STUDIES ON THE ACTION OF STREPTOMYCIN

by

A. HUNTLEY BLAIR

B.A., University of British Columbia, 1956

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in the Department

of

BIOCHEMISTRY

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

SEPTEMBER, 1957
ABSTRACT

The mode of action of streptomycin has been investigated with specific emphasis on the effect of the antibiotic on adaptive enzyme formation by *Escherichia coli*. The first phase of this study was to investigate the possibility that streptomycin interferes with the action of a metabolite required by *E. coli* for enzyme synthesis and growth. A resistant variant of *E. coli*, which exhibited partial dependence on the drug for enzyme formation, was used to test for the presence of a metabolite in other variants which did not exhibit this dependence. Subcellular fractions derived from susceptible and resistant cells were tested for the presence of a substance which would satisfy the streptomycin requirement of partially dependent organisms for enzyme induction. All attempts to demonstrate such a metabolite were unsuccessful.

In the second phase of the study, the effect of streptomycin on the protein and nucleic acid content of partially dependent cells during enzyme induction was studied. By the standard techniques employed, it was shown that the antibiotic did not affect either the gross protein or nucleic acid content of the cells during the period in which the formation of a specific enzyme was demonstrated.
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representative. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of BIOCHEMISTRY

The University of British Columbia,
Vancouver 8, Canada.

Date Sept. 13, 1957
CONTENTS

Acknowledgements ........................................ iii
Introduction .................................................. 1
Materials and Methods ........................................ 10
Results and Discussion ........................................ 18

1. The Search for a Metabolite in E. coli ................. 18

The Preparation of Dihydrostreptomycin-treated
Susceptible E. coli ........................................ 18

Fig. I: The Effect of Dihydrostreptomycin
on the growth of streptomycin-
susceptible E. coli ... to follow page 21

The Examination of Dihydrostreptomycin-treated
E. coli for Evidence of a Metabolite ....................... 22

The Use of Dihydrostreptomycin-Resistant
Organisms to Detect a Metabolite ......................... 24

Fig. II: Partial Dependence ... to follow page 25

Fig. III: Partial Dependence ... to follow page 25

II. An Examination of the Effect of Dihydrostreptomycin on
the Protein and Nucleic Acid Content of Resistant
E. coli during Enzyme Induction .......................... 36

Conclusions .................................................. 41

Summary .................................................... 45

Bibliography ................................................ 46
ACKNOWLEDGEMENTS

The author gratefully acknowledges personal assistance from the National Research Council in the form of a Bursary and two Summer Supplement allowances.

Acknowledgement is made to Dr. W.J. Polglase for his interest and advice during the course of this study.
INTRODUCTION

Since the discovery of the antibiotic, streptomycin, and the elucidation of its molecular structure, much attention has been focused on the problem of determining its mode of action. Many diverse metabolic processes have been implicated as the site which is attacked by the drug. Many of these studies were hindered by two factors:

1. The criteria used to measure the effect of streptomycin (i.e., oxygen uptake) were indirect in nature and thus it was difficult to interpret the results in an unequivocal fashion.

2. There is difficulty in distinguishing the key interaction which is responsible for the cessation of bacterial multiplication from secondary interactions due to the chemical nature and size of the streptomycin molecule.

Respiration studies on several bacteria have yielded varied and contradictory results. In 1947 Benham found that streptomycin stimulated the endogenous metabolism of a susceptible strain of Eberthela typhosa and failed to inhibit the oxidation of carbohydrates or carbohydrate intermediates by this organism (1). In contrast to this observation, Henry and co-workers reported that streptomycin had no effect on the endogenous respiration of S. aureus and inhibited that of B. cereus (2). Upon testing various substrates, these workers found
that the oxidation of glycerol or lactate by *S. aureus* was inhibited by streptomycin, whereas the oxidation of either glucose, pyruvate, glutamate, or glycerate was unaffected. With *B. cereus*, however, the oxidation of all substrates tested was inhibited. All of these inhibitions exhibited a lag period and seldom reached 100% inhibition. Permeability factors did not seem to be involved since the antibiotic did not affect the permeability of susceptible cells to pyruvate. Under anaerobic conditions, it was shown by measuring carbon dioxide and acid production that with *S. aureus*, streptomycin did not inhibit the metabolism of any substrate. Investigations dealing with *E. coli* showed that anaerobic dissimilation of pyruvate was inhibited but not that of glucose, as determined by carbon dioxide production (3). Geiger noted that streptomycin had no effect on the oxidation of glucose, malate, fumarate, oxalacetate, or pyruvate (4). He found that the oxidation of amino acids by *E. coli* was stimulated by prior oxidation of fumarate, oxalacetate, or certain other compounds. Streptomycin prevented this stimulatory effect. Geiger postulated that the action of an intermediate, connected with amino acid metabolism, formed during the period of prior oxidation of fumarate, or oxalacetate, was interfered with by the antibiotic. This work done by Geiger led to the series of investigations carried out by Umbreit and his associates (5, 6, 7, 8, 9).

They attempted, through the interpretation of oxygen uptake
studies, to learn the nature of the effect of streptomycin on the interaction between fumarate and subsequent threonine oxidation. Evidence was obtained that the process inhibited was connected with terminal respiration (5). Umbreit and co-workers suggested that the intermediate formed from fumarate which, according to Geiger (4), was not formed in the presence of streptomycin, was actually oxalacetate (7). These workers (7) found that, in cells which had been aged to remove oxalacetic decarboxylase, pyruvate and oxalacetate were oxidized separately at a slow rate whereas a mixture of the two was rapidly oxidized. This rapid oxidation was inhibited by streptomycin. Thus, they concluded that the sensitive reaction was the condensation of oxalacetate and pyruvate (7). However, they were unable to isolate the enzyme responsible for the condensation. Since the formation of citrate was not inhibited by streptomycin in susceptible organisms, Umbreit postulated an alternate pathway by which pyruvate enters the terminal respiration system (8). He suggested that this alternate pathway involved the formation of a seven carbon phosphorylated compound (9) similar to one isolated from rat liver by Rapoport and Wagner (10). Umbreit and co-workers obtained some evidence for a similar compound in E. coli but were unable to isolate it or to relate it to any known metabolic pathway. Incorporation of radioactive phosphorus into the fraction of the trichloracetic acid extract supposed to contain this compound was reduced by streptomycin (9).
Paine and Clark found, using resting cell suspensions of susceptible \textit{E. coli}, that streptomycin had no effect on endogenous respiration or oxygen uptake in the presence of glucose as a substrate \cite{11}. However, using fumarate and glutamate as substrates, they observed marked inhibition of oxygen uptake in the presence of the antibiotic. To attempt to correlate these results with the "killing" ability of streptomycin, these investigators determined the number of viable cells remaining at intervals during the incubation. It turned out that bacteria were killed most rapidly in the presence of glucose even though oxygen uptake continued undiminished. The rate of killing was substantially less with the other substrates. They drew the significant conclusion from this and other similar data that the inhibition of oxygen uptake seemed to bear no particular relation to the killing effect of the antibiotic, but rather, was related to the substrate being metabolized \cite{12}. In other words, the killing action of streptomycin is a function of the rate of metabolism of the organisms, rather than being connected primarily with its varied effects on oxygen uptake. These results do not support Umbreit's concepts.

