rate of energy formation could explain both the higher rate of growth and the stronger catabolite repression observed in gluconate-grown cultures of the Sm-dependent mutant.

Neidhardt (1960) has studied a mutant of *A. aerogenes* which lacked glucose repression but not gluconate repression. This mutant (JF-4) grew as well as the wild-type parent strain on all carbon sources except glucose. An investigation of glucose metabolism by strain JF-4 revealed that this mutant was unable to metabolize glucose by a direct oxidation. This loss in glucose oxidase activity diminished the overall rate of glucose dissimilation by the cell (but not the yield of energy from glucose) thereby virtually eliminating glucose repression. In contrast, the Sm-dependent mutant of *E. coli* B (DB), which also lacks glucose repression for a number of catabolite repression-sensitive enzymes, grew more slowly on all carbon sources investigated than did the Sm-sensitive parent strain (Tables IX and XIV). Furthermore, this mutant was approximately one-third less efficient than the wild-type organism in utilizing glucose or gluconate aerobically for cell synthesis (Table XV). These data suggest that loss of glucose repression by strain DB, unlike strain JF-4, results from a general impairment of energy metabolism probably at the level of terminal oxidation.

While energy formation is impaired in the Sm-dependent mutant, this catabolic deficiency appears to be independent of antibiotic concentration (Table XVII). Catabolite repres-
sion, however, was enhanced in cultures of the same Sm-dependent mutant growing on limiting-DHSm and this effect was dependent on the concentration of antibiotic in the medium (Fig. 19). Moreover, the increase in catabolite repression correlated with both an increase in the concentration of the ATP pool (Fig. 21) and a decrease in the growth rate of the mutant. Thus, the physiological changes observed in strain DB during growth on limiting DHSm are consistent with the hypothesis that the ribosome is the antibiotic-dependent site in Sm-dependent mutants (Spotts and Stanier, 1961; Likover and Kurland, 1967a).

The experimental results presented in this section suggest that a relationship exists between the growth rate of the Sm-dependent mutant on limiting-concentrations of antibiotic and the degree of catabolite repression in the antibiotic-deprived cell. For example, the nature of the carbon source determines the concentration of DHSm required for the dependent culture to attain the half-maximal growth rate (Table XII), and there is a direct relationship between the growth rate of the mutant on limiting-antibiotic and the degree of repression of certain glucose-sensitive enzymes (Fig. 20). These results indicate that catabolite repression may play an important role in establishing the quantitative requirement for antibiotic for growth of Sm-dependent E. coli. In order to incorporate these results into the hypothesis which indicates protein synthesis to be the primary process affected by Sm (or DHSm) one may
postulate that, in vivo, the effect of the addition of the antibiotic on protein synthesis is augmented by the secondary effect of catabolite repression. In the Sm-dependent cell, the limitation of antibiotic initially may impair ribosomal function without immediately affecting catabolic processes. The result of limitation of anabolism without a corresponding decrease in the rate of catabolism would be severe repression of the glucose-sensitive enzymes, ultimately resulting in a decreased rate of both catabolic and anabolic reactions. In Sm-dependent cells it would appear that catabolite repression is, quantitatively, an important factor in limiting growth at low concentrations of antibiotic since the nature of the carbon source plays a major role in determining the concentration of DHSm required for growth.
GENERAL DISCUSSION

