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INTRODUCTION

The purpose of this project was to study the genetic control in Escherichia coli of four physiologically unrelated metabolic activities, all of which seem to be under the influence of one gene. The phenotypic characteristics involved are:

(i) leucyl-, phenylalanyl-tRNA-protein transferase activity;

(ii) proline oxidase activity;

(iii) L-serine deaminase activity, and

(iv) the ability to utilize maltose as carbon and energy source.

The phenomenon investigated here is the following. A mutant lacking leucyl-, phenylalanyl-tRNA-protein transferase has been shown to have an altered regulation of a catabolic enzyme, proline oxidase (Soffer, 1974). In this work we show that this mutant also has alterations in its ability to use maltose and its expression of L-serine deaminase activity. The work in this thesis is designed to show that all of these characteristics are influenced by the product of a single pleiotropic gene. Examples of genes as pleiotropic as this are not common in E. coli. It is hoped, therefore, that this discovery will shed light on one of the fundamental regulatory mechanisms of E. coli.

In the first part of this introduction, the enzyme leucyl-, phenylalanyl-tRNA-protein transferase (called transferase in here) will be

described in some detail and its relation with the other metabolic characteristics discussed. This will be followed in part two by a discussion of current knowledge of proline oxidase, L-serine deaminase and maltose utilization. The introduction will end with part three, a short review of pleiotropic regulatory mechanisms.

Part 1

Leucyl-, Phenylalanyl-tRNA-Protein Transferase and Its Metabolic Interrelations

a) Leucyl-, Phenylalanyl-tRNA-Protein Transferase

Leucyl-, phenylalanyl-tRNA-protein transferase is one of a group of enzymes known as aminoacyl-tRNA-protein transferases (Soffer, 1974). This group of soluble enzymes catalyzes the transfer of amino acid residues from aminoacyl-tRNA into peptide linkage with specific amino-terminal residues of protein or peptide receptors. These enzymes are superficially similar to the peptidyl transferases involved in de novo protein synthesis. However, the latter group of enzymes transfers amino acids to peptides bound to ribosomes, and this transfer reaction only occurs in the presence of template mRNA, Mg^{++} and guanosine triphosphate (GTP). The aminoacyl-tRNA-protein transferases studied by Soffer have no requirements for ribosomes, template mRNA, Mg^{++} or GTP.

The first evidence for the existence of aminoacyl-tRNA-protein transferases was found in experiments in which partially fractionated soluble extract of rat liver cells, devoid of ribosomes, incorporated certain species of amino acids from aminoacyl-tRNA into proteins or peptides (Kaji and Novelli, 1963). In later experiments, partially

purified enzymes were found to function well in the incorporation assay, even in assay mixtures containing no template mRNA, Mg^{++} or GTP (Soffer, 1969). The transferase activities were further differentiated from protein-synthesizing enzymes in that they were unaffected by chloramphenicol which inhibits protein synthesis.

The transferase, with which this work is concerned, the leucyl-, phenylalanyl-tRNA-protein transferase of E. coli, has been purified more than 300-fold and its characteristics have been studied in some detail. As the name implies, this enzyme catalyzes the transfer of leucine and phenylalanine from leucyl-tRNA and phenylalanyl-tRNA to protein acceptors. Since the two catalytic activities increased in specificity to the same extent during purification and their kinetics of thermal inactivation were identical, it is believed that one enzyme catalyzes the transfer of both amino acids (Leibowitz and Soffer, 1970).

The transferase also shows specificity with respect to protein acceptors. Leibowitz (1971) added purified protein preparations to enzyme assay mixtures to test for their activity as acceptors. Among twenty-five proteins assayed, only those proteins containing an amino-terminal arginine residue (such as β-casein A_2), were found to be able to accept stoichiometric quantities of phenylalanine or leucine. Tryptic digests of these acylated proteins showed that the leucine and phenylalanine were attached to the amino-terminal arginine by a peptide linkage. Later, peptides with an amino-terminal lysine were also found to be acceptors with lysine being the accepting site for the amino acids transferred (Soffer, 1973). Thus, an amino-terminal arginine or lysine is

most probably an absolute determinant of acceptor specificity in the reaction catalyzed by the transferase.

R.L. Soffer in 1974 attempted to find out what the naturally occurring acceptor substrates in the cell were. This involved the isolation of a mutant strain lacking transferase activity. To find this, strain W4977, a proline auxotroph derived from E. coli K12, was heavily mutagenized with nitrosoguanidine and the survivors were screened for transferase activity. The lysate of one strain out of 1200 survivors screened was found to have no detectable transferase activity (less than 0.3% of wild type activity). Since a mixture of the lysates of the mutant and the parent strains did show transferase activity comparable to that in the wild type, the possibility that an inhibitor accounted for the loss of transferase activity in the mutant was ruled out.

One would expect that the transferase-deficient mutant would have the same potential acceptor proteins as the parent, strain W4977. However, since the mutant has no transferase, these potential acceptors would not be acylated. Thus an extract of W4977 may contain a mixture of acylated and non-acylated acceptors, while an extract of the mutant would contain only non-acylated acceptors. These non-acylated acceptors could be acylated if transferase were supplied. Soffer (1974) took advantage of this to identify the acceptors of transferase. He mixed the cell lysates with purified transferase and C^{14}-labelled phenylalanyl-tRNA and observed acylation of various proteins in the lysates. Because there was much more incorporation of radioactivity in the mutant than in the parent lysate, he concluded that a significant quantity of protein is normally acylated in vivo. The analysis of the labelled acceptors by

gel electrophoresis showed that more than 75% of the potential soluble acceptor molecules migrated with a mobility corresponding to a molecular weight in the reduced denatured form of less than 12,000.

