THE 503nm PIGMENT OF ESCHERICHIA COLI: PROPERTIES AND FUNCTION

by

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ABSTRACT

The streptomycin (Sm)-dependent mutants of four Escherichia coli strains (known to be catabolite-repression negative) were found to have a 25-35% lower aerobic efficiency (yield of protein from glucose) than the parent wild-type organisms. A non-dependent revertant derived from the Sm-dependent mutant of E. coli B was also lower in efficiency. In contrast, the anaerobic protein yields (although lower than that from aerobic growth) were identical for both types of cells.

In both wild-type and mutant strains the glucose-reduced/peroxide-oxidized difference spectra of whole cells showed the same content of cytochromes and flavin. However, Sm-dependent strains lacked the 503nm pigment which was present in all wild-type strains. These observations suggested that the 503nm pigment (P503) (of previously unknown function) might play a role in energy metabolism.

Addition of gluconate to air-oxidized cells produced the P503 peak prior to the appearance of cytochrome and flavin absorption bands. Addition of succinate, glycerol, lactate or acetate produced cytochrome and flavin spectra but not P503. Addition of the amino acid L-methionine to air-oxidized cells elicited the P503 band rapidly but the other components of the difference spectrum did not appear until later. No other amino acid tested had this specific effect on P503.

When wild-type cells were grown on limiting glucose-salts medium containing 2,4-dinitrophenol (500 μM), the yield of cell protein was decreased and formation of P503 was inhibited. Also, growth under these conditions resulted in derepressed levels of fumarase, aconitase and, unexpectedly, glucose 6-phosphate dehydrogenase.

From these observations the general hypothesis was developed that P503 participates in an oxidative energy-yielding pathway in which the initial substrate is reduced nicotinamide adenine triphosphate (NADPH), (the first product of gluconate metabolism in glucose-grown E. coli).

The synthesis of P503 was observed with glucose or gluconate as carbon source. Less P503 was synthesized when succinate, glycerol or lactate was the carbon source, in which cases generation of NADPH would be less efficient.

When L-methionine was present in medium containing glucose as carbon source, the synthesis of P503 was inhibited. Other amino acids did not inhibit synthesis of P503. The unique response of P503 to methionine suggests regulation of the pigment by this amino acid.

In agreement with observations of other investigators, P503 was found to be transient and labile, and could not be detected in cells which had been frozen and thawed or in cell extracts.

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ABBREVIATIONS USED

P503	pigment absorbing maximally at 503nm
A_{420} (600) or absorbance$_{420}$	absorbance at 420nm (600nm)
ΔA	(i) difference in absorbance between two suspensions or solutions, one oxidized and the other reduced or (ii) change in absorbancy
E	efficiency of growth (µg/ml increase in protein per µg/ml glucose consumed)
Sm	streptomycin
DHSm	dihydrostreptomycin
$NADP^+$ (NADPH)	nicotinamide adenine dinucleotide phosphate (reduced)
NAD^+ (NADH)	nicotinamide adenine dinucleotide (reduced)
FAD	flavin adenine dinucleotide
FMN ($FMNH_2$)	flavin mononucleotide (reduced)
ATP	adenosine triphosphate
Pi	inorganic phosphate
PP	pyrophosphate
DNP	2,4-dinitrophenol
TBP	1,3,5-tribromophenol
CM	chloramphenicol
EDTA	ethylenediamine tetraacetic acid
PEP	phosphoenolpyruvate

Abbreviations Used (continued)

G6-P	glucose 6-phosphate
TCA	tricarboxylic acid cycle
HMP	hexosemonophosphate
E-D	Entner-Doudoroff
E-M	Embden-Meyerhof
B_{12}	vitamin B_{12}
f-met	formyl methionine
$tRNA_M^{met}$	transfer ribonucleic acid $^{\text{methionine}}_{\text{methionyl}}$
$tRNA_F^{met}$	transfer ribonucleic acid $^{\text{methionine}}_{\text{formyl}}$
aa's	amino acids
Km	Michaelis Menton constant
Vmax	maximum velocity

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INTRODUCTION

The occurrence of a pigment absorbing maximally around 503nm has been reported in several organisms. Its presence in baker's yeast (Saccharomyces cerevisiae) was noted by Lindenmayer (1959), Lindenmayer and Smith (1964), Nosoh (1964) and Nosoh and Itoh (1965). Several strains of bacteria, including Bacillus megaterium (Kepes, 1964), Azotobacter chroococcum and A. agile (Goucher and Kocholaty, 1957), Escherichia coli (Kepes, 1964; Olden and Hempfling, 1970), Rhizobium japonicum (Appleby, 1969), and finally the photosynthetic bacterium Rhodopseudomonas sp. (Cooper, 1956) were also found to possess this pigment. In all cases, the presence of P503 was detected via spectrophotometric methods. In view of the existence of these observations, it seems rather odd that little attention has been given to this pigment. Perhaps an explanation for this paucity of information can be found when one studies the conditions under which P503 is found.

The pigment is absent in cells grown on an enriched or complex medium; only when a restrictive environment (minimal salts) surrounds the organism is P503 formed. Thus Labbe, Volland and Chaix (1967) noted the lack of P503 when yeast cells were grown on nutrient agar or peptone broth, and its appearance when a synthetic medium was used. Similarly, Nosoh and Itoh (1965) reported that E. coli and Bacillus subtilis grown in nutrient broth and excess glucose failed to exhibit a 503nm peak. Furthermore, the intensity of its absorption has been found to be dependent upon the physiological state of the culture. Lindenmayer (1959) observed the peak to vary greatly in height with the age and treatment of the cells. Whereas logarithmically growing cells showed a large P503 peak, stationary phase cells had

a substantially decreased level of the pigment (Lindenmayer, 1959; Labbe et al., 1967; Nosoh, 1964). The latter author noted that addition of a reducing agent such as sodium dithionite to oxidized cells resulted in a 503nm band in the absorption spectrum. However, with a more concentrated reducing agent, the 503nm band disappeared completely. It is not surprising, therefore, that the pigment has received little attention by investigators of microbial metabolism.

In contrast to the cytochromes, the transient, unstable nature of P503 has discouraged attempted isolation or structural determination. Reduction of the pigment by glucose results in its appearance along with the cytochromes and flavin; however, the P503 peak begins to decrease and eventually disappears in 10-15 minutes while the spectrum of the cytochrome components remains constant. Lindenmayer and Smith (1964) observed the disappearance of the pigment on rupture of the cells; this result discouraged more direct studies. The only successful attempt was reported by Labbe et al. (1967) who claimed that P503 was present in the soluble fraction in yeast cells homogenized under nitrogen.

The structure of P503 has not yet been determined. The possibility of its being cytochrome-like or flavin-like has been ruled out due to several observations: (1) The disappearance of the P503 peak does not result in a concomitant appearance (or increase) of any other absorption band either in the visible (700-450nm) or Soret (~425nm) region of the spectrum (Lindenmayer, 1959); (2) The 503nm pigment does not have an absorption spectrum of a hemoprotein; whereas the latter shows several bands (α,β, and γ) varying in absorption intensity, P503 has a single peak (Lindenmayer and Smith, 1964); (3) The cytochrome system and flavoproteins react rapidly on exhaustion or addition of oxygen while changes

observed with P503 are much slower (Lindenmayer and Smith, 1964). These workers suggested that the absorbancy and reactivity of P503 was in agreement with properties possessed by the semiquinone form of ubiquinone. Several findings are inconsistent with this view. Ubiquinones (or coenyme Q) are a class of homologues derived from 2,3-dimethoxy-5-methyl-benzoquinone; substitution by varying lengths of side chain isoprenoid units at position 6 of the quinone ring accounts for the different homologues formed. In microorganisms, the presence and amount of coenzyme Q has been correlated with respiratory rates. Thus the formation of coenzyme Q was found in two facultative organisms, E. coli and Saccharomyces cerevisiae, to be adaptive and induced only by growth under aerobic conditions (Lester and Crane, 1959; Sugimura and Rudney, 1960). The 503nm pigment, however, is synthesized to the same extent under both aerobic and anaerobic atmospheres (Lindenmayer, 1959; Nosoh, 1964). A second point against the identity of P503 as a semiquinone of ubiquinone is that Labbe et al. (1967) located the 503nm pigment in the soluble fraction of yeast cells; on the other hand, Cox et al. (1970) indicated that ubisemiquinone exists in the membrane fraction in E. coli. Furthermore, the latter compound was stable, unlike P503.

Several publications in the literature have referred to the semiquinoid intermediate of flavin possessing an absorption band around 490-510nm. Although a semiquinone of free FMN does absorb at 503nm in a 1 M HCl solution (pH 0), the form present under physiological conditions (neutral pH) absorbs at 565nm (Beinert, 1956a; 1956b). In 1957, Beinert extended his studies to include three enzyme-bound flavoproteins (a fatty acyl CoA dehydrogenase, L-amino acid oxidase of snake venom, and the "old yellow

enzyme of yeast"- acyl dehydrogenase, C_4 to C_{16}). Investigations of difference spectra suggested to him that the spectral characteristics of free flavins during oxidation-reduction at neutral pH were almost identical with those observed with fatty acyl CoA dehydrogenase-flavoprotein (the latter enzyme having been reduced by octanoyl CoA). Both spectra showed a broad absorption band between 500-650nm with a peak at 560-570nm. A noticeable difference was that the band absorbing maximally at 565nm had approximately 20 times the intensity with enzyme-bound FAD as with free FAD. The band having an absorption maximum at 565nm during reduction and re-oxidation of free flavins was ascribed to a semiquinone form of the flavin (Beinert, 1956b). In addition, the intermediate formed upon reduction of the acyl dehydrogenase with dithionite as substrate was concluded to be a semiquinone form of the enzyme-bound flavin (Beinert, 1957). The formation and disappearance of the latter intermediate was found by Beinert to be inversely related to the absorption characteristics of the flavin band at about 450nm.

In the case of L-amino acid oxidase, the absorption band ranged from 520-650nm with a maximum value at about 545nm.

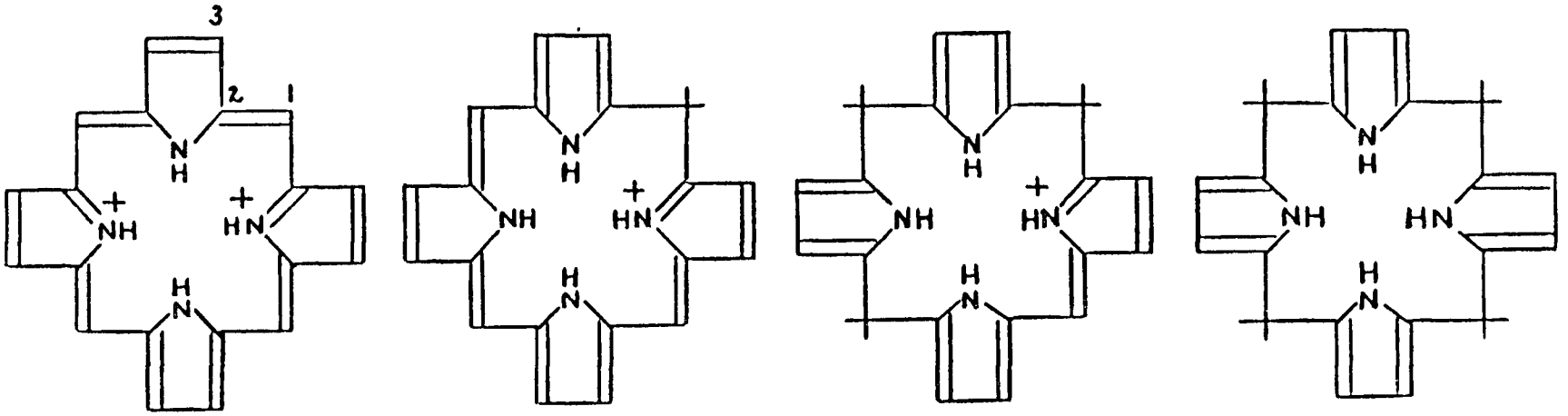
Spectrophotometric analysis of the enzyme-substrate complex of D-amino acid oxidase (Yagi and Ozawa, 1962; 1963) indicated an absorption spectrum possessing a characteristic shoulder at 490nm. In their work, benzoate was used as a substitute for the real substrate since it does not undergo dehydrogenation to reduce FAD^+, thereby enabling them to study the complex of apo-enzyme + FAD + substrate-substitute. Since the shoulder at 490nm was observed only in the enzyme-substrate model, not in the holoenzyme (apo-enzyme + FAD), they concluded that the band at 490nm was characteristic for the artificial complex composed of the

apo-enzyme, coenzyme and substrate-substitute.

Labbe et al. (1967) and Olden and Hempfling (1970) favoured a protoporphomethene structure for P503 on the basis of the striking similarity in the properties of both compounds. The photoreduction of porphyrins proceeds through two stages from porphyrin (absorption peaks at 400, 550, 590nm) to dihydroporphyrin (absorption peaks at 440, 735nm) to tetrahydroporphyrin or protoporphomethene (absorption peak at 500nm) (Figure 1). Certain reducing agents such as sodium dithionite can reduce the protoporphomethene beyond the second stage to the colourless hexahydroporphyrin or porphyrinogen (Mauzerall, 1962). This third stage of reduction accounted for the ability of dithionite, sulfite, potassium cyanide and cysteine to cause the disappearance of the 503nm peak (Labbe et al., 1967; Nosoh, 1964). The former group suggested that the mechanism involved the linkage of sulfite and cyanide to the methene bridge to form the colourless structure. No evidence thus far has been established to refute this protoporphomethene identity of P503. A positive identification awaits the actual isolation and purification of the pigment.

If the chemical nature of the 503nm pigment remains unknown, its function in cellular metabolism has been even more obscure. No functional hypotheses have been advanced prior to the present investigation.

Our interest in P503 resulted from investigations on the decreased efficiency of growth in Sm-dependent mutants compared to the wild-type parents observed only during aerobic growth. The growth of microorganisms in relation to their energy supply (the amount of substrate utilized) under aerobic versus anaerobic conditions has been well documented (Bauchop and Elsden, 1960; Kormančíková, Kovác and Vidová, 1969; Coukell, 1969; Cox et at., 1970). A linear relationship exists between the cell



porphyrin	dihydroporphyrin or phlorin	tetrahydroporphyrin or porphomethene	hexahydroporphyrin or porphyrinogen
(absorption peaks at 400, 550, 590nm)	(absorption peaks at 440, 735nm)	(absorption peak at 500nm)	(colourless)

Figure 1. Structures of chemical or photoreduced porphyrins. The structures were taken from Mauzerall (1962). Alkyl substituents on the ring carbons and hydrogens on the methene bridges are omitted.

mass formed and amount of glucose consumed at low concentrations of substrate. This ratio (termed variously: growth yield constant, molar growth yield, or simply yield constant) is substantially higher during aerobic growth than under anaerobic conditions in wild-type E. coli cells.

A preliminary survey of four E. coli strains (B, K12, UL and CRX) indicated that although the Sm-dependent mutants were just as efficient as their wild-type parents during anaerobic growth, their aerobic efficiency was as much as 25-35% less than the wild-type organisms. A deficiency was therefore implicated in an aerobic route of energy metabolism.

The Sm-dependent mutants had previously been found to differ from wild-type cells in the following manner: (1) decreased aerobic efficiency (Coukell, 1969), (2) excretion of valine in the medium when glucose was the substrate (Bragg and Polglase, 1962; Tirunarayanan, Vischer and Renner, 1962), and (3) derepressed levels of the catabolite repressible enzymes citrate synthase, fumarase, and aconitase (Coukell, 1969).

In view of the numerous aspects of deficiency exemplified by the Sm-dependent phenotype, it was of interest to do comparative studies of hydrogen metabolism in the wild-type versus Sm-dependent mutant.

An analysis of the reduced/oxidized difference spectra of wild-type, Sm-dependent, and non-dependent revertant (derived from the Sm-dependent mutant) indicated that the cytochromes a (600-650nm), cytochrome b (560nm), and flavin trough (460nm) were present to the same extent in all three cases. The only difference observed was that the large symmetrical peak at 503nm (P503) in wild-type cells was absent or negligible in the Sm-dependent and revertant mutants. Since no role for this pigment, or its participation in any reaction was yet known, this study was directed to its characterization in an attempt to ascertain its function.

METHODS AND MATERIALS

I. Organisms

i. Wild-type strains:

Most of the research was carried out on *E. coli* B (ATCC 11303). For comparative purposes, *E. coli* strains K12, UL, and CRX were also used. Strain K12 was obtained from Dr. J. Stock, Department of Microbiology, University of British Columbia, Vancouver; strain UL was isolated at the Department of Bacteriology, University of Laval, Quebec City; strain CRX was obtained from Laboratory of Hygiene, Ottawa, Ontario. Stock strains were stored on minimal salts-agar slopes and subcultured every month.

ii. Glycogen-less mutant:

E. coli B-SG1, a mutant of *E. coli* B lacking adenosine diphosphate-glucose: α-4-glucosyl transferase and thus incapable of synthesizing glycogen, was originally isolated and characterized by Dr. J. Preiss, Department of Biochemistry, University of California, Davis.

iii. Methionine-less mutant:

E. coli K12-met$^{(-)}$ (ATCC 25019), a mutant of *E. coli* K12 requiring L-methionine for growth, was used in later experiments after the discovery that L-methionine affected the production and formation (synthesis) of P503 in a unique manner.

iv. Streptomycin mutants:

a. Dependent:

Spontaneous Sm-dependent mutants of *E. coli* strains B, K12, CRX, and UL were isolated from the wild-type organisms as described by Coukell

and Polglase (1965).

b. Non-dependent revertant:

E. coli Br4 is a Sm-sensitive revertant obtained from Sm-dependent E. coli B by M.B. Coukell.

c. Resistant (indifferent):

Sm-resistant mutants of E. coli B, K12, CRX, and UL were isolated from the wild-type organisms as described by Coukell and Polglase (1965).

II. Growth of Cultures

i. Media:

All cultures were grown on a basal salts medium, pH 7.0, consisting of K_2HPO_4 (0.7%), KH_2PO_4 (0.3%), $(NH_4)_2SO_4$ (0.1%) and $MgSO_4.7H_2O$ (0.02%) as described by Davis and Mingioli (1950), but without citrate. The pH of the medium was adjusted to 7.5 with aqueous sodium hydroxide for growth of cultures overnight. The carbon sources (glucose, gluconate, glycerol, succinate, and lactate) and growth supplement (L-methionine), autoclaved separately in concentrated solution, were added to the medium to obtain the final concentration required. Sm-dependent strains were grown on medium supplemented with 1 mg of dihydrostreptomycin (Merck, Sharp, and Dohme, Montreal, Quebec) / ml.

ii. Measurment of cell growth:

A Beckman B spectrophotometer with light path of 1.0 cm was used to determine turbidity of the cultures. The change in absorbancy of the cultures was measured at 420nm (or at 600nm when cells were grown on minimal medium supplemented with 2,4-dinitrophenol). Distilled water was used as blank.

Throughout the growth period, protein determinations were made (see

METHODS IV, i), and standard curves of A_{420} versus protein (μg/ml) were plotted for the wild-type, Sm-dependent, and Sm-resistant (±dihydrostreptomycin) mutants of each strain. In addition, for wild-type E. coli B, a graph relating A_{600} to protein was prepared. In all cases, the absorbancy was directly proportional to cell protein.

iii. Growth and harvesting of cells for difference spectra:

a. Aerobic growth with glucose:

Wild-type, non-dependent revertant, Sm-dependent, and Sm-resistant (indifferent) cells were grown under aerobic conditions as follows. A culture of cells, grown aerobically overnight in a 37° shaking water bath on 0.2% glucose-salts medium (plus supplementation where required in particular experiments) was inoculated into 500 ml of fresh salts medium (plus supplementation when required) and grown with aeration on 0.2% glucose from A_{420} 0.1 to 1.0. The suspension was then cooled on ice, harvested by centrifugation (12,000 g for 15 minutes) at 4°, washed in 0.01 M potassium phosphate buffer (pH 7.0) and resuspended in 2 litres of fresh salts medium to give A_{420} 0.10-0.20. The cells were grown on limiting glucose (0.04%, w/v), harvested just at completion of growth, washed once in 200 ml of phosphate buffer, and resuspended in buffer at a protein concentration of 6.25 mg/ml. To effect the depletion of substrate as well as to oxidize the electron transport system, the suspension of cells was aerated for 1-1 1/2 hr at 37° prior to analysis of the difference spectra.

b. Inhibitors:

Wild-type E. coli B was grown as before on limiting glucose supplemented with inhibitors as follows. Stock solutions of 10^{-2} M 2,4-dinitro-

phenol (The British Drug Houses Ltd., Poole, England), 1.3×10^{-2} M 1,3,5-tribromophenol (gift of D.J. Rainnie), 10^{-4} M chloramphenicol (Parke, Davis & Co., Detroit, Michigan), and 10^{-1} M hydroxylamine-hydrochloride (Matheson Coleman & Bell, Norwood, Ohio) were prepared and the appropriate volume (ml) added to the medium to give the final concentration desired in each case.

c. Variation of carbon source :

To determine the effect of various carbon sources as growth supplement on P503 formation (see Table XV and Table XVI), the cells were first adapted for growth on the appropriate compound. Thus 0.2% gluconate (sodium salt; Eastman Organic Chemicals) or 0.2% glycerol (The Nichols Chemical Co.) was substituted for glucose and the cells treated as in METHODS II, iii, a.

In the case of succinate, 10 ml of glucose-grown cells were inoculated into 100 ml minimal medium (pH 6.0) containing 0.8% succinate (disodium salt; Fisher Scientific Co.) and grown overnight at 37° in a shaking water bath. (The pH after overnight growth was 7.0 and therefore no re-adjustments were necessary). 10 ml of this overnight culture were used to re-inoculate 100 ml of fresh 0.8% succinate-salts medium, and the cells grown overnight a second time. Without harvesting, a portion of the second overnight culture was diluted to A_{420} of 0.10-0.20 in 2 litres of fresh medium containing 0.067% (or 0.53%) succinate. The pH during the final growth (monitored at 30 minute intervals) remained constant at 7.0.

For experiments involving lactate as carbon source, 5 ml of cells grown on 0.5% lactate (sodium salt; Fisher Scientific Co.) were inoculated into 100 ml of minimal salts-lactate (1.0%) medium for overnight

growth. Without harvesting, a portion of the overnight culture was diluted to A_{420} of 0.10 in 2 litres of fresh medium containing 0.2% lactate. In all cases, the cells were harvested as described in METHODS II, iii, a, at the end of growth (when carbon source was limiting) or during log phase (when excess carbon source was used).

d. L-methionine and its analogues as growth supplement :

Two per cent stock solutions of L-methionine (Calbiochem, Los Angeles), casein hydrolysate (vitamin and salt free; Nutritional Biochemicals Corp., Cleveland, Ohio), L-ethionine (Sigma Chemical Co., St. Louis, Missouri), DL-ethionine (Sigma Chemical Co.), DL-norleucine (Nutritional Biochemicals Corp.), and DL-selenomethionine (Sigma Chemical Co.) were prepared, and the appropriate volume added to the final growth medium to give the desired concentration.

The 18 L-amino acids (± methionine) were weighed out in the proportions given for casein hydrolysate (i.e., the 21 amino acids minus tryptophan, glutamine, and asparagine) (West and Todd, 1955) and dissolved in 50 ml of heated medium. This amino acid mixture was subsequently made up to a final volume which resulted in a total concentration of 0.10%. Alanine, glutamic acid, valine, phenylalanine, serine, aspartic acid, arginine, histidine, threonine, and isoleucine were purchased from Calbiochem, Los Angeles. Glycine, tyrosine, and lysine monohydrochloride were supplied by Nutritional Biochemicals Corp., Cleveland, Ohio. Leucine, cystine, and hydroxyproline were obtained from Mann Research Laboratories Inc., New York, N.Y. The experimental conditions described in METHODS II, iii, a were followed

for growth of cultures.

e. Other compounds as growth supplement:

Wild-type E. coli B was grown as before on limiting glucose (0.04%)-salts medium supplemented with one of the following compounds: folic acid (0.01% and 0.10%) (Sigma Chemical Co.); ascorbic acid (0.01% and 0.05%) (Calbiochem, Los Angeles); glycine (0.01% and 0.10%) (Nutritional Biochemicals Corp.); L-cysteine (10^{-3} M) (Mann Research Laboratories Inc., New York); 2-deoxy-D-glucose (0.04%) (20% aqueous solution; Sigma Chemical Co.).

f. Anaerobic growth with glucose:

The cells were prepared as described in METHODS III, iii. At the end of anaerobic growth, the 37° water bath was replaced by an ice-salt bath and nitrogen gas flow continued for 1 hr. The cells were then harvested, washed in 0.01 M potassium phosphate buffer, pH 7.0, and the difference spectra studied immediately.

iv. Growth and harvesting of cells for enzyme assays:

Overnight cultures were centrifuged, washed, and resuspended in fresh salts-glucose (0.2%) medium (supplemented with 250 μM 2,4-dinitrophenol in particular experiments; see Table IX). Suspensions were grown from A_{420} of 0.10 to 0.80 (or A_{600} of 0.06 to 0.50 for DNP-containing media). The cells were harvested, washed in 0.01 M potassium phosphate buffer, pH 7.0, then resuspended in 500 ml of 0.2% glucose-salts medium in separate flasks supplemented with various concentrations of 2,4-dinitrophenol, 1,3,5-tribromophenol, or chloramphenicol as indicated in Table XIII and Table XIV. The cells were then grown with aeration at

37° until they reached A_{420} of about 0.80 (or A_{600} of 0.47) at which point they were chilled in ice, harvested by centrifugation, washed in 0.01 M potassium phosphate buffer (pH 8.0), and stored at 0° as packed cells.

To obtain sonic extracts, the cells were resuspended to a final concentration of 1 g wet weight of cells / 15 ml of 0.10 M potassium phosphate buffer (pH 8.0) and the suspension kept in ice. Three ml of this cell suspension were pipetted into a small plastic tube. With the plastic tube held in an ice-water bath, the cells were disrupted by a 30 second treatment in a Branson model W 1350 Sonifier operated at 100 watts.