Metabolic control by catabolite repression was relaxed in a Sm-dependent mutant of *E. coli* B (strain DB) grown on a non-limiting concentration of DHSm (>100 μg./ml.). The observations which led this conclusion were that a number of glucose-sensitive enzymes were derepressed in the Sm-dependent mutant compared with wild-type *E. coli* B (Table VII). The relaxation of catabolite repression in this mutant was attributed to a reduced efficiency in aerobic carbohydrate metabolism since it was found (Tables VIII and XV) that the yield of Sm-dependent cells from glucose or gluconate was about one-third less than the yield of wild-type *E. coli* B from the same carbon sources. A direct measurement of ATP synthesis in exponential cultures of strains SB and DB (Fig. 15) confirmed the supposition that energy metabolism is impaired in the Sm-dependent mutant. The decreased rate of growth of the Sm-dependent mutant of *E. coli* E (strain DE) on glucose and on glycerol compared to the wild-type parent (Table IX) as well as the decreased catabolite repression in this mutant (Figs. 9 and 11) suggest that a similar defect in energy metabolism may exist in this additional strain of *E. coli*. When strain DB was grown on a rate-limiting concentration of antibiotic (<100 μg./ml.), however, catabolite repression (as determined by the specific activities of a number of glucose-sensitive enzymes) was enhanced (Tables XIII and XVI). The
degree of increase in catabolite repression was inversely related to the limiting concentration of antibiotic on which the Sm-dependent mutant was grown (Fig. 19). This increase in catabolite repression correlated with the slight increase in the concentration of the ATP pool observed in antibiotic-limited cultures (Fig. 21). Thus, there are two distinct and contrasting observations on Sm-dependent *E. coli* to be understood. First, the impaired carbohydrate metabolism which *decreases* catabolite repression in cells grown on non-limiting concentrations of antibiotic and second, the effect of antibiotic-limitation which *increases* catabolite repression.

While the latter observation can be understood in terms of a decreased rate of protein synthesis resulting from impaired ribosomal function (Goodman and Spotts, 1967; Likover and Kurland, 1967b), the former observation can be understood only if one postulates a second mutation resulting in a decreased efficiency in energy metabolism. Alternatively, the presence of DHSm (or Sm), while permitting maximal anabolic activity in the Sm-dependent cells may simultaneously interfere with carbohydrate metabolism. Cell yield studies in the presence of limiting and non-limiting concentrations of DHSm indicated that the decreased efficiency of carbohydrate metabolism in strain DB was independent of antibiotic concentration (Table XVII). Therefore, while the impaired energy metabolism of strain DB appears to be the result of a second mutation, further study will be necessary to establish the exact nature of this
catabolic defect. The fact that the formation of AHA synthetase was derepressed in Sm-dependent mutants obtained from 5 different strains of *E. coli* (Coukell and Polglase, 1965; Fig. 5) and that all Sm-dependent mutants examined excreted valine suggest that impaired carbohydrate metabolism may be a general property of Sm-dependent mutants (see later discussion).

Results of a number of experiments performed with strain DB growing on rate-limiting concentrations of DHSm indicated that catabolite repression was an important factor in limiting growth at low concentration of antibiotic (Table XII and Fig. 20). This finding suggests that catabolite repression may also play a role in the action of DHSm (or Sm) on antibiotic-sensitive cells. Experimental results which suggest that catabolite repression may increase significantly in Sm-sensitive cells treated with Sm, were provided by the report of Engelberg and Artman (1964) that when Sm was added to susceptible cultures of *E. coli*, the synthesis of the glucose-sensitive β-galactosidase was preferentially and completely (96%) inhibited by a concentration of antibiotic which only partially (66%) inhibited protein synthesis. Similar results with chloramphenicol (Palmer and Moses, 1967) and 5-fluorouracil (Horowitz and Kohlmeier, 1967) have been attributed to increased catabolite repression resulting from a reduction in the rate of protein synthesis. In support of the concept that catabolite repression may augment the direct action of Sm on the sensitive ribosome, one may cite the observations (Lightbown,
1957) that inhibition of growth by Sm is less effective under anaerobic conditions where catabolite repression also is diminished (Cohn and Horibata, 1959). Furthermore, the addition of Sm to growing sensitive cells does not result in an immediate decrease in viability (Engelberg and Artman, 1964) even though it has been established that the antibiotic is taken up by the cell extremely rapidly (Szybalski and Mashima, 1959; Engelberg and Artman, 1964). The time lag between the uptake of Sm by the culture and the decrease in viability could be the period during which the secondary effect of accumulation of catabolite co-repressor takes place. Rosenblum and Bryson (1953) studied the effect of the rate of bacterial metabolism on the action of Sm in wild-type *E. coli* B and concluded that metabolically active cells were more sensitive than non-active cells to the bactericidal action of Sm. Of particular significance to the present work was their observation that washed cells suspended in saline were relatively resistant to the bactericidal action of Sm but were sensitive when glucose was present in the suspension.