The mutation that caused the disappearance of transferase activity was tentatively mapped by using 18 F' strains whose episomes comprised almost the entire E. coli genome. Sixty recombinants resulting from crosses between the mutant and each of the donors were assayed for transferase activity. Only one strain, that carrying an episome spanning from 45 min. to 54 min., was found to transfer the ability to produce the transferase. Thus the gene responsible for the production of transferase activity must be between 45 to 54 min. of the E. coli chromosome. It is not known whether the gene transferred in this experiment is the structural gene for transferase or a regulatory gene. The transferase-positive recombinants lost the transferase activity on subculturing, and so they may have carried non-integrated episomes.

b) Evidence for a Metabolic Relation between Transferase and Proline Oxidase

The lack of transferase seemed to cause disturbances in proline catabolism (Deutch and Soffer, 1975). When the transferase-deficient strain (MS845) was grown in minimal medium with glycerol and 2mM proline, it exhibited a long lag which lasted up to twelve hours. It also ceased growth prematurely with a final yield of about 40% of that of the parent (Deutch and Soffer, 1975). When the proline concentration in the minimal medium was increased to 20mM, the mutant grew after a short lag and the premature cessation of growth was not seen. This indicated that the mutant required

a high level of proline in order to attain the same growth as the parent. The "high proline-requiring" character was later found to be correlated with a four- to five-fold increase in proline oxidase in the mutant, proline oxidase being an enzyme catalyzing the degradation of proline (as described later in this introduction). Both W4977 and MS845 carried a mutation in proline biosynthesis and hence could not synthesize their own proline. The elevated proline-degrading activity in strain MS845 probably dissimilated the proline supplied to such an extent that not enough was left for the biosynthetic needs of the cells in a medium with a low concentration of proline. With glucose as the carbon source, MS845 showed a 50% reduction in proline oxidase activity and grew well with lower exogenous proline supplies. This finding agrees with the above explanation for the abnormal growth of the mutant in medium with low concentration of proline.

Since the mutant exhibited a long lag before resuming growth when transferred from a stationary phase culture to a fresh culture with a low concentration of proline, repeated subculture of the strain in this medium, each time at the lag phase, should enable a selection of "low proline-requiring" cells (which did not exhibit a long lag phase). According to this rationale, Soffer and Savage (1974) "cycled" the mutant in minimal medium with glycerol and isolated several "low proline-requiring" strains after eight cycles. These strains had low proline oxidase and had transferase activity. Thus, it is likely that the change in transferase and proline oxidase activity were affected by a common factor. Deutch and Soffer (1975) suggested that proline oxidase might be an acceptor protein of the transferase activity and that a post-translational modification of this

protein by aminoacylation might result in an abrupt change of its catalytic activity. This hypothesis has not been directly tested, because the enzyme is membrane-bound and exceedingly difficult to purify.

c) A Relation Between Transferase and L-Serine Deaminase

In E. coli, several amino acid-degrading enzymes are induced by exogenous addition of leucine. A three-fold increase in the activities of threonine deaminase, serine deaminase and threonine dehydrogenase in cells grown in the presence of leucine was reported (Pardee, 1955; Newman et al, 1976). Of particular interest here is the fact that leucine is not a substrate of these enzymes and that there is no direct relation between the biosynthetic pathway of leucine and that of threonine or serine. In vitro, the activities of the enzymes were not increased to any significant extent by the presence of leucine (Newman, unpublished data). There seemed to be a possibility that "induced" serine deaminase may actually have been activated by incorporating a leucine molecule through the catalytic action of the transferase. In our laboratory, the parent strain W4977, the mutant, MS845, and a transferase-positive strain, R18, derived by Soffer from "cycling" MS845 in minimal medium with glycerol, were assayed for L-serine deaminase activity. Serine deaminase activity was found to be nine- to ten-fold higher in MS845 than in the parent strain, while R18 showed exceedingly low activity of the enzyme (Newman, unpublished data). These results are contrary to the prediction that "induced" serine deaminase is "leucinylated serine deaminase", but do indicate that there is some relationship between transferase and L-serine deaminase. Another two leucine-induced enzymes, threonine

deaminase and threonine dehydrogenase, were also tested, but there were no significant differences in these enzymes in the three strains.

The transferase-negative mutant, MS845, showed one more deficiency. It could not utilize maltose as carbon and energy source. Both W4977 and R18 could ferment maltose (Newman et al, unpublished data).

d) Summary - The Pleiotropic Effect of Transferase Deficiency

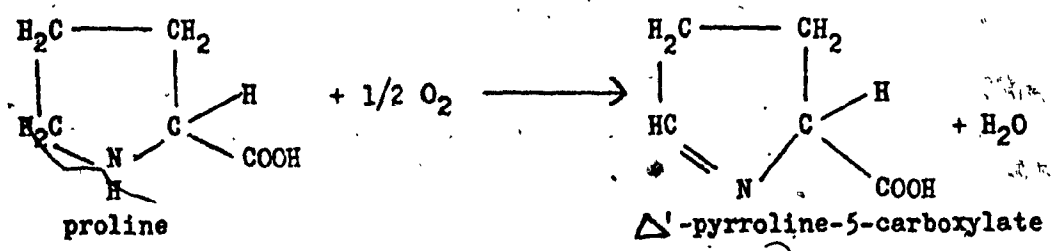
Strain MS845 differs from its parent with respect to transferase activity, proline oxidase activity, serine deaminase activity and the ability to utilize maltose as carbon and energy source. A transferase-positive strain, R18, derived from MS845 has regained, to a large extent, the phenotype of the wild type. These observations suggest the hypothesis that the four metabolic activities may be influenced by the expression of one gene, so that a mutation in that gene results in simultaneous changes in the four metabolic activities. The work in this thesis is intended to confirm this hypothesis.

Part 2

Description of Enzymes

a) Proline Oxidase

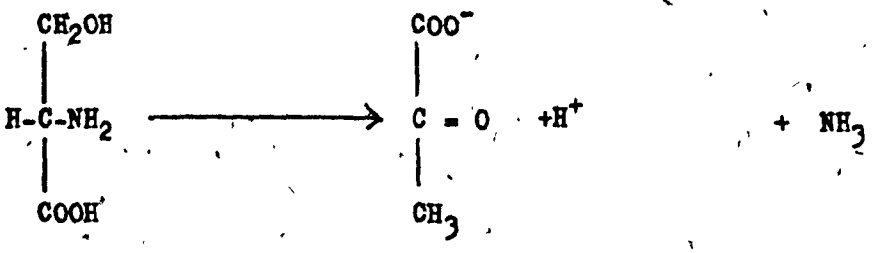
Proline oxidase is a membrane-bound enzyme present in both procaryotic and eucaryotic cells. It catalyzes the oxidation of proline to Δ'-pyrroline-5-carboxylate (PCA) (Frank, 1964):



In E. coli, proline oxidase is inducible by proline and is subjected to catabolic repression (Dendinger and Brill, 1970). The biological role of the enzyme has not been determined. Since the product of the catalyzed oxidation reaction, Δ^1-pyrroline-5-carboxylate can be further oxidized to glutamate, proline oxidase may play a role in regulating the level of intracellular proline: conversion of proline to glutamine would entail no loss of carbon. The structural gene for proline oxidase has not been mapped.

b) L-Serine Deaminase

L-serine deaminase is a soluble enzyme catalyzing the conversion of serine to pyruvate and ammonia:



L-Serine Pyruvate Ammonia

L-serine deaminase is not induced by its substrate (Pardee, 1955). Nevertheless, the level of L-serine deaminase in the cell is affected by various components of the growth medium (Isenberg, 1974). These include glycine, leucine and ammonium sulfate. Leucine causes a two-fold induction of L-serine deaminase. A combination of leucine and glycine induces the enzyme still further. The enzyme is not subjected to catabolite repression, but the enzyme level in cells grown in medium with ammonium sulfate is lower than that in medium without ammonium sulfate. The

biological role of serine deaminase is not known. It may be a detoxifying enzyme for maintaining L-serine at a low level such that it cannot inhibit L-threonine deaminase (Fraser, 1975), but, if this were the case, one would expect it to be induced by L-serine. The fact that L-serine deaminase is not induced by L-serine makes this hypothesis much less convincing. The structural gene for L-serine deaminase has not been mapped.

c) Maltose Utilization

Exogenous maltose and maltodextrin are taken into E. coli cells by an active transport system called maltose permease. An amylomaltase converts the endogenous maltose into maltodextrin, after which a maltodextrin phosphorylase catalyzes the phosphorylation of the maltose moieties in the maltodextrin to form glucose-1-phosphate, which is then metabolized via glycolysis (Palmer et al, 1973). The genes which specify these enzymes have been mapped in two regions, malA and malB, of the E. coli chromosome. The malA region, at 74 minutes, in the genetic map of E. coli contains:

(i) an operon composed of two structural genes - malP coding for the maltodextrin phosphorylase and malQ coding for the amylomaltase, and

(ii) a regulatory gene malT.

The malB region at 90 minutes in the genetic map contains one or more genes involved in maltose permeation (malB) and a lamB gene involved in the synthesis of cell surface receptors specific of phage lambda (Schwartz, 1966; Bachman, 1976). The malB and lamB are most likely to be in the same operon, as they are induced and repressed simultaneously (Hofmung, 1974).

The regulation of the transcription of these genes is of considerable complexity. The expressions of malP, malQ, malB and lamB seem to be co-ordinately controlled. Syntheses of maltodextrin phosphorylase, amylomaltase, maltose permease and the receptors for phage lambda are all induced by the presence of maltose. This control may be mediated by a product of the regulatory gene malT. A mutation in malT affects all four characteristics - the malT mutant has lowered and uninducible maltodextrin phosphorylase and amylomaltase activities, cannot take up maltose and is resistant to phage lambda. Thus, it seems that the gene product of malT (74 min.) has a regulatory effect on both the malP-Q operon (74 min.) and the malB-lamB operon (90 min.), (Schwartz, 1971).

Schwartz (1971) found that in a malT mutant, a further mutation of a gene located between malB and leu results in the unmasking of a new permeation system for maltose. The relation of this permease to the preceding one is not at present understood.

Part 3

A Brief Review of Pleiotropic Regulatory Mechanisms

The hypothesis presented in this work is that a single gene influences several diverse metabolic characteristics. The evidence presented here is genetic in nature. No idea of the mechanism by which the effect of this gene is mediated can be given at this time. However, in both eucaryotic and procaryotic cells, the mechanisms of some of such multiple effect of regulatory genes are known. Some of these will be reviewed in the following paragraphs.

In procaryotic cells, the structural genes of several enzymes,

usually metabolically related, may be clustered into a single regulatory unit, the operon. The best known case is the co-ordinate control of the lac operon, which consists of a promoter, an operator and the structural genes of three enzymes involved in lactose metabolism (Jacob and Monod, 1961). The levels of the three enzymes in the cells are determined by the rate of transcription of the operon as a single unit. This rate is, in turn, under the control of the lac repressor, a product of a regulatory gene located outside the operon. The amount of these enzymes in the cells can then be modified by factors affecting the regulatory gene, the promoter or the operator. Genes located in different loci of the chromosome can also be co-ordinately controlled by the expression of a single regulatory gene. In E. coli, the genes responsible for arginine biosynthesis have been found distributed among five unlinked operons. Nonetheless, there is strong evidence that one regulatory gene controls the levels of enzymes belonging to all the operons (Jacoby and Govini, 1969).

In the case of the lac operon and the arg operons, the genes involved specific enzymes which are obviously metabolically related. In the case of the malB- lamB operon mentioned previously, two very different activities: the maltose permease activity and the formation of the receptors for phage lambda, are co-ordinately controlled (Hofnung, 1974).

Even more diverse metabolic activities are known to be controlled by the concentration in the cells of one single substance, adenosine 3', 5'-cyclic monophosphate (cyclic AMP). E. coli cells which cannot produce cyclic AMP cannot ferment any of a large number of carbohydrates (Perlman and Pastan, 1969). The lac operon, for example, cannot be transcribed in the absence of cyclic AMP (Perlman and Pastan, 1969).

This is one of the several known cases in which the same genes are regulated by several factors, in this case, a specific factor (the inducer-β-galactoside) and a general one (the nutritional state of the cells as signified by the endogenous concentration of cyclic AMP).

The mechanism by which cyclic AMP produces its effect in bacteria has recently been elucidated. The transcription of several genes, such as the lac operon (De Crombrugghe, 1971) and the gene for D-serine deaminase (McFall, 1973) require the binding to the promoter of a cyclic AMP receptor protein (CRP) before the binding of RNA polymerase. The CRP will bind to these promoters only if it has previously bound a molecule of cyclic AMP (De Crombrugghe, 1971). Therefore the endogenous concentration of cyclic AMP will affect the rate of synthesis of all enzymes coded by "CRP-dependent" operons. Since glucose has the effect of lowering the level of cyclic AMP in the cells, either by inhibiting its synthesis or accelerating its breakdown (Buettner, 1973), the rate of biosynthesis of those enzymes coded by the "CRP-dependent" operons will be retarded in the presence of glucose, resulting in a phenomenon known as catabolite repression. Thus a mutation of either the gene coding for the enzyme adenyl cyclase, responsible for catalyzing cyclic AMP biosynthesis (Perlman, 1969), or the gene coding for CRP (Zubay, 1970) results in a loss of ability of the cells to synthesize the "catabolite-repressible enzymes". The physiology of the cells is then greatly altered as a result of the single mutation.

The compound guanosine 5'-diphosphate 3'-diphosphate (ppGpp) has also been suggested as a pleiotropic effector of the "stringent response" in bacteria (Cashel, 1974). Thus E. coli cells starved of amino acids

show a decrease in rate of protein and RNA synthesis, a decrease in polysome formation and an increase in rate of protein degradation - all these being attributed to the cellular concentration of ppGpp. Thus the "stringent response" will not be observed in a strain that carries a mutation in a gene involved in ppGpp accumulation under amino acids starvation. A similarly diverse response of mammalian cells to serum deprivation has been described as the "pleiotypic response" (Kram, 1973). Cyclic AMP seems to be the effector of this pleiotypic response.