III. Determination of Efficiency of Cell Growth

i. Definition:

The efficiency of growth (E) on minimal salts medium was defined as the µg/ml increase in protein per µg/ml glucose consumed.

ii. Amount of protein formed from glucose by cells growing aerobically:

Cells were grown aerobically on 0.2% glucose-minimal salts medium from A_{420} of 0.10 to 0.80, harvested during log phase, washed in 0.01 M potassium phosphate buffer, pH 7.0, then resuspended in fresh medium (minus glucose) to give A_{420} of 0.10 and incubated in a 37° shaking water bath for 30 minutes. After the absorbancy was re-measured, 400 µg/ml of glucose were added and a sample (5 ml) removed immediately for initial glucose and protein determination. Thereafter, portions were removed at 15 minute intervals throughout the growth period, cooled immediately in

an ice-salt bath, and the cells centrifuged down at 12,000 g for 20 minutes. The pellet was resuspended in distilled water for protein determination while the supernatant was analyzed for glucose content (see METHODS IV, i and ii). To ensure complete exhaustion of glucose, sampling was continued for 1 hr after the absorbancy had become constant.

iii. Amount of protein formed from glucose by cells growing anaerobically:

Glucose-starved cells were prepared as described in METHODS III, ii. After the 30 minute incubation period, the cells were first grown aerobically from A_{420} of 0.10 to 0.40 in minimal salts medium containing 650 µg glucose / ml, then transferred into a 2 litre Erlenmeyer flask fitted with a two-hole stopper. L-grade (99.99% pure) nitrogen gas (Canadian Liquid Air Ltd., Vancouver, British Columbia) was bubbled through an inlet tube vigorously for 10 minutes, then at a slower rate. A glass exit tube, placed 1.3 cm above the culture, allowed gases to escape. With anaerobic conditions being maintained, the cells were grown at 37° until the glucose was exhausted ($A_{420} \simeq 1.0$). Portions of 5 ml were removed at 15 minute intervals, initially, and thereafter at 7 1/2 minute intervals (when growth was more rapid) throughout anaerobic growth. In the sampling procedure, a 10 ml Hamilton syringe with plastic tubing attached to its needle was inserted through the gas-exit tube after the trapped air had been expelled from the syringe. This procedure was followed until the end of growth. All samples for protein and glucose determinations were treated as previously described.

iv. Amount of protein formed from glucose by cells growing aerobically and anaerobically on medium supplemented with 2,4-dinitrophenol :

The experimental details were as described in METHODS III, ii and iii, the medium being supplemented with 250 μmoles 2,4-dinitrophenol. The absorbancy was followed at 600nm. The cells were transferred to anaerobic conditions at A_{600} of 0.20 (equivalent to cells having an absorbance of 0.40 at 420nm).

IV. Chemical Analyses

i. Protein :

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951). A standard protein curve relating the absorbancy at 500nm to concentration of bovine Y-globulin from 0-400 μg/ml (California Corp. for Biochemical Research, Los Angeles, California) was prepared, and all samples (in duplicate) were diluted with distilled water so that the protein concentration was in the range of 20 to 300 μg/ml.

ii. Glucose :

Glucose was determined by using Glucostat, a commercial preparation purchased from Worthington Biochemical Corp., Freehold, New Jersey. The lyophilized enzyme preparation and chromogen, contained in separate vials, were dissolved together in 50 ml of distilled water (Glucostat reagent). A standard curve was prepared of A_{400} versus glucose concentration, from 0 to 300 μg/ml. All samples (in duplicate) were diluted with distilled water so that the glucose concentration was in the range

of 0-300 µg/ml. A 0.5 ml volume of each sample was pipetted into a test-tube. Potassium phosphate buffer, 2.5 ml of 0.10 M (pH 7.0), followed by 2.0 ml of the Glucostat reagent were added to the sample and the contents mixed thoroughly on a Vortex mixer. After incubation of this mixture for 30 minutes in a 37° water bath, 2 drops of 6 N HCl were added to stabilize the solution at room temperature, followed by 5.0 ml of distilled water. Absorbancy readings were taken at 400nm and the glucose concentration obtained from the standard curve.

V. Determination of Difference Spectra

A Cary 15 spectrophotometer with an absorbance scale of 0.1 (that is, showing a maximum absorbance of 0.1) was used. In most cases, the spectra were scanned from 700nm to 430nm. In the Figures of difference spectra the curves were corrected for slight deviation of the baseline.

The nomenclature assigned to bacterial respiratory pigments has been reviewed extensively in the literature (Smith, 1961; Bartsch, 1968; Kamen and Horio, 1970). The cytochrome pigments (hemoproteins) have been grouped into the classes a, b and c depending upon the type of iron-porphyrin prosthetic group and protein as well as upon the nature of the bond formed between the two portions of the molecule. Further breakdown of the nomenclature to a_1, a_2 and so on was necessitated in the bacterial system to differentiate among the numerous forms found in various microorganisms.

Generally, the absorption maxima of the cytochromes in the literature have been determined from the reduced spectrum since sharp bands appear in the visible region, 500-650nm. In contrast, the oxidized

spectrum shows broad, diffuse bands (Smith, 1961).

E. coli is known to possess the following cytochromes which absorb in the visible spectrum: (1) cytochrome a_1, the absorption maximum of its α band occurring at 590nm; (2) cytochrome a_2 having an absorption maximum at 630nm; (3) cytochrome b_1 showing α and β peaks at 560nm and 530nm respectively. In log-phase cells, the terminal oxidase of E. coli is cytochrome o. The absolute absorption spectrum of this component is still unknown. Stationary-phase cells possess both cytochromes a_2 and o as the terminal oxidases. In this thesis, cytochromes $a_1 + a_2$ will be referred to as cytochromes a (absorption peaks between 600-650nm) and cytochrome b_1 as cytochrome b (absorption peak at 560nm).

In mitochondria of eukaryotes, spectroscopic studies of the region from 430-500nm have indicated overlapping absorption bands due to flavoproteins and non-haem iron proteins (Ragan and Garland, 1971). The similarity of their spectral characteristics has made it extremely difficult to determine the contribution by either group of compounds alone. If the situation in mitochondria is poorly understood, even less is known about the non-haem iron proteins of prokaryotic organisms. Although the trough at 460nm, then, may be due in part to compounds other than flavin, it will be referred to in this thesis as "flavin trough".

The reduced/oxidized difference spectra were obtained in one of the two following ways depending upon the growth conditions of the cultures.

i. Cells grown with limiting carbon source:

In most experiments cells were grown on a limiting carbon source

and harvested at the end of the log phase. The final suspensions were air-oxidized for 1-1 1/2 hr prior to analysis of the difference spectra. Cell suspension, 2.3 ml, was placed in cuvettes in both the upper and lower compartments of the spectrophotometer. A baseline was obtained by scanning the spectrum from 700 to 430nm. To establish that cells in the upper cuvette were oxidized, 0.1 ml of a 0.3% solution of hydrogen peroxide was added to the lower cuvette, while 0.1 ml of distilled water was added to the upper cuvette (the latter addition being made to maintain an equal concentration of cells in both the upper and lower cuvettes). When the spectrum was again scanned from 700 to 430nm and the resulting baseline was identical to that obtained prior to addition of the hydrogen peroxide, it was assumed that the upper cuvette did indeed contain oxidized cells. An amount of 0.1 ml of a 10% solution of the substrate (sugar or acid) or 0.1 ml of a 0.2% solution when the substrate was an amino acid, was then added to the upper cuvette to produce the reduced/oxidized difference spectra, the concentration of the cell suspension in the bottom cuvette having been adjusted appropriately by the addition of 0.1 ml of distilled water. Repeated, consecutive scanning (7 to 15 times) of the difference spectrum was carried out from 700 to 430nm for approximately 30 minutes.

ii. Cells grown with excess carbon source:

In certain cases when cells were grown on an excess of carbon source and harvested during log phase (see Table XVI), the reduced/oxidized difference spectra were obtained by addition of 0.1 ml of a 0.3% solution of hydrogen peroxide to the bottom cuvette. The concentration of the cell suspension in the upper cuvette was adjusted by addition of 0.1 ml of

distilled water. The spectrum was scanned repeatedly as in METHODS V, i.

VI. Enzyme Assays

Enzyme activities were determined on freshly prepared crude sonic extracts (see METHODS II, iv) using a Cary 15 spectrophotometer (direct scale). The reaction mixtures were kept in a 25° water bath. The cell extracts were placed in ice. The specific activities, designated as units of enzyme / mg of protein, were obtained from several enzyme assays and the average value taken.

i. Fumarase:

Fumarase (EC 4.2.1.2) activity was determined using the method of Hanson and Cox (1967). Cell extract, 0.025 ml, (diluted when necessary to contain 50-250 µg of protein) was added to 0.975 ml of reaction mixture consisting of 50 µmoles potassium phosphate buffer (pH 7.2) and 15 µmoles L-malic acid. The rate of formation of fumarate from L-malate was followed at a wavelength of 240nm using a blank of 0.975 ml distilled water mixed with 0.025 ml cell extract. A unit of activity is defined as ΔA_{240} of 0.001 / min.

ii. Aconitase:

Aconitase (EC 4.2.1.3) activity was determined as outlined by Hanson and Cox (1967). Cell extract, 0.025 ml, (50-250 µg protein) was added to 0.975 ml of reaction mixture containing 50 µmoles potassium phosphate buffer (pH 7.2) and 15 µmoles sodium DL-isocitrate. The rate of formation of cis-aconitic acid from isocitrate was followed at a wavelength of 240nm against a buffer-cell extract blank. A unit of activity is defined as ΔA_{240} of 0.001 / min.

iii. Glucose 6-phosphate dehydrogenase:

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was determined by measuring the rate of reduction of $NADP^+$ by glucose 6-phosphate at 340nm. The reaction mixture contained the following in 1.0 ml: glucose 6-phosphate, 5 µmoles; $MgCl_2$, 10 µmoles; $NADP^+$, 0.41 µmoles; glycylglycine buffer (pH 7.5), 50 µmoles; crude cell extract (100-500 µg protein). A unit of activity is defined as that amount of enzyme effecting the formation of 1 nmole of NADPH / min.

iv. Glucokinase:

Glucokinase (EC 2.7.1.2) was determined by measuring the rate of reduction of $NADP^+$ in a glucose 6-phosphate dehydrogenase-coupled reaction system at 340nm. The reaction mixture contained the following in 1.0 ml: tris-HCl buffer (pH 7.0), 100 µmoles; glucose, 4 µmoles; ATP, 2 µmoles; $MgSO_4$, 4.5 µmoles; $NADP^+$, 420 nmoles; glucose 6-phosphate dehydrogenase, 1 unit; crude cell extract (100-500 µg protein). A unit of activity is equivalent to the formation of 1 nmole of NADPH / min.

v. Isocitric dehydrogenase:

Isocitric dehydrogenase (EC 1.1.1.42) was determined by measuring the rate of reduction of $NADP^+$ by isocitrate at 340nm. The reaction mixture contained the following in 1.0 ml: tris-HCl buffer (pH 7.5), 50 µmoles; $MgCl_2.6H_2O$, 10 µmoles; sodium DL-isocitrate, 10 µmoles; $NADP^+$, 0.41 µmoles; crude cell extract (100-500 µg protein). A unit of activity is defined as that amount of enzyme forming 1 nmole NADPH / min.

VII. Attempts to Obtain a Peak at 503nm in Crude Cell Extracts or Permeabilized Cells

The following methods were used in attempts to prepare cell extracts or to increase cell permeability so that P503 might be studied directly: (i) ultrasonication; (ii) French press; (iii) EDTA-tris- HCl; (iv) toluene; (v) lysozyme; (vi) warming; (vii) heat denaturation of proteins. Since a 503nm peak could not be detected following any one of these treatments, no details of methods will be given here.

The previously listed techniques proved to be too harsh for the survival of the 503nm peak; therefore, the lability of P503 was tested by subjecting the cells to freezing and thawing as follows: 5 ml portions of the final air-oxidized cell suspensions were pipetted into 3 separate test-tubes. The samples were quick-frozen in dry ice-ethanol and subsequently quick-thawed in a 37° water bath. The 3 test-tubes were thus treated 1, 3 and 5 times consecutively, followed immediately by a spectral analysis. Although the cells remained oxidized even after 5 consecutive exposures to freezing and thawing, reduction by glucose elicited the control heights of only the cytochrome b and flavin bands. Since the 503nm peak could not be detected after this extremely mild treatment, further efforts to obtain this peak in permeabilized cells or in crude cell extracts by an alternate treatment of whole cells were abandoned. See PART C: I, DISCUSSION.

PART A: Yield of Cell Protein from Glucose

RESULTS

I. Amount of Protein Formed from Glucose by Cells Growing Aerobically

When grown on minimal salts and limited glucose under aerobic conditions, the streptomycin (Sm)-dependent mutant of E. coli B was found to produce 35% less cell weight than the wild-type organism (Coukell and Polglase, 1969). A proportional difference was observed when the efficiencies (µg/ml increase in cellular protein per µg/ml glucose consumed) were determined for the Sm-dependent and wild-type cultures of E. coli strains B, CRX, K12 and UL (Table I). The per cent decrease in efficiency ranged from 24.3 to 37.6. A non-dependent revertant (SBr4), derived from the Sm-dependent mutant of E. coli B, showed a 42.7% decrease, slightly less efficient than its parent.

The corresponding Sm-resistant (indifferent) mutants of these strains grown on minimal salts-limiting glucose medium ($\pm$ dihydrostreptomycin) did not show a consistent pattern of efficiency with respect to the wild-type organism (Table II), but rather a different one for each strain. In addition, the presence or absence of dihydrostreptomycin did not affect all strains in the same manner.

II. Amount of Protein Formed from Glucose by Cells Growing Anaerobically

When the wild-type and Sm-dependent mutant of E. coli strains B and CRX were grown on minimal salts-limited glucose medium under an anaerobic nitrogen atmosphere, both strains of organisms were found to have the same decreased protein yield relative to aerobic growth of approximately 0.10 (Table III). Figure 2 further emphasizes the effect of changing

Table I. Protein yields from glucose for wild-type and streptomycin (Sm)-dependent (and non-dependent revertant) mutant of Escherichia coli strains B, CRX, K12 and UL grown aerobically

	E*			Decrease in E (%)**	
Strain	wild-type	Sm-dependent	non-dependent revertant+	Sm-dependent	non-dependent revertant
B	0.314	0.196	0.180	37.6	42.7
CRX	0.292	0.200	-	31.5	-
K12	0.289	0.210	-	27.3	-
UL	0.300	0.227	-	24.3	-

*E is the efficiency of growth, defined as the μg/ml increase in protein per μg/ml glucose consumed. Values of E are the averages of several experiments.

**Per cent decrease in E was calculated using the wild-type values as reference in each case.

+Non-dependent revertant (SBr4) is a sensitive strain derived from Sm-dependent E. coli B.

Table II. Protein yields from glucose for Sm-resistant (indifferent) mutants (± dihydrostreptomycin, DHSm) of E. coli strains B, CRX, K12 and UL grown aerobically

Strain	E*		Decrease in E (%)**	
	Sm-resistant (-DHSm)	Sm-resistant (+DHSm)	Sm-resistant (-DHSm)	Sm-resistant (+DHSm)
B	0.216	0.183	31.2	41.8
CRX	0.205	0.230	29.8	21.2
K12	0.255	0.248	11.8	14.2
UL	0.260	0.225	13.3	25.0

*E is the efficiency of growth as defined in Table I.

**Per cent decrease in E was calculated using wild-type values of Table I as reference in each case.

Table III. Protein yields from glucose for wild-type and Sm-dependent strains of E. coli B and CRX during aerobic and anaerobic growth

Strain		E^*	
		Aerobic	Anaerobic
B:	wild-type	0.314	0.111
	Sm-dependent	0.196	0.100
CRX:	wild-type	0.292	0.114
	Sm-dependent	0.200	0.110

*E is the efficiency of growth as defined in Table I. Values are the averages of several experiments.

Log-phase cells were harvested, washed, and oxidized in air (see METHODS). The cells were resuspended in minimal salts-glucose (0.065%) medium and grown aerobically. When the A_{420} reached 0.40, the culture was transferred to anaerobic conditions as described under METHODS. Samples were removed throughout both the aerobic and anaerobic growth periods for glucose and protein determinations.

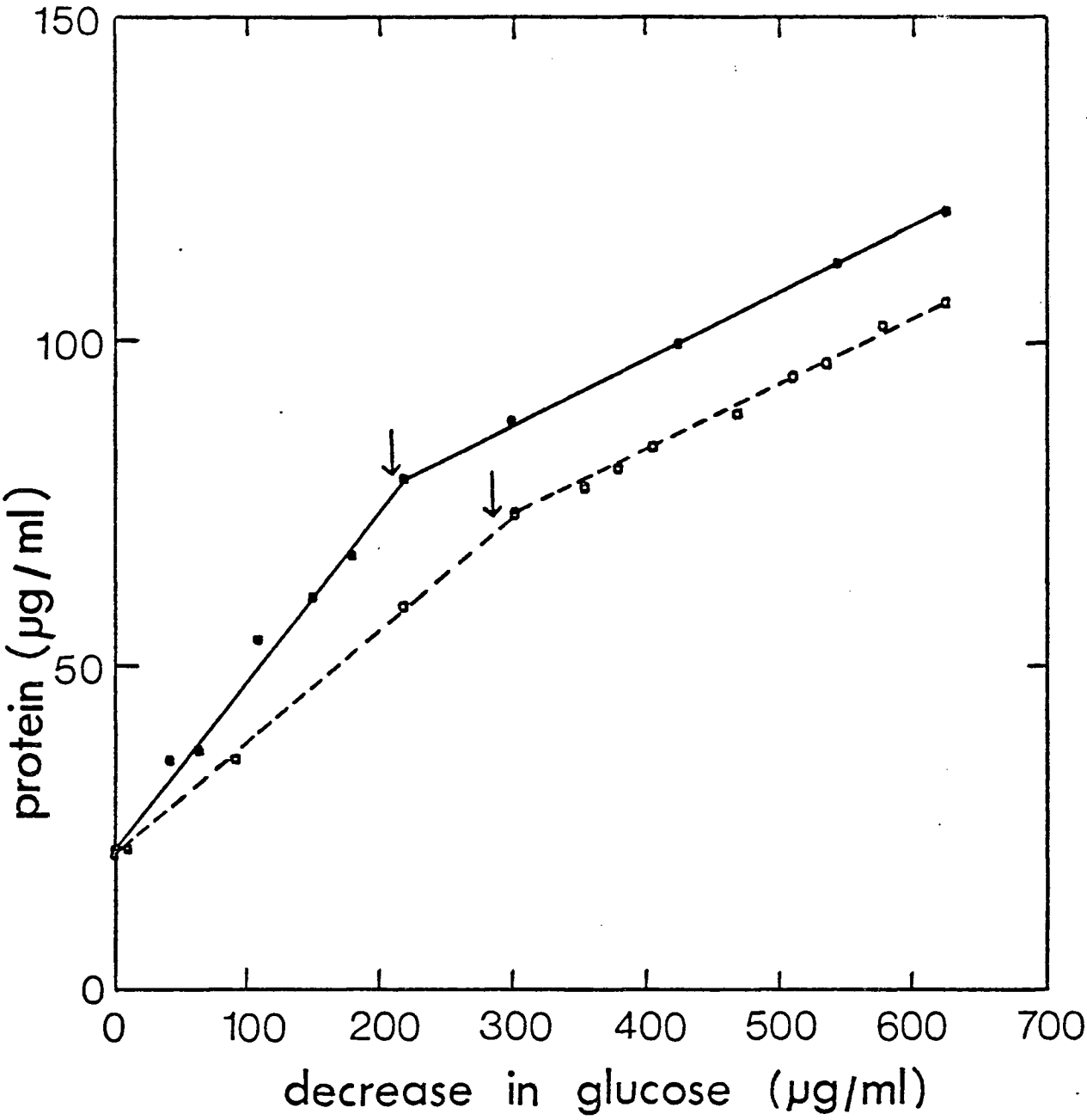


Figure 2. Yield of cell protein from glucose for wild-type E. coli B (●—●) and the streptomycin-dependent mutant (○- - -○). The initial concentration of glucose was 650 µg/ml. After a preliminary period of aerobic growth, the atmosphere was changed (at the arrows) to N_2 gas. Samples of culture were removed at intervals throughout the aerobic and anaerobic stages for protein and glucose determinations. Experimental details are given in the METHODS.

E. coli B from aerobic to anaerobic growth conditions. The slopes obtained aerobically of the wild-type and Sm-dependent mutant are divergent while the slopes obtained anaerobically are parallel.

III. The "Aerobic Increment" of Protein Yield from Glucose

Since the difference in efficiency of growth between the Sm-dependent and wild-type organisms was observed only under aerobic conditions, the "aerobic increments" were compared in the two cases (Table IV). The aerobic protein yield for Sm-dependent E. coli B exceeded the anaerobic yield by an increment of 0.096. For wild-type cells, the aerobic increment was 0.203, approximately twice the value of the Sm-dependent mutant.

DISCUSSION

All four Sm-dependent E. coli strains tested resulted in a similar pattern of 25-35% decreased aerobic growth efficiency compared to their corresponding wild-type parent. Under anaerobic conditions, however, both cell types produced identical protein yields. The metabolic deficiency of the Sm-dependent mutants therefore implicated an aerobic energy-yielding process. In view of these findings, it was of interest to calculate the difference in the "aerobic" portion of growth for the two types of organisms. From Table IV, it can be seen that the ratio of the aerobic increments of wild-type to Sm-dependent E. coli B is 2.11, indicating that the former possesses the ability of producing double the aerobic energy of the latter.

Several Sm-dependent strains had previously been characterized in

Table IV. Calculation of "aerobic increment" of protein yield for the wild-type and Sm-dependent strains of *E. coli* B

	μg/ml protein increase / μg/ml glucose consumed		
Strain	Aerobic	Anaerobic	Increment
wild-type	0.314	0.111	0.203
Sm-dependent	0.196	0.100	0.096

$$\text{Ratio of "aerobic increments"} \quad \frac{\text{wild-type}}{\text{Sm-dependent}} = 2.11$$

The aerobic increment is defined as the difference in protein yield obtained from cells grown aerobically versus anaerobically on glucose.

this laboratory and were found to have the following properties in common: (1) an impairment in their aerobic energy metabolism (Coukell, 1969); (2) excretion of valine into the medium when grown on glucose (Bragg and Polglase, 1962; Tirunarayanan, Vischer and Renner, 1962); (3) de-repression of the catabolite repressible enzymes, citrate synthase, fumarase and aconitase (Coukell, 1969).

In order to understand the reason for this difference in energy yield, it was necessary to choose which direction further studies would follow- substrate level energy-yielding reactions, or hydrogen metabolism. For the following reasons, hydrogen metabolism seemed more appropriate: (1) Since the anaerobic energy yields on limiting glucose were the same for the wild-type and Sm-dependent organisms, it was unlikely that the impairment was at the substrate level; (2) The cells were grown not on an excess, but rather a limiting amount of glucose, so that intermediate metabolites would not be expected to accumulate to the extent that would be expected when an excess of substrate is present; (3) Sm-dependent cells grown aerobically on limiting succinate as carbon source also resulted in a decreased yield of cells when compared to the wild-type. Further investigations were therefore focussed on the metabolism of hydrogen.

PART B: Difference Spectra

RESULTS

The mechanism underlying the production of a peak at 503nm may involve reduction of the pigment by a substrate or another process (such as ligand formation between some compound and P503). However, since the cell suspensions were air-oxidized initially and the difference spectra obtained by addition of a substrate to the top cuvette (while the bottom reference cuvette contained cells oxidized by hydrogen peroxide), the appearance of the 503nm peak was interpreted as being the result of reduction of the pigment (P503) by substrate.

When the reduced/oxidized difference spectra of the wild-type, Sm-dependent, and non-dependent revertant of E. coli B were studied (Figure 3), the usual complement of cytochromes was found to be present- the cytochromes a (600-650nm), cytochrome b (560nm), and the flavin trough (460nm). (An explanation for the nomenclature of the pigments is given in METHODS V). But in addition, the wild-type strain had a very large, symmetrical peak at 503nm which was missing or negligible in both the dependent and revertant mutants. Similarly the wild-type strains of E. coli CRX and K12 possessed the 503nm pigment, while the corresponding Sm-dependent mutants showed only a trace.

Measurements of the peaks (or trough) are tabulated in Table V. For purposes of comparison, the cytochromes b of all strains were normalized to that of wild-type E. coli B (x 1000). By calculating the ratios of heights of peaks (or trough) in the wild-type to the Sm-dependent mutant, it is seen that most of the values do not deviate

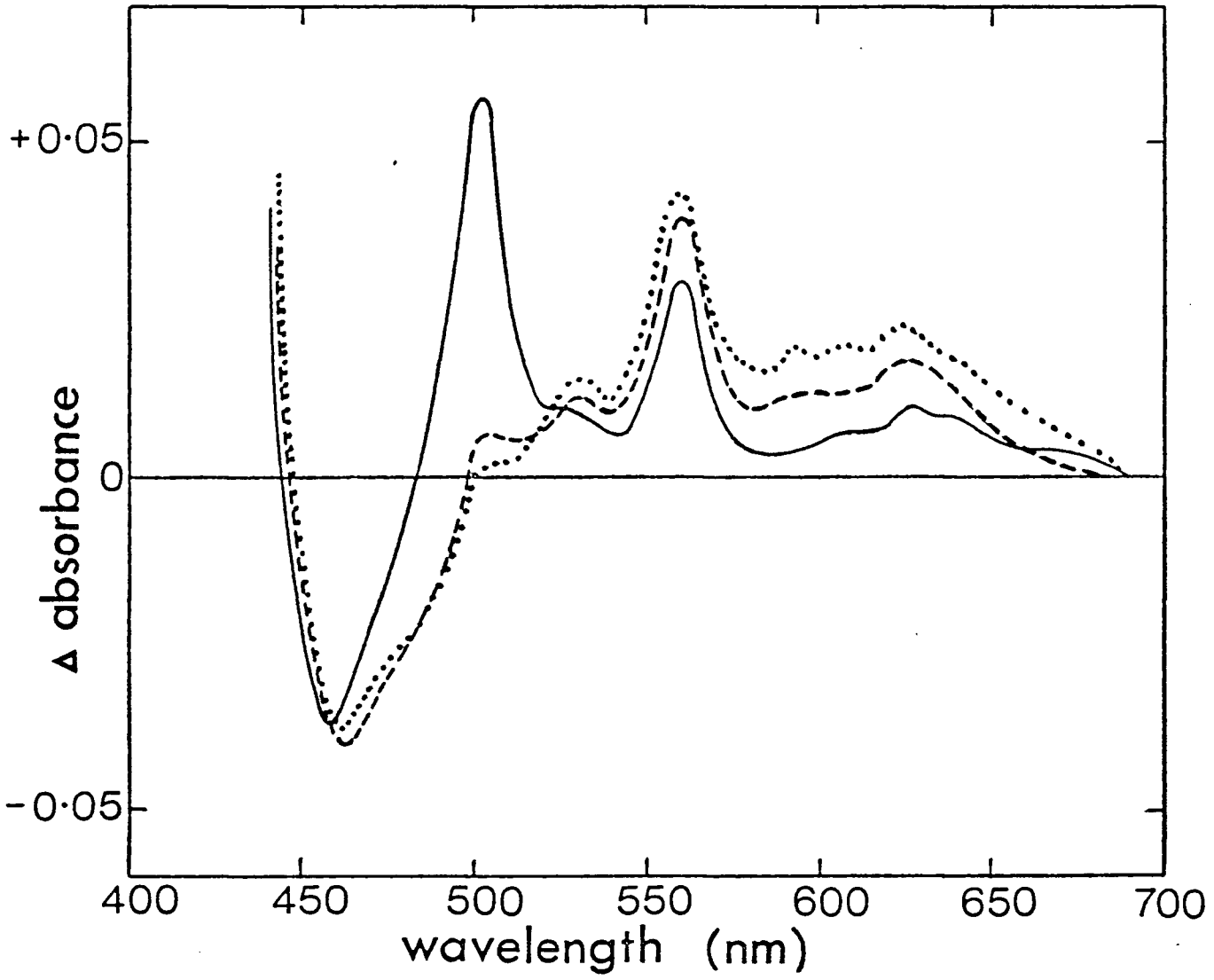


Figure 3. Reduced/oxidized difference spectra after addition of glucose to air-oxidized cell suspensions of E. coli B. Cells were grown on minimal salts medium and prepared as described under METHODS. ——— , wild-type; – – – , Sm-dependent; ····· , non-dependent revertant (SBr4).