As a result of the extreme variability of catabolite repression in strain DB, the concentrations of a number of glucose-sensitive enzymes involved in general intermediary metabolism, changed dramatically during growth on different concentrations of antibiotic (Tables XIII and XVI). One of these enzymes, AHA synthetase, was of particular interest in the present study because of its proposed role in the phenomenon
of valine excretion by Sm-dependent mutants (Bragg and Polglase, 1965; Coukell and Polglase, 1965). An investigation of the regulation of AHA-synthetase formation and activity in E. coli B revealed that two unexpected properties of this enzyme were responsible for extracellular valine production by the antibiotic-dependent mutant. First, AHA synthetase was found to be sensitive to catabolite repression (Fig. 3) and second, there was incomplete feedback inhibition of this enzyme by valine (Fig. 5). Catabolite repression of AHA synthetase is relaxed in the Sm-dependent mutant of E. coli B (DB) growing exponentially on glucose and on a non-limiting concentration of DHSm (Fig. 7). A considerable part of the pyruvate produced from glucose metabolism will therefore be converted to valine and ultimately will lead to valine excretion (Table X). Starvation of the same mutant for antibiotic, however, severely represses AHA-synthetase formation (Table XVI) and in this way inhibits the production of extracellular valine (see Bragg and Polglase, 1964a).

While control of AHA-synthetase formation by catabolite repression has been studied only in E. coli B, indirect studies suggest that this property of AHA synthetase may not be restricted to this strain. For example, a survey of AHA-synthetase activity in Sm-sensitive, resistant and dependent organisms of 4 different strains of E. coli disclosed that the formation of this enzyme was derepressed in all Sm-dependent mutants when compared to the activities in the
antibiotic-sensitive and resistant cells (Coukell and Polglase, 1965). In the present work, catabolite repression has been examined in only one of these previously studied Sm-dependent mutants (strain DE) but the results indicate that catabolite repression in this strain is also "relaxed" as in *E. coli* strain DB (Figs. 9 and 11). Furthermore, since valine excretion by strain DB was attributed to both the derepression of AHA synthetase in this mutant and the inability of this enzyme to be completely inhibited by valine, then the production of extracellular valine by a Sm-dependent mutant of a *Mycobacterium* sp. (Tirunarayanan et al. 1962) suggests that the AHA synthetase of this organism may also possess the unusual properties described for the enzyme of *E. coli* B.

It may be concluded from the present study that many of the biochemical and physiological changes which are observed in Sm-dependent mutants of *E. coli* following antibiotic-starvation or supplementation are mediated through the action of the antibiotic on catabolite repression rather than through a direct effect of the antibiotic on protein synthesis.
SUGGESTIONS FOR FUTURE WORK

During the course of this study, a number of questions arose which were either not examined since they were off the main line of the original problem or were examined but were left unanswered at the conclusion of the present work. It is the author's belief that until these questions are answered, the significance of the findings described here cannot be evaluated fully.

1. What is the nature of the impairment in energy metabolism in strain DB? Is energy metabolism impaired in all Sm-dependent mutants and if so, what is the relationship between defective energy metabolism and Sm-dependence?

2. Can severe catabolite repression alone inhibit the growth of certain micro-organisms and if so, by what mechanism?

3. Do the AHA synthetases of micro-organisms other than *E. coli* exhibit sensitivity to catabolite repression and incomplete feedback inhibition and what is the survival value of these properties?

4. What is the mechanism by which end-product repression and catabolite repression function together to regulate the Krebs-cycle and the "ilva" pathway?
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