Paine and Clark made a further observation on \textit{S. aureus} which argues against the terminal respiration system of Umbreit as the site of action of this drug \cite{12}. This organism does not manifest a terminal respiration system under anaerobic conditions and acts on pyruvate by reducing it to lactate. Nevertheless streptomycin exerts a bactericidal effect on such cells either in the resting or the growing state. Henry made a similar observation in his earlier studies \cite{2}.
Thus, it can be seen that indirect studies of this type have not led to a systematic and consistent body of knowledge. Too many distantly related metabolic processes contribute to the overall respiration of the cell to permit unequivocal conclusions to be drawn as to the site of action of streptomycin.

Numerous other effects of streptomycin have been reported. Olitzki found that hydrogen sulfide production by several species of bacteria from cysteine, cystine, and methionine etc. was inhibited by the antibiotic (14). Non-multiplying organisms exposed to streptomycin for twenty-four hours at 37 ° remained viable but lost their ability to produce hydrogen sulfide. This ability was regained on subculture. The effect of streptomycin on cysteine desulfhydrase in cell-free extracts from E. coli was examined by Artman and co-workers (15). He showed that the inhibition was non-competitive and irreversible. The inhibition of succinic dehydrogenase from E. coli has also been reported (16). In this case the inhibition is reversed by biotin. Lichstein and Gilfillan discovered that Saccharomyces fragilis is sensitive to streptomycin when grown on a medium containing \( \beta \) - alanine as a precursor for coenzyme A (17). However, when pantothenate is supplied in the medium the organism is no longer susceptible. So far, this finding has not been extended to any other organisms. The diamine oxidase of several organisms has been shown by Owen and co-workers to be inhibited in susceptible variants but not in resistant variants (18). This enzyme is also inhibited by a variety of other substituted, synthetic
amines (10). With regard to the physical properties of bacteria, streptomycin has been found to combine with the surface groups of the rough form of E. coli, thereby decreasing the charge on the cells and resulting in agglutination (20). This combination and agglutination is readily reversed by washing with 0.1M sodium chloride or by resuspending the cells in fresh broth.

These various effects would appear to be secondary phenomena arising from the chemical properties of the streptomycin molecule rather than the result of the primary biochemical lesion. (This is not to say that the primary lesion is not a manifestation of the structural features of the molecule). In view of the properties of this molecule, it is only to be expected that such a structure would have various repercussions in a biological system apart from that directly responsible for killing the cell. For example, the two highly charged guanido groups may tend to disrupt the polar group interactions responsible for maintaining the configuration of macromolecules in general. Also, the molecule contains two moieties, hitherto unknown in nature—a branched chain sugar, and glucosamine in the L-configuration. The ultimate aim of the studies on streptomycin is to elucidate the mode of action in terms of these molecular features.

The effect of streptomycin on nucleic acids has received considerable attention due to the fundamental role played by nucleic acids in protein synthesis and cell multiplication. In this connexion, the first observation was made by Cohen in 1946 (21, 22). He found that desoxyribo-
nucleic acid from the T2 bacteriophage of E. coli was precipitated by streptomycin. These "streptomycin nucleates" and complexes of virus and antibiotic were dissociated by 1.0M sodium chloride as were thymus nucleohistones and nucleoprotamines which also contain many guanido groups. After treatment of the virus with desoxyribonuclease, streptomycin is no longer capable of causing the formation of a precipitate. On the basis of their work, Di Marco and Boretti suggested that the action of streptomycin in vivo might involve a competition between the antibiotic and protamine for nucleic acid (23).

The process of enzyme induction has also been implicated in the search for the site of action of streptomycin. Fitzgerald, Bernheim and Fitzgerald found that, under certain conditions, the antibiotic inhibited the oxidation of benzoic acid by a strain of Mycobacteria (24). In this organism, the enzyme responsible for benzoic acid oxidation is an adaptive enzyme. Streptomycin had no effect on oxidation of the acid by cells which had been grown on media containing benzoate as a nutrient. However, the drug inhibited oxidation by cells which had not been grown on benzoate and which showed the characteristic lag period due to adaptation when incubated with the substrate. They concluded that the adaptive process rather than the catalytic action of the enzyme itself had been affected. These workers extended their observations to include hydroxybenzoic acid which is also metabolized via an induced enzyme system. Streptomycin also prevented adaptation to this substrate.
Further support for the consideration of this interference with enzyme induction as an important aspect of the mode of action of streptomycin was supplied by the work of Polglase on adaptive enzyme formation in non-proliferating _E. coli_ (25, 26, 27, 28). It was found that dihydrostreptomycin (which has the same antibiotic activity as streptomycin) inhibited enzyme induction by arabinose, lactose, and glucuronic acid (25). These results, based on oxygen uptake studies, were confirmed by the investigation of the formation of _β_-galactosidase, an enzyme for which there is a specific method of assay (26). Dihydrostreptomycin (DHSM) was found to inhibit induction of _β_-galactosidase in susceptible cells grown on glucose, but it had no effect on the enzyme activity in such cells grown on lactose. In the resistant variant, the induction of enzyme was not inhibited by DHSM. The formation of _β_-galactosidase in dependent organisms was enhanced by prior incubation with DHSM (27). Thus, the adaptive behavior of susceptible, resistant, and dependent strains of non-proliferating _E. coli_ under the influence of the antibiotic is consistent with the effect of the antibiotic on growth. Also, the concentrations of DHSM employed in the studies of adaptive enzyme formation in non-proliferating cells were of the same order of magnitude as the concentrations affecting growth of the respective variants. Unlike the reports on streptomycin-inhibition of certain specific enzymes, the inhibition of adaptive enzyme formation is a general phenomenon, not restricted to a particular enzyme. These facts provide corroborative evidence that the inhibition of enzyme induction is fundamentally connected with the primary site of action of the
antibiotic.

The present investigation is a continuation of work in which the general objective is to delineate the site of antibiotic action of streptomycin with specific emphasis on the effect of the drug on enzyme formation. The first phase is devoted to a search for a possible metabolite capable of antagonizing the action of streptomycin. The second phase is concerned with the effect of streptomycin on the protein and nucleic acid content of an E. coli variant which showed antibiotic dependency for enzyme formation.
MATERIALS AND METHODS

Cultures

Two variants of *E. coli* were used in these experiments:

1. A dihydrostreptomycin (DHSM) - sensitive variant in which growth is prevented by approximately 50 units per ml of DHSM.