Table V. Height (or depth) of pigment peaks (or trough) obtained from the reduced/oxidized difference spectra of wild-type (S) and Sm-dependent (D) (or non-dependent revertant, SBr4) strains of E. coli B, CRX, and K12

Strain	B					CRX			K12		
	SB (a)	DB (b)	SBr4 (c)	ratio a/b	ratio a/c	SCRX (d)	DCRX (e)	ratio d/e	SK12 (f)	DK12 (g)	ratio f/g
a cytochromes (600-650nm)	9.9	13.4	15.4	0.74	0.64	9.3	10.4	0.90	12.1	11.7	1.03
cytochrome b (560nm)	29.2*	29.2	29.2	1.00	1.00	29.2	29.2	1.00	29.2	29.2	1.00
P503 (503nm)	55.8	4.9	1.2	11.40	46.60	26.4	1.0	26.40	15.6	3.6	4.33
flavin trough (about 460nm)	-36.0**	-30.2	-25.8	1.19	1.44	-51.8	-51.5	1.01	-21.0	-29.6	0.71

*The value for cytochrome b in wild-type E. coli B was used as reference to standardize the cytochrome b of all the other strains.

**A minus sign (-) indicates a trough in the reduced/oxidized difference spectrum (see Figure 3).

from 1.00 to the same extent as that found in the case of the 503nm pigment. The height of the P503 peak in wild-type E. coli B exceeds that found in the Sm-dependent organism by a factor of 11.40, and that in the non-dependent revertant by 46.60. The ratio of P503 peak heights in wild-type versus Sm-dependent E. coli varied with the strain.

The situation is not as clear-cut for the Sm-resistant (indifferent) organisms of the same E. coli strains (Table VI). No consistent pattern emerges for the presence or absence of a 503nm peak, either with or without dihydrostreptomycin. The P503 peak, when present, was more persistent (less transient) than in the wild-type strains. A comparison between Tables II and VI indicates a correlation, nevertheless, of decreased efficiency accompanied by a substantial decrease in height of the 503nm pigment.

It should be mentioned at this point that the 503nm pigment is transient in the wild-type organism, and its decrease and eventual disappearance can be followed with time. Under the same conditions, the cytochromes and flavin trough remain unchanged in the reduced/oxidized difference spectra. Addition of more glucose after the disappearance of the 503nm band does not cause the subsequent re-appearance of this peak. The reversible nature of the reduction-oxidation of P503 was shown by air-oxidizing the cells after glucose reduction had produced the difference spectra. Reduced P503 underwent re-oxidation during aeration again of the cells, and could be reduced a second time by addition of glucose.

Table VI. Height (or depth) of pigment peaks (or trough) obtained from the reduced/oxidized difference spectra of wild-type (S) and Sm-resistant (R) (±DHSm) strains of E. coli B, CRX, and K12 (The same format is used as in Table V)

Strain	B			CRX			K12		
	SB	RB (-DHSm)	RB (+DHSm)	SCRX	RCRX (-DHSm)	RCRX (+DHSm)	SK12	RK12 (-DHSm)	RK12 (+DHSm)
a cytochromes (600-650nm)	9.9	10.3	11.8	9.3	8.7	13.2	12.1	4.7	5.2
cytochrome b (560nm)	29.2	29.2	29.2	29.2	29.2	29.2	29.2	29.2	29.2
P503 (503nm)	55.8	11.3	15.8	26.4	0.0	6.1	15.6	8.5	9.2
flavin trough (about 460nm)	-36.0	-39.2	-37.4	-51.8	-48.0	-51.2	-21.0	-56.4	-54.3

DISCUSSION

In all comparisons between the wild-type and Sm-dependent strains, only the P503 band was found to differ significantly while the cytochrome and flavin bands remained fairly constant. These results suggested that the lack of P503 (or its decrease) and impaired energy metabolism observed in Sm-dependent mutants of *E. coli* (as well as in the non-dependent revertant) were related phenomena.

The 503nm pigment could not have been a precursor of another component in the reduced/oxidized difference spectra since its disappearance with time did not affect the size of other peaks (or trough). In fact, re-runs of the spectra resulted in perfectly superimposable peaks with the exception of the 503nm band. The property of reversible reduction-oxidation shown to be inherent in P503 would imply its participation in an electron transport system.

Wild-type *E. coli* B was used in subsequent detailed studies of P503 since appropriate conditions had already been worked out for the consistent appearance of a large and symmetrical peak at 503nm.

PART C: Characterization of P503

I. Attempts to Isolate P503

RESULTS

The 503nm peak was not detectable in cells which had been treated as stated in METHODS VII (i.e., disrupted, permeabilized or frozen-thawed cells).

DISCUSSION

The evidence thus far has only indirectly implicated a relationship between P503 and energy metabolism. It would have been advantageous at this point to study the characteristics of P503 directly in crude cell extracts or in permeabilized cells. Several compounds which otherwise do not penetrate the E. coli cell membrane could then be added to verify or disprove our theory. In addition, if P503 proved to be stable upon cell disruption, one might succeed in its isolation, purification, and structural characterization.

In 1964, Lindenmayer and Smith tried disrupting yeast cells but found the 503nm pigment to be very labile. Similarly, our endeavours proved to be futile. The reason for the disappearance of the 503nm band is not known. It may be that the cofactor(s) necessary for its stability is(are) diluted out upon cell permeabilization or rupture. Neu, Ashman and Price (1967) found that EDTA treatment released large molecular weight substances such as nucleotides into the medium. Another possibility may be that disruption of the cells changes the stable conformation of the pigment. Whether the structure of the pigment is

destroyed, or changed to a non-absorbing form is also unknown. If the structure of P503 were similar to protoporphomethene (Labbe, Volland and Chaix, 1967), then its absorption and fluorescence in visible light could depend on the resonating character of the conjugated double bonds, as is the case for porphyrins (Mauzerall, 1962). When the double bonds of the methylene bridges are reduced, the porhyrins are converted to colourless porphyrinogens.

In view of the extreme lability of P503, our emphasis was shifted to a more productive approach, and further investigations involved the characterization of P503 in whole cells.

II. Production of the 503nm Peak

1. Effect of sugars and acids on production of the 503nm peak:

RESULTS

In wild-type E. coli B cells containing P503, various sugars and acids were tested for their ability to produce the 503nm peak. Figure 4 shows the sequential effect of gluconate addition. It requires approximately 2 minutes to scan the spectrum from 700-430nm. Repeated consecutive scanning of the difference spectrum showed initially only a large peak at 503nm. Subsequently, flavin and cytochrome b were reduced to their steady-state levels. Figure 5 shows the effect of succinate as substrate under the same conditions. Whereas the flavin followed by cytochrome b was reduced immediately, only a trace of P503 was detected after the appearance of the other pigments. In contrast, reduction by glucose (as control) elicited the entire spectrum on the first run.

Other compounds as reductants are shown in Table VII for wild-type E. coli B and B-SG1. Strain B-SG1 is incapable of synthesizing glycogen (Preiss and Greenberg, 1965; Harvey, 1970), so that all substrates added would be metabolized and none would be stored as glycogen for later use. Any difference, then, between E. coli B and B-SG1 might reflect the utilization of another metabolic route after back-reaction (glycogen synthesis) has occurred. However, since both strains were similarly affected by the substrates, one can assume that the added compounds were directly metabolized. Reduction by glucose produced a high 503nm peak. With gluconate as reductant, the peak of P503 was slightly smaller. Glycerol and acetate did not elicit a P503 band. With succinate and

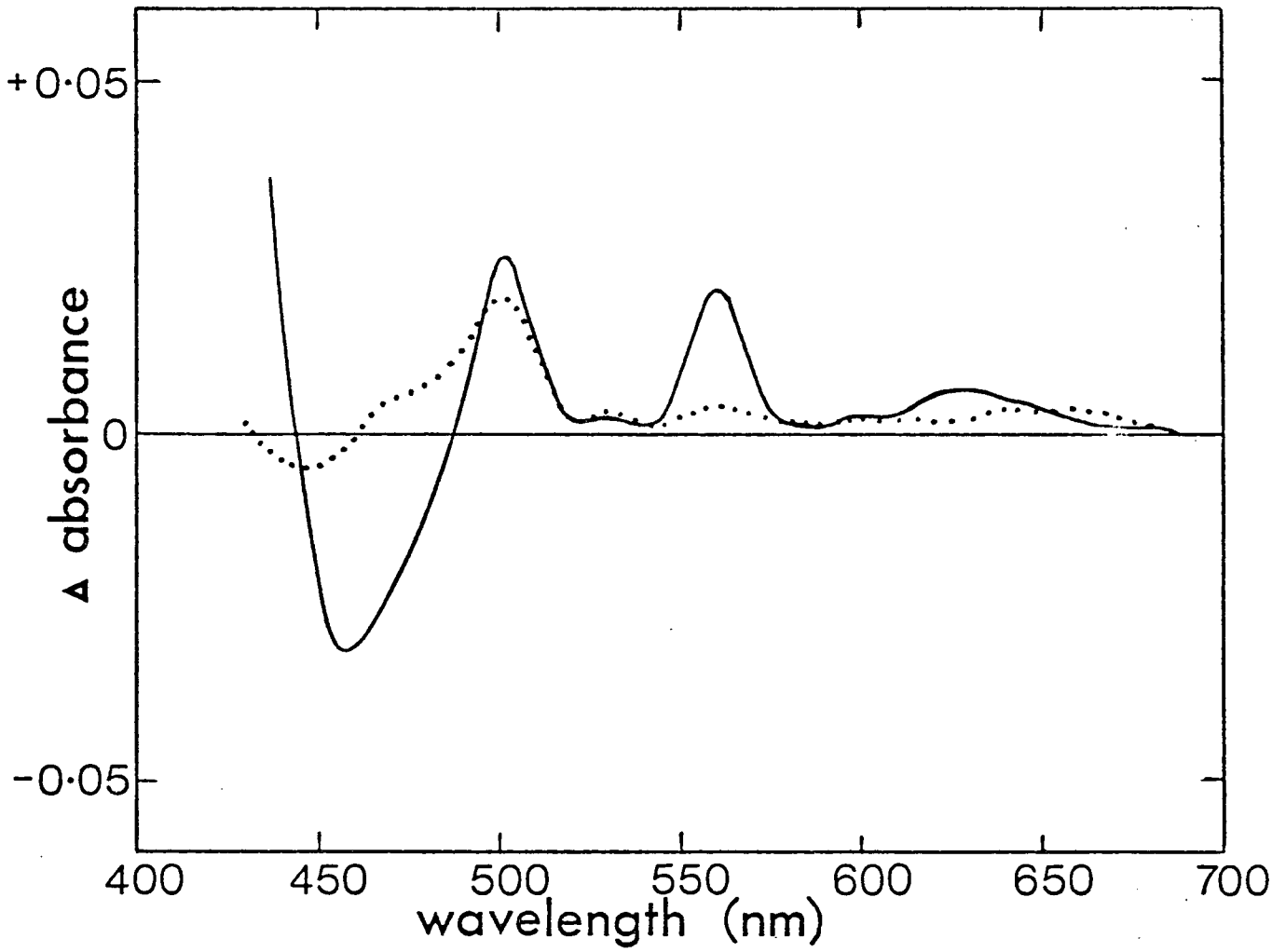


Figure 4. Reduced/oxidized difference spectra after addition of gluconate to air-oxidized suspension of wild-type E. coli B: ····, after 2 minutes; ——, after 5.2 minutes.

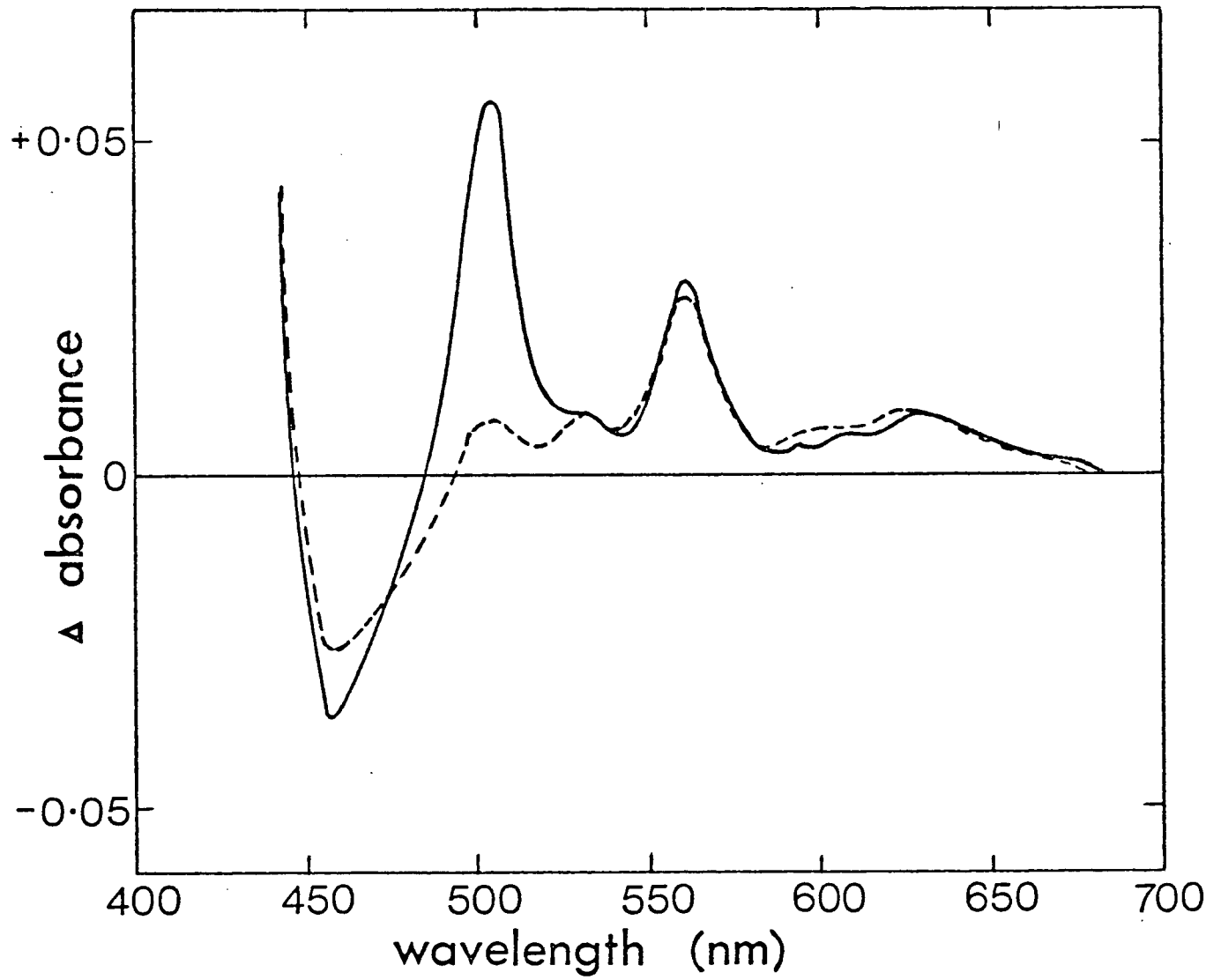


Figure 5. Reduced/oxidized difference spectra after addition of glucose (——) or succinate (- - -) to air-oxidized suspensions of wild-type E. coli B.

Table VII. Effect of various sugars and acids on production of the 503nm peak in wild-type E. coli B and B-SG1

Strain	Sugar or acid	Height of 503nm peak* (units)	Order of appearance of 503nm peak with respect to cytochrome peaks
B	gluconate	43.8	first
	glucose	55.8	simultaneous
	glycerol	0.0**	A
	succinate	4.3**	after
	lactate	2.6**	simultaneous
	acetate	0.0**	A
B-SG1	gluconate	35.9	first
	glucose	56.6	simultaneous
	succinate	3.4**	after
	lactate	2.9**	simultaneous
	acetate	0.0**	A

*In each case, the height of cytochrome b was normalized to the control value (29.2 units) obtained via reduction by glucose (see Table V).

**The 503nm peak was small or absent after 10-15 minutes. Subsequent reduction by glucose resulted in a large peak at 503nm.

A- The 503nm peak was absent although the cytochromes were present.

Cells containing P503 were grown on minimal salts-glucose (0.04%) medium, harvested, washed once in phosphate buffer, and air-oxidized for 1 hr prior to analysis of the difference spectrum. Various sugars and acids were tested as indicated for their ability to reduce the 503nm pigment.

lactate reduction of the cells, P503 was hardly detectable in both strains. After allowing 10-15 minutes to elapse following the addition of glycerol, succinate, lactate, or acetate, glucose was added. A large peak at 503nm appeared rapidly, indicating that the cells indeed possessed P503, but that the substrates added initially were unable to cause the immediate appearance of its spectrum.

DISCUSSION

There appears to be a high level of specificity involved in reduction to produce a peak at 503nm. If the absorbance peaks or trough obtained 5.2 minutes after the addition of gluconate are considered to represent steady-state reduction levels of 100%, then gluconate as reductant (involving $NADP^+$ as the first electron acceptor) caused the rapid appearance of 76% of the 503nm peak within the first 2 minutes; in contrast, only 19% of the cytochrome b peak and 14% of the flavin trough were observed. The situation with succinate as reductant (involving flavin as the first electron acceptor) showed different absorption characteristics. Whereas the cytochrome b and flavin bands appeared immediately, the 503nm band, observed only after several scans through the spectrum, was small compared to the height elicited by glucose (control). These results suggest that the oxidation-reduction potential of P503 is lower (more negative) than that of cytochrome b or flavin, and that the 503nm pigment accepts electrons at the level of pyridine nucleotide (NADPH).

Thirty per cent of the glucose and gluconate, taken up by phosphorylation via the phosphoenolpyruvate phosphotransferase system (Simoni, Levinthal, Kundig, Kundig and Roseman, 1967; Fraenkel, 1968;

Roseman, 1969), follow the hexosemonophosphate shunt (Model and Rittenberg, 1967; Wang et al., 1958). In this pathway, glucose would give a maximum yield of 2 molecules of NADPH per molecule of glucose, while gluconate would yield only 1 molecule of NADPH per molecule gluconate. The steady-state level of NADPH might be lower and less rapidly attained with gluconate so that the extent of reduction to form the 503nm peak would be correspondingly less.

Succinate has been shown to be transported in E. coli by a highly specific inducible system (Kay and Kornberg, 1971). Since there was no accumulation of the dicarboxylic acid concomitant with its uptake from the medium, these workers concluded that the energy required for this translocation was supplied by its rapid removal, through oxidative metabolism, subsequent to its entry into the cells. Its immediate oxidation to fumarate is linked to a flavin coenzyme.

As discussed later (when the nutritional aspects concerning P503 formation are considered), glycerol enters the metabolic scheme at the level of dihydroxyacetone phosphate; being uncharged, it was thought initially to freely penetrate bacterial cells (Packer and Perry, 1961; Koch, Hayashi and Lin, 1964) before being rapidly metabolized. More recent investigations (Sanno, Wilson and Lin, 1968; Berman and Lin, 1971) have established that entry of glycerol into E. coli is not by simple diffusion but rather by facilitated diffusion whereby a specific membrane carrier (permease) catalyzes the equilibrium of intracellular and extracellular substrate concentrations without energy coupling. Lactate is converted to pyruvate via the NAD^+-dependent lactic dehydrogenase (Kline and Mahler, 1965) and further to phosphoenolpyruvate via phosphoenolpyruvate synthase (Kornberg and Smith, 1967; Cooper and Kornberg, 1967).

The former group found pyruvate transport to be controlled by a gene (or genes) in E. coli, but noted that regulation of uptake for pyruvate was not the same as for lactate. The entrance of lactate into cells is most likely unhindered by the membrane. The cells are freely permeable to acetate. The C_2 compound as sole carbon source results in the induction of the glyoxylate bypass for its dissimilation (Kornberg, 1966). However, this anaplerotic pathway is repressed during growth on glucose (Holms and Bennett, 1971), so that subsequent oxidation of acetate occurs by operation of the TCA cycle. This was the experimental condition used in our experiments. The three compounds- glycerol, lactate, and acetate, entering the metabolic path at a lower level and presumably not generating high concentrations of NADPH during their breakdown or further metabolism would not be expected to produce a large peak at 503nm as shown by the results.

2. Effect of L-amino acids on production of the 503nm peak

RESULTS

The specificity involved in the appearance of the 503nm peak caused by sugars and acids prompted an investigation of the effect of L-amino acids as reductants under similar conditions. The results of a systematic analysis are shown in Table VIII. The L-amino acids listed from alanine to valine (added in solution to the cells) gave the absorption spectrum of P503, the cytochromes and flavin simultaneously after an initial lag period of 10-14 minutes. In each case, the 503nm peak was small compared to that of cytochrome b (29.2 units). Lysine produced no 503nm peak; after a 10 minute lag, the flavin and cytochromes were reduced. Histidine produced no spectrum at all, even after 20 minutes. Addition of L-methionine (Figure 6) or casein hydrolysate (which contains the 21 amino acids excluding tryptophan, glutamine and asparagine) resulted in the reduction of P503 within 0.9 minutes; the flavin and cytochromes were reduced after 10 minutes. Of the L-amino acids tested, only methionine and casein hydrolysate were able to cause immediate appearance of the peak at 503nm. The other amino acids had no significant effect on this pigment.

DISCUSSION

Wild-type *E. coli* is capable of synthesizing all the necessary amino acids when grown on a minimal salts-glucose medium, and is therefore independent of an exogenous supply of amino acids. However, when an amino acid is added to the growth medium, the organism utilizes the appropriate transport system(s) for its uptake. Piperno and Oxender (1968) reported four distinct transport systems to be operative in *E. coli* K12 for

Table VIII. Effect of L-amino acids on production of the 503nm peak in wild-type E. coli B (The format is used as in Table VII)

Amino acid	Height of 503nm peak* (units)	Order of appearance of 503nm peak with respect to cytochrome peaks
alanine	10.8	simultaneous
arginine	12.0	"
aspartic acid	9.0	"
glutamic acid	14.4	"
glycine	16.2	"
leucine	10.0	"
phenylalanine	9.5	"
proline	8.4	"
serine	7.3	"
tyrosine	7.8	"
valine	9.9	"
lysine	0.0	A*
histidine	-	- (no spectrum)
methionine	32.9	first
casein hydrolysate	29.5	first

*See Table VII.

Methionine and casein hydrolysate gave the absorption peak of P503 within 0.9 minutes, followed by the cytochromes after a 10 minute lag.

The L-amino acids from alanine to valine gave the absorption peaks of both P503 and the cytochromes simultaneously after a 10-14 minute lag.

Lysine gave no absorption peak at 503nm; the cytochromes were reduced after 10 minutes.

Histidine produced no spectrum at all.

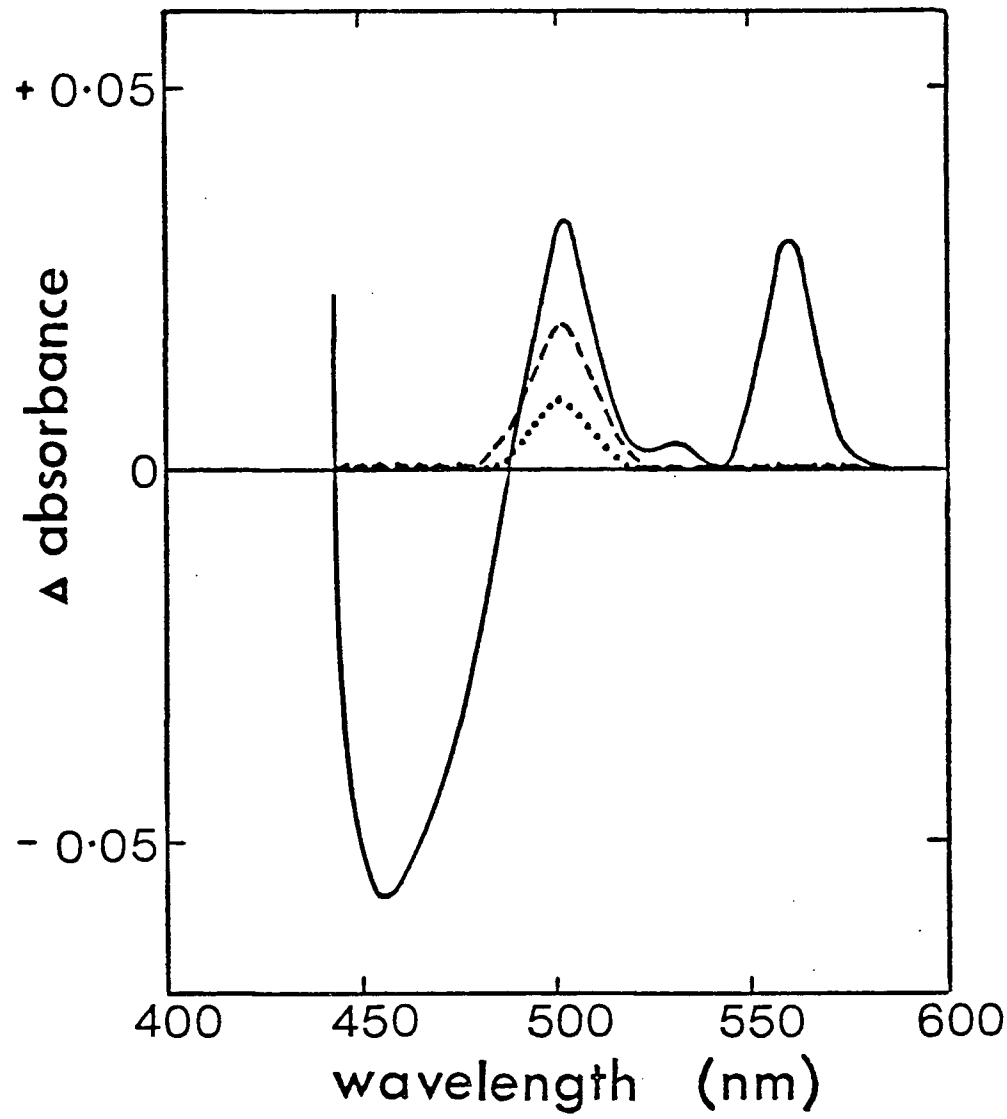


Figure 6. Reduced/oxidized difference spectra after addition of L-methionine to air-oxidized suspensions of wild-type E. coli B: ····, after 1 minute; – – –, after 6 minutes; ———, after 10 minutes.

neutral amino acid uptake, with very little overlap between them. Each system transported the following specific groups of amino acids: (i) leucine, isoleucine and valine; (ii) alanine, glycine and serine; (iii) phenylalanine, tyrosine and tryptophan; (iv) methionine. The only neutral amino acid found to have a specific transport system was L-methionine.