While cyclic AMP in procaryotes is believed to influence the rate of transcription of certain genes, other pleiotropic effectors act at other points in metabolism. One other mechanism by which a compound can regulate the activities of several enzymes is by influencing the activity of a protein-modifying enzyme, a phenomenon known as "enzyme-catalyzed chemical modification of enzyme" (Holzer, 1969). The enzymes involved in glycogen synthesis and degradation are subjected to inverse controls of this type (Holzer, 1972). In nutritional conditions favouring the synthesis of glycogen, glycogen phosphorylase, which catalyzes the degradation of glycogen, is inactivated by a dephosphorylation. Glycogen synthetase, which polymerizes glucose-1-phosphate to glycogen, is simultaneously activated, also, by a dephosphorylation. When physiological conditions require glycogen breakdown, glycogen phosphorylase is activated by phosphorylation. A similar phosphorylation of the glycogen synthetase, however, leads to an inactivation of the enzyme. The phosphorylations and dephosphorylations of these two enzymes are under the catalytic actions of other enzymes, the activities of which are ultimately influenced by endogenous cyclic AMP and other effectors (Killilea, 1975).

Another example of metabolic regulation through post-translational modification of enzymes is the adenylylation of glutamine synthetase in E. coli. Glutamine synthetase is responsible for the conversion of glutamic acid to glutamine. Glutamine synthetase exists in an adenylylated form, and a deadenylylated form. Upon adenylylation, the conformation of the protein changes and becomes less catalytically active. Deadenylylation of the protein will convert it back to an active form. The adenylylation and deadenylylation of glutamine synthetase are catalyzed by a single adenylyltransferase (ATase). The ability of ATase to catalyze adenylylation or deadenylylation is controlled by a number of metabolites which include glutamate, α-ketoglutarate, UTP, ATP and a regulatory protein, PII. The regulatory protein exists in two forms. One form, PII_A, promotes the ATase-catalyzed adenylylation of glutamine synthetase, while the other form, PII_D, promotes the ATase-catalyzed deadenylylation. When conditions favour glutamine synthesis, e.g., when α-ketoglutarate is present in high concentration, glutamine synthetase is activated by a deadenylylation. The process probably involves the conversion of PII_A to PII_D, which stimulates ATase-catalyzed deadenylylation of the enzyme. The regulation of glutamine synthetase in this way is particularly interesting because glutamine synthetase itself, in Klebsiella aerogenes, is the effector for the transcription of several genes, including its own structural gene, a phenomenon known as autogenous regulation (Streicher, 1975; Calhoun, 1975). When glutamine synthetase is present in high concentration in the active deadenylylated form, the transcription of other enzymes such as proline oxidase and tryptophanase is increased (Prival, 1973; Magasanik, 1976). Thus the adenylylation of glutamine not only decreases

its activity, but also decreases the transcription of the genes specifying other enzymes. It can be visualized from these facts that the genes coding for glutamine synthetase, for the regulatory protein PII and the adenylytransferase are important in regulating several diverse metabolic activities in bacterial cells.

It is clear then that, in the past few years, several systems have been described in which one gene product may influence several metabolic activities. The gene described in this thesis appears to be one further example of this type of control.

MATERIALS AND METHODS

Culture Methods

All strains of bacteria were kept at 4°C on yeast tryptone slants. Cultures were transferred to fresh slants once every thirty days.

Liquid cultures were incubated at 37°C in Erlenmeyer flasks aerated by shaking in a gyrotory water bath shaker (New Brunswick Scientific Co., Model G76). However, for screening large number of bacterial strains for serine deaminase activity, cultures were grown in 5 ml. of liquid medium in pyrex screw cap tubes (12 mm. in diameter), aerated in a rotor drum (New Brunswick Scientific Co., Model TC-4).

Media

Minimal medium: 0.54% K_2HPO_4, 1.26% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.2% $MgSO_4.7H_2O$, and 0.001% $CaCl_2$ at pH 6.4 in distilled water. Other additions to the growth medium were made according to the requirements of the experiments as stated in the text.

Minimal medium agar: Minimal medium with 2% agar.

Soft agar: Minimal medium with 0.8% agar.

Yeast tryptone agar: 0.5% yeast extract, 0.5% NaCl, 1% tryptone and 2% agar.

Luria broth: 0.5% yeast extract, 0.5% NaCl, 1% tryptone in water.

LB agar: Luria broth and 2% agar.

Nutrient agar: (Difco No. 000-1-02-7) and MacConkey agar (Difco No. 0818-02-0) were prepared according to the directions given by the manufacturer.

All media were autoclaved at 15 lb./in.^{2} for 15 min. at 121°C.

Sources of Strains

Strain W4977 was obtained from R.L. Soffer of Albert Einstein College of Medicine who had previously obtained it from Dr. A. Ganesan of Stanford University. It is a proline auxotroph of E. coli strain K12. It is incapable of fermenting xylose (Xyl^-), lactose (Lac^-), and mannitol (Mtl^-), due to independent mutations. When cultured on a MacConkey maltose plate, it differed from a wild type maltose-fermenting organism (Mal^+) in that it lacked the red halo which usually appeared around a Mal^+ strain.

Strain MS845 obtained from R.L. Soffer is a mutant isolated after N-methyl-N'-nitro-N-Nitrosoguanidine treatment of strain W4977 and was chosen for its absence of leucyl-, phenyalanyl-tRNA protein transferase. It retained genetic markers of W4977 (Xyl^-, Lac^-, Mtl^-).

Strain MS845-II is a derivative of MS845 isolated during this work. MS845 was unable to grow in minimal medium with glucose (2%), and proline (50 µg./ml.), but could grow in the same medium with 500 µg./ml. proline. This "high proline-requiring" character was lost when the strain was in possession of Dr. E.B. Newman, most probably due to its being at a selective disadvantage. All available cultures of MS845 no longer show the "high proline-requiring" character. The MS845 derivative

which grows well in minimal medium with glucose (2%) and proline (50 µg./ml.) was named MS845-II.

Strain R18 was obtained from Dr. R.L. Soffer. It was isolated from strain MS845 by repeatedly subculturing MS845 in minimal medium with glycerol.

Strain Porl obtained from Dr. R.L. Soffer is a derivative of MS845 with low proline oxidase. It was isolated after a penicillin treatment of MS845 cultured on proline as the carbon source and was selected for its inability to grow on proline as the sole carbon source. The strain is transferase-negative (Soffer communication).

Strain Porl7 obtained from Dr. E.B. Newman is a mutant isolated after an ultraviolet treatment of MS845 and was selected for its ability to grow on minimal medium with glucose (2%) and proline (50 µg./ml.).