2. A DHSM-resistant variant which is capable of growth in medium containing 1000 units per ml of DHSM.

Media and Growth Conditions

The following basal medium was used for the growth of all cultures:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Weight (gms.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄·3H₂O</td>
<td>7.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.0</td>
</tr>
<tr>
<td>sodium citrate 3H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.2</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>H₂O</td>
<td>to make 1 liter</td>
</tr>
</tbody>
</table>

Glucose, from a 10% sterile solution, was added to this basal medium, after autoclaving, to give a final concentration of 0.2%. Yeast extract was added to the medium as a supplement (0.2%) except
where a chemically defined medium was desired, as for the growth experiments. Where necessary, DHSM was introduced, from a sterile aqueous stock solution, as the sulfate salt. The antibiotic activity of DHSM is equal to that of streptomycin, the parent compound. (1 unit of DHSM = $10^{-6}$ g of the free base). The cultures were grown for 20-22 hours in Roux flasks at 37°C, after which, harvesting was accomplished by centrifugation at 2500 rpm followed by one washing with 0.85% saline. Cells were stored under refrigeration for 24 hours before use. Such cells may be described as "semi-starved". Growth experiments, described under Part I of the results, were carried out either in Roux flasks or colorimeter tubes, using chemically defined medium. When Roux flasks were used, aliquots were taken for turbidity measurements using the Beckman Model B Spectrophotometer. In the case of experiments carried out in colorimeter tubes, the tubes were placed directly in the Klett-Summerson Colorimeter for turbidity determinations using the blue filter. All incubations were carried out at 37°C.

**Sonic-Disruption of Bacterial Cells**

For many purposes in these investigations, cells disrupted by sonic oscillations were required. This treatment was carried out by suspending the cells in 0.022 M potassium phosphate buffer (pH 7.5) and placing the suspension in a Raytheon 9 kc sonic oscillator. The disintegration of the cells was carried out for varying periods of time depending on
the particular experiment involved. Such sonic-disrupted cell preparations are, hereafter, called "sonicates".

Examination of Sonicates and Growth Supernatants by Chromatography

The growth supernatants were concentrated to a small volume under vacuum at 40°C so as to minimize decomposition of labile components. The concentrates were then spotted on sheets of Whatman No. 1 filter paper. The volume of solution used in making up each spot was such that all spots represented the same weight of bacteria (as determined by the optical density of the culture). In the case of the sonicates, equivalence was affected by dilution of the samples in accordance with the optical density of the original suspension of organisms. Thus, valid comparisons were possible between a given sample and its control. The solvent system employed was butanol:acetic acid:water; 250:60:250. The chromatograms were run overnight at room temperature during which time the solvent front advanced approximately 14 inches. The papers were dried at room temperature prior to development. The developing reagents employed were: acidic potassium permanganate (28); benzidine (29); p-anisidine (30); aniline-phthalate (31); ninhydrin (32). The chromatograms were also photographed under ultra-violet light.

Induction of β-Galactosidase

(A) Six Hour Induction:

When a complete rate curve showing β-galactosidase
formation was desired the following procedure was adopted. A suspension of organisms in phosphate buffer (pH 7.5) was adjusted in cell concentration to optical density 0.85 and then divided into two equal portions. After being brought to equilibrium in a water bath at 37°C, DHSM was added to one portion to bring the concentration to 100 units per ml. The volume of the other suspension was increased by a corresponding amount. Any other additions of test substances were made at this point. After a period of preincubation, usually one hour, lactose was added to both portions to bring the final concentration to 2 μmoles per ml. During the subsequent incubation samples were taken at regular intervals for analysis as described under "Enzyme Assay". For certain experiments where it was desired to obtain concentrated samples for other analyses during the enzyme induction, a heavier initial suspension was employed and the concentration of inducer was increased correspondingly.

(B) Three Hour Induction:

In experiments where one point on the enzyme induction curve was sufficient to show the effect of an experimental parameter, a less elaborate and time-consuming procedure was used. In these experiments, incubations were carried out in small flasks containing a buffered solution of the bacterial fraction under test. A uniform suspension of the organisms to be induced was made up in buffer and aliquots introduced into each flask so as to eliminate any variation in the results due
to uneven distribution of cells. Each flask contained the following:

- Cell suspension in phosphate buffer (pH 7.5) 0.5 mls
- Lactose (aqueous solution - 100 μ moles per ml) 0.5 mls
- Phosphate buffer + bacterial fraction, etc. 9. mls
- Phosphate buffer 10 mls

Preincubation with DHSM was carried out for 0.5 hours and the incubation itself was carried out for three hours at 37°. At the end of this period the contents of each flask were immediately treated as described under "Enzyme Assay". This procedure is tantamount to sampling at the three hour point of the six hour induction (see figure II).

**Enzyme Assay**

(A) Preparation of Sample:

The sample taken for analysis (generally 10 mls) was placed in the sonic oscillator immediately after removal from the incubation medium. Sonic oscillations were applied for a period of three minutes. The sonicates were maintained at 0° until assayed.

(B) Assay:

The method used to carry out the assay of β-galactosidase has been described by Polglase (26) and is based on that of Lederberg (33). An aliquot of the sonicate buffered with pH 7.5 phosphate buffer was placed in a cuvette (3 mls) and 0.2 mls of 0.005M ω-nitrophenyl-β-D-galactoside (ONPG) was added. The rate of hydrolysis at room temperature was measured with the spectrophotometer set at a wavelength of 420 mμ.
The slope of the first order rate curve was taken as a measure of the β-galactosidase concentration.

**Protein Determination**

Bacterial protein was determined by the procedure of Lowry et al. (34) which involves the use of the Folin phenol reagent. The precipitation of protein from the bacterial suspensions was carried out with trichloracetic acid, the final concentration of the acid in this case being 5%. After two washings with 5% trichloracetic acid, the precipitate was dissolved in 1.0N sodium hydroxide and made up to volume with 1.0N alkali. A 0.5 ml aliquot of this solution was taken for analysis and the reagent volumes modified accordingly. In adding the Folin reagent it was found necessary to standardize the addition procedure carefully in order to obtain reproducible values. This reagent decomposes rapidly in the basic solution. It was also found that the color of the final solution continued to increase slowly even after standing for thirty minutes. Thus, samples must be measured for color intensity after precise time intervals. Commercial egg albumin was used as the working standard in these determinations. The color intensity was measured at 750 μm with the Beckman Model B Spectrophotometer.
Isolation of Nucleic Acids

The procedure adopted for the isolation of nucleic acids was based on that of Schneider (35) which has been used by other investigators for bacterial tissue (36). A suitable aliquot of the bacterial suspension was treated at 0° with perchloric acid to precipitate the protein and nucleic acids. In each case the final concentration of perchloric acid was 6%. The precipitate was washed twice with 2 mls of 6% perchloric acid. This initial treatment served to remove the "acid-soluble fraction". The precipitate was then extracted with successive portions of 5 mls each of: (1) 80% ethanol (2) 100% ethanol and (3) ethanol:ether (1:1). The residue from the lipid extraction was then heated at 90° for one hour in the presence of: 20 volumes of 6% perchloric acid. Two washings with 6% perchloric acid were combined with the hot extract. After being made up to volume, the hot perchloric acid extract was used for analysis as described below.