The addition of L-amino acids to whole cells of wild-type E. coli B resulted in a lag varying from 10-14 minutes prior to the appearance of the difference spectra. This delay might reflect the induction of the appropriate transport system and/or accumulation of the amino acid into pools prior to its metabolism. Ames (1964) reported the existence of specific transport systems in Salmonella typhimurium for histidine and some of the aromatic amino acids, in addition to a less specific system which could transport all the aromatic amino acids. The eventual reduction of the cytochromes (except in the case of histidine) indicated, then, that the cells were permeable to all the L-amino acids added, and that the latter were metabolized after their uptake into the cells. One might assume that histidine was accumulated but not metabolized, so that no reduction of the cytochromes occurred.

In general, the uptake of C^{14}-amino acids was found by Piperno and Oxender (1968) to be very rapid. Most of the radioactive label was taken up within 30 seconds, and steady-state levels were reached in 1-2 minutes. The amino acids, L-leucine and glycine, were transported four-fold faster than L-alanine and L-methionine. After 2 minutes, however, 98% of the radioactive L-isomers of leucine, isoleucine, phenylalanine and valine were still intact inside the cells; in contrast, only 50% of the radioactivity was recovered in glycine, L-alanine and

L-methionine. These workers concluded from the results that the former group of amino acids were not metabolized rapidly, while the latter group of amino acids underwent extensive metabolism.

Since the time for transporting L-methionine into E. coli cells is longer than for other amino acids, the relatively minute quantity that enters the cells within the first minute must be affecting the P503 system either prior to its metabolism or at an early stage in its metabolism.

Thus far, glucose, gluconate, and L-methionine have been found to cause the appearance of a 503nm band quickly. While glucose and gluconate also caused reduction of the cytochrome system fairly quickly, L-methionine behaved differently in that a long lag persisted prior to the appearance of the cytochromes. A plausible explanation can be found when one studies the metabolic fate of the three compounds. L-methionine is converted into S-adenosylmethionine at the expense of ATP. As stated earlier, glucose and gluconate are phosphorylated prior to their uptake by bacterial cells. The transport mechanism, well documented by several workers (Simoni, Levinthal, Kundig, Kundig and Roseman, 1967; Fraenkel, 1968; Roseman, 1969), has been shown to proceed via the phosphoenolpyruvate (PEP) phosphotransferase system. In both cases, PEP is produced at the expense of ATP (Roseman, 1969). In addition, the metabolism of these two sugars involves the early production of hydrogen atoms, resulting in the reduction of the cytochromes. This is not the case for L-methionine; hydrogen atoms are not produced immediately, and consequently cytochromes are not reduced until much later.

As speculated later, it is conceivable that NADPH is oxidized via P503 to generate ATP. Then the addition of methionine would lower the

ATP concentration and stimulate as a compensatory response, the formation of ATP via P503, thus generating the spectrum of this pigment. Under the conditions used for preparing cells, i.e., growth on limiting glucose, harvesting, and aeration in buffer for 1 hr at 37°, NADPH was found to be present at a concentration in excess of 0.20 μmoles of NADPH per gram of cell protein (Polglase, 1972).

3. Effect of L-methionine analogues on production of the 503nm peak

RESULTS

In view of the singular effect of L-methionine on eliciting a peak at 503nm, several of its analogues (Figure 10) were used to test the functional groups and stereospecificity of action (Table IX). The isomeric D-methionine behaved similarly to L-lysine, causing reduction of the flavin and cytochromes but not of P503 after 12 minutes. The analogues, L-ethionine, DL-norleucine, DL-methionine sulfone, L-methionine sulphoxide and DL-selenomethionine had the same effect as the majority of the other amino acids; only after a lag of 10 minutes did the entire difference spectra appear simultaneously with a 503nm band. N-acetyl L-methionine produced no spectrum at all. Addition of L-ethionine did not produce a peak at 503nm immediately. Addition of L-ethionine followed by L-methionine gave the same result as L-methionine alone. The effect of L-methionine on P503 was thus shown to be very specific. No substitutions or deletions in the sulphur or methyl moiety were allowable.

DISCUSSION

A survey of L-methionine analogues further emphasized the specificity of the effect of L-methionine on P503. A change in configuration from the L- to D-form, replacement of the methyl group with ethyl, oxidation of the sulphur moiety to sulfone or sulphoxide, omission of the sulphur and addition of methyl, substitution of sulphur with selenium- all resulted in analogues lacking the ability possessed by L-methionine to elicit a P503 peak immediately following its addition to the cells. Consequently, neither the sulphur nor the methyl group per se were the sole requirements; rather, the molecule as a whole seemed to be necessary. The appearance of the P503 peak before the cytochromes upon addition of L-ethionine followed by L-methionine

Table IX. Effect of L-methionine analogues on production of the 503nm peak in wild-type E. coli B (The same format is used as in Table VII)

Amino acid or analogue	Height of 503nm peak* (units)	Order of appearance of 503nm peak with respect to cytochrome peaks
L-methionine	32.9	first
D-methionine	0.0	A*
L-ethionine	29.2	simultaneous
DL-norleucine	29.2	"
DL-methionine sulfone	7.8	"
L-methionine sulfoxide	10.1	"
DL-selenomethionine	18.3	"
N-acetyl L-methionine	-	- (no spectrum)
**L-ethionine; then L-methionine	46.7	first

*See Table VII. D-methionine reduced the cytochromes after a 12 minute lag, but not the 503nm pigment.

L-ethionine reduced both the 503nm pigment and the cytochromes after a 10 minute lag.

**L-ethionine, followed by L-methionine, gave the same result as L-methionine alone.

N-acetyl L-methionine produced no spectrum at all.

indicated that this analogue was not effective as a competitor.

If two compounds share a common transport system, then the addition of one compound to the medium can increase the loss of a second compound previously accumulated (Wilbrandt and Rosenberg, 1961). Structural analogues can be useful in ascertaining the characteristics and diversity of transport systems present, by their ability to participate in this type of "counter-transport".

With the exception of the alanine-glycine-serine transport system, the other three systems for neutral amino acid uptake were found to be highly stereospecific (Piperno and Oxender, 1968). When E. coli cells were preloaded with C^{14}-L-methionine then washed with unlabelled L-methionine, 30% of the radioactivity was lost. This was not the situation, however, when the cells were washed with L-norleucine; 96% of the label remained within the cells. Likewise, the D-isomer of methionine and L-ethionine were tested and found to be transported by systems different from that for L-methionine. In Table IX then, the addition of L-methionine after L-ethionine would not have resulted in loss of any L-ethionine which had already been accumulated by the cells. As the results indicated, the presence of both L-methionine and its analogue did not alter the effect of the amino acid alone on P503. This point will be discussed further in the next section concerning growth of cells in the presence of both L-methionine and DL-ethionine (or DL-norleucine).

Properties of the various structural analogues of L-methionine and the recent research published in this area is briefly reviewed in the DISCUSSION section under Effect of L-methionine and its analogues as growth supplement on P503 formation (synthesis).

4. Effect of other compounds on production of the 503nm peak

RESULTS

The four compounds ascorbic acid, folic acid, betaine and 2-deoxy-D-glucose were tested for their ability to reduce the 503nm pigment by addition of 0.1 ml of a 0.2% solution to the top cuvette (as described under METHODS V, i). Ascorbic acid (sodium salt; Calbiochem) as reductant resulted in the simultaneous appearance of a small 503nm peak, the cytochrome bands and flavin trough. Folic acid (Sigma Chemical Co.) was also tested as a hydrogen donor but failed to produce a 503nm peak at all. Its metabolism caused the flavin and cytochrome b to become reduced after 10 minutes. The identical result (as folic acid) was obtained upon addition of betaine anhydrous powder to the upper cuvette. With 2-deoxy-D-glucose (20% aqueous solution from Sigma Chemical Co.) the entire spectrum was produced rapidly, similar to the metabolism of glucose except for a markedly smaller P503 band. As far as production of the 503nm peak was concerned then, these additional four compounds were not effective.

DISCUSSION

The ability of ascorbic acid to undergo reversible oxidation to the dehydro-form is well known; dehydroascorbic acid can in turn be reduced by glutathione. The vitamin is also known to donate electrons via N,N,N',N'-tetramethyl-p-phenylenediamine (Howland, 1963; Wilson and Brooks, 1970) or directly (Klingenberg, 1968) to cytochrome c in mitochondrial systems. The photoreduction of porphyrins was studied by Mauzerall (1962). The second stage of reduction involves the formation of tetrahydroporphyrin (porphomethene), having an absorption maximum

at 500nm. Ascorbic acid (pH 7.0) caused reduction of the porphyrin beyond the second stage to form hexahydroporphyrin or porphyrinogen (a colourless compound). This suggests that ascorbate may produce a more highly reduced (leuco) form of P503 than does glucose. Nosoh (1964) reported that reduction by ascorbate, cysteine, glutathione, potassium cyanide and dithionite could flatten out the 503nm peak. His difference spectra of substrate-treated cells minus nontreated growing cells showed a trough at 503nm with potassium cyanide, cysteine, and sodium dithionite ($Na_2S_2O_4$). Since a 503nm trough was also observed by Nosoh in the difference spectrum of "growing cells upon standing at 30° for 4 hours minus growing cells before standing", his results might be interpreted in the following manner: addition of cysteine, cyanide, and dithionite to the top cuvette resulted in reduction of P503 to its colourless form. The "growing cells" in the bottom cuvette were reduced so that a 503nm band was present, resulting in a 503nm trough in the difference spectrum. Similarly, the cells (in the top cuvette) left standing for 4 hours probably became depleted of endogenous substrate, while those cells (in the bottom cuvette) used immediately without standing still had a supply of substrate.

Since the tetrahydro-derivative of folic acid is a coenzyme involved in methylation reactions, the vitamin was used to study the possible involvement of methyl transfer with appearance of the P503 band. If the effect of L-methionine were due to the donation of its methyl group, then a compound such as betaine (possessing a labile methyl group and known to transmethylate homocysteine to form methionine in animals) might also produce a peak at 503nm. However, the

results with folic acid and betaine were both negative. The evidence appeared to be against participation of methyl transfer in production of the 503nm peak.

2-Deoxy-D-glucose was previously thought to undergo phosphorylation at carbon #6 (forming 2-deoxyglucose 6-phosphate at the expense of ATP) without being metabolized further. If the immediate effect of L-methionine were to utilize ATP in forming S-adenosylmethionine, thereby causing a decrease in the level of ATP and, in turn, stimulating the P503 pathway as a compensatory mechanism, one should observe the rapid appearance of a P503 peak upon addition of 2-deoxyglucose as well. This was not the case, however, and a trace of P503 appeared simultaneously with the flavin and cytochrome b. Contrary to expectations, 2-deoxyglucose was being metabolized. These rather confusing results were explained when it was noted that Dietz and Heppel (1971) reported that E. coli cells might be capable of metabolizing 2-deoxyglucose. These workers found that 2-deoxyglucose inhibited growth of E. coli and also caused temporary growth stasis when glycerol or succinate was the carbon source. In contrast, cells grown in the presence of gluconate or pyruvate resisted these effects of 2-deoxyglucose. This compound was unable to compete with glucose for entry into the cells, and consequently had no effect on growth when glucose was the source of carbon. It was also reported that 2-deoxyglucose inhibited phosphorylation and fermentation of glucose in lysozyme lysates (Dietz and Heppel, 1971) of E. coli B, but not in intact cells. In an earlier publication, Pogell, Maity, Frumkin and Shapiro (1966) studied the fate of ^{32}P-labelled 2-deoxyglucose 6-phosphate after it was taken up by E. coli cells. Since only 50% of the compound chromatographed similarly to unchanged 2-deoxyglucose-

6-phosphate, they concluded that the nature of the label gave no information on whether the 2-deoxyglucose was metabolized or not. Since phosphorylation by ATP was obviously not the sole reaction of 2-deoxyglucose, this compound did not provide a test of the hypothesis that appearance of the 503nm peak alone (as observed with methionine) might occur as a result of a change in the energy charge.

III. Formation (Synthesis) of P503

I. Effect of 2,4-dinitrophenol on formation of P503

RESULTS

2,4-Dinitrophenol (DNP) has been used extensively in studies of the uncoupling of oxidative phosphorylation in mitochondria since the first experimental observation of its action (Loomis and Lipman, 1948). Ideas regarding mechanism of action, still being investigated by numerous laboratories, appear to differ depending on which hypothesis (Mitchell's chemiosmotic hypothesis (Mitchell, 1961; 1967) or the chemical hypothesis (Boyer, 1968) implicating a phosphorylated high-energy protein intermediate or "coupling factor") is favoured for oxidative phosphorylation. This topic will not be dealt with in this thesis, since another possible reaction of DNP (namely, the reduction of its nitro group(s)) provides a more satisfactory explanation of the results. Stockdale and Selwyn (1971) reported that phenols have two effects- one on the coupling activity and the other on inhibition of respiration, the two phenomena depending on different properties of the phenol.

Varied effects of DNP in E. coli have been reported, depending on its concentration and the growth conditions. Thus, 1.3×10^{-3} M to 2.4×10^{-3} M DNP was found to inhibit respiration by 40-85% (Packer and Perry, 1961). A lower concentration, 10^{-4} M to 5×10^{-5} M DNP resulted in stimulation of respiration by 130% (Bovell, Packer and Helgerson, 1963). DNP has also been reported to inhibit energy-linked membrane processes such as uptake of substrate (Pavlasova and Harold, 1969; Kay and Kornberg, 1971; Pogell, Maity, Frumkin and Shapiro, 1966).

When wild-type E. coli B was grown aerobically on minimal salts-

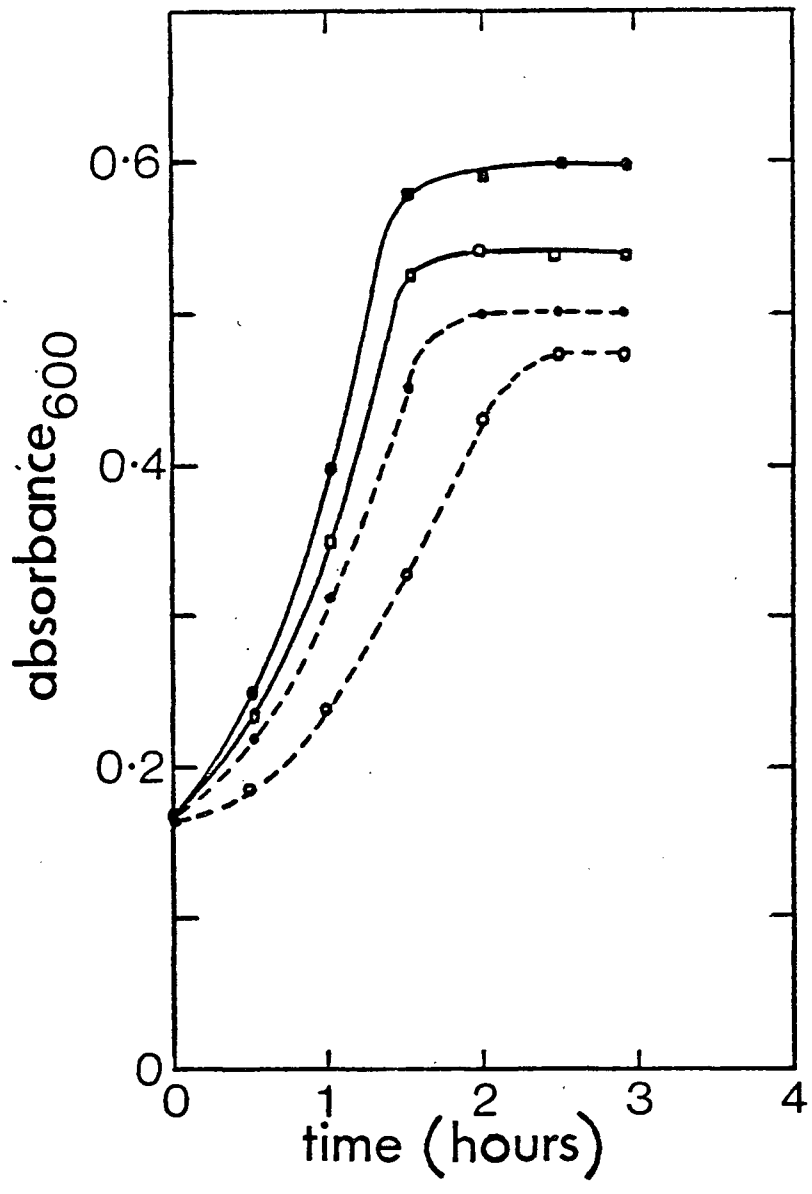


Figure 7. Growth of wild-type E. coli B on minimal salts-limiting glucose medium supplemented with various concentrations of 2,4-dinitrophenol: ●—●, none (control); □—□, 100 μM; ●- -●, 250 μM; ○- -○, 500 μM.

limiting glucose medium supplemented with DNP (Figure 7), the growth rate and yield of cell protein decreased with increasing concentration (100 μM to 500 μM) of the supplement. The increase in turbidity of cell cultures was followed at 600nm so that the yellow colour of the DNP-supplemented medium would not contribute to the absorbancy readings. Table X shows the decrease in efficiency from the control value, ranging from 4.1% at 10.0 μM DNP to 30.9% at 500 μM DNP. An increase in doubling time accompanied this decrease in growth efficiency. There was no growth of cells when DNP was present at 1000.0 μM. At a concentration of 500 μM DNP, the efficiency of growth was similar to that found for Sm-dependent E. coli B (Table 1). In addition, the doubling time of 87.5 minutes was in agreement with the value of 85 minutes obtained by Coukell (1969) for the Sm-dependent mutant.

In order to characterize this effect of DNP further, the growth efficiency was determined under anaerobic conditions (Table XI). The decrease in efficiency (from 0.314 to 0.251) observed when cells were grown aerobically was not seen when cells were grown under a nitrogen atmosphere. For both control and DNP-grown cells, the anaerobic efficiency was approximately 0.100. DNP was therefore affecting an aerobic (and not anaerobic) energy-yielding route. A difference in the effect of DNP under aerobic versus anaerobic conditions was also reported by Pavlasova and Harold (1969); they found that anaerobic generation of ATP in general was not inhibited by uncouplers, but that utilization of metabolic energy for the active transport of galactosides was prevented. A possibility was that DNP increased the permeability of the membrane to protons, thereby abolishing the proton gradient across the membrane, a condition perhaps

Table X. Effect of various concentrations of supplement on the efficiency (E) of growth for wild-type *E. coli* B

supplement	Concentration of supplement in the medium (μM)	Efficiency (μg/ml protein increase per μg/ml glucose consumed	Decrease in E* (%)	Doubling time (minutes)
DNP (2,4-dinitro-phenol)	0.0	0.314	control	54.6
	10.0	0.301	4.1	56.2
	100.0	0.251	20.0	57.5
	250.0	0.244	22.3	68.2
	500.0	0.217	30.9	87.5
	1000.0	-	-	-
TBP (1,3,5-tri-bromophenol)	0.0	0.314	control	54.6
	50.0	0.221	29.6	72.8
	75.0	0.217	30.9	74.5
	100.0	0.206	34.4	93.9
	250.0	0.158	49.7	161.0
	350.0	-	-	-
CM (chloram-phenicol)	0.0	0.314	control	54.6
	1.0	0.310	1.3	74.3
	2.5	0.259	17.5	135.7
	5.0	0.199	36.6	281.2
	10.0	-	-	-
$NH_2OH.HCl$ (hydroxylamine-hydrochloride)	0.0	0.314	control	54.6
	10.0	0.294	6.4	61.2
	100.0	0.230	26.8	115.8
	500.0	-	-	-

*Per cent decrease in E was calculated using the control value as reference in each case.

-Cells did not grow at these supplement concentrations.

Cells were grown on glucose (0.2%)-salts medium for 4 hours in a shaking water bath, harvested, washed once in phosphate buffer, then re-suspended in minimal glucose-salts medium supplemented with various concentrations of compounds as indicated. Initial and final samples were removed for glucose and protein analyses as described in METHODS.

Table XI. Effect on efficiency (E) during aerobic and anaerobic growth on 2,4-dinitrophenol (DNP)-supplemented medium for wild-type E. coli B

Supplement	E*	
	Aerobic	Anaerobic
None (control)	0.314	0.111
DNP (100 μM)	0.251	0.100

*E is the efficiency of growth defined as μg/ml increase in protein per μg/ml glucose consumed.

Exponential cells starved for glucose were prepared as in METHODS. The washed cells were resuspended in minimal salts-glucose (650μg/ml) medium (±DNP supplement) and grown aerobically. When the A_{600} reached 0.20, the culture was transferred to anaerobic conditions as described under METHODS III, (iii). Portions of the culture were removed for glucose and protein determinations throughout the entire aerobic and anaerobic growth period.

essential in the maintenance of an energized membrane conformation.

An investigation of the reduced/oxidized difference spectrum (Figure 8) indicated that compared with control cells (solid line), growth with 10 μM DNP decreased the height of P503 by one-half, and growth with 250 μM DNP eliminated the 503nm peak completely.

At this point, it should be emphasized that since the cells were grown in medium containing DNP, the latter affected the formation (synthesis), and not the functioning, of the 503nm pigment. Experiments, in which cells grown without added DNP were subsequently treated with DNP just prior to spectral analysis, indicated no effect upon reduction of P503 by glucose. P503 synthesis was therefore inhibited by growth in the presence of DNP. In some cases, cells were grown on 250 μM DNP prior to the actual experiment. When control cells treated in this manner were subsequently grown without DNP supplementation, the 503nm peak re-appeared, indicating that the effect of DNP was not only reversible, but also exerted only during its presence in the medium. It was possible, then, by growing wild-type E. coli on 250-500 μM DNP, to eliminate the 503nm pigment and to decrease the aerobic yield of cell protein simultaneously.

The marked effect of DNP on the 503nm pigment prompted an investigation of other compounds, in order to ascertain whether DNP was specific in its elimination of P503 or whether a general effect on energy metabolism was involved. All compounds which behave like DNP in their uncoupling effect have been shown to possess a phenolic -OH group; for example, thyroxine and halogenophenols (Parker, 1958; Wilson, Ting and Koppelman, 1971). The degree of dissociation of a particular uncoupler was correlated to its potency as an inhibitor of oxidative phosphorylation, highly

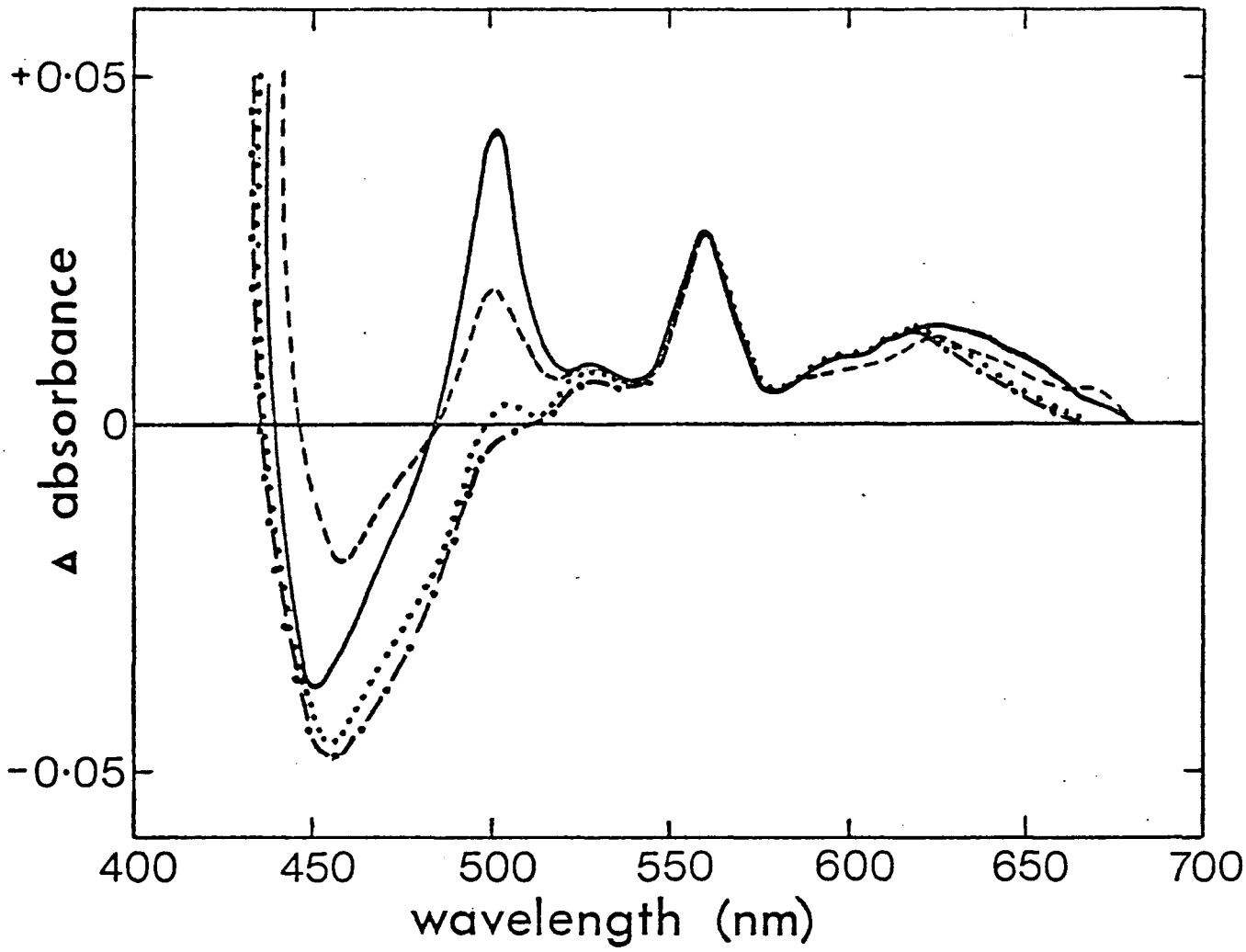


Figure 8. Reduced/oxidized difference spectra after addition of glucose to air-oxidized suspensions of wild-type E. coli B grown in medium supplemented with 2,4-dinitrophenol: ——, none (control); - - -, 10 μM; ••••, 100 μM; —·—, 250 μM.

dissociated phenols possessing greater biological activity. Hempfling (1970a) found that 300 μM 2,4-dibromophenol (DBP) abolished the esterification of Pi in intact E. coli cells when all three sites of oxidative phosphorylation were functional, but did not affect the oxidation of NADH. It was desirable to test this analogue in place of DNP to determine whether the nitro groups of the latter compound could be participating in a reaction other than the well known effect as an uncoupler of oxidative phosphorylation. However, DBP was not available from the usual chemical companies. The tribromo-derivative was therefore used in place of DBP. In view of the potency of a compound (as uncoupler) depending upon the ionization of the hydroxyl group, one would assume that TBP, possessing an extra Br- constituent, would have a greater tendency to withdraw electrons from the aromatic ring than the dibromo-derivative, consequently resulting in a greater potential toward ionization. Stockdale and Selwyn (1971), investigating the effects of ring substituents on the activity of phenols as inhibitors and uncouplers of mitochondrial respiration, compared the compounds DNP, DBP, and TBP. They concluded that in addition to the mediation of proton transport across the membrane, these phenols affected one or more components of the electron transfer system, an effect not involved in uncoupling.