Strain K10 is a prototrophic strain of E. coli obtained from A. Caren of Yale University.

Strain THEK7 is a transductant of MS845-II by a phage ø80dmalA and was isolated by M. Schwartz by its ability to ferment maltose.

Strain CU4 was obtained from Dr. H.E. Umbarger of Purdue University. It is a prototrophic E. coli K12.

Phage lambda vir is a virulent lambda phage obtained from M. Levinthal, Purdue University.

P1-Cm, a P1 phage carrying a gene for chloramphenicol resistance, was obtained from M. Levinthal, Purdue University.

Strain CU1008, carrying a deletion of ilvA, a gene specify threonine deaminase, was obtained from M. Levinthal.

The strains used in this study and their characteristics are listed in Table 1.

TABLE 1

List of Bacterial Strains and Their Relevant Characteristics

Strain	Genotype and/or Relevant Characteristics	Source
K10	Prototroph	A. Caren
CU4	Prototroph	H.E. Umbarger
W4977	Pro mal-1 lac xyl mtl	R.L. Soffer
MS845	As W4977, transferase-deficient, high proline requirement	R.L. Soffer
MS845-II	As MS845 - lowered proline requirement (see text)	E.B. Newman
Porl7	MS845 derivative, transferase-positive	E.B. Newman
THEK4	MS845-II derivative, transferase-positive	This work
THEK5, 6	MS845-II derivative, Mal^+	This work
THEK7	MS845-II transduced to Mal^+ with phage Ø80dmalA	M. Schwartz
MS845-II/W4977	MS845-II transduced to Mal^+ with phage PI-Cm grown on W4977	This work
Porl	MS845-II derivative, with low proline oxidase activity	R.L. Soffer
Porl/W4977	Porl derivative transduced to Mal^+ with PI-Cm grown on W4977	This work
W4977/CU4	W4977 tranduced to Por^{+*} with PI-Cm grown on CU4	This work
MS845/CU4	MS845 transduced to Por^+, with PI-Cm grown on CU4	This work
Porl7/CU4	Porl7 transduced to Por^+, with PI-Cm grown on CU4	This work
CU1008	ilvA deletion	M. Levinthal

*Por^+ - proline independent

Measurement of Cell Density and Absorbance of Various Chemicals

Cell density of a culture, indicated by its turbidity, was measured in a Klett-Summerson Photoelectric Colorimeter (Klett Mfg. Co. Inc., New York, N.Y., Model 800-3) using a No. 42 filter. All absorbances of various chemicals were measured with the same instrument. The filter used is specified in each method.

Mutagenization of Cells by Ultraviolet Irradiation

A culture at mid-log phase was adjusted to a turbidity of 50 with sterile minimal medium. A 10 ml. portion of this in a petri dish was then rotated slowly at a distance of 10 cm. under an ultraviolet lamp (Universal U.V. lamp Gelman-Camag, Switzerland, Model 51402) at 250 mμ for 60 sec.

Enzyme Assays

Assay of Proline Oxidase

Proline oxidase activity was assayed by a modification of the method of Dendinger and Brill (1970). The oxidation of L-proline by proline oxidase yields Δ'-pyrroline-5-carboxylate which can react with O-aminobenzaldehyde to form a yellow derivative, thought to be a dihydroquinazolium compound (for structure, see Appendix I). This product can be determined quantitatively by reading its absorbance (No. 42 filter). To assay for proline oxidase, cells grown to mid log phase were chilled and centrifuged at 10,000 x g for 5 min. at 0°C in a refrigerated centrifuge (International Equipment Co., Needham Hts., Mass., Model B-20). The pellet obtained was centrifuged and resuspended once in minimal medium without ammonium sulfate; centrifuged again and then resuspended in 0.1M sodium cacodylate buffer at pH6.6 and centrifuged once more. This pellet was resuspended in the

same cacodylate buffer to a turbidity of 400 Klett units (No. 42 filter). One ml. of this cell suspension was added to a prechilled 250 ml. Erylermeyer flask containing 2 ml. of 1 M proline solution in the same cacodylate buffer, 0.2 ml. o-aminobenzaldehyde (30 mg. in 5 ml. 20% ethanol) and 0.02 ml. of toluene. The flask was incubated at 37°C with fast shaking. The reaction was then terminated by adding 0.4 ml. of 20% cold TCA. Cell debris in the mixture was removed by centrifugation at 10,000 x g for 5 min. The absorbance of the yellow supernatant was determined (No. 42 filter). The assay was carried out in triplicate. Controls without proline were included in each enzyme assay. Activity is expressed as the difference between the average of the optical densities of the incubation mixtures and that of the controls at the end of the experiment, normalized for 100 Klett units of cells incubated. The actual turbidity of the cell suspension (400 Klett units) was determined by diluting the cell suspension 4-fold with distilled water and then reading in the Klett colorfmeter (at a more sensitive range). The assay at first gave variable results, most probably due to the fact that o-aminobenzaldehyde is not very soluble in 20% ethanol. The assay was much improved by grinding o-aminobenzaldehyde in a mortar before dissolving in 20% ethanol. In later experiments, the substrate solution was made by dissolving proline in distilled water instead of cacodylate buffer. This did not adversely affect the results.

Assay for Serine Deaminase

L-serine deaminase was assayed by the method of Isenberg and Newman (1974), which is a slight modification of the method of Pardee and Prestige (1955). This is based on the detection of pyruvate formed from serine by means of a reaction between pyruvate and 2, 4-dinitrophenylhydrazine (DNPH). The incubation mixture contained 0.1 ml. of L-serine (20 mg./ml.), 0.3 ml. of washed cells suspended in 0.05 M phosphate buffer, (pH 6.4), 0.02 ml. of

toluene. This mixture was incubated for 35 min. at 37°C; 0.9 ml. of DNPH (250 µg./ml. in 4.1% HCl) was added, and the mixture was incubated for a further 20 min. at room temperature. Following the incubation with DNPH, 1.7 ml. of 10% NaOH was added and the absorbance was determined (No. 54 filter), using pyruvate as standard. Included in this assay was one set of assay mixtures to which no substrate was added. This allowed a measure of endogenous α-ketoacid production. Activity is expressed as the difference between the amounts of ketoacid formed in the assay mixtures with and without substrate, related to the amount of protein in the cells added to the assay mixtures.

Proteins were determined by the method of Lowry et al (1951), using trypsin as a standard.