Determination of Nucleic Acids

(A) Ribose Nucleic Acid (RNA):

RNA was determined on an aliquot of the hot perchloric acid extract by utilizing the color reaction of orcinol with pentoses as proposed by Bial (37). In this case, the modification developed by Mejbaum (38) was followed. The reaction mixture was heated for forty-five minutes instead of twenty minutes as originally specified (39).
Standard solutions of commercial RNA and DNA were also run. The slight color due to DNA was subtracted from the total green color measured at 670 m\(\mu\) with the Beckman Model B Spectrophotometer.

(B) Desoxyribose Nucleic Acid (DNA):

A second aliquot of the hot perchloric acid extract was analysed for desoxyribose by the diphenylamine method of Seibert (40). The color intensity was measured at 595 m\(\mu\) with the Beckman Model B Spectrophotometer. In most cases the amount of DNA extractable by hot perchloric acid was too small to give reliable results so that in these instances it was not possible to draw any rigorous conclusions. Similar difficulty in studying DNA from \textit{E. coli} by routine procedures has been experienced by Bolton (41).
The fact that DHSM interferes with bacterial growth as well as its action in blocking certain specific processes suggests the possibility that a metabolite may be caused to accumulate at the point of DHSM-blockage. Since no evidence was available to suggest the chemical nature of such a hypothetical metabolite, a group of non-specific chemical techniques was employed to determine whether any difference between normal susceptible E. coli, and susceptible E. coli treated with DHSM could be demonstrated. If such a difference were found it would constitute evidence for the presence of an accumulated metabolite. The method of demonstration would also provide an indication of its chemical nature and suggest methods of isolation. DHSM-treated cells were obtained under two conditions. In one case the cells were grown in the presence of DHSM and in the other, cells which had entered the stationary phase of growth were incubated in the presence of the antibiotic.

The Preparation of DHSM-treated Susceptible E. coli

(A) Susceptible E. coli Grown in the Presence of DHSM:

It was reasoned that, under conditions in which the concentration of DHSM was such that growth of the culture was impaired but not completely stopped, accumulation of a metabolite would be most likely.
Therefore, it was necessary to investigate the effect of DHSM on susceptible organisms in various phases of growth to determine suitable conditions, under which a limited degree of cellular reproduction would still be possible. Preliminary experiments were carried out to determine the effect of DHSM, in varying concentrations and at different times of addition, on the growth of susceptible E. coli.

A Roux flask containing 100 mls of synthetic medium was inoculated with one ml of a twenty-four hour tube-culture. The suspension was then incubated at 37° for twenty-three hours. After ten hours of normal growth DHSM was added so as to obtain a concentration of 50 units per ml. At intervals, samples of the growing culture were removed, using aseptic techniques, for measurement of the turbidity. A control flask inoculated from the same tube was included, to which no DHSM was added. It turned out that under these conditions, DHSM has no effect either on the growth of the organisms or their morphological characteristics as determined by microscopic examination of cells fixed by Gram's stain. The optical density readings were plotted and the growth curve obtained was the same as that of normal organisms which is shown in figure I.

Next, the effect of a wider range in the concentration of DHSM on growth was investigated. For this purpose, 100 mls of synthetic medium were inoculated as before. Nine ml portions of this
suspension were transferred to colorimeter tubes. The concentrations of DHSM introduced ranged from 30 to 1000 units per ml. The time of addition of the drug varied between 0 and 9 hours after the start of the incubation period. Control tubes containing no DHSM were also incubated. Turbidity measurements were made over a period of twenty-four hours and the results were plotted to obtain growth curves for each set of conditions. As the results were found to be subject to a certain amount of variation for a given set of conditions, they have been reported in tabular form using an arbitrary scale of four (see Table I).

**TABLE I**

*Effect of DHSM on the Growth of DHSM-Susceptible E. coli*

<table>
<thead>
<tr>
<th>Time of addition (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHSM conc. (units per ml)</td>
<td>1000</td>
<td>####</td>
<td>####</td>
<td>####</td>
<td>####</td>
<td>###</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>####</td>
<td>####</td>
<td>####</td>
<td>###</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>####</td>
<td>####</td>
<td>###</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>####</td>
<td>###</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>#</td>
<td>#</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
</tbody>
</table>

Key: # = slight inhibition; ## = moderate inhibition; ### = high inhibition; #### = total inhibition.

As shown in Table I, a growth response of approximately one half the control response is obtained when 50 or 100 units per ml of DHSM are added after five hours of incubation. To confirm this finding, a more
precise determination was carried out, using 100 mls of growth suspension instead of 9 mls. The results of this experiment are shown in figure I where it will be seen that the above conditions permit growth to the extent desired for metabolite accumulation. On microscopic examination of cells fixed with Gram's stain, it was found that DHSM caused the cells to take the form of elongated rods, many of which were joined end to end to produce thread-like colonies. *E. coli* normally exists as a short rod.

(B) Susceptible *E. coli* from the Stationary Phase of Growth Incubated with DHSM:

In testing the metabolite hypothesis, it is necessary to take into account the possibility that the metabolite may be in the form of a factor, essential for the operation of some metabolic process, which is displaced from a complex by DHSM. If this were the case, the most desirable experimental situation to demonstrate it would be one in which complete displacement of the factor could be achieved. Thus, for testing the metabolite hypothesis from this point of view, a culture of susceptible organisms was allowed to grow for eighteen hours, by which time it would be in the stationary phase of growth. At this point, a large quantity (500 units per ml) of DHSM was introduced and the incubation was extended for a further 6.5 hours before the cells were harvested. A control culture was also incubated under the same conditions except for the addition of DHSM. In this case, no morphological difference was
GROWTH OF SUSCEPTIBLE E. COLI IN THE PRESENCE OF DHSM

Fig. 1 The effect of dihydrostreptomycin (DHSM) on the growth of streptomycin-susceptible E. coli.

Curve A. no DHSM added to growth medium.
B. growth in the presence of 50 units per ml.
C. growth in the presence of 100 units per ml.
observed between DHSM-treated and control organisms.

Both conditions (A) and (B) utilized separately, supply systems suitable for examining either aspect of the hypothesis, but in conjunction they offer a wider scope for investigation.

The cultures resulting from the respective treatments described under conditions (A) and (B) were prepared for subsequent investigation in the following manner. First, after harvesting the organisms, the growth media were filtered through an ultrafine sintered glass funnel to remove any remaining cells. The resulting "growth supernatants" were stored under refrigeration until used. The harvested cells were suspended in distilled water and subjected to sonic disintegration for a period of ten minutes. The cell debris was removed from the resulting sonicates by centrifugation under a force of 12,000 g. The sonicates were then frozen until used.

Examination of DHSM-treated E. coli for Evidence of a Metabolite

(A) By Paper Chromatography:

The sonicates and growth supernatants, obtained as described above, were subjected to partial fractionation by paper chromatography. The wide range of reagents used in the development of the chromatograms obtained, produced identical patterns among both sonicates and growth supernatants. In each case the test material derived from DHSM-treated cells gave rise to the same distribution of
spots, with regard to both Rf value and intensity, as its corresponding control. The only exception to this finding occurred in a chromatogram of the growth supernatant from cells treated with DHSM during growth (condition A) which had been developed with the aniline phthalate reagent. In this case, a brown spot appeared in the pattern given by DHSM-treated cells with no corresponding spot in the pattern derived from the normal cells used in the control. However, this spot was traced to the presence of unmetabolized glucose still present in the growth medium owing to the retarded growth of the organisms.