As seen in Table X, growth of cells on minimal salts-limiting glucose supplemented with 50-250 μM TBP resulted in a gradual decline in efficiency values, accompanied by an increase in doubling time. Similar experiments with chloramphenicol (CM) as supplement indicated the same trend at a concentration 100-fold less than in the case of DNP or TBP. Hydroxylamine-hydrochloride ($NH_2OH.HCl$) also inhibited growth of the cells. Corresponding

measurements of the height of the 503nm peak for each case, however, indicated a difference in the effects exerted by the inhibitors (Table XII). Hydroxylamine-hydrochloride had no effect on P503 height (in contrast to the finding by Kepes (1964) that P503 reacts with hydroxylamine to form a colourless compound), so that its effect must be exerted on some cellular process other than the P503 pathway of energy metabolism. (In mitochondria, Yoshikawa and Orii (1970) and Takemori, Sekuzu and Okuni (1960) have shown hydroxylamine to inhibit the cytochrome oxidase reaction at 3.1×10^{-5} M and 10^{-3} M respectively). TBP eliminated P503 even at its lowest concentration, while chloramphenicol had a gradual effect. These two latter compounds were tested further, and the results will be discussed later in this section.

Thus far, wild-type E. coli B grown on 500 μM DNP was seen to "mimic" the Sm-dependent phenotype with respect to a decreased aerobic energy yield (~30%) and lack or decrease of the 503nm pigment. Coukell and Polglase (1969) found that Sm-dependent E. coli B had de-repressed levels of catabolite repressible enzymes. For purposes of comparison, these values were included in Table XIII. When the enzyme levels were checked for de-repression in the non-dependent revertant (SBr4), similar trends were noted. Wild-type cells grown with excess glucose in salts medium containing 100-500 μM DNP showed progressively greater derepressed enzyme levels. At 500 μM DNP, the specific activities of fumarase and aconitase were increased over five-fold. Glucokinase and isocitrate dehydrogenase activities were not affected by DNP, in agreement with values for Sm-dependent E. coli B (Coukell and Polglase, 1969).

An unexpected result was the four-fold increase in glucose 6-phos-

Table XII. Effect of various concentrations of supplement on the height of P503 in wild-type E. coli B

Supplement	Concentration of supplement in the medium (μM)	Height of P503*
DNP (2,4-dinitro-phenol)	0.0	1.00 (control)
	10.0	0.48
	100.0	0.10
	250.0	0.00
	500.0	0.00
	1000.0	-
TBP (1,3,5-tri-bromophenol)	0.0	1.00 (control)
	50.0	0.00
	75.0	0.00
	100.0	0.00
	250.0	0.00
	350.0	-
CM (chloramphenicol)	0.0	1.00 (control)
	1.0	0.44
	2.5	0.12
	5.0	0.00
	10.0	-
$NH_2OH.HCl$ (hydroxylamine-hydrochloride)	0.0	1.00 (control)
	10.0	0.92
	100.0	0.92
	500.0	-

*Height of P503 is expressed as a fraction of the control value, designated as 1.00.

-Cells did not grow at these supplement concentrations.

Experimental conditions were as described in Table X.

Table XIII. Specific activities of enzymes from wild-type (SB), wild-type supplemented with 2,4-dinitrophenol (SB-DNP), Sm-dependent (DB), and non-dependent revertant (SBr4) strains of E. coli B

	Specific activity (units/mg of protein)						Ratio of enzyme activities		
				SB-DNP			SB-DNP (500μM) / SB	DB / SB	SBr4 / SB
Strain	SB	DB*	SBr4	2,4-dinitrophenol					
Supplement...	None	None	None	(100μM)	(250μM)	(500μM)			
fumarase	481	2545	1872	622	2400	2569	5.35	5.30	3.90
aconitase	113	789	543	246	547	577	5.10	6.98	4.80
glucose 6-phosphate dehydrogenase	116	137	84	169	403	452	3.88	1.18	0.72

*Results for Sm-dependent E. coli B were taken from Coukell (1969).

The minimal salts medium contained glucose at an initial concentration of 0.2%. Enzymes were assayed in ultrasonically-treated extracts of exponential cells prepared as described in METHODS. Specific activities (units of enzyme / mg of protein) are averages from several determinations.

phate dehydrogenase activity, an enzyme not known to be sensitive to catabolite repression. From the ratios given in the last two columns of Table XIII, the activity of this enzyme in control cells is seen to be similar to that in the Sm-dependent mutant (DB/SB = 1.18) and slightly higher than that in the non-dependent revertant (SBr4/SB = 0.72).

The similarity in the effect of 1,3,5-tribromophenol (TBP) and chloramphenicol (CM) to 2,4-dinitrophenol (DNP), both in decreased efficiency of growth and in elimination of P503, suggested an examination of enzyme levels. From Table XIV, it can be seen that TBP (at all concentrations used) had virtually no effect on fumarase or glucose 6-phosphate dehydrogenase. CM, on the other hand, at 1/100th the concentration of the other compounds, caused a general inhibition of protein synthesis, decreasing the levels of both enzymes.

When DNP, CM, TBP and $NH_2OH.HCl$ were compared with respect to the three criteria of efficiency, P503 formation, and de-repressed enzyme levels, the effects of each compound were distinctly different.

DISCUSSION

The overall effect of 2,4-dinitrophenol (DNP) as supplement was unique, and involved the following four aspects in common with Sm-dependent mutants: (1) slower growth rate; (2) lower cell protein yield (30%); (3) inhibition or impairment of P503 formation; (4) de-repression of catabolite repressible enzymes. The only difference between Sm-dependent and wild-type cells grown with DNP was de-repression by DNP, as well, of glucose 6-phosphate dehydrogenase.

It is conceivable that NADPH is a co-repressor of glucose 6-phos-

Table XIV. Specific activities of enzymes from wild-type E. coli B grown on glucose supplemented with 1,3,5-tribromophenol or chloramphenicol

	Specific activity (units/mg of protein)						
Supplement...	None	1,3,5-tribromophenol (50μM)	(100μM)	(250μM)	chloramphenicol (1.0μM)	(2.5μM)	(5.0μM)
fumarase	481	453	440	423	588	244	139
glucose 6-phosphate dehydrogenase	116	132	130	137	101	75	91

Cultures were grown on 0.2% glucose-salts medium supplemented as indicated. Extracts were prepared for enzyme assays as in Table XIII.

phate dehydrogenase. If this is the case, de-repression of this enzyme could result from rapid removal of its product, NADPH, which may transfer its hydrogen to 2,4-dinitrophenol (an effect of DNP different from its role as an uncoupler of oxidative phosphorylation). The medium containing DNP did undergo a change in colour from yellow to brown when wild-type E. coli cells were grown in it- an indication of possible reduction of the nitro group(s) to the amino form.

The amount of DNP available for NADPH oxidation was calculated for 1 litre of medium. An initial concentration of 0.04% or 0.40/180 = 2.2×10^{-3} moles of glucose was present. Several studies have estimated that 24-30% of the glucose is metabolized by the hexosemonophosphate shunt (Caprioli and Rittenberg, 1969; Model and Rittenberg, 1967; Wang et al., 1958). If 30% is taken as the maximum utilization of the pathway, then $2(6.6 \times 10^{-4})$ or 13.2×10^{-4} moles of NADPH + H^+ would be formed. Since 3 moles of NADPH + H^+ are required for the reduction of a nitro group to the amine, then the amount of DNP (5.0×10^{-4} moles) which eliminated P503 exceeds that required to react with all of the NADPH which might be produced. That is, in the reduction of one nitro group per DNP molecule, 5.0×10^{-4} moles of DNP would react with 15.0×10^{-4} moles of NADPH whereas not more than 13.2×10^{-4} moles of NADPH would be expected to be formed. Thus, enough DNP was present in the medium to remove the NADPH which could be formed by the HMP shunt.

It was found (Tables III and IV) that wild-type cells were more efficient than Sm-dependent mutants only when grown aerobically. DNP likewise affected only aerobic efficiency. Since in both cases the impairment in energy metabolism was accompanied by the disappearance or elimination of P503, one would conclude that P503 is involved in an

aerobic route of energy metabolism.

This leads to the general hypothesis: an oxidative pathway is present in wild-type E. coli which generates ATP from NADPH via an intermediate pigment, P503 (Figure 12). This pathway accounts for 25% to 35% of the total energy or 50% of the solely aerobic energy produced by cells grown on a minimal salts medium with glucose as carbon source.

In Sm-dependent mutants, the mutation results in a P503 deficiency. In DNP-grown wild-type cells, the P503 deficiency was produced in some way by the DNP. In either case, the proposed NADPH→P503→ATP pathway was absent.

Results which correlate with those obtained in the present work have been reported by several investigators and can be understood on the basis of the general hypothesis cited above. Mandelstam (1961) found DNP to relieve catabolite repression in non-growing E. coli to different degrees, depending on the carbon source. Succinate, lactate, and pyruvate, which are metabolized aerobically, produced incomplete catabolite repression; DNP was able to reverse this repression. Complete repression by gluconate was only patially released by DNP, and glucose repression was not reversible by DNP. If one postulates that the effect of DNP was indeed to oxidize NADPH, a possible candidate for catabolite co-repressor, then removal of NADPH by DNP explains all of Mandelstam's results with non-growing E. coli. The substrates succinate, lactate, and pyruvate would not generate much NADPH during their metabolism, so that repression would be incomplete; any NADPH formed, however, could be removed by DNP. Gluconate, generating 1 molecule of NADPH per molecule of gluconate metabolized, would provide enough NADPH to cause complete

repression, but DNP could partially remove the effector. Glucose, producing twice as much NADPH, would also cause complete repression; but enough NADPH would probably remain even with addition of DNP, so that the latter compound would be ineffective in releasing this repression.

In a report on the oxidative pathway of carbohydrate metabolism, not known to us until after the publication of our preliminary results and speculations (Kamitakahara and Polglase, 1970), Scott (1956b) found a four-fold de-repression of glucose 6-phosphate dehydrogenase when E. coli was grown in medium containing DNP. It is interesting at this time to review her observations and to determine whether or not they are in agreement with our hypothesis: (1) Scott found that 2×10^{-4} M DNP as supplement affected the yield of cells, but had no effect on the rate of removal of glucose from the medium. This observation can be readily explained if DNP were accepting hydrogen and re-oxidizing NADPH. Glucose would still be taken up from the medium since a product (NADPH) is removed, but the amount of NADPH left for reducing power in biosynthetic reactions would be less, thereby affecting the overall yield of cells; (2) When cells grown on DNP were removed, washed, and re-grown without DNP, the glucose 6-phosphate dehydrogenase activity was again decreased to the normal level (Scott, 1956b). Our results were identical, suggesting that the effect of DNP was exerted only during its presence in the medium; (3) The effect of DNP on growth and enzyme activity were determined when the carbon sources for growth were arabinose, lactate and gluconate (Scott, 1956b). She reported that if arabinose or lactate were supplied to the cells, no significant effect of DNP on growth or extractable enzyme activity was perceptible.

Gluconate as carbon source de-repressed glucose 6-phosphate dehydrogenase but was less effective than glucose. These results are consistent with the hypothesis that DNP causes de-repression of glucose 6-phosphate dehydrogenase by re-oxidizing the NADPH formed in the hexosemonophosphate pathway; (4) In order to determine whether 6-phosphogluconate could be split without oxidation of NADPH, Scott (1956b) determined the activities of the phosphogluconate degrading enzyme (Entner-Doudoroff pathway) versus 6-phosphogluconate dehydrogenase (HMP shunt) during growth on phosphogluconate.She (Scott, 1956b) found that the most active extract had activities of 0.012 units and 0.16 units respectively. Although these results indicated that the rate of the phosphogluconate degrading enzyme was less than 1/10th that of the 6-phosphogluconate dehydrogenase, since the products of the reactions were not determined, Scott could not estimate the importance of the Entner-Doudoroff pathway in the metabolism of gluconate; (5) When cells were grown on glucose with DNP, the activity of the phosphogluconate degrading enzyme (Entner-Doudoroff path) was seen to increase, the effect being greater with prolonged time of exposure to the drug. In addition, the increase obtained in glucose 6-phosphate dehydrogenase activity with DNP as supplement (when glucose or gluconate was the carbon source) was never accompanied by a simultaneous increase in 6-phosphogluconate dehydrogenase, the first enzyme specific to the HMP shunt. Our findings were similar; growth of cells in the presence of 0-500 μM DNP had no effect on this enzyme. These two results suggest that the accumulation of 6-phospho-gluconate results in the induction of the Entner-Doudoroff enzymes for rapid removal of this product. (Eisenberg and Dobrogosz (1967)

reported an inducible Entner-Doudoroff pathway in gluconate-grown E. coli cells). Since this pathway is not known to generate energy during cleavage of 6-phosphogluconate to pyruvate plus triose-phosphate, a lower efficiency would be expected, and this was found to be the case; (6) Ascorbic acid or oxidized glutathione with cysteine were found by Scott to increase NADPH oxidase activity, resulting in an increase in the rate of re-oxidation of NADPH. In view of the previously mentioned report by Nosoh (1964) that reduction of the cells by ascorbate, cysteine, glutathione, and dithionite resulted in the flattening out of the 503nm peak, an explanation for both Nosoh and Scott's observations might be that ascorbate, and oxidized glutathione with cysteine convert the pigment to a non-absorbing colourless form. The mechanism might be analogous to that operative with sodium dithionite which can effect the complete reduction of the pigment past the 503nm absorbing stage to the leuco form; (7) DNP was shown by Scott to affect the cells anaerobically as well. This result was in contrast to our findings. No details were given in Scott's article as to the manner in which anaerobic conditions were produced or maintained. This is a crucial point, since it was discovered in preliminary experiments that nitrogen gas of 95% purity proved to be unsatisfactory in eliminating aerobic growth.

Scott's explanation for her observed increase in glucose 6-phosphate dehydrogenase with DNP was that the drug blocked other pathways of metabolism of glucose 6-phosphate (G6-P), resulting in a pile-up of the substrate so that more enzyme was formed in response to the increased G6-P concentration. Alternatively, our explanation is that

the reduced pyridine nucleotide, NADPH, a product and possible co-repressor of G6-P dehydrogenase, would be removed by its reduction of DNP (perhaps to monoamino-mononitrophenol or even further to di-aminophenol), thereby releasing the repression of the enzyme by its product.

Previously (point 5, Scott, 1956b), a possible interpretation was presented concerning the shunting off of NADPH and 6-phospho-gluconate via the inducible Entner-Doudoroff pathway when DNP was present in the growth medium. A similar interpretation might account for observations of Coukell (1969) which could not be explained at that time. Coukell reported an enhancement in growth rate (that is, a decrease in doubling time from 91 to 74 minutes) in Sm-dependent cells when gluconate replaced glucose as the carbon source, accompanied by greater repression of the catabolite repressible enzyme, acetohydroxyacid synthetase (that is, a decrease in specific activity from 14.1 to 7.85). The latter observation was unexpected, since his results with glucose-grown cells indicated the Sm-dependent mutant to be de-repressed with respect to the glucose-sensitive enzymes compared to wild-type cells. (Also, the wild-type strain had the same growth rate on glucose and gluconate). These two observations of enhanced growth rate and increased catabolite repression of aceto-hydroxyacid synthetase would tend to suggest that gluconate metabolism by the mutant was more efficient than glucose metabolism; however, determination of cell yield on the two sugars indicated that the mutant was 38% less efficient than the wild-type strain, whether grown on glucose or gluconate (µg dry cells formed / µmole carbon source consumed was 59.7 and 59.3 respectively). Coukell concluded that the first two

observations were therefore not a result of increased yield of energy from gluconate.

An examination of the metabolic pathways (Figure 12) indicates that gluconate as carbon source via the HMP shunt results in the formation of one less NADPH molecule per molecule of carbon source consumed, than in the case of glucose. If gluconate is dissimilated via the Entner-Doudoroff pathway, then no NADPH is formed. The chief source of energy would be ATP formed in the TCA cycle from the pyruvate generated in two steps in the Entner-Doudoroff pathway. Although the growth rate is enhanced, since there are no energy-generating steps for the Sm-dependent strain via the Entner-Doudoroff path or via the postulated ATP-generating path of NADPH→P503, the overall efficiency should be the same on glucose and gluconate.

It was postulated previously that ATP is generated from NADPH during oxidation of the latter compound via the P503 pathway. It should be emphasized that this pathway is assumed to be operative as a result of its induction by NADPH. The wild-type organism may then form ATP from NADPH while the Sm-dependent mutant lacks this source of ATP. Indeed, the rate of ATP formation was found to parallel the growth rate in the Sm-dependent organism (Coukell, 1969) whereas the rate of ATP production in the wild-type strain exceeded the rate of growth.

Studies on the reduction of nitrite (NO_2) and nitroaryl groups by nitrite reductase have been previously reported in non-denitrifying organisms. (Denitrifying bacteria such as Pseudomonas aeruginosa are facultative, and can utilize nitrate or nitrite (in place of oxygen) as a hydrogen acceptor in energy-yielding oxidative reactions). Lazzarini

and Atkinson (1961) characterized a cyanide-sensitive NADPH-specific nitrite reductase from E. coli which was not stimulated by FMN, FAD, or a variety of metal ions. Three moles of NADPH were consumed per mole of nitrite reduced, the reduction product being ammonia. Purified nitrite reductase contained three additional activities: cytochrome c reductase, hydroxylamine reductase, and sulphite reductase, all specific for NADPH. In deep-standing (anaerobic) cultues of E. coli, two other nitrite reductases were detected, one in which NADH served as the electron donor, and one in which $FMNH_2$ was the donor. Mager (1960) reported that NADPH-specific sulphite reductase and NADPH-specific hydroxylamine reductase (both FAD dependent) of E. coli, perhaps catalyzed by the same enzyme, were feed-back repressed by methionine, cysteine, or cystine. Since the Km for sulphite was 100-fold less than for hydroxylamine, he concluded that sulphite was the "true" physiological substrate and that NH_2OH reduction was an incidental capacity of the same enzyme. This point will be considered further in the discussion of our experiments with L-methionine. Taniguchi, Sato and Egami (1956) stated that nitrate with terminal nitrate reductase could serve as a cellular oxidant instead of oxygen plus the terminal respiratory oxidase, which are the normal oxidants under aerobic conditions. Although they found nitrate metabolism to occur anaerobically as well as aerobically, the anaerobic process did not proceed beyond the nitrite stage unless oxygen was added. Thus E. coli was found to possess two different types of nitrate metabolism: one being the anaerobic reduction of nitrate to nitrite, and the other being the aerobic reduction of nitrate to nitrite and further to ammonia. They found, however, that stoichiometric quantities of the expected intermediates such as hydroxylamine were not obtained; some unidentified

intermediary product(s) or compound(s) derived from nitrite were therefore assumed to be accumulated. Silver and McElroy (1954) showed that wild-type Neurospora mycelia could catalyze the reduction of m-dinitrobenzene to nitrophenylhydroxylamine and finally to nitroaniline. The culture medium turned a deep amber during growth. As early as 1935, Greville and Stern identified the reduction product of 2,4-dinitrophenol in E. coli cells as 4-nitro-2-aminophenol, the reduction requiring 6 hydrogen equivalents per molecule of DNP reduced. No diaminophenol was detected at this time.

It is not known whether the metabolism of nitrite proceeds in micro-organisms via an inorganic or organic pathway. De la Haba (1950) postulated that nitrate or nitrite may first be bound in an organic form ($R-NO_3$ or $R-NO_2$) prior to its reduction to an amino compound ($R-NH_2$) which then transfers the amino group by transamination, thereby effecting the synthesis of amino acids. In organisms such as E. coli, more than one pathway has been found to exist for the dissimilation of nitrate (Taniguchi, Sato and Egami, 1956). Although the physiological significance of the reduction of nitro-aromatic compounds by microorganisms is still obscure, one might suggest that such a process would provide a means of detoxication by removal of the nitro-compound. The corresponding amino-form has been shown to be non-toxic (Cain, 1958). The participation of one particular enzyme system over another would then depend upon the environmental conditions surrounding the organism- any subsequent change in the medium (for example, aerobic to anaerobic) causing an alternate pathway to come into play.

In studies on catabolite repression, Prevost and Moses (1967) found that glucose and gluconate were the only sugars whose addition to

E. coli growing exponentially on glycerol resulted in severe inhibition of β-galactosidase synthesis. Sixty minutes after the addition of the glucose or gluconate, the cells recovered and synthesis of the enzyme resumed at the normal rate. These workers suggested that the effector exerting this catabolite repression might be either directly produced metabolically from glucose, or its concentration might be changed when glucose is added to the cells, even though the effector is derived from another source. When the levels of intermediates of glucose metabolism were followed during the experiments, an increase was observed in the pool sizes of glucose 6-phosphate, 6-phosphogluconate, fructose 1,6-diphosphate, and NADPH immediately upon the addition of glucose. Sixty minutes after the addition of glucose, the pool sizes of all four compounds decreased. On the contrary, they found no change in the concentrations of ATP, NADH, and several other phosphorylated intermediates. From these results, they concluded that the activity of the pentose phosphate cycle was of central importance in determining the sensitivity of the cells to catabolite repression by glucose. The rapid production of NADPH was presumed to exceed both the cells' requirement for the reduced coenzyme, as well as the cells' ability to oxidize NADPH in terminal respiration. An accumulation then resulted in a metabolic imbalance which persisted until the cells were able to achieve a new steady metabolic state in the changed environment caused by adding glucose. They stated, furthermore, that under anaerobic conditions, the activity of the pentose phosphate cycle would be negligible. In view of the general hypothesis that ATP is readily formed from NADPH via P503, it is equally plausible that catabolite repression results from the maintenance of a high energy charge via this pathway.

2. Effect of anaerobic growth on formation of P503

RESULTS

The 503nm pigment has previously been found in anaerobically grown cells of Saccharomyces cerevisiae (Lindenmayer, 1959; Lindenmayer and Smith, 1964; Nosoh, 1964). Similar results were obtained when wild-type E. coli B was grown initially under aerobic conditions, then changed to an anaerobic nitrogen atmosphere for the remainder of the growth period (Figure 9). P503 and the cytochromes were present but in an oxidized state after anaerobic growth, indicating they were not functional and did not accept electrons in the absence of oxygen.

DISCUSSION

Cells were grown aerobically for a short period initially so that a sufficient amount of energy would have accumulated to carry them over the change to anaerobiosis. When approximately half the amount of P503 was formed under these conditions, it was suspected that perhaps P503 was being formed during the aerobic phase of the experiment and being diluted out during anaerobic growth. This possibility was dismissed, however, when cells were grown in DNP-supplemented medium (known to eliminate P503) prior to anaerobic growth. Chloramphenicol was added at the end of the experiment (to inhibit further protein synthesis) and the cells were chilled in an ice-salt bath in the anaerobic atmosphere prior to harvesting. The results were identical to those obtained by the first method of anaerobic growth. P503 and the cytochromes were formed under anaerobic conditions but remained in an oxidized state.