Assay for Leucyl-, Phenylalanyl-tRNA Protein Transferase

The assay was carried out according to the method of R.L. Soffer (1974). It measures the amount of ^{14}C-leucine or ^{14}C-phenylalanine transferred from labelled leucyl-tRNA or phenylalanyl-tRNA to a product insoluble in hot TCA. The charged tRNA used for the assay was prepared by incubating stripped tRNA (Nutritional Biochemicals), with either ^{14}C-leucine (324 µc/µm), or ^{14}C-phenylalanine (486 µc/µm) and an extract of E. coli (Muench, 1969). In the assay, cells were grown overnight in 10 ml. of Luria Broth at 30°C. Unfractionated lysates were prepared using EDTA, lysozyme, Brij 58 and DNase according to the method of Godson et al (1967). The reaction mixture (75 µl) contained 50 mM 2-mercaptoethanol, 0.2M KCl, 0.3 mg./ml. chloramphenicol, 0.5 mg./ml. of α-casein and approximately 0.2 n moles/ml. of ^{14}C-leucyl-tRNA or 0.15 n moles/ml. of ^{14}C-phenylalanyl-tRNA and 25 ul. of cell lysate corresponding to approximately 1 x 10^8 cells.

The radioactive aminoacyl-tRNA in each assay was added such that each assay mixture contained approximately 10,000 to 15,000 cpm. The assay mixtures were incubated for 60 min. at 37°C. After an addition of 3 ml. of cold TCA, the mixture was further incubated for 15 min. at 90°C. The precipitates were then collected by vacuum filtration on glass filters (Millipore Corporation, AP200, 2500). These were then dried under a 300 watt-150 volt heat lamp for one hour. The filters were then placed in 20 ml. screw-top glass liquid scintillation vials (Amersham/Searle, Toronto, Ont., Model 3326), with 10 ml. of scintillation fluid (0.5% 2, 5-diphenyloxazole and 0.02% p-bis [2-(5-phenyoxazolyl)] - benzene in toluene). The radioactivities were determined by a liquid scintillation counter (Nuclear Chicago Co., Montreal, Que., Model Unilux II). Included in the experiment is a control in which no cell extract was added to the assay mixture. This allowed a measure of radioactivity incorporated into the insoluble material by mechanism other than transferase activity. Another control consisted of an assay mixture containing no cell extract and filtered right after the addition of 3 ml. of cold TCA. This enabled an estimation of tRNA-bound radioactivity put into the assay mixture. Strains which have been described as transferase-positive transferred 1000 cpm or more, while those that were described as transferase deficient transferred less than 200 cpm. In this work, any strain transferring more than 600 cpm was considered as transferase-positive, any strain transferring less than 200 was considered as transferase-deficient.

Assay for the Ability to Utilize Carbohydrates

Bacteria were streaked simultaneously on minimal medium agar with glucose (0.2%) and proline (50 µg./ml.) and agar with maltose (0.2%) and

proline (50 μg./ml.). Strains that could grow on plates with glucose and proline, but not on plates with maltose and proline, were regarded as unable to utilize maltose as the carbon and energy source.

The ability of bacterial strains to ferment carbohydrates was also determined with MacConkey plates. These plates contained Eosin Y and methylene blue and deoxycholate (DOC) in addition to yeast extract, tryptone and any carbohydrate desired. Strains that could ferment the carbohydrate formed acid and turned red (Eosin Y reaction) and were surrounded by a halo (DOC precipitation). Strains which grew at the expense of yeast extract and tryptone only did not form acid and appeared to be white.

Transduction Experiments

Preparation of Phage Lysate

Strains were cultured in Luria Broth and then streaked on a LB plate supplemented with 5×10^{-3} M $CaCl_2$ and 12.5 μg./ml. chloramphenicol. A P1-Cm phage lysate was then streaked across the "bacterial streaks". The plate was incubated at 32°C. This procedure is based on the fact that cells which are lysogenized by P1-Cm become chloramphenicol resistant and so can form colonies. This lysogenic state is maintained at 32°C, but not at 34°C or above. After two days of incubation, a single colony from each strain was then cultured in 6 ml. of Luria Broth supplemented with 10^{-2} M $MgSO_4$ for about 12 hours at 32°C. The culture was then aerated for one or two hours until the cell density reached about 2×10^8 cells/ml. and then shifted to 42°C for 35 min., still with good aeration in order to induce phage synthesis. The culture was shifted back to 32°C for two to three hours until it became clear. 0.2 ml. of chloroform was then added to the

culture to kill any remaining bacteria. After vigorous shaking, the cell debris was centrifuged and the supernatant was removed to a sterile tube to which chloroform was added.

Titration of Phages

Phage lysates were serially diluted in Luria Broth containing 10^{-2}M $MgSO_4$. 0.1 ml. of each of these dilutions was added to 0.2 ml. of a fresh overnight Luria culture of a phage-sensitive bacteria strain (K10). 2 ml. of soft agar at 42°C were added to the mixture, which was then poured over on a Luria Broth plate containing 5×10^{-3}M $CaCl_2$. The plate was incubated face up at 37°C for eight to ten hours. The number of plaques on each plate was counted and, from these numbers, the phage density of the original lysate was determined.

Transduction

The recipient strain for transduction was cultured in 5 ml. of Luria Broth at room temperature until the cell density reached approximately 2×10^8 cells/ml. $CaCl_2$ was added to the culture to a final concentration of 5×10^{-3}M. A 0.1 ml. portion of the culture was plated on a selection plate to check for contamination and for spontaneous mutants of the phenotype to be selected. This was followed by the addition of phage lysate to the culture at a multiplicity of infection of 0.2 (i.e., for every five bacteria, one phage particle was added). After 30 min. of incubation at room temperature, the cells were centrifuged and resuspended in 0.5 ml. 0.9% NaCl. 0.1 ml. duplicate samples of this cell suspension were plated on a selection plate and then incubated at 32°C. If the transductant colonies grew too slowly on the selection medium, the plates were transferred to 37°C on the third day after the transduction to hasten the

growth. The transductants were purified by streaking for single colonies on the same type of selection plates.

RESULTS

The purpose of this project was to study the genetic controls of four metabolic activities in E. coli. These metabolic activities are:

(i) leucyl-, phenylalanyl-t-RNA-protein transferase activity;

(ii) proline oxidase activity;

(iii) serine deaminase activity, and

(iv) the ability to utilize maltose as the carbon and energy source.

For genetic study which involves screening large populations, simple and time-saving assays for phenotypes are essential. Tests for maltose-fermenting ability and serine deaminase activity are comparatively simple. The original method for assaying proline oxidase, as developed by Dendinger and Brill (1970), on the other hand, is tedious and time-consuming. A simplified method is used in this study and Part 1 of this Results section is devoted to a demonstration of the reliability of the method as a test for the amount of enzyme activity present in the strains.