(B) By Ultraviolet Spectrophotometry:

Absorbance measurements on all sonicates in the range from 230 m\(\mu\) to 340 m\(\mu\) yielded similar curves with a maximum at 257 m\(\mu\). Curves obtained with growth supernatants failed to show a peak in this wavelength band.

Thus, up to the point to which these experiments were carried, they failed to yield any evidence for a metabolite or "factor" accumulating under the influence of DHSM, either in growing or resting suspensions of susceptible \textit{E. coli}. This approach was discontinued in favor of a recently observed phenomenon which gave promise of demonstrating a "metabolite" effect. This test-system involved a DHSM-resistant variant not yet described.
The Use of DHSM-resistant Organisms to Detect a Metabolite

Description of the T-1 Variant

As well as the DHSM-susceptible variant of E. coli, there are also resistant and dependent strains which are genetically distinct and interconvertible under the proper conditions. The resistant strain under consideration was derived originally from a susceptible variant and is capable of growth in media containing 5000 units per ml of DHSM. For experimental purposes, medium supplemented with yeast extract and containing no DHSM, is inoculated from a stock culture of resistant organisms which have been grown for twenty hours in the presence of 500 units per ml of DHSM. The culture resulting from this first transfer in the absence of the antibiotic is used to inoculate Roux flasks containing supplemented medium. After twenty to twenty-two hours of growth the cells are harvested and are designated as "T-1" organisms. They are, in effect, DHSM-resistant cells which have been grown in the absence of the drug. The properties of this variant are complicated by the fact that it possesses a certain degree of DHSM-dependence. This is shown by the finding that growth, as measured by turbidity, takes place at a greater rate in the presence of DHSM than in its absence. (However, it is not the same as the dependent variant, which will not grow at all unless DHSM is present). Also, the process of enzyme induction in these organisms is greatly influenced by DHSM. It is this latter property which gives rise to the possibility of using this variant
as a tool in the search for a metabolite.

To determine the effect of DHSM on the induction of $\beta$-galactosidase in the T-1 variant, a rate curve of enzyme formation was plotted from data obtained during a six hour induction period as described in the section on methods. As shown in figure II, when DHSM is present in the induction medium, enzyme synthesis proceeds rapidly after a short lag period and the enzyme concentration reaches a maximum after approximately three hours. The level then remains constant for at least three more hours, probably due to the exhaustion of nitrogen-containing precursors of enzyme protein. However, in the absence of the antibiotic, the rate of enzyme synthesis is very slow and only after several hours does the enzyme concentration begin to approach that found in the presence of DHSM. This result confirms the observation of Peretz and Polglase (28).

In a second, similar experiment, sodium glutamate (0.2 mg per ml) was included in the incubation medium as a source of nitrogen. In this case, the extent of $\beta$-galactosidase formation was much greater, both with and without DHSM, owing to the presence of the exogenous nitrogen source (see figure III). In the presence of DHSM, the enzyme content of the cells did not level off, but continued to rise, far outdistancing enzyme production in the absence of the antibiotic. The continued rise in the $\beta$-galactosidase concentration in the presence
Fig. II. Partial Dependence. The effect of dihydrostreptomycin (DHSM) on the induction of \( \beta \)-galactosidase in streptomycin-resistant *E. coli* grown in the absence of streptomycin. Lactose was added at zero time.

Lower curve: no DHSM

Upper curve: 100 units per ml added one hour before inducer.

Enzyme units: slope of ONPG hydrolysis curve \( \frac{\text{O.D.}}{\text{minutes}} \) \( \times 10^4 \)
Fig. III. Partial Dependence. The effect of dihydrostreptomycin (DHSM) on the induction of β-galactosidase in streptomycin-resistant E. coli grown in the absence of streptomycin. Lactose was added at zero time.

Lower curve: no DHSM
Upper curve: 100 units per ml added one hour before inducer.
Enzyme units: slope of ONPG hydrolysis curve \( \frac{\text{O.D.}}{\text{minutes}} \times 10^4 \)
of glutamate may be attributed to the continued presence of nitrogenous precursors. It must be noted that under these conditions, general metabolism and growth would also be stimulated.

The role of DHSM in this system is to permit a more rapid synthesis of enzyme since protein is still synthesized in its absence, although at a slower rate. That is, the effect is on the rate of formation of enzyme rather than on the ultimate quantity formed. Information bearing on the mechanism by which DHSM performs its role in increasing the rate of enzyme induction was obtained through the observation, by Peretz and Polglase, that a sonicate of the susceptible variant, will, if present in the induction medium, exert an effect similar to that of the antibiotic itself (42). It was postulated that some specific factor was present in the sonicate which fulfilled the role of DHSM in speeding the rate of protein synthesis. A hypothetical explanation of this finding, taking into account the peculiarities of the T-1 organism, may be set forth as follows. It presumes the existence of a factor (F) in susceptible organisms which plays an essential role in adaptive enzyme formation, perhaps in the form of a charged complex in some nucleic acid-protein interaction. By virtue of its distinctive properties, in particular its highly polar guanido groupings, the DHSM molecule would replace F in the aforementioned complex. However, the presence of DHSM instead of F in the complex would prevent its normal functioning in enzyme synthesis. To extend this concept to deal with the T-1 organism,
it is postulated that the cellular chemistry has been modified so as to permit DHSM to carry out the role of F. However, it is assumed that F is still active, but owing to the reduced requirement for F, its synthesizing mechanism is less active. (This view is compatible with the reduced rate of growth of resistant organisms observed in DHSM-free media). Thus, the internal pool of F would be smaller. This line of reasoning may also be extended to cover the dependent variant by postulating that in this case DHSM only, will fulfil the function originally carried out by F, F being absent from the cell or, at least, non-functional. Returning to the consideration of adaptive enzyme formation in resistant E. coli, the role of DHSM may now be explained in terms of this hypothesis. In the presence of the drug, the addition of inducer would initiate the enzyme-forming mechanism and bring both F and DHSM into play. However, in the absence of the antibiotic, the low intracellular level of F would permit only a low rate of enzyme synthesis. The synthesis of further F would gradually permit more protein to be formed. The role of susceptible sonicates in restoring the enzyme synthesizing capacity of T-1 cells through a DHSM-like effect could be explained by the fact that susceptible organisms contain normal amounts of F which would be utilized in protein synthesis. Finally, the T-1 system appeared to provide a test system which could be used to study this metabolite or factor, apparently present in susceptible organisms. Unlike the experiments described earlier, it provided the first evidence in favor of the
metabolite concept.

Further work was then carried out, based on this observa-
tion, to confirm the presence of a metabolite and to isolate it. Owing
to the fact that the preparation of rate curves based on the six hour
induction period is time consuming and unnecessary for the purpose of
showing a metabolite effect, the shorter three hour procedure was adopted.
To accurately test each bacterial fraction for activity in the T-1 system,
the following set of incubations was carried out using aliquots from the
same suspension of T-1 organisms.