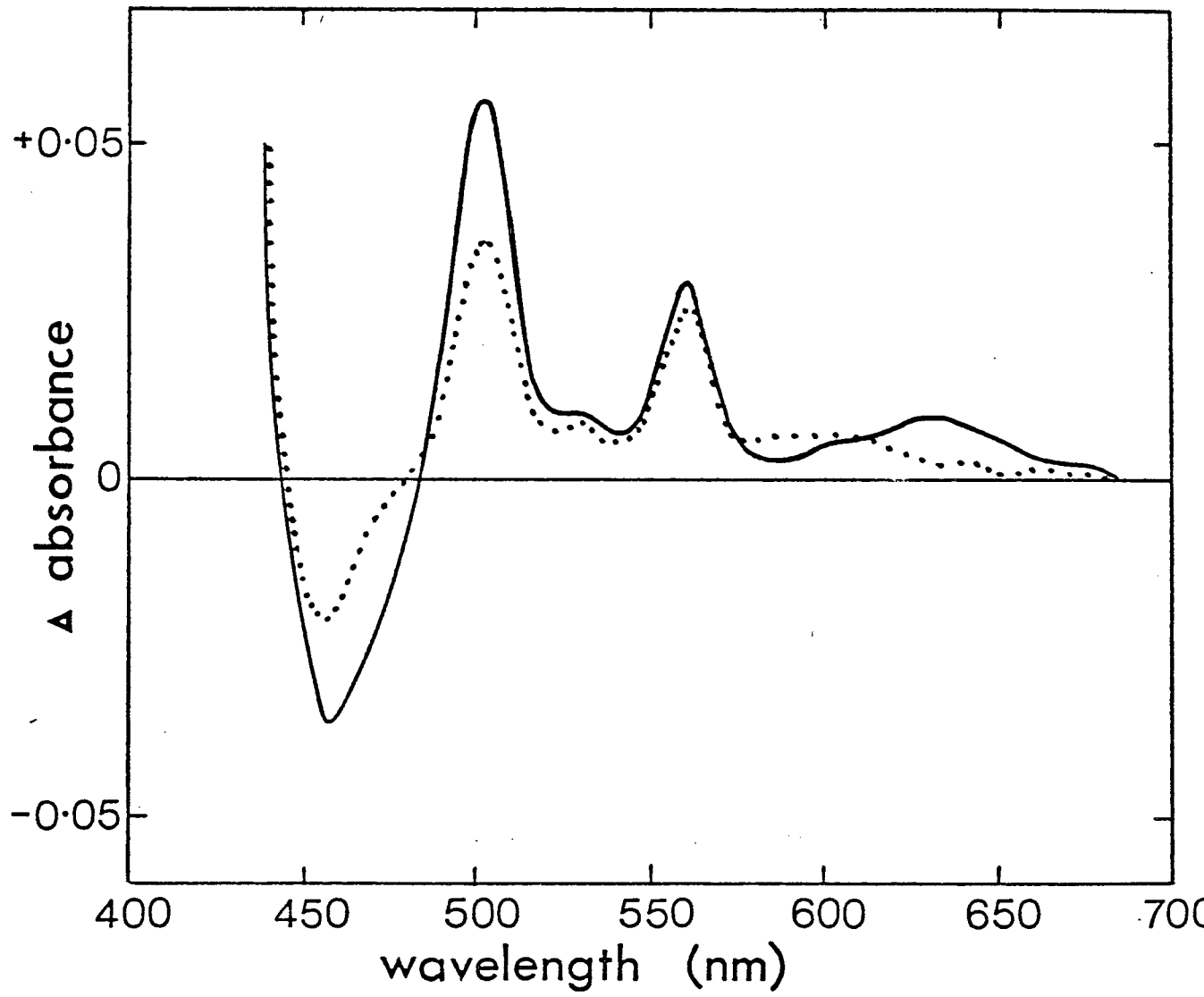


Figure 9. Reduced/oxidized difference spectra after addition of glucose to wild-type E. coli B grown anaerobically under a nitrogen (N_2) atmosphere. Cells were first grown aerobically from A_{420} 0.10 to 0.40 in minimal salts medium containing 650 µg glucose/ml, then transferred to anaerobic conditions as described under METHODS. At the end of growth, the 37° water bath was replaced by an ice-salt bath, and N_2 flow continued for 1 hr. The cells were then harvested, washed, and analyzed immediately. Since the cytochromes and P503 were still oxidized under these conditions, glucose was added to produce the difference spectra. ——, aerobically grown control cells; ····, anaerobically grown cells.

The formation of these pigments anaerobically is not surprising in view of several observations. An adaptive change in the type and amount of cytochrome content from anaerobiosis to aerobiosis was first established by Ephrussi and Slonimski (1950), and Chin (1950) in yeast cells. These workers reported the absence of the "normal" cytochrome system in anaerobically grown cells of Saccharomyces cerevisiae, and the appearance of these enzymes upon exposure of the cells to oxygen under non-growing conditions. This result implied that the inducer of the active enzymes was molecular oxygen, and that the oxygen-binding compound was synthesized in the absence of oxygen.

More recently, Chen and Charalampous (1969), Tuppy and Birkmayer (1969), and Henson, Perlman, Weber and Mahler (1968) found that under anaerobic conditions, yeast cells possess cytochrome oxidase apoenzymes which are converted to active enzymes during de-repression and addition of oxygen in the absence of cell division. In eukaryotes, several cases of the interdependence of mitochondria and cytoplasm have been reported (Chen and Charalampous, 1969; Birkmayer, 1971). The protein-synthesizing machinery of the cytoplasm was responsible for synthesis of the apoenzymes, and only when aerobic conditions were met did the mitochondria synthesize the active oxidase. In this case, oxygen served not only as an inducer initiating the cytochrome oxidase formation, but also as a cosubstrate in the biosynthesis of heme a.

Perhaps a similar but simpler situation prevails in E. coli cells. In contrast to yeast, reports on the oxygen-mediated regulation of respiratory activity or the content of respiratory enzymes in bacteria are scarce and conflicting. Hino and Maeda (1966) found that the respiratory activity

of anaerobically grown E. coli could be increased by aerating the cells in the absence of cell proliferation. Since the amount of cell nitrogen remained constant, they concluded that the general synthesis of proteins did not occur; however, several amino acids were required for this development of respiratory activity, suggesting that specific synthesis of protein(s) responsible for the respiration of E. coli occurred. The increase in respiratory activity reached the level of aerobically grown cells when the culture was aerated in buffer containing casamino acids, without a concomitant increase in the content of cytochromes b_1, a_1, or a_2. They inferred from these results that cytochromes were not the limiting factor for overall rate of respiration in E. coli, and that other terminal oxidase systems might be present.

Little is known about the structural and functional changes in the membrane system of E. coli during the change from anaerobiosis to aerobiosis. E. coli and other facultative bacteria do not possess extensive membrane systems (Gray, Wimpenny, Hughes and Mossman, 1966). Anaerobically grown cells were found by these workers to retain many of the membrane-bound components that are characteristic of aerobes (cytochromes oxidase and b_1), but to form, in addition, soluble enzymes similar to those typical of anaerobes (soluble cytochrome c). In 1966, Gray, Wimpenny and Mossman reported that the amount of NADH oxidase was decreased during anaerobic growth, while adequate amounts of cytochromes a_1, a_2, and b_1 were still present. They stated that this decrease in NADH oxidase was probably due to a deficiency in some electron carrier other than these cytochromes. In addition, Cavari, Avi-Dor, and Grossowicz (1968) observed a relationship between the development of the respiratory system and the

energy-conserving phosphorylative mechanism of E. coli during the transition from anaerobic to aerobic growth. Upon exposure to oxygen, the NADH oxidase activity of anaerobically grown cells increased to the aerobic level in 10 minutes although the cytochrome content was re-established after 60 minutes. This development of NADH oxidase activity was paralleled by the synthesis of protein and ribonucleic acid (but not deoxyribonucleic acid). The implication of their results was that the respiratory chain between NADH and the cytochromes was linked only when oxygen was present.

Our results were similar to those of Gray, Wimpenny, Hughes and Mossman (1966) in that the cytochrome spectra were obtained under both aerobic and anaerobic conditions. Since prokaryotes do not possess compartmentalization of various functions and have only one type of protein-synthesizing apparatus, the cytochromes and P503 may be produced under anaerobic conditions, but remain non-functional until the system is exposed to oxygen. Such a mechanism would prepare the cells for immediate utilization of high energy aerobic routes in preference to the lower energy-yielding anaerobic pathways whenever oxygen is made available to them.

3. Nutritional effects on formation of P503

RESULTS

Since the height of the 503nm peak and its order of appearance with respect to the cytochrome bands in the difference spectra were found to be dependent upon the carbon source used as reductant (Table VII), wild-type cells of *E. coli* B were grown on a variety of sugars and acids to determine their effect on formation (synthesis) of P503 (Table XV). The presence of P503 was tested by glucose reduction of air-oxidized suspensions, since this method was shown previously to produce the maximum height of the P503 peak.

Glucose-grown, glucose-reduced cells showed a very large 503nm peak and were used as the control. Gluconate as carbon source yielded a slightly smaller peak. Growth on glycerol, succinate, and lactate resulted in a smaller but still noticeable 503nm pigment. The cytochromes and flavin were not affected by changes in carbon source. Supplementation of succinate medium with iron (ferric chloride) did not affect the results, indicating that iron was not limiting under the growth conditions utilized. Monitoring of pH throughout the final growth on succinate showed no deviation from pH 7.0. When cells were grown on 0.04% glucose plus 0.04% 2-deoxy-D-glucose, glucose reduction produced one-half the amount of P503 of control cells (without added 2-deoxyglucose). A correspondingly lower growth rate was observed. In general, P503 was formed in variable amounts on all carbon sources tested.

When the concentration of glucose or of gluconate in the medium was increased, an effect was observed on the amount of P503 formed (Table XVI). With glucose, the height of the P503 peak decreased from 38.9 units

Table XV. Effect of various carbon sources as growth supplement on P503 formation (synthesis) in wild-type E. coli B

Sugar or acid supplement	Concentration of supplement in the medium (%)	Per cent of P503 formed*
glucose	0.04	100.0
gluconate	0.04	74.3
glucose + gluconate	0.02 0.02	77.0
succinate	0.07 0.53	59.8 30.9
glycerol	0.05 2.00	32.4 6.4
lactate	0.20	30.2

*The height of the 503nm peak was taken as an indication of the amount of P503 formed. The per cent of P503 formed was determined relative to the control (0.04% glucose) value of 100.0%.

The cytochromes and flavin spectra were not affected.

Cells were grown on minimal salts medium supplemented with various carbon sources as indicated. In all cases, the presence of P503 was tested by glucose reduction of air-oxidized suspensions. For experimental details, see METHODS.

Table XVI. Effect of concentration of carbon source in medium (glucose or gluconate) on P503 formation (synthesis) in wild-type E. coli B

Supplement	Concentration of supplement in the medium (%)	Height of P503* (units)	Height of cytochrome b** (units)	Ratio of height P503/ height cytochrome b
glucose	0.05	38.9	29.2	1.36
	0.25	31.3	"	1.07
	0.75	27.1	"	0.93
	1.25	14.7	"	0.52
	1.75	13.0	"	0.45
	2.00	0.0	"	0.00
gluconate	0.05	22.8	29.2	0.78
	0.16	13.3	"	0.46
	0.20	10.2	"	0.35
	0.50	6.8	"	0.23
	2.00	0.0	"	0.00

*The height of the 503nm peak was taken as an indication of the amount of P503 formed.

**The height of cytochrome b was normalized to 29.2 units in each case (see Table V).

Cells were grown on 0.2% glucose (0.2% gluconate), harvested during log phase, washed once in phosphate buffer, then resuspended in minimal salts medium supplemented with the concentrations of glucose or gluconate as indicated. During log phase (see METHODS), cells were harvested, washed, and resuspended in 0.01 M phosphate buffer. The reduced/oxidized difference spectra were obtained by addition of hydrogen peroxide to the bottom cuvette.

(at 0.05% glucose) to 0.0 units (at 2.00% glucose); cytochrome b remained constant in height. Gluconate was effective in decreasing P503 synthesis at lower concentrations. Gluconate at a concentration of 0.16% or glucose at 1.75% gave the same ratio of 0.45 for height of P503 / height of cytochrome b. The rates of growth with excess carbon source were identical throughout the range of concentrations tested.

DISCUSSION

In microorganisms, a change in the nutritional environment is accompanied by an adaptive change in the metabolic route utilized for dissimilation of the nutrient (Kornberg, 1961; Sanwal, 1970). Since prokaryotes lack the physical compartmentation found in higher organisms (eukaryotes), they have evolved precise regulatory mechanisms to channel metabolic intermediates into the appropriate anabolic, catabolic, or amphibolic pathway in response to their energy and growth requirements. The degree to which these pathways are linked with P503 may be reflected in the height of P503 obtained. Generally, the P503 peak was found to decrease in height (but not be eliminated) with carbon sources whose metabolism does not involve NADPH production at an early stage.

During the process of glucose and gluconate oxidation, NADPH is synthesized via the hexosemonophosphate (HMP) shunt. Numerous workers have followed the break-down of radioactively labelled glucose in an attempt to determine the extent to which various pathways are utilized. Specific yields of ^{18}O-labelled CO_2 produced from E. coli grown in minimal medium on (1-^{18}O)glucose, (6-^{18}O)glucose, and (1-^{18}O)gluconate indicated that 25% of the sugar was channelled through the HMP shunt (Model and Rittenberg, 1967). In similar studies, Caprioli and Rittenberg (1969)

reported a value of 30% and Wang et al. (1958) 28%. The remainder of the carbon source (up to 70%) was found to be metabolized via the Embden-Meyerhof (E-M) path of glycolysis. The Entner-Doudoroff (E-D) path was of little significance under these conditions (Wang et al., 1958; Scott and Cohen, 1953; Scott, 1956a). Only when the HMP pathway is blocked or not required for ribose and NADPH production does the E-D route come into play. This inducible pathway provides a means of producing pyruvate and glyceraldehyde 3-phosphate more directly than via glycolysis, but is not known to result in any form of energy production prior to the TCA cycle. Conflicting evidence was reported by Eisenberg and Dobrogosz (1967), who favoured the E-D path as the primary route of gluconate metabolism in E. coli. However, since they found no significant differences in the levels of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in cell-free extracts of glucose-grown and gluconate-grown cells, one can assume that the utilization of the HMP shunt remained constant; induction of the E-D pathway allowed rapid production of pyruvate for a more direct entrance into the TCA cycle. Nevertheless, NADPH will be produced even though the main route of gluconate metabolism may be the E-D path.

Glycerol metabolism involves the induction of glycerol kinase and α-glycerophosphate dehydrogenase (Koch, Hayashi and Lin, 1964); its conversion to dihydroxyacetone phosphate and subsequent entrance into the glycolytic or glucogenic pathway does not result in NADPH production. A smaller amount of P503 was detected in the reduced/oxidized difference spectra of glycerol-grown cells when compared to the control case (glucose-grown).

Growth of E. coli B on lactate gave a doubling time of 120 minutes (Scott, 1956a). Dissimilation of the C_3 compound occurs via the induction of phosphoenolpyruvate (PEP)-synthase, an enzyme catalyzing the conversion of pyruvate to PEP (Kornberg and Smith, 1967; Cooper and Kornberg, 1967). Lactate, therefore, does not participate in NADPH generation during its metabolism; a lower amount of P503 was observed.

When succinate serves as a carbon source, the glucogenic enzyme PEP carboxykinase is induced, catalyzing the step from oxalacetate to PEP (Sanwal, 1970). The immediate reactions of succinate involve its oxidation to fumarate (via the flavin-linked dehydrogenase) and further to malate. At this stage, either of two routes may be followed: (1) malate $\longrightarrow$ oxalacetate (involving NADH) or (2) malate $\longrightarrow$ pyruvate (generating NADPH). Katsuki, Takeo, Kameda and Tanaka (1967) reported that the $NADP^+$-linked malic enzyme was operative in E. coli cells grown on minimal medium whereas the NAD^+-linked malic enzyme was active when an enriched medium was available. The participation of these enzymes is therefore dictated by the energy state and biosynthetic requirements of the cell. It follows then that E. coli grown under our conditions of minimal salts-succinate would possess de-repressed levels of the $NADP^+$-specific malic enzyme. It is therefore expected that succinate-grown cells would eventually accumulate enough NADPH to elicit a P503 band. The 503nm pigment could be detected in succinate-grown cells.

Less P503 was formed at high than at low glucose or gluconate concentrations suggesting a link between P503 and catabolite repression. These sugars have been reported to produce severe repression singly (Prevost and Moses, 1967; Mandelstam, 1961). But the simultaneous presence of both

glucose and gluconate should be most effective in causing catabolite repression (Hsie, Rickenberg, Schulz and Kirsch, 1969; Perlman, de Crombrugghe and Pastan, 1969; Moses and Sharp, 1970). Since P503 was as large in this case (glucose and gluconate together) as in the control, its relationship to catabolite repression is neither simple nor straight forward. NADPH is an attractive candidate for catabolite co-repressor. Repression has been found to depend on the rapid metabolism of the carbon source, resulting in a metabolic imbalance of some compound. Model and Rittenberg (1967) reported the HMP shunt to function maximally during log phase when reducing power is needed for biosynthetic purposes. By stationary phase, as bacterial growth ceases, the pathway would no longer be needed as a source for NADPH or pentose so that the HMP route decreases in activity. Similar observations have been found by several independent groups. Allen and Powelson (1958) and Scott and Cohen (1953) observed that on limiting carbon source, rapid growth resulted in the dissimilation of glucose via the HMP shunt; at the onset of stationary phase, a shift occurred from the HMP pathway to the E-M route. Concentrations of the nicotinamide adenine dinucleotide coenzymes were reported to fluctuate between bacteria of different physiological types (obligate anaerobes, facultative anaerobes, and obligate aerobes) and with the nutritional and cultural nature of the medium surrounding microorganisms (London and Knight, 1966). E. coli grown aerobically on minimal medium and limiting glucose possessed the highest concentration of the NADP-coenzyme (0.84 μmoles / gm dry wt); a change to anaerobiosis, excess glucose, or completely defined medium decreased this value to 0.58, 0.24, and 0.48 respectively. The NAD-coenzyme

remained fairly constant under all conditions at ~2.5 μmoles / gm dry wt. It is interesting that Sanwal (1970) reported the level of NADH (opposite to that of NADPH) to be high in early log phase (5.5 μmoles / gm dry wt), decrease during mid log (3.0) and increase again by stationary phase (4.5). In keeping with the activity of the HMP shunt, Hempfling (1970b) found that E. coli B grown on glucose possessed a lower efficiency of phosphorylation when harvested during the log period (due to the presence of glucose) than cells harvested in the stationary phase of growth. These results suggested to him that part of the enzymatic or structural apparatus of oxidative phosphorylation was subject to catabolite repression.

Whether P503 is sensitive to, or whether it affects catabolite repression is not known. Indeed, the two may not even be connected. In view of our hypothesis relating P503 with NADPH, the observed results may reflect the action of the reduced coenzyme as opposed to the pigment itself. If the amount of P503 present in growing cells reflected the energy state and relative proportion of metabolic routes utilized, then one would expect the height of P503 to vary also. Labbe et al., (1967) and Nosoh (1964), using a synchronous culture of yeast cells, found the 503nm peak was highest during log growth. When the culture was continued to the stationary phase, the 503nm band was lowered appreciably. Depending upon the time of harvesting, therefore, one might obtain the same results seen in Table XVI. Again the distinction between limited versus excess carbon source for growth must be emphasized. The constancy in growth rate even with increasing concentrations of glucose (or gluconate), in contrast to the decline in P503, may be indicative of the cells' need for the utilization of the P503 pathway. Only under limiting conditions, when a given

substrate is broken down and oxidized fully, is a high energy-yielding path needed, and therefore used. Under enriched conditions (such as an excess of substrate), other routes such as glycogen synthesis are also utilized and the path of P503 is no longer needed immediately, nor is it used. Labbe, Volland and Chaix (1967) noted the lack of a P503 peak when cells were grown on enriched media such as nutrient agar or peptone broth. Similar findings were reported by Nosoh and Itoh (1965). E. coli and Bacillus subtilis grown in complex medium (8 g nutrient broth and 40 g glucose per litre) showed no P503 band. Absence of this pigment was confirmed by difference spectrophotometry. Since Nosoh (1964) had previously observed that respiration seemed to be essential to obtain the 503nm band, the respiratory activity was determined on complex medium. The high values obtained (1965) led to their conclusion that P503 is absent in bacterial cells under favourable conditions. The activity of the HMP shunt was reported by Model and Rittenberg (1967) to be high during logarithmic growth on glucose in minimal medium and significantly less as cells entered the stationary phase. The identical behavioural pattern of the appearance of the P503 band and the activity of the HMP shunt is remarkable. This group also reported the shunt activity to be less when the minimal medium was enriched with casein hydrolysate or yeast extract- again paralleling observations made with P503. Since the amino acids and other compounds necessary for growth and division of the cells are already present in the medium, the cells need not build up stores of NADPH for reducing power in biosynthetic reactions. Consequently, P503 would not be induced and additional pathways producing high energy ATP would not be required.

4. Effect of L-methionine and its analogues as growth supplement on P503 formation

RESULTS

Olden and Hempfling (1970) mentioned in a brief abstract that E. coli grown in the presence of 1 mg/ml methionine had no P503. It was of interest to study this observation in more detail, especially since previous results (Tables VIII and IX) indicated a specific effect of L-methionine on eliciting a P503 band in non-growing cells. Table XVII summarizes the effect on P503 synthesis when E. coli B is grown on minimal salts-limited glucose medium supplemented with L-methionine and/or its analogues (Figure 10). In the case of ethionine, norleucine and selenomethionine, a large amount of P503 was formed; no effect of concentration was noticeable. Growth on L-methionine, at a concentration of 0.01%, repressed P503. In the presence of both L-methionine and an analogue (ethionine or norleucine), P503 formation was markedly decreased when compared to the effect of the analogue alone. This decrease was due to L-methionine, even though it was present at a concentration 1/10th that of the analogue.

Other individual amino acids such as L-cysteine and glycine as growth supplement had no effect on P503 formation (L-cysteine, however, adversely affected the growth rate). Thus, a more complete mixture was tested. Casein hydrolysate proved to be just as effective as L-methionine in its ability to abolish P503 synthesis. When all the amino acids of casein hydrolysate were added to the growth medium in the appropriate proportions (West and Todd, 1955) including L-methionine, then the P503 band did not appear. However, when L-methionine was excluded from the mixture, then a peak at 503nm was once again produced. From these results, it would appear that

Table XVII. Effect of L-methionine and its analogues as growth supplement on P503 formation (synthesis) in wild-type E. coli B (The same format is used as in Table XVI)

Supplement	Concentration of supplement in the medium (%)	Height of P503* (units)
DL-ethionine	0.01	73.0
"	0.10	71.7
DL-norleucine	0.01	94.2
"	0.10	98.7
DL-selenomethionine	0.01	50.5
"	0.05	52.7
L-methionine	0.01	0.0
"	0.10	0.0
L-methionine + DL-ethionine	0.01 + 0.10	12.8
L-methionine + DL-norleucine	0.01 + 0.10	29.2
casein hydrolysate	0.05	0.0
"	0.10	0.0
**18 aa's plus L-methionine	0.10	0.0
**18 aa's without L-methionine	0.10	61.7

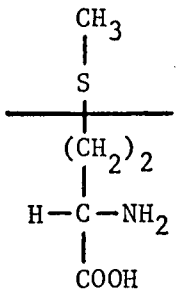
*See Table XVI.

**The amino acids (aa's) ± L-methionine were used in the proportions given for casein hydrolysate (West and Todd, 1955) (i.e., the 21 aa's minus tryptophan, glutamine and asparagine).

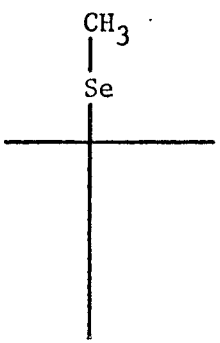
The cytochrome and flavin spectra were not affected by the supplements.

Cells were grown on 0.2% glucose, harvested, washed in phosphate buffer, air-oxidized, then resuspended in minimal salts-glucose (0.04%) medium supplemented with L-methionine or its analogues at the concentrations indicated. At the end of log phase, the cells were harvested, washed, and air-oxidized for 1 hr. The reduced/oxidized difference spectra were obtained by addition of glucose in each case.

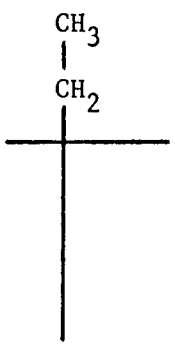
methionine



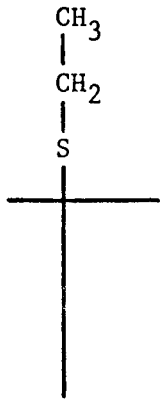
selenomethionine



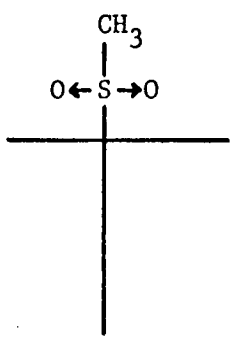
norleucine



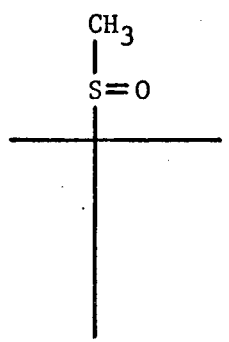
ethionine



methionine sulfone



methionine sulfoxide



N-acetyl methionine

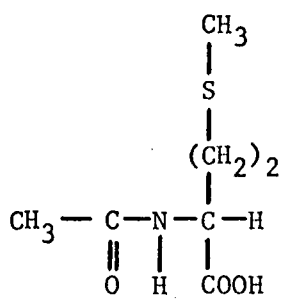


Figure 10. Structural analogues of methionine. The first 5 analogues have the same structure as methionine in the portion below the horizontal line. The last analogue differs from methionine only by the addition of an acetyl group attached to the nitrogen.

the lack of P503 formation when cells were grown on casein hydrolysate was in fact due to the presence of L-methionine.

The presence of L-methionine and/or a structural analogue in the growth medium was found to influence the rate of growth (Table XVIII) with respect to control conditions (glucose alone). In all cases, the semilog plot of increase in absorbance at 420nm versus time indicated that exponential cell growth was maintained when an analogue was included in the medium. The doubling time decreased slightly with L-methionine or casein hydrolysate supplementation. When all the amino acids of casein hydrolysate excluding L-methionine were added to the medium, the doubling time of 51.8 minutes was similar to the control (glucose-salts) value of 53.8 minutes. The inclusion of L-methionine in the mixture resulted in a marked decrease in generation time to 39.0 minutes-equivalent to an increase in growth rate of 27.4%. Growth in medium supplemented with DL-ethionine or DL-norleucine was slightly slower than the control when these compounds were present at a concentration of 0.01%, and much less at 0.10%. The addition of 0.01% L-methionine as well, to the medium containing 0.10% DL-ethionine or DL-norleucine countered the effect of the analogue alone, such that the new growth rate was similar to the control value. DL-selenomethionine exhibited a similar pattern of slowing down the growth rate, its effect being enhanced at the higher concentration (0.05%). Of the analogues tested, the amount of decrease in growth rate was maximal in the case of selenomethionine. The increase in growth rate with 0.01% L-methionine was not accompanied by a simultaneous increase in growth efficiency. In fact, 0.01% or 0.10% L-methionine as supplement in limiting glucose-salts medium produced the

Table XVIII. Effect of L-methionine and its analogues as growth supplement on rate of growth (doubling time) of wild-type E. coli B

Supplement	Concentration of supplement in the medium (%)	Doubling time (minutes)
none (control)*	-	53.8
L-methionine	0.01	49.3
casein hydrolysate	0.10	46.1
**18 aa's plus L-methionine	0.10	39.0
**18 aa's without L-methionine	0.10	51.8
DL-ethionine	0.01	58.7
"	0.10	76.2
DL-ethionine + L-methionine	0.10 + 0.01	58.7
DL-norleucine	0.01	58.8
"	0.10	80.0
DL-norleucine + L-methionine	0.10 + 0.01	53.8
DL-selenomethionine	0.01	68.5
"	0.05	85.8

*Control cells were grown on 0.04% glucose.