Part 2 of this section summarizes the phenotypic differences between the parent W4977, the transferase-deficient mutant MS845 and a revertant, Porl7. Part 3 of this Results section deals with the isolation of another revertant from the mutant and a variety of attempts to transduce transferase-negative strains. Part 4 is devoted to a study of the regulation of proline oxidase in the strains under study.

Part 1

Validity of the Enzyme Assay Measuring Proline Oxidase Activity

The enzyme assay developed by Dendinger and Brill (1970) makes use of the fact that the enzyme catalyzes the oxidation of proline into Δ^1-pyrroline-5-carboxylate, which will react with o-aminobenzaldehyde to give a soluble yellow complex. The amount of product can be determined by the absorbance of the solution at 420 nm. The total enzyme activity in an assay mixture is indicated by the increase in absorbance of the assay mixture during the period of the assay. The assay is done on whole cells rather than on cell extract because proline oxidase is a membrane-bound enzyme. In the method of Dendinger and Brill, toluene is added to the cells 10 min. before proline is added, and then the mixture is incubated for a further 45 min. with proline. In the method employed here, toluene, proline and o-aminobenzldehyde are all added at time zero of the assay. The incubation period is also shortened to 35 min. To show that this method allows an accurate measure of proline oxidase activity in E. coli cells, the following experiments were carried out on E. coli strain K10 grown on minimal medium with glycerol (0.2%) and proline (200 µg./ml.)

Part 1a

Response to Enzyme Concentration

When the substrate is at saturating concentration and the enzyme concentration is not too high, the enzyme activity measured should be directly proportional to the amount of enzyme present. In this experiment, different volumes of cells from a cell suspension at a turbidity of 400 Klett units were added in duplicate to a set of assay mixtures.

The optical density (OD) of the assay mixtures (No. 42 filter) after a 40 min. incubation was recorded. The graph in Fig. 1 shows a plot of the final OD of the assay mixtures against the volume of cells added. It can be seen that the relation between the final OD of the assay mixtures and the volume of cells added was approximately linear from 0.2 ml. of cell suspension to 1.0 ml. Since the amount of enzyme is proportional to the amount of cells, it can be seen that the OD readings obtained by the present assay give a valid indication of the amount of enzyme in the assay mixtures.

Part 1b

Enzyme Response to Time

Under optimal conditions, the amount of substrate converted to a product should be directly proportional to the time of action of the enzyme, at least for short time periods. To test if this is true for the enzyme incubated under the method employed, identical assay mixtures containing 1 ml. of cell suspension at a turbidity of 300 Klett units were incubated for 0, 10, 20, 40, 60 and 80 min. in duplicate and the final OD of the assay mixtures was determined. The graph in Fig. II shows a plot of the average final OD of the assay mixtures incubated for any given times against the incubation periods. The graph indicates that the absorbance of the assay mixtures increased linearly with the duration of incubation from 10 min. to at least 40 min. The linear nature of the plot indicates that within this time the enzyme had not been subjected to significant degradation. Absorbance of the assay mixtures did not increase linearly in the first 10 min. of incubation. This was probably because the cells had not been completely perforated by the action of toluene. Based on

Figure 1: Proline Oxidase Assay: Activity versus Concentration of *E. coli* Cells.

Cells of *E. coli* K10 were grown in minimal medium with glycerol (0.2%) and proline (200 µg./ml.), harvested and resuspended in buffer to a turbidity of 400 Klett units. 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml. of cells were added to the assay mixtures containing 0.55M of proline. The OD of the assay mixtures was determined after a 40 min. incubation period. The OD of the assay mixture incubated without cell extract was 70 Klett units. All readings plotted were corrected by this value.