(1) T-1 cells in buffer
(2) T-1 " " " + lactose
(3) T-1 " " " + " + DHSM

The contents of the flasks were analysed for $\beta$-galactosidase as described
under "Methods". The endogenous enzyme production was given by (1).
This value (which was quite small) was subtracted from (2) and (3). Thus,
by comparing the corrected values from (2) and (3), a measure of the
enhancing effect of DHSM on enzyme production was obtained for the par-
ticular culture of T-1 organisms used for the test. With the characte-
ristics of the test organisms thus defined, it was possible to evaluate the
results obtained in a similar set of determinations carried out with the
test material present, viz.:

(4) T-1 cells + buffer + test material
(5) T-1 " " " + " + lactose
(6) T-1 " " " + " + " + DHSM
By comparing (5) and (6) with (2) and (3) after subtracting the corresponding endogenous values, it is possible to draw valid conclusions as to the DHSM-effect of the test material.

The Testing of Susceptible Sonicates for Metabolite Activity

As stated above, some factor present in the sonicate of susceptible E. coli exerted a DHSM-effect on the T-1 system in that it increased the enzyme concentration at three hours to a level approaching that found with DHSM present (42). The first step in attempting to isolate this activity was to fractionate the sonicate and test the activity of the fractions in the T-1 system. This was accomplished as follows. A twenty-two hour culture of susceptible organisms was harvested and the cells suspended in phosphate buffer (pH 7.5). Sonic oscillations were applied to the suspension for a period of ten minutes. The resulting sonicate was then centrifuged at 12,000 g for twenty minutes to remove cell debris and any remaining whole cells, which give rise to false high activities. The clear sonicate was separated into two fractions by ultracentrifugation under a force of 150,000 g for two hours. During a run the centrifuge rotor was refrigerated to avoid decomposition of labile constituents of the sonicate. The resulting supernatant and residue were then tested for activity by adding suitable aliquots to the medium in which the three hour inductions were carried out.
### TABLE II

The Effect of Susceptible Sonicate Fractions on the T-1 System

<table>
<thead>
<tr>
<th>Test Fraction (derived from ultracentrifugation of susceptible sonicates)</th>
<th>Relative Enzyme Production (enzyme activity in the presence of DHSM with no added test fraction = 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in the presence of lactose</td>
</tr>
<tr>
<td>1. buffer control</td>
<td>0.0</td>
</tr>
<tr>
<td>supernatant fraction</td>
<td>1.5</td>
</tr>
<tr>
<td>residual fraction</td>
<td>4.0</td>
</tr>
<tr>
<td>2. buffer control</td>
<td>0.0</td>
</tr>
<tr>
<td>supernatant fraction</td>
<td>0.2</td>
</tr>
<tr>
<td>residual fraction</td>
<td>0.2</td>
</tr>
<tr>
<td>3. buffer control</td>
<td>0.2</td>
</tr>
<tr>
<td>supernatant fraction</td>
<td>5.3</td>
</tr>
<tr>
<td>residual fraction</td>
<td>0.6</td>
</tr>
</tbody>
</table>

As shown in Experiment 1, Table II, most of the activity appeared in the residual fraction. However, subsequent attempts to duplicate these results failed. One determination gave results which reversed these -- the major portion of the high activity lay in the supernatant fraction (Experiment 3). Experiment 2 gave results which showed equal, smaller activities in both fractions. It is to be noted from Experiment 3 that the activity of the supernatant fraction is far greater than that of DHSM and that the two in combination give rise to a further marked enhancement in activity. These findings coupled with the fact that the results of the experiments were erratic suggested that non-specific
effects, apart from any attributable to the metabolite, were tending to mask any specific metabolite action. Also, the issue was confused by the fact that some activity was dialysable. This would argue against a large molecule such as would be found in the residue from ultracentrifugation. However, these findings could be interpreted as showing that a specific factor was produced in variable quantities, being associated with a macromolecular fraction as well as being present in a dissociated form. In an effort to better define the system by instituting more rigorous controls, use was made of a hitherto undescribed variant of E. coli related to the T series.

**Description of the T-25 Variant**

It has been shown (figure II) that there is a marked lag in $\beta$-galactosidase formation by T-1 organisms unless DHSM is present. However, if further transfers of the cultures are carried out in DHSM-free medium, this lag gradually decreases until, after 25 -- etc. transfers, the rate curve for enzyme induction corresponds to that obtained with T-1 organisms in the presence of DHSM (42) (see figure II). This organism will be designated as the "T-25" variant. The behavior of this variant when induced with lactose during a three hour period is shown by the results for the buffer control in Table V. The properties of this variant may also be explained in a fashion consistent with the hypothesis outlined during the discussions of the T-1 variant. Unlike the T-1 organism in
which it is postulated that the F-generating mechanism is restricted because of the effect of DHSM, the T-25 cell would have reinstated its full F-synthesizing capacity and would no longer show any degree of DHSM-dependence. The T-25 variant is still fully resistant and will grow in the presence of the drug. Thus, it would be expected that sonicates of this organism would also exert a DHSM-like action on the T-1 system similar to that of susceptible sonicates. Further, any effects due to the fact that susceptible sonicates are derived from a genetically different organism should be eliminated. Therefore, the effect of T-25 sonicates on the T-1 system was investigated.

The Testing of T-25 and T-1 Sonicates for Metabolite Activity

The effect of the T-25 sonicate on the T-1 system and the effects of the two fractions obtained from the T-25 sonicate by centrifugation at 150,000 g are shown in Table III. These results show the same erratic behavior exhibited by susceptible sonicates. The non-specific nature of the effects of sonicates in general was then confirmed by examining T-1 sonicates themselves, in the same manner. The final design of the experiment was such that a comparison of the effects of both T-1 and T-27 sonicates on the T-1 system was possible. These results are shown in Table IV. All sonicates have a similar effect on the induction of β-galactosidase. No evidence of a specific factor present in any sonicate has been obtained from the T-1 test system.
### TABLE III

**The Effect of the T-25 Sonicate and Sonicate Fractions on The T-1 System**

<table>
<thead>
<tr>
<th>Test Fraction (derived from ultracentrifugation of T-25 sonicates)</th>
<th>Relative Enzyme Production (enzyme activity in the presence of DHSM with no added test fraction = 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in the presence of lactose</td>
</tr>
<tr>
<td>1. buffer control T-25 whole sonicate</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>6.3</td>
</tr>
<tr>
<td>2. buffer control supernatant fraction residual fraction</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>3. buffer control supernatant fraction residual fraction</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>3.3</td>
</tr>
</tbody>
</table>

### TABLE IV

**The Effect of the T-1 and T-27 Sonicates on the T-1 System**

<table>
<thead>
<tr>
<th>Test Material (whole sonicates and fractions derived from ultracentrifugation of the sonicates)</th>
<th>Relative Enzyme Production (enzyme activity in the presence of DHSM with no added test material = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in the presence of lactose</td>
</tr>
<tr>
<td>1. buffer control T-1 whole sonicate T-27 whole sonicate</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>2. buffer control T-1 supernatant fraction T-1 residual fraction</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>0.65</td>
</tr>
</tbody>
</table>
The "Excess Metabolite" Theory of the Mode of Action of Streptomycin

During the development of the metabolite-antimetabolite concept of the mode of action of sulfanilamide, it was shown that resistant bacteria had developed the capacity to produce large excess quantities of p-aminobenzoic acid which overcame the blocking action of the drug. However, if the drug then ceased to be present, the large excess of p-aminobenzoic acid proved to be inhibitory in itself. Thus, the situation exists where a normal, essential, metabolite is inhibitory in excess amounts.