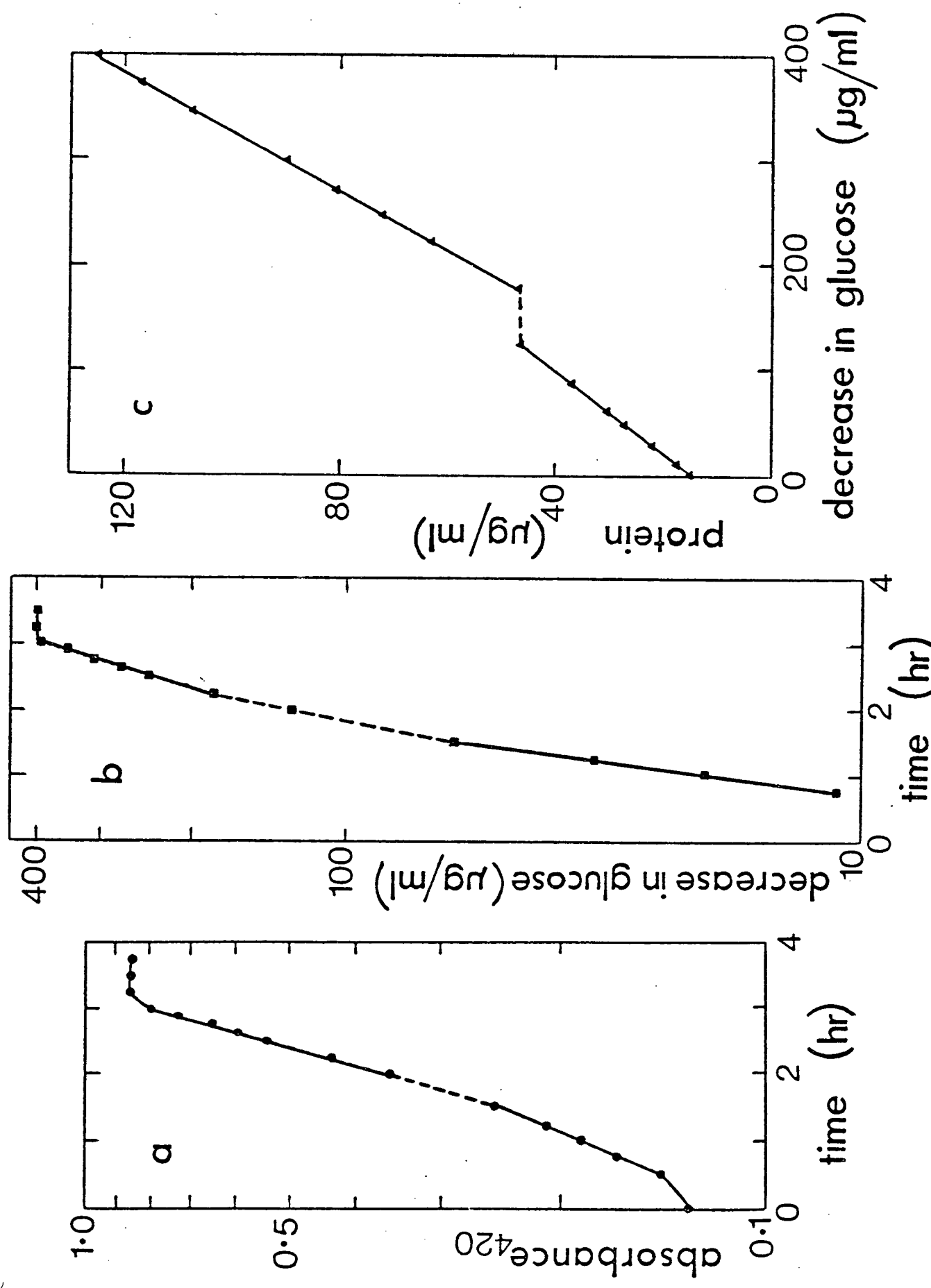
**See Table XVII.

Semilog plots were prepared of increase in absorbance$_{420}$ with time for the experiments described in Table XVII, and the doubling times (minutes) calculated in each case.

same efficiency as the control (0.04% glucose alone).

The unique effect of L-methionine supplementation in causing the elimination of P503 in wild-type *E. coli* B prompted an investigation of a methionine-requiring (met^-) auxotroph, *E. coli* K12W-6. In order to ascertain generally if an optimal concentration of L-methionine existed which resulted in the highest efficiency of growth, cells were grown on minimal salts-limiting (0.04%) glucose medium supplemented with L-methionine from 0.001% to 0.10%. Although the growth rate for this mutant was essentially the same in all cases, L-methionine at a concentration of 0.02% appeared to result in a slightly higher efficiency in cell yield. A peculiar observation was the biphasic nature of the arithmetic plot of efficiency (μg/ml increase in protein versus μg/ml glucose consumed). Two slopes were obtained in all cases, the initial slope (0.270) being less than the second (0.380). Although the efficiency for wild-type K12 was found to be 0.289, this strain was not the parent of the met^- mutant, so that the two organisms could not be compared. This unexplainable biphasic property was thought at first to be an artifact; however, its occurrence in all experiments involving this mutant would tend to suggest otherwise. In fact, semilog plots of growth rate ($absorbance_{420}$ versus time) and rate of glucose consumption (μg/ml glucose consumed versus time) also were biphasic. Although the initial rates were less than the final for growth and efficiency, glucose consumption showed the opposite effect; namely, consumption was more rapid at the beginning of growth. Replacement of L-methionine by DL-ethionine resulted in a decreased growth rate and lower efficiency but the biphasic nature of the three plots persisted (Figure 11).

Figure 11. Biphasic nature of growth, glucose consumption, and protein yield from glucose for the met$^-$ mutant, E. coli K12W-6. Slope changes at broken lines. Cells were grown on minimal salts-0.04% glucose medium supplemented with 0.02% L-methionine. (a) semilog plot of increase in absorbance$_{420}$ versus time (hours); (b) semilog plot of decrease in glucose (µg/ml) versus time (hours); (c) arithmetic plot of protein yield (µg/ml) versus glucose consumed (µg/ml).


a
absorbance420
1·0
0·5
0·1
0
2
4
time (hr)
b
decrease in glucose (µg/ml)
400
100
10
0
2
4
time (hr)
c
protein (µg/ml)
120
80
40
0
0
200
400
decrease in glucose (µg/ml)

The reduced/oxidized difference spectra of the met^- mutant indicated no P503 peak with L-methionine supplementation; however, substitution of L-methionine by DL-ethionine in the medium produced the 503nm band. Since these studies with the met^- mutant did not further elucidate the effect of L-methionine on P503, our focus was shifted once again to wild-type *E. coli* B.

For several reasons (explained in the DISCUSSION) L-methionine was thought to be a modulator of enzyme activity, the target enzyme being either glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase. Brief experiments were therefore carried out to determine whether this amino acid exerted an inhibitory effect on either enzyme. The substrate concentration (glucose 6-phosphate or 6-phosphogluconate) was varied in the reaction mixture from 1/20 to 1/100 the normal amount, and controls (minus L-methionine) were run to establish the minimum substrate concentration allowable before it became limiting, thereby resulting in a decrease in enzyme activity. L-methionine (from equimolar amounts to 10x the concentration of the substrate) was then added to cell extracts prior to addition of the reaction mixture to eliminate the possibility of $NADP^+$ or glucose 6-phosphate (or 6-phosphogluconate) protecting the active or allosteric site and/or preventing any binding of L-methionine to the enzyme. The results were negative for both enzymes; neither enzyme activity was affected by the presence of L-methionine.

The possibility that S-adenosylmethionine was the active compound responsible for inhibition was also tested; however, no effect was observed on either enzyme. The other substrate, $NADP^+$, was varied in a similar manner, keeping glucose 6-phosphate (or 6-phosphogluconate) constant at

1/25 (or 1/50) the normal concentration. The results were again negative.

It should be emphasized that these investigations on the effect of L-methionine or S-adenosylmethionine on enzyme function were not carried out in detail, nor were they repeated. Their sole purpose was to determine if a general inhibition could be obtained. If the results had indeed indicated a possible inhibitory effect, then further detailed enzyme studies would have been in order. Only a qualitative statement can be made, therefore, that L-methionine alone does not inhibit either glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase, so that the collective effects of L-methionine on P503 cannot be explained on the basis of an effect of the amino acid on these HMP enzymes.

DISCUSSION

The utilization of structural analogues of L-methionine by E. coli has been investigated by numerous laboratories in recent years. L-selenomethionine is most likely the closest analogue resembling L-methionine, replacement of the sulphur (in methionine) by selenium causing little disturbance of the molecular conformation. In fact, the analogue has been shown to substitute for L-methionine in diverse reactions. Nisman and Hirsch (1958) studied the activation (for protein synthesis) of L-methionine and its analogues by enzymatic fractions of E. coli. Whereas D-methionine showed no activation, and DL-ethionine and norleucine stimulated PP^{32} exchange only to 30 and 15% of the methionine rate, DL-selenomethionine proved to be a better substrate than L-methionine itself. The same authors reported the formation of Se-adenosylselenomethionine catalyzed by S-adenosylmethionine synthetase. Se-adenosylselenomethionine can serve as a methyl donor for bacterial RNA and DNA

methylations (Wu and Wachsman, 1971), as well as for the biosynthesis of creatine and choline (Mudd and Cantoni, 1957). Furthermore, it has been shown to be an excellent substrate for spermidine biosynthesis (Pegg, 1969) although another analogue, S-adenosylethionine, proved to be a poor substitute. The question then arises whether selenium can replace all the sulphur in E. coli. Cowie and Cohen (1957) found no selenoglutathione formed. β-Galactosidase (containing 80 cystine and 150 methionine residues per mole protein) was isolated and analyzed from E. coli grown on Na selenate (Huber and Criddle, 1967). Since none of the cystine or cysteine moieties of the enzyme were replaced by their selenium analogues while 80 out of 150 of the methionine residues had been replaced by the corresponding selenium compound, these workers concluded that the use of selenium in amino acids was restricted to the formation of selenomethionine. (Perhaps substitution by seleno- cystine and cysteine would prove to have a drastic effect on the chemical properties in contrast to selenomethionine). The newly-formed β-galactosidase was unchanged in its catalytic properties (Km and Vmax) and pH optimum. However, properties depending on the tertiary structure of the enzyme were found to be affected, the Se-enzyme being less stable to heat and urea denaturation than the S-enzyme. Cowie and Cohen (1957) showed selenomethionine could replace methionine completely in proteins of E. coli and still allow exponential growth and active enzyme synthesis; however, the concentration of the analogue in the growth medium was found to influence the growth rate. While a concentration of 10^{-5} M selenomethionine gave the same rate of growth as the control (Coch and Greene, 1971; Cowie and Cohen, 1957), increasing amounts (10^{-4} to 10^{-2} M)

caused a progressive decrease in the final rate and level of growth. Our results (Table XVIII) confirmed their observations, 0.01% (or 5.1×10^{-4} M) increasing the doubling time from 53.8 to 68.5 minutes, and 0.05% (or 2.6×10^{-3} M) still further to 85.8 minutes. Perhaps the greatest detrimental effect exhibited by this analogue (more than with ethionine or norleucine) can be attributed to its having the closest resemblance to the natural amino acid (substitution of the sulphur by selenium constituting a more subtle change), consequently replacing methionine in more processes than the other analogues.

The other analogues tested, differing more in structure and chemical properties, would be expected to alter enzyme functions much more drastically if they are substituted for L-methionine in proteins. For example, introduction of an extra methylene group into the methionine side chain (as in ethionine) would most likely interfere with spatial relationships necessary for the active three dimensional structure of the enzyme. Thus Spížek and Janeček (1969) found that increasing levels of ethionine led to a decrease in β-galactosidase activity, an enzyme not known to involve methionine in its catalytic mechanism (Wallenfels and Malhotra, 1960). Since the changed β-galactosidase was as effective as the normal enzyme with respect to its cross-reacting ability (antigen-antibody precipitation), the effect of ethionine as a replacement must have resulted in formation of an inactive protein.

The ability of an analogue to decrease the synthesis of methionine in organisms by feedback repression would be one indication of its similarity to the natural amino acid (with respect to shape, size, and ionic configuration), such that the structural requirements of the

biosynthetic enzymes would be satisfied. Rowbury and Woods (1961) found that growth in the presence of L-methionine repressed its biosynthesis in E. coli; the effect was reversible, and the cells regained this activity when incubated in a growth medium without L-methionine. An analysis of several analogues showed effectiveness of repression in the following order: DL-methionine sulphoxide > DL-norleucine > DL-methionine sulphone > DL-ethionine. The effect of methionine sulphoxide was attributed to its conversion to methionine, since Sourkes and Trano (1953) showed that the analogue could be reduced by E. coli in the presence of molecular hydrogen via the hydrogenase system. In contrast, the 60% repression exerted by the sulphone was assumed to be legitimate since this compound was not reduced via the hydrogen-hydrogenase system nor could it support the growth of methionine auxotrophs.

An alternate determinant of the similarity of an analogue to a natural amino acid is its ability to displace the amino acid from internal pools. Richmond (1962) found that L-methionine-C^{14} could be displaced by L-norleucine but not by D-norleucine or D-methionine in E. coli. These results suggested that structural requirements were involved both in holding an amino acid in the pool and in the ability of an exogenous compound to displace this amino acid from the pool. Richmond also found that whereas L-norleucine could displace both valine and methionine from internal pools, neither of the natural amino acids was able to displace the other. Thus norleucine was sufficiently similar to both valine and methionine to be taken up at their sites.

In contrast to the findings of Rowbury and Woods (1961), Munier and Cohen (1959) found that norleucine did not inhibit methionine biosynthesis, but rather inhibited the incorporation of methionine into proteins. The

inhibition of growth via norleucine was therefore a result of the synthesis of abnormal proteins; replacement of internal- and N-terminal methionine as well as leucine residues was thought to produce a catalytically inactive enzyme. The ability of norleucine to replace methionine in the initiation of protein synthesis in E. coli was confirmed by Trupin, Dickerman, Nirenberg and Weissbach (1966), who observed not only that $tRNA_M^{met}$ and $tRNA_F^{met}$ could be acylated with norleucine, but further that norleu-$tRNA_F^{met}$ could also be converted to f-norleu-$tRNA_F^{met}$ in a reaction analogous to the formylation of met-$tRNA_F^{met}$. Kerwar and Weissbach (1970) found f-norleu-$tRNA_F^{met}$ could substitute for f-met-$tRNA_F^{met}$ in subsequent reactions leading to the incorporation of the formylated species into newly synthesized protein; no differences were observed in binding to ribosomes, puromycin reaction, and deformylation. They noted, however, that norleucine could not substitute for methionine in reactions involving the metabolism of the amino acid.

Of the analogues utilized in our methionine studies, the N-acetyl compound appears to be the least investigated; very little has been published on its properties. Brief mention was made by Davis and Mingioli (1950) that this compound could substitute for methionine to support the growth of B_{12} auxotrophs (methionine could apparently replace the vitamin in the growth medium).

The unique effect of L-methionine on prevention of P503 synthesis (even at the low concentration of 0.01%) was unexplainable. Under circumstances in which a particular functional group is responsible for promoting a given reaction, removal of the required moiety or its replacement by an alternate (but similar) compound usually pinpoints the target. In the

situation concerning P503, however, none of the analogues was a successful substitute.

Plausible explanations were therefore sought as to what effect the presence of L-methionine might have on the bacterial cells. The results obtained thus far with L-methionine and its structural analogues suggested that L-methionine was behaving like an effector or modulator of enzyme activity, possibly of glucose 6-phosphate- or 6-phosphogluconate dehydrogenase: (1) Addition of L-methionine to glucose-grown cells caused the appearance of a peak at 503nm in less than 0.9 minutes (the shortest time it was possible to measure), suggesting an effect either by its presence per se, or by its "active" form, S-adenosylmethionine; (2) The stereospecific nature of the effect of L-methionine on eliciting a P503 peak in non-growing cells as well as on formation of the pigment would suggest a stereospecific recognition of L-methionine by some allosteric site on an enzyme; (3) The low concentration of L-methionine required (and its being effective against a ten-fold greater concentration of its analogue) would imply a low Km value, catalytic nature, and physiological range- all involved with enzyme functions; (4) The sulphur or methyl moiety per se were not significant; rather, the complete structure was essential, possibly implicating a three-dimensional conformation required in a recognition process or for its fit into a groove in an enzyme; (5) Presence of L-methionine in the medium did not increase the efficiency of growth although the rate of growth was enhanced. The possibility of a role of L-methionine in controlling an enzyme function prompted investigations on glucose 6-phosphate- and 6-phosphogluconate dehydrogenase, the two enzymes responsible for formation of NADPH. The increase in G6-P dehydrogenase activity in contrast to th constancy of 6-phosphogluconate dehydrogenase under the influence

of DNP was interpreted as indicating the latter enzyme to be under stringent control. The modern concept of feedback regulation of the first enzyme in a biosynthetic pathway also favoured 6-phosphogluconate dehydrogenase (the first enzyme specific to the hexosemonophosphate shunt) as the more likely candidate. The limited experiments testing this possibility were all negative, and it appeared that methionine or S-adenosylmethionine did not regulate either of the dehydrogenases. On the other hand, if their effect depended on the presence of other factors such as coenzymes or a particular cell energy charge in the inhibitory mechanism, the lack of appropriate conditions may have been responsible for the negative results obtained.

The influence of methionine versus its analogues on the growth rate of E. coli was yet another property peculiar to methionine. The dramatic effect observed when the medium was supplemented with 18 amino acids $\pm$ methionine emphasized this point even further. The increase in growth rate without a concomitant increase in growth efficiency suggested an effect of methionine due to its presence per se. This phenomenon was mentioned earlier by Davis and Mingioli (1950) but was not discussed by them. They found that growth of wild-type E. coli in minimal medium was accelerated by the addition of either methionine or B_{12}; other amino acids and vitamins did not singly produce this acceleration. In view of the specificity in the relationship between P503 and methionine, an explanation may arise only after the complete story of P503 is elucidated.

The limited studies on the methionine auxotroph, E. coli K12W-6, indicated an optimal concentration of methionine (0.02%) which resulted in the highest efficiency of growth. Various laboratories utilizing a

met^- mutant in their investigations have reported supplement concentrations ranging from 0.002% to 0.20%. Mansouri, Decter and Silber (1972) studied the regulation of one-carbon metabolism in E. coli. They found that the interconversion of serine and glycine, mediated by serine hydroxymethyltransferase, was controlled by the level of methionine in the medium. Thus low levels of methionine (1.3×10^{-5} M) enhanced the specific activity, whereas higher levels (1.3×10^{-4} M) repressed enzyme activity. Since the addition of methionine or S-adenosylmethionine to the serine hydroxymethyltransferase assay mixture had no effect on specific activity, regulation by methionine was considered to be not via feedback inhibition; rather, the level of the amino acid in the growth medium was instrumental in causing the repression-derepression of the enzyme. It is not therefore surprising that an optimal concentration of methionine does exist.

The semilog plots of growth rate and glucose consumption as well as the arithmetic plot of efficiency were biphasic. Whereas the final slope in the two cases, growth rate and efficiency, was greater than that during the initial phase, glucose consumption was observed to be more rapid at the beginning of growth. A possible explanation for this difference might be that the cells were low in energy initially and rapid metabolism of glucose occurred to build up a supply of ATP and NADPH for biosynthetic reactions. Once the higher steady state level was reached, less glucose was needed, and protein synthesis increased, accounting for greater efficiency and a faster growth rate.

Growth of the methionine auxotroph on ethionine decreased both the growth rate and the efficiency. Since the mutant can use neither inorganic sulphate nor cysteine as a source of methionine, it follows that ethionine must replace all the methionine residues in proteins. As discussed previously, ethionine is not sufficiently similar to methionine in structure that it can replace the amino acid without causing adverse effects. Furthermore, Beaud and Hayes (1971) found that ribosomes of an auxotroph grown on ethionine in place of methionine were submethylated, resulting in the formation of 30S and 50S subunits defective in the capacity to associate at a high Mg^{++} concentration (10 mM) to form 70S particles.

The lack of P503 in wild-type E. coli grown in the presence of L-methionine was observed in the met^- mutant. Since the pigment was formed when ethionine was used, one can conclude that the mutation to methionine dependence did not affect the P503 system.

The question still remains as to the mechanism underlying the ability of methionine to prevent formation of P503. It was previously mentioned that Lazzarini and Atkinson (1961) reported a NADPH-specific nitrite reductase in E. coli. The purified enzyme was found to contain three additional activities: cytochrome c reductase, hydroxylamine reductase, and sulfite reductase, all specific for NADPH. In addition, Mager (1960) reported the NADPH-specific sulfite reductase and NADPH-specific hydroxylamine reductase (perhaps the same enzyme) to be feedback repressed by cysteine or cystine and to a lesser extent by methionine. Lampen, Roepke and Jones (1947) showed that the second (and not the first) step of sulfite reductase ($HSO_3^- \longrightarrow H_2S$) was catalyzed by an enzyme specifically linked to NADPH. The initial stages of sulfite assimilation prior to attachment

of the sulphur atom to the carbon skeleton of serine (to form cysteine) requires much energy in the form of ATP as well as NADPH. Perhaps then the energy-sparing effect and/or a specific repression by methionine of the enzyme catalyzing the step NADPH $\longrightarrow$ P503 (a justified possibility) is responsible for the lack of P503 formation.

With regard to the "energy-sparing" effect of L-methionine, one might argue a similar result should then be observed via supplementation with cysteine or cystine. Thus cysteine, an intermediate in the biosynthesis of methionine, should accelerate methionine synthesis and consequently facilitate growth. This was not observed; contrary to expectations, the presence of L-cysteine in the medium increased the doubling time from 53.8 (control value) to 146 minutes. The severe repression of cysteine on sulfite reductase might affect the synthesis of some other essential sulphur compound. On the other hand, a completely separate process altogether may be affected. Schachet and Squire (1971) reported that 7.5×10^{-3} M cysteine caused very rapid inactivation of bovine adrenal glucose 6-phosphate dehydrogenase. Although this action of cysteine was not observed in our experiments, the difference may have been due to a concentration effect. The reasons for the adverse effect of cysteine on cell growth remains obscure.

Another possible site of enzyme control by L-methionine may be between NADPH $\longrightarrow$P503. The possibility of methionine repressing the NADPH $\longrightarrow$P503 step is not only attractive, but also quite feasible. The previously mentioned studies of Mansouri, Decter and Silber (1972) showed methionine to control the activity of serine hydroxymethyltransferase. Furthermore, methionine has been shown to regulate other folate-dependent

reactions including methyl transferase (catalyzing 5-methyltetrahydrofolate + homocysteine ⟶ methionine) and reductase (catalyzing 5,10-methylenetetrahydrofolate ⟶ 5-methyltetrahydrofolate). Indeed the diversity of reactions already found to be controlled by methionine supports the possibility of its regulatory action over yet another enzyme.

5. Effect of other compounds as growth supplement on P503 formation

RESULTS

Growth of wild-type E. coli B on limiting glucose supplemented with 0.01 to 0.05% ascorbic acid, 0.01 to 0.10% folic acid, or 0.04% 2-deoxyglucose had no effect on either growth rate or P503 formation. In all cases, a large amount of P503 was synthesized.

GENERAL DISCUSSION AND CONCLUSIONS

A study of four Escherichia coli strains (B, K12, UL and CRX) indicated a consistent pattern of 25-35% decreased aerobic efficiency in the Sm-dependent mutant compared to the wild-type parent. A non-dependent revertant (SBr4), derived from the Sm-dependent mutant of E. coli B, showed a similar decreased aerobic efficiency. The anaerobic protein yield proved to be identical in all cases although lower than under aerobic conditions. Subsequent calculation of the solely aerobic portion of growth revealed that wild-type cells produced double the amount of energy that could be ascribable to aerobic metabolism, as that produced by dependent cells.

In view of previous findings regarding the less efficient, uncontrolled nature of metabolism peculiar to this mutant (namely, excretion of valine when grown on glucose (Bragg and Polglase, 1962; Tirunarayanan, Vischer and Renner, 1962) as well as de-repressed levels of catabolite repressible enzymes (Coukell, 1969)), investigations were initiated on hydrogen metabolism in an attempt to elucidate the mechanism(s) responsible for this decrease in energy yield. The only difference noticeable in the reduced/oxidized difference spectra of aerobically grown cells was that the large, transient, symmetrical peak at 503nm in wild-type E. coli was absent or markedly decreased in Sm-dependent mutants (and the non-dependent revertant); the cytochromes and flavin were identical. Consequently, the remainder of the thesis was focussed on characterizing the properties of this 503nm pigment (P503) in wild-type E. coli B.

Numerous and varied attempts to obtain a peak at 503nm in permeabilized cells or in crude extracts were futile; the pigment proved to be quite labile under all conditions and could not be detected. Further

investigations were therefore carried out on whole cells.

Subsequent studies involved characterizing either (1) reduction of the pigment in non-growing cells or (2) its formation (synthesis) during growth. When the effects of several types of compounds on production of the 503nm peak were compared, a high level of specificity was found. In the case of sugars and acids, whereas reduction by gluconate produced only a substantial P503 peak in the first few minutes prior to the steady-state levels of the cytochrome and flavin bands, succinate as substrate resulted in the initial appearance of flavin followed by cytochrome b with only a trace of P503. Glucose (as control) elicited the entire spectrum immediately. Other substrates such as glycerol, lactate, and acetate showed an absence or negligible amount of P503 although cytochrome b and flavin were constant. For all cases in which little (or no) P503 peak was obtained, the addition of glucose after a 10-15 minute lag caused the rapid appearance of the 503nm peak, indicating that the cells did possess P503 but that the substrates added initially were unable to elicit its appearance. These results implicated a relationship between P503 and NADPH, such that the reduced coenzyme might transfer its hydrogen to the 503nm pigment.

A similar survey of L-amino acids as reductants singled out the effect of methionine. Addition of L-methionine or casein hydrolysate (but no other amino acids) produced only a P503 band within 0.9 minutes; after a lag of 10-14 minutes, the cytochromes and flavin were reduced. Structural analogues of L-methionine were then studied to test both the stereospecificity of action as well as the various functional substituents. The results indicated that no changes whatsoever were allowable, further emphasizing the uniqueness of the effect of L-methionine on P503. Among other compounds tested ascorbic

acid, folic acid, betaine, and 2-deoxy-D-glucose were also ineffective in eliciting a large P503 peak in non-growing cells.