By analogy, the action of DHSM in the T-1 system could be explained on the basis that the slow rate of enzyme formation by T-1 organisms in the absence of the antibiotic, is due to the inhibitory effect of some essential metabolite present in excess amounts because of a compensatory mechanism active in resistant organisms. DHSM would serve to "neutralize" this excess, thus permitting rapid enzyme formation. Furthermore, according to this line of reasoning, T-25 cells would be characterized by a return of this essential metabolite to normal levels. If this hypothesis were true, the effect of T-1 and T-25 sonicates on the T-1 system should be inhibitory. No inhibitory effects were observed. A further test of this hypothesis would be obtained from the effect of T-1 and T-25 sonicates on the induction of \( \beta \)-galactosidase in T-25 organisms. If an inhibitory excess of a normal
metabolite were involved, T-1 sonicates should inhibit induction and T-25 sonicates should have a slight enhancing effect due to non-specific factors. The results in Table V show that both T-1 and T-25 sonicates increase enzyme production slightly. DHSM produces a slight reduction in enzyme formation in the presence of the T-1 sonicate whereas theory would predict the opposite effect. Thus, no evidence has been obtained to support this theory.

**TABLE V**

The Effect of T-1 and T-25 Sonicates on the Induction of β-Galactosidase

in T-25 Organisms

<table>
<thead>
<tr>
<th>Test Sonicate</th>
<th>Relative Enzyme Production (enzyme activity in the presence of DHSM with no added test sonicate = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in the presence of lactose in the presence of lactose + DHSM</td>
</tr>
<tr>
<td>buffer control</td>
<td>1.1</td>
</tr>
<tr>
<td>T-1 whole sonicate</td>
<td>1.5</td>
</tr>
<tr>
<td>T-25 whole sonicate</td>
<td>1.6</td>
</tr>
</tbody>
</table>
II. An Examination of the Effect of DHSM on the Protein and Nucleic Acid Content of Resistant Escherichia Coli during Enzyme Induction

β-galactosidase production involves the formation of a specific protein which is not demonstrable in the cell prior to induction. Experiments were undertaken to determine whether any changes in the gross protein and nucleic acid content of the T-1 organisms, being induced with and without DHSM, could be observed by standard techniques.

First, T-1 organisms were induced with lactose as described in "Methods". The suspension was divided into two portions, one of which was preincubated with DHSM. Samples were taken for analysis at the start of the incubation, and after three hours, at which time the difference in enzyme content of the two suspensions was at a maximum. Protein and nucleic acid determinations were carried out on these samples as described under "Methods". The results are shown in Table VI. DHSM apparently has no effect on the cellular concentration of either protein or nucleic acid during enzyme induction under these conditions.
### TABLE VI

Protein and Nucleic Acid Content of Resistant (T-1) E. coli During Induction of β-Galactosidase with Lactose

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Total Protein no DHSM</th>
<th>RNA no DHSM</th>
<th>DNA no DHSM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHSM present</td>
<td>DHSM present</td>
<td>DHSM present</td>
</tr>
<tr>
<td>0</td>
<td>1.69</td>
<td>0.409</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>1.69</td>
<td>0.409</td>
<td>0.065</td>
</tr>
<tr>
<td>3.5</td>
<td>1.71</td>
<td>0.357</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td>1.71</td>
<td>0.357</td>
<td>0.073</td>
</tr>
</tbody>
</table>

The T-1 organisms used in this induction are derived from a stationary phase culture which has been washed and stored at refrigerator temperature overnight. This treatment vastly decreases the viability of the cells. Endogenous metabolic reserves are at a reduced level and the cells are termed ”semi-starved”. With no nitrogen source present in the induction medium, growth of the organisms is impossible. Thus the process of enzyme induction is not complicated by other metabolic processes concerned with growth and multiplication. This property renders the T-1 system an excellent one for studying the induction process. However, it was considered useful to carry out the same experiment with the inclusion of a nitrogen source in the medium, thus permitting more extensive metabolism. An analysis of the soluble protein content of the cells was carried out.
as well as the determination of the total protein as before. For this purpose, the cells were subjected to sonic oscillations and centrifuged at 12,000 g for twenty minutes. The proteins were precipitated from the clear supernatant with trichloracetic acid in the usual manner. The results of these analyses are included in Table VII.

TABLE VII

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Total Protein</th>
<th>Soluble Protein</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no DHSM no DHSM</td>
<td>no DHSM no DHSM</td>
<td>no DHSM no DHSM</td>
<td>no DHSM no DHSM</td>
</tr>
<tr>
<td>0</td>
<td>4.69</td>
<td>4.69</td>
<td>1.07</td>
<td>1.09</td>
</tr>
<tr>
<td>4</td>
<td>5.08</td>
<td>5.07</td>
<td>1.10</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Similarly, in the presence of glutamate DHSM did not affect the protein or nucleic acid content of the cells. In interpreting these results it is necessary to consider the fact that the system is no longer restricted but is being supplied with a much larger supply of nitrogenous precursors which permit more extensive metabolism. It would appear that the inductive process is not accompanied by any changes in the protein or nucleic acid concentration under these conditions.
As Cohen had reported (21, 22), DHSM will cause nucleic acids and nucleoproteins to precipitate. It has also been found that, when sonicates of cells containing β-galactosidase are treated with DHSM, the resulting precipitate exhibits considerable enzyme activity (43). Experiments were undertaken to examine this precipitate, by the same analytical techniques employed with the whole cells, to determine whether the presence of DHSM during enzyme induction caused any change in its properties. It was thought that this would provide a more sensitive means of comparing cells induced with and without DHSM. Sonicates were prepared from T-1 cells induced with lactose as described elsewhere and the cell debris removed. DHSM was added to each sonicate to bring the final concentration to 5000 units per ml. The resulting precipitates were centrifuged down and washed with a dilute DHSM solution. The analyses were carried out by the usual techniques. The compositions of the two precipitates were found to be identical by the techniques employed (Table VIII). There was no indication of any difference in the properties of the nucleic acids or proteins from the two sources with regard to their behaviour towards DHSM as a precipitating agent.
TABLE VIII

Comparison of the Compositions of the DHSM - precipitates from Resistant E. coli Induced with Lactose

(average values of duplicate determinations expressed as mg)

<table>
<thead>
<tr>
<th>Component</th>
<th>DHSM - Precipitate</th>
<th>No DHSM present during induction</th>
<th>DHSM present during induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.952</td>
<td>0.938</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>0.624</td>
<td>0.624</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>0.14</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSIONS

The first phase of this study was to investigate the possibility that streptomycin antagonizes, displaces, or otherwise interferes with the action of a metabolite required by E. coli for enzyme synthesis and growth. Conditions have been sought, under which the presence of such a metabolite would be most likely to be detected—namely, in a situation where metabolic activity is proceeding in a retarded fashion under the influence of the drug. In experiments designed to test for the presence of a metabolite both in growing and in resting cells of susceptible E. coli, streptomycin has been found not to affect the composition, as determined by chromatography or spectrophotometry, of sonicates or extracellular material prepared from susceptible organisms. Thus, it is concluded that no substance, detectable by the chromatographic techniques employed, accumulated in susceptible E. coli in the presence of streptomycin.