Experiments concerning the effect of growth conditions and supplements furnished information regarding the formation or synthesis of the 503nm pigment during growth. Anaerobically grown wild-type E. coli B possessed P503, the cytochromes, and flavin; however, since these were present in an oxidized state, it was concluded that they were nonfunctional anaerobically and did not accept electrons in the absence of oxygen.

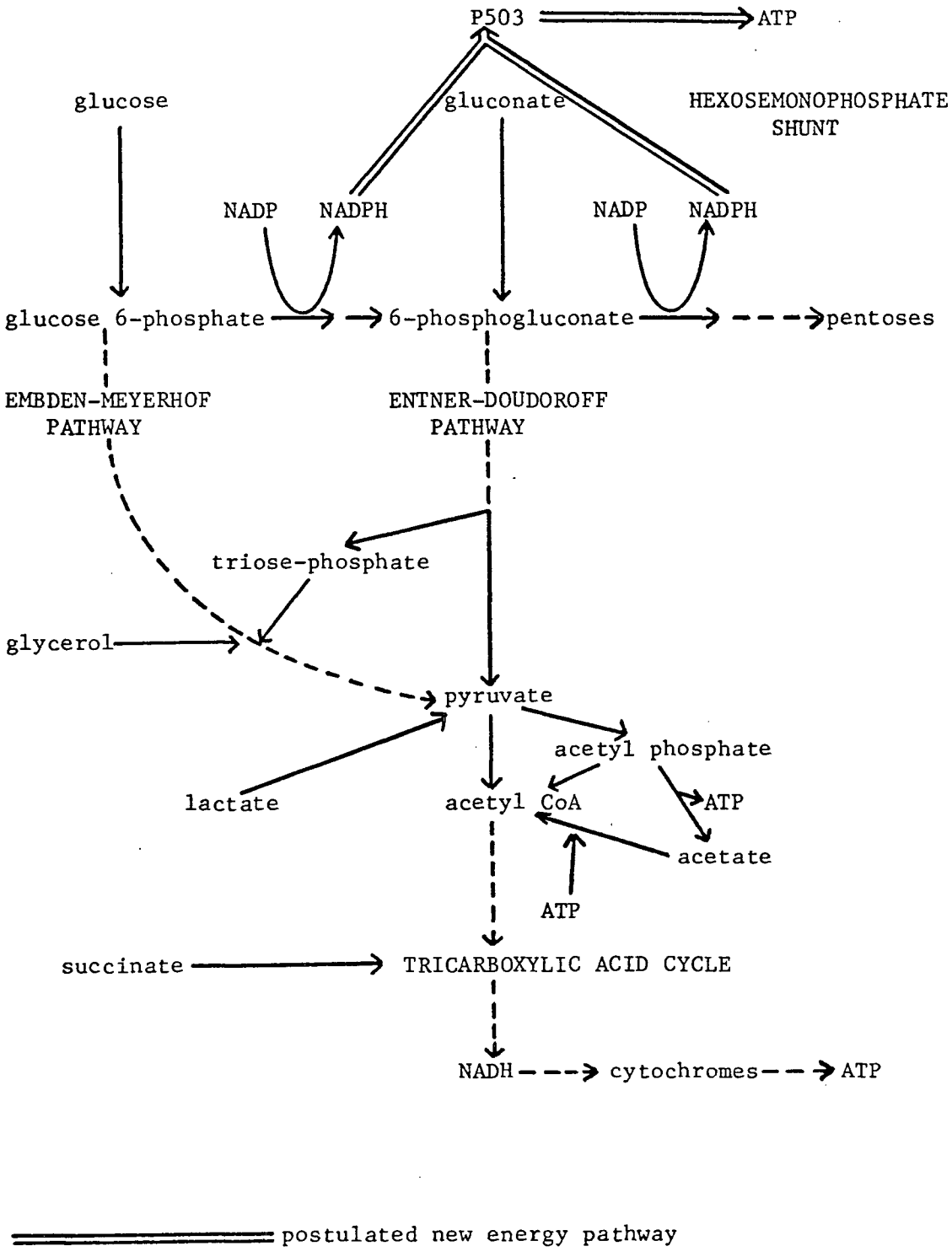
Inclusion of increasing concentrations of 2,4-dinitrophenol (DNP) in the minimal salts-limiting glucose medium resulted in a progressive decrease in aerobic growth rate, yield of cell protein, and height of the 503nm peak. At 500 μM DNP, wild-type cells were found to resemble the Sm-dependent mutant and non-dependent revertant in the following ways: (1) decreased aerobic efficiency (30%), (2) enhanced doubling time (87.5 minutes), (3) lack of P503 and (4) de-repressed levels of the catabolite repressible enzymes fumarase and aconitase. An unexpected result was the four-fold increase in glucose 6-phosphate dehydrogenase as well- an enzyme not known to be sensitive to catabolite repression. No effect of DNP was observed under anaerobic conditions or when the phenol was added to control cells just prior to analysis of the difference spectrum. Furthermore, its effect was reversible and re-growth of DNP-grown cells in the absence of DNP resulted in synthesis of the 503nm pigment once again. Other inhibitors including 50.0-250.0 μM 1,3,5-tribromophenol, 1.0-5.0 μM chloramphenicol, and 10.0- 100.0 μM hydroxylamine-hydrochloride did not have the same effect as DNP, emphasizing the rather unique quality of the last compound. The collective results

obtained with DNP were compatible with the following interpretation: DNP might undergo reduction by oxidizing NADPH. Removal of the NADPH, a possible co-repressor of glucose 6-phosphate dehydrogenase, would then displace the equilibrium of the enzyme, thereby increasing its activity. In addition, removal of the reduced coenzyme would prevent induction of the 503nm pigment, previously suggested as receiving hydrogen from NADPH. The decreased growth efficiency under the influence of DNP observed only in aerobiosis (and not anaerobiosis) which accompanied the elimination of P503 could implicate P503 in a major aerobic pathway of energy metabolism.

The following general hypothesis was subsequently put forth: an oxidative pathway which generates ATP from NADPH via an intermediate pigment, P503, and which accounts for 25-35% of the total energy or 50% of the solely aerobic energy with glucose as carbon source, is present in wild-type E. coli cells grown on a minimal salts medium (Figure 12). In Sm-dependent cells and DNP-grown wild-type cells, a deficiency in P503 (caused by mutation in the former and some effect of the drug in the latter case) resulted in elimination of the NADPH $\longrightarrow$ P503 $\longrightarrow$ ATP pathway.

A survey of compounds (analogous to that conducted on the reduction of the 503nm pigment) further elucidated the nature of P503 synthesis. Glucose and gluconate as carbon source resulted in the formation of a significant peak at 503nm. In contrast, succinate, glycerol, or lactate as sole carbon and energy source produced a smaller P503 peak. Glucose and gluconate metabolism involve the synthesis of NADPH during the process of their dissimilation via the hexosemonophosphate shunt,

Figure 12. Pathways of carbohydrate metabolism in Escherichia coli



whereas the latter three compounds (succinate, glycerol, and lactate) do not generate NADPH at an early step in their metabolism. A concentration effect of glucose and gluconate on P503 formation was also observed, such that increasing concentrations of either sugar caused a progressive decrease in height of the 503nm peak. Although a possible relationship between P503 and catabolite repression was suggested, the connection was obscure and no conclusions could be made in this regard.

The previously discovered uniqueness of L-methionine on reduction of the 503nm pigment in non-growing cells was extended to the effect of this amino acid as supplement on P503 synthesis in growing cells. No other L-amino acid singly, nor a mixture of the 18 amino acids of casein hydrolysate (minus methionine), possessed the ability of methionine to prevent the formation of P503; indeed, no structural analogue of L-methionine, even at a ten-fold higher concentration, was able to duplicate the effect of this amino acid, implicating stereospecificity and chemical integrity as being requisite in both reduction and formation (synthesis) processes. Another distinction of L-methionine was the enhanced growth rate of the cells during its presence in the medium without a concomitant increase in growth efficiency (yield of protein). Further studies to elucidate the explanations underlying these observations indicated that L-methionine (or its active form, S-adenosylmethionine) alone was not exerting its effect by inhibiting either glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase. Other cofactors or the energy state of the cells might be involved in the mechanism of inhibition.

At this point, it should be clearly stated that methionine holds a singular position in the realm of amino acids. As more biochemical knowledge is accumulated, the diversity of reactions in which methionine participates is being extended. This amino acid is required not only for incorporation into proteins, but also for various methylation reactions, synthesis of polyamines, and initiation of protein synthesis. Although its relationship to P503 is still obscure, a consideration of the total influence of this amino acid supports its role as an effector in the control of an enzyme function, possibly between NADPH$\longrightarrow$P503. The final proof that L-methionine is a modulator of such a reaction, however, must await a demonstration of its effect in a purified cell-free system, a seemingly difficult task in view of the extreme lability of the 503nm pigment.

The chemical identity of the 503nm pigment has not yet been established. Although such a study would perhaps entail its prior isolation, one might evaluate the overall characteristics observed in support of or in contrast to those structures already proposed in the literature. The likeness of P503 to a cytochrome- or flavin-type compound has been favoured by several investigators, while rejected by others. Nosoh (1964) reported that the appearance of the 503nm peak was related to the disappearance of a 519nm peak and vice versa; in fact, he found the change in height of the 503nm band to be accompanied always by a change in various cytochrome bands. Olden and Hempfling (1970) stated that the pigment absorbing at 503nm could not be attributed to a cytochrome, but that it appeared to be in kinetic equilibrium with flavin. On the other hand, Lindenmayer (1959) and Lindenmayer and Smith (1964) found no

other absorption band to appear paralleling the change in P503. Two results obtained from our work would tend to support the dissimilarity of P503 with respect to both the cytochromes and flavin: (1) Whereas the height of the 503nm peak was seen to increase, decrease, and eventually disappear upon reduction by glucose, the peaks of the cytochromes and flavin trough were constant; families of curves recorded from 700-400nm during the progressive decline of the 503nm peak showed perfectly superimposed bands for the other pigments; (2) The time lapse between the appearance of the steady-state level of the 503nm peak versus that of the cytochromes (and flavin) upon reduction by gluconate and L-methionine (approximately 3 and 10-14 minutes respectively) suggests that the 503nm pigment has a lower (more negative) redox potential than the respiratory pigments. Other properties of P503, namely the single absorption band as well as the reactivity, were in agreement with observations reported by Lindenmayer and Smith (1964).

In view of the instability and formation anaerobically of P503, it is unlikely that P503 is the semiquinone of ubiquinone. The absorption characteristic of the semiquinone form of free flavin or enzyme-bound flavin is also dissimilar to that obtained for the 503nm pigment.

One final proposal was the protoporphomethene (or tetrahydroporphyrin) nature of P503 (Labbe, Volland and Chaix, 1967; Olden and Hempfling, 1970). The ability of porphyrins to undergo stepwise reduction (Mauzerall, 1962) through two stages to protoporphomethene (500nm), and further with certain reducing agents to a third colourless form (porphyrinogen), makes this group of compounds a very favourable candidate. There are several points, however, that must be explained before

one can state this structure of P503 with certainty: (1) The porphyrin (400, 550, 590nm) and dihydroporphyrin (440, 735nm) forms were not detected in any of the spectral runs so that a precursor-type relationship could not be established; (2) Mauzerall (1962) reported the half-life of the tetrahydroporphyrin (500nm) to be approximately 20 hours at 100°C. In contrast, our experiments with P503 indicated the pigment to be transient, even when cell suspensions were heated to 80° after the appearance of P503, in an attempt to denature enzymes and "trap" the structure in its 503nm absorbing state; (3) Mauzerall (1962) found reduction by sodium dithionite (and sulfite) to give a 500nm peak (tetrahydroporphyrin), but reduction by ascorbic acid to cause reduction beyond the second stage to a colourless compound; (4) Labbe et al. (1967) and Mauzerall (1962) suggested the formation of a complex between sulfite and the methene bridge of tetrahydroporphyrin to produce the colourless structure. Although such a linkage is feasible and could explain the effect of cysteine, cyanide, and L-methionine as well, the difference with the seleno- analogue of methionine does not satisfy this proposed mechanism.

The first point might be explained in part by the time factor- the inability of the instrument to record the appearance of bands at several wavelengths simultaneously. Perhaps an analysis using a dual wavelength spectrophotometer set at the absorption maxima of two different reductive forms might reveal such a stepwise sequence. In addition, the 550 and 440nm bands might be obscured by the cytochrome b and broad flavin absorption pattern; however, one would then expect to see a shoulder in the peak (or trough) which disappeared with time. This observation was not made.

The comparatively long half-life of tetrahydroporphyrin tends to rule out this compound, since if P503 had this structure, one would expect the pigment to be found in cell extracts, especially if P503 were obtained first via reduction by glucose and the cells then plunged immediately into an 80° bath followed by disruption of cells. Several experiments on stabilizing or "trapping" the pigment in the 503nm absorbing form were all negative.

Assuming that "chemical" reduction is more complete than the substrate or "enzymatic" process (Beinert, 1956a), it is puzzling that Mauzerall obtained only partial reduction with dithionite and complete reduction with ascorbic acid, the latter compound stated by him to be a "mild" reducing agent. Although the concentration factor might account for the difference in extent of reduction, the concentrations were not given for either of these reductants.

Selenomethionine has been a useful substitute for methionine in experiments determining the importance of the sulphur atom, due to the subtlety of the change. Selenium and sulphur are similar in their physical and chemical properties (Jáuregui-Addell, 1966; Rosenfeld and Beath, 1964). Since the bivalent type of linkage is common to both compounds, one would not expect the replacement of sulphur by selenium to affect covalent bonding. The high degree of specificity involved in the dual effect of methionine on the 503nm pigment (reduction and prevention of its formation) is more in line with a regulatory role of the amino acid as mentioned previously. Only when a structure is found possessing all the characteristics inherent in the 503nm pigment will a definite chemical identification be achieved.

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APPENDIX

I should like to comment on the objections raised by the external examiner, Dr. W.P. Hempfling, so that a few points necessary to the understanding of the hypothesis might be clarified.

(1a) Objection: Dry weight, instead of protein determinations, should have been used to calculate the growth yield, since the original observations of Bauchop and Elsden related dry weight to amount of ATP formed. Some groups have found protein content of the cell to vary as a function of the growth rate. If dry weight is not included, however, the author should list the growth rates.

Answer: Coukell (1969) found that at low substrate concentrations (i.e. limiting glucose), the increase in absorbance at 420nm was directly proportional to the µg/ml increase in dry weight as well as to the µg/ml increase in total cellular protein, up to an A_{420} of 1.0. In our preliminary studies, using 0.04% glucose as C source, repeated protein determinations of the wild-type, Sm-dependent, and resistant ($\pm$DHSm) organisms of each strain indicated that the increase in A_{420} was also directly proportional to the µg/ml increase in protein.

In addition, under these experimental conditions (i.e., minimal salts and limited glucose), Coukell reported that a linear relationship existed in the plot of µg/ml increase in cell weight versus µg/ml glucose consumed. Our plots of µg/ml increase in protein versus µg/ml glucose consumed showed a similar linear relationship. It follows, then, that the use of protein determinations in growth yield calculations is justified. Since determinations of protein were reproducible and sensitive, yet simpler and faster than in the case of cell weight, we chose to use the former method in estimating the efficiency values.

It should also be mentioned that the growth rates (i.e. doubling times) were given for the wild-type and Sm-dependent mutant of E. coli B, the strain

used in most of the experiments.

(1b) Objection: Coliform bacteria may produce acetate during aerobic glucose metabolism, and it is possible that the lower growth yields for some of the bacterial strains are due to incomplete oxidation of glucose. Product assays were not done. The experiments using limited glucose somewhat temper this objection.

Answer: The last statement itself counters the criticism. The cells were grown not on an excess, but rather a limiting amount of glucose, so that intermediate metabolites would not be expected to accumulate to the extent expected when an excess of substrate is present. Experiments, in which absorbance measurements were continued for 2 1/2 hours after the glucose had been exhausted, indicated no further increase in the A_{420}. If acetate were an end product of incomplete glucose metabolism, one would then have expected the induction of the glyoxylate pathway after the exhaustion of glucose, and a resumption of growth.

In addition, Sm-dependent E. coli B grown aerobically on succinate as C source also resulted in a decreased cell yield compared to the wild-type organism.

Finally, in preliminary studies, determination of the CO_2 produced indicated that the Sm-dependent organism produced twice as much CO_2 as the wild-type when grown on limiting glucose. This result would imply complete oxidation of glucose.

(1c) Objection: Some measure of variance of the growth yield data should be included so that the significance of the differences of 25-30% can be evaluated.

Answer: This criticism would be justified if growth yield were determined on only a few occasions. However, cell yield experiments, using cell weight, had been carried out by Coukell many times; similarly, the protein yields had been estimated in our experiments, on each strain, for

well over six months. In addition, in every experiment, the control cells were checked for growth efficiency as well as the presence of P503. The results were consistent, the value recorded in the thesis being the average of all the determinations. In view of the numerous repetitions by both groups, the difference of 25-35% found in the growth yield between the Sm-dependent and wild-type organisms is significant and reliable.

(2) Objection: The P503 content may vary with time course of growth on different C sources, so that the cellular content of P503 should have been monitored during growth.

Answer: Kropinski, from our group, did monitor the P503 content during growth. He found that the pigment was produced early during log phase and that the steady-state level remained high throughout growth.

In our studies using different C sources of growth, the experimental conditions were kept constant to determine the specific effect of the particular sugar or acid. Thus, cells were adapted for growth on the appropriate compound prior to the actual experiment. After the cells were harvested, the suspensions were air-oxidized for 1 hour in buffer to deplete any endogenous substrates which might obscure the results. In all cases, the presence of P503 was tested by the addition of glucose as reductant, since glucose had previously been found to elicit the highest 503nm peak. Therefore, if any synthesis of the 503nm pigment had been allowed on the particular C source used, this method would give consistent results which could be used in comparison studies.

(3) Objection: The author did not test her hypothesis of NADPH oxidation through P503; therefore, an experiment is suggested. The growth yield of the wild-type should be measured in the presence and absence of methionine. If the P503 energy conservation pathway hypothesis is correct, then the addition of methionine should decrease the growth yield by an appropriate amount. The experiment should be carried out

with U-C^{14}-methionine to estimate the amount of methionine carbon incorporated which results in more oxidative metabolism of glucose. In another experiment U-C^{14}-glucose might be used so that an assessment of soluble products could be made. Should the growth yield not change or should it increase, the hypothesis would be untenable.

Answer: From the suggested experiment, Dr. Hempfling reveals, unfortunately, that he does not fully understand the hypothesis presented in the thesis, and has misinterpreted the implications of it.

First, the hypothesis relating NADPH oxidation through P503 was developed from studies comparing the growth efficiencies of the Sm-dependent mutant and the wild-type organism. When the presence or absence of P503 was observed to be the only difference between the two cell types, further studies were done using 2,4-dinitrophenol. This drug was found to decrease the growth efficiency, eliminate P503, and cause de-repression of the catabolite repressible enzymes. Its effect on glucose 6-phosphate dehydrogenase as well, implicated the reduced pyridine nucleotide, NADPH. The investigations on the specificity of the C sources as reductants in eliciting the spectrum of P503 also pointed to NADPH. A direct study of NADPH as reductant could be performed only on permeabilized cells, since cells are impermeable to this coenzyme. However, all attempts to obtain a P503 peak in disrupted or permeabilized cells were negative. Due to the extreme lability of P503, indirect evidence for the relationship between NADPH and P503 was necessitated using whole cells.

Secondly, as stated in the thesis (commencing on page 99, line 24), supplementation of the growth medium with 0.01% L-methionine increased the growth rate, but not the growth efficiency. The latter was found to be identical to the control condition (0.04% glucose alone). This result implies that the action of methionine was as the intact molecule; in fact, the conclusion was that methionine appeared to be acting as an effector

of an enzyme function, possibly diverting the metabolic route from the P503 system.

Methionine may have a small "sparing" effect since it can be incorporated into proteins, but it is added in such a minute quantity to the medium compared to the amount of glucose, that it would probably not be used as a C source. In addition, the structural analogues, which have been reported to substitute for L-methionine in various reactions such as protein synthesis, methylation of DNA and RNA, and biosynthesis of choline and spermine, were not effective in substituting for L-methionine with regard to the P503 system, suggesting again that the three-dimensional structure of the complete molecule is essential.

The last statement of the objection is incorrect. If the growth yield increases, then the hypothesis would be tenable. The P503 system is assumed to be operative as a result of its induction by the presence of excess NADPH. In the Sm-dependent mutant, a possibility for its excretion of valine was that the excess NADPH formed might be removed by the synthesis of valine (the latter amino acid serving as a neutral hydrogen acceptor). If methionine somehow prevents the over-production of NADPH (and therefore the induction of the P503 system), then growth of Sm-dependent mutants in medium supplemented with methionine should eliminate the wasteful production of NADPH, so that the intermediate product of glucose metabolism, pyruvate, would be metabolized via the TCA cycle, and not be used for the synthesis of valine. An increase in efficiency should therefore be observed, although perhaps not to the same extent as in the wild-type organism. This prediction can be tested using either the Sm-dependent mutant, or DNP-grown wild-type cells, since

both conditions were shown to be equivalent with respect to the P503 system. Since the completion of this thesis, such an experiment has been done by Joyce Boon. Wild-type cells were grown on medium supplemented with DNP and methionine simultaneously. A decrease was observed in the DNP-reduction products, implying that methionine did divert the route from the NADPH $\longrightarrow$ DNP path. A slight increase in efficiency was also observed. These experiments are still being conducted, using varying concentrations of methionine, so that a definite value for the increase in efficiency cannot be stated at this time.

The constancy in the efficiency of wild-type cells supplemented with methionine (as stated previously) is not unexpected, since although the P503 path is no longer available, this route is assumed to be used only when an excess of NADPH is present, and addition of methionine prevents the wasteful over-production of NADPH, thereby conserving on energy. The glycolytic path for ATP synthesis, as well as the TCA cycle, are presumed to be the same in both wild-type, and wild-type supplemented with methionine.

SUGGESTIONS FOR FUTURE WORK

Before the complete story of the 503nm pigment can be elucidated and its functional significance in cellular metabolism established, much more research must be done. Despite the widespread occurrence of this pigment, little attention has been given to it in the literature. It is the author's hope that the findings reported in this thesis will contribute in part to the understanding of the role of this pigment, and instigate renewed interest in this area. Due to the shortage of time, several points relevant to the support or contradiction of the hypothesis were not able to be examined fully. These suggestions are outlined below.

(1) The isolation, purification, identification and quantitative determination of the reduction products of 2,4-dinitrophenol would enable one to ascertain whether the reduction of DNP does, in fact, account for all the NADPH which is channelled off and removed prior to the reduction of P503. The results would provide a check on the calculations presented in the thesis.

(2) A comparison of wild-type control cells versus DNP-grown wild-type cells with regard to the activity of a DNP-reductase might be carried out by following NADPH oxidation at 340nm. The following questions might then be asked: (a) Is there a sufficient difference of activity in the two conditions to account for the excess NADPH being oxidized?

(b) Is DNP-reductase "induced" during growth of the cells on DNP or is the enzyme always present?

Since DNP added to cell suspensions just prior to spectral analysis has

no effect on P503, the enzyme should be inducible. The kinetic parameters and optimal condition for the DNP-reductase could also be investigated.

(3) The possibility of a connection between P503 and catabolite repression was suggested by the decrease and eventual elimination of P503 when wild-type cells were grown on increasing concentrations of glucose or gluconate. Gluconate was effective in causing this decreased synthesis of P503 at a lower concentration than in the case of glucose. Is the relationship between catabolite repression and (a) P503, (b) NADPH or (c) the energy charge (adenylate ratio) of the cell?

(4) The suggestion was made that L-methionine added to the growth medium might control the NADPH $\longrightarrow$ P503 $\longrightarrow$ ATP reaction sequence. At what point is its effect exerted, and what is the mechanism involved?

(5) A possible explanation for the excretion of valine by Sm-dependent mutants was that the excess NADPH could not be channelled off via the P503 system (since this pathway was impaired), thereby resulting in an accumulation of the reduced coenzyme. The utilization of NADPH for valine biosynthesis would off-set this increase in NADPH. If the action of methionine is, indeed, to inhibit this NADPH $\longrightarrow$ P503 $\longrightarrow$ ATP path, or to divert metabolism away from the P503 system, then growth of Sm-dependent (or DNP-wild-type) cells in the presence of methionine might prevent excess accumulation of NADPH. Such an effect would predict three things: (a) Sm-dependent cells grown with methionine should excrete less valine or none at all; (b) since no wasteful NADPH is made, the efficiency should increase; (c) less DNP should be reduced.

(6) Various attempts to obtain P503 in disrupted or permeabilized wild-type cells were negative. If further studies were continued along this line, one might try using the Sm-resistant (indifferent) strain of

E. coli B. Although the height of the 503nm peak was not as high in this mutant as in the wild-type organism, it was more persistent (less transient). If one did succeed in this regard, a large area of research would be opened for scrutiny. (a) Addition of NADPH versus NADH to permeabilized cells would test the specificity of P503 directly. (b) If the P503 system generates ATP (as stated in the hypothesis), then this pathway should not be operative when the cell has a high enough energy charge. The energy charge explanation, given as a possible effect of L-methionine in eliciting the 503nm peak rapidly, could then be tested by addition of ATP plus L-methionine simultaneously to the cell. The added ATP should help maintain the energy charge of the cell, so that the utilization of the P503 path would not be needed as a compensatory response. Alternatively, one could add S-adenosylmethionine to determine whether the effect of L-methionine is exerted via its "active" form, or as the amino acid per se. (c) The cofactor requirements for the stabilization of the pigment could be investigated. (d) If the appropriate conditions were found for the stabilization of P503, the possibility would then arise of its isolation and purification. (e) The isolation of P503 would, in turn, enable its structural determination.

(8) The distribution of P503 in biological systems, both in eukaryotes and prokaryotes, might be determined to ascertain whether a pattern results which may contribute to its further functional significance.

(9) Mutant studies might provide a test of certain explanations which were advanced to support the hypothesis. For example, (a) E. coli mutants which lack 6-phosphogluconate dehydrogenase would most likely metabolize all the gluconate provided via the Entner-Doudoroff path. Since an excess of NADPH would not then be expected to accumulate, the P503 system should

not be induced. (b) E. coli mutants lacking glucose 6-phosphate dehydrogenase would dissimilate glucose via the Embden-Meyerhof path, and none should be metabolized via the HMP shunt. One would then expect to see a decreased level of the 503nm peak with glucose. (c) E. coli mutants lacking phosphoglucose isomerase would be unable to convert G6-P to F6-P in the Embden-Meyerhof path, thereby forcing glucose metabolism to proceed via the HMP shunt and E-D path. The greater use of the HMP shunt would then predict a higher level of P503.