During this investigation, the phenomenon of partial dependence on streptomycin for adaptive enzyme formation has been studied. Partial dependence is shown by resistant E. coli cells which have been grown for at least twenty hours without the antibiotic (T-1 cells). The dependence on streptomycin for enzyme formation, manifested by these T-1 cells during the first three hours of exposure to inducer, provides an excellent tool for the detection of a natural
metabolite which might fulfil an essential role in enzyme synthesis in cells which are not streptomycin-dependent. One would naturally expect to find such a metabolite to be present in those cells which are not dependent on the drug for enzyme synthesis -- for example, wild-type E. coli cells which are streptomycin-susceptible. It would also be expected to be found in resistant organisms which, by virtue of repeated transfers to medium not containing streptomycin, have lost their partial dependence on the antibiotic for protein synthesis (so-called T-25, 26 --- etc., cells). Neither in susceptible E. coli, nor in "normal" resistant E. coli (T-25 etc.) has any evidence been found for a metabolite capable of permitting a normal rate of enzyme formation in T-1 cells. It can be stated that no such metabolite is present within the limitations of the methods used in this investigation. The search for the metabolite was confined to extracts made from the cells since, concurrently, an investigation of extracellular components was being carried out (42) which has yielded date indicating the absence of such a metabolite in culture filtrates from several variants of E. coli.

The possibility that partial dependence on streptomycin is due to the production of an inhibitor of enzyme formation by T-1 cells, which would exert its effect on the induction process in the absence of the antibiotic, has also been investigated. This inhibitor would logically be assumed to be absent from resistant organisms (T-25 etc.)
which no longer exhibit retarded enzyme formation. No evidence was found for an inhibitor in T-1 cells, of adaptive enzyme formation by T-25 cells. It is concluded that the previous growth in the presence of streptomycin has not elicited the production of an inhibitor of enzyme synthesis in T-1 organisms, the effect of which is reversed by the continued presence of the drug. It has not been possible to demonstrate, in *E. coli*, the presence of any metabolite for enzyme synthesis which is antagonized or displaced by streptomycin.

The observation that the effect of streptomycin on adaptive enzyme formation parallels its effect on growth in *E. coli* has led to the investigation of the effect of the antibiotic on other measurable properties of the T-1 (partially dependent) organism during enzyme induction, in particular the protein and nucleic acid content of the cells. This study comprises the second phase of the present investigation. It has been found that, during the period of the production of enzyme by the cells, streptomycin has no effect on these other properties as measured by standard techniques. These results have been corroborated by similar analyses on the nucleic acid-protein fraction precipitable by streptomycin from T-1 cells taken during the phase of formation of enzyme. The analyses showed no difference between the material precipitated by the antibiotic during enzyme production whether the induction was carried out in
the presence of the drug or not. The fact that the gross protein content of T-1 organisms remains constant during induction, whether or not streptomycin is present, may be explained in one of two ways. One explanation, in view of the high sensitivity of the enzyme assay method (hydrolysis of \( \sigma' \)-nitrophenyl-\( \beta \)-D-galactoside), is that the standard techniques employed for determination of a change in protein or nucleic acid content are not sensitive enough to determine the small amount of enzyme protein synthesized. It is, at present, impossible to calculate the amount of protein which should be formed during \( \beta \)-galactosidase induction since this enzyme has not been purified.

An alternative explanation is that the antibiotic exerts its effect at a late stage in protein synthesis -- i.e. at a stage when the extended polypeptide chain has been formed on a template but has not received its final, configurational specificity or, has not been released from the template. We would conclude that there is no doubt that streptomycin affects protein synthesis but that this effect is not demonstrable by analysis of the gross cellular protein or nucleic acid content.
SUMMARY

1. No evidence for a metabolite accumulating under the influence of streptomycin in susceptible *E. coli* was found, using various chromatographic techniques.

2. No evidence for a factor or metabolite, which would satisfy the streptomycin requirement of partially dependent (T-1) *E. coli* for adaptive enzyme formation, was found in either susceptible organisms or resistant organisms which did not exhibit such an antibiotic requirement. It was also shown that the requirement for streptomycin of resistant *E. coli* was not due to the manifestation of an inhibitor in the absence of the drug.

3. By analysis of the gross, cellular protein and nucleic acid content by standard techniques, it was shown that streptomycin has no effect on the cell content of protein and nucleic acid in T-1 organisms during the induction of a specific enzyme.
BIBLIOGRAPHY

(1.) Benham, R.S., Science 105, 69 (1947).

(2.) Henry, J., Henry, R.J., Housewright, R.D., Berkman, S., J. Bact. 56, 527 (1948).

(3.) Barkulis, J.L., J. Bact. 61, 375 (1951).


(5.) Umbreit, W.W., J. Biol. Chem. 177, 703 (1949).


(9.) Umbreit, W.W., J. Bact. 66, 74 (1953).


(16.) Miura, Y., and Iwamoto, T. Kagaku (Science) 26, 362 (1956).
(17.) Lichstein, H.S., and Gilfillan, R.F.,

(18.) Owen, C.A., Karlson, A.G., Zeller, E.A.,

(19.) Zeller, E.Z., Owen, C.A., Karlson, A.G.,
J. Biol. Chem. 188, 623 (1951).

(20.) McQuillen, K.,

(21.) Cohen, S.S.,
J. Biol. Chem. 166, 393 (1946).

(22.) Cohen, S.S.,

(23.) DiMarco, A., Boretti, G., Enzymologia, 14, 141 (1950).

(24.) Fitzgerald, R.J., Bernheim, F., Fitzgerald, D.,
J. Biol. Chem. 175, 195 (1948).

(25.) Roote, S.M., and Polglase, W.J.,

(26.) Polglase, W.J.,

(27.) Polglase, W.J., Peretz, S., Roote, S.M.,

(28.) Peretz, S., and Polglase, W.J.,
Antibiotics Annual 533 (1956-7).

(29.) Bacon, J.S.D., and Edelman, J.,

(30.) Hough, L., Jones, J.K.N., Wadman, W.H.,

(31.) Partridge, S.M.,

(32.) Toennies, G., and Kolb, J.J.,


(35.) Schneider, W.C., J. Biol. Chem. 161, 293 (1945).


(37.) Bial, M., Deut. med. Wochschr. 29, 253; 29, 477 (1903).


(40.) Seibert, F.B., J. Biol. Chem. 133, 593 (1940).