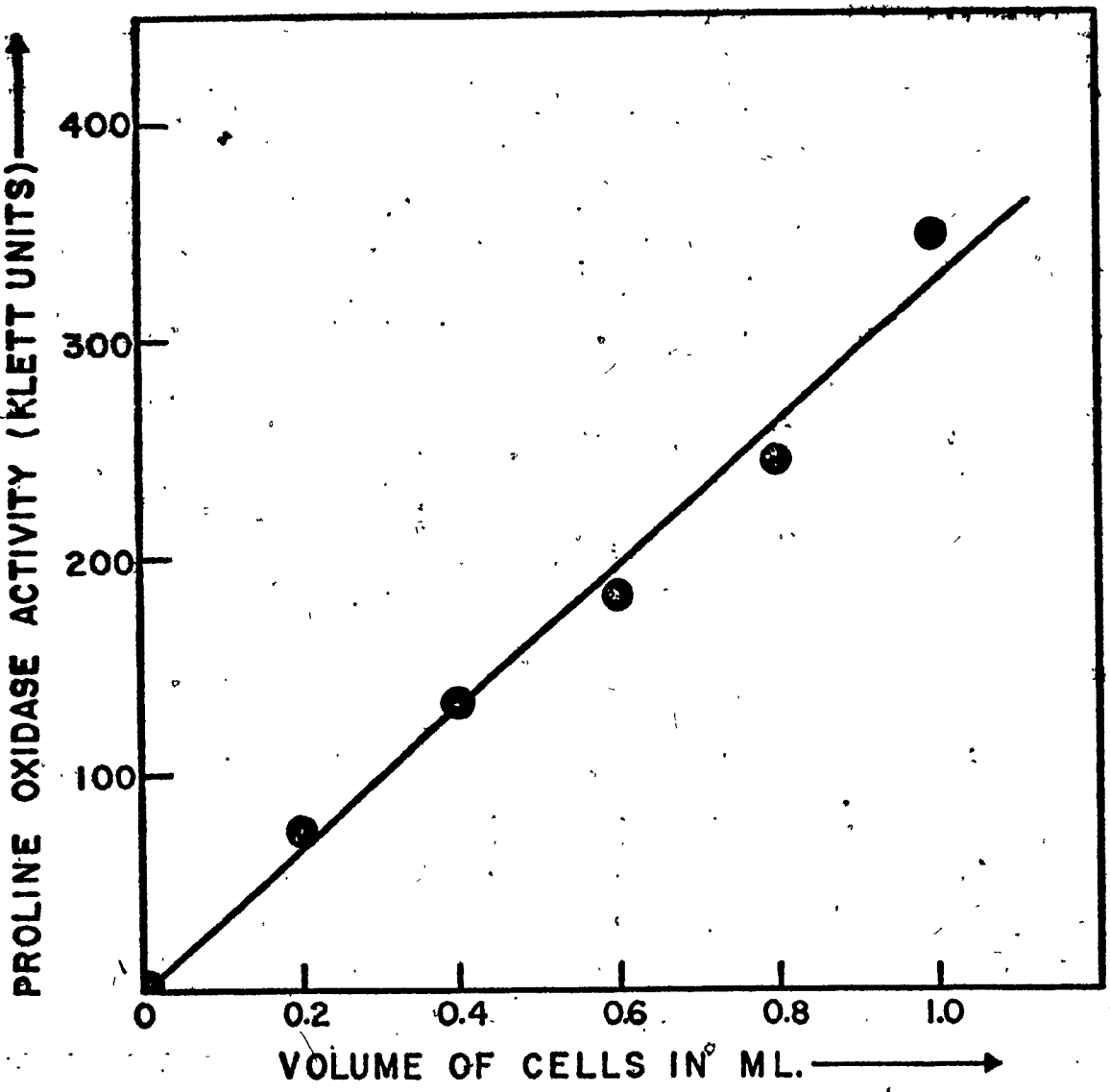
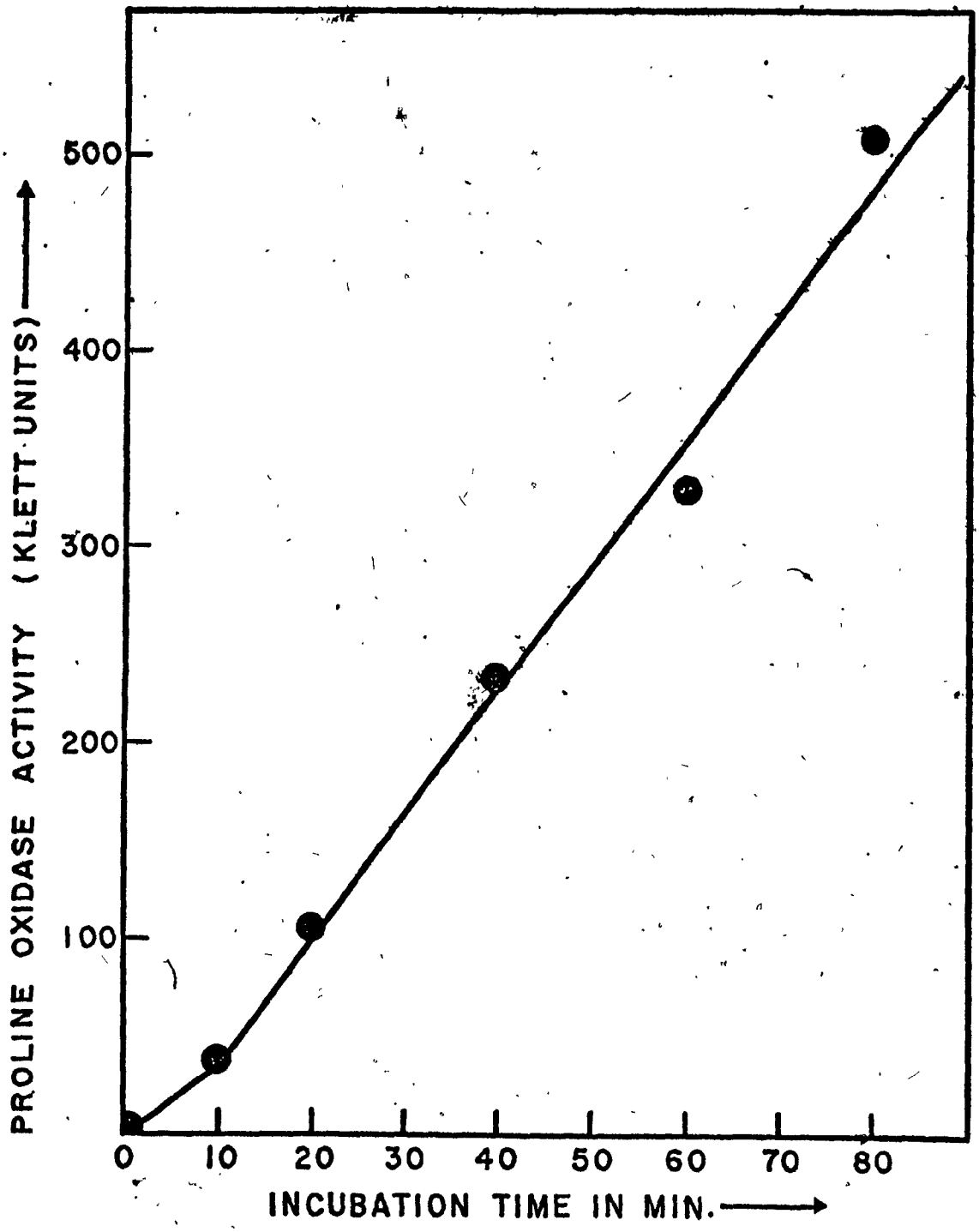

PROLINE OXIDASE ACTIVITY (KLETT UNITS)
400
300
200
100
0
0.2
0.4
0.6
0.8
1.0
VOLUME OF CELLS IN ML.

Figure 2: Proline Oxidase Assay: Activity versus Incubation Time.

Cells of E. coli K10 were grown in minimal medium with glycerol (0.2%), and proline (200 µg./ml.), harvested and resuspended in buffer to a turbidity of 300 Klett units. Assay mixtures containing 1 ml. of the cell suspension and 0.55M proline were incubated for 0, 10, 20, 40, 60 and 80 min., and the OD was determined. The OD of the assay mixture incubated for 0 min. was 90 Klett units. All readings plotted were corrected by this value.


PROLINE OXIDASE ACTIVITY (KLETT UNITS)
500
400
300
200
100
0
10
20
30
40
50
60
70
80
INCUBATION TIME IN MIN.

these observations, a 35 min. incubation period was chosen for the standard assay.

Part 1c

Response to Substrate Concentration

In order to assay the enzyme activity in a cell suspension, the substrate concentration in the assay mixture must be at a level that saturates the amount of enzyme throughout the incubation period. To find a saturating concentration of substrate for the assay, various concentrations of substrate were used in a set of assay mixtures containing 1 ml. of cell suspension at a turbidity of 300 Klett units and incubated for 40 min. The graph in Fig. 3 shows a plot of the OD of the assay mixtures against the concentration of proline tested. The graph indicates that the absorbence of the assay mixtures increased as the concentration of the substrate increased up to about 0.2M, but did not increase significently when the substrate concentration was raised still higher. Therefore a concentration of 0.55M proline was selected as the standard concentration of substrate for the following assays. This concentration of proline is well above the saturation level for the enzyme (Fig. 3), so that there should be no changes in reaction rate due to changes in proline concentration during a 35 min. incubation, i.e., proline does not become rate-limiting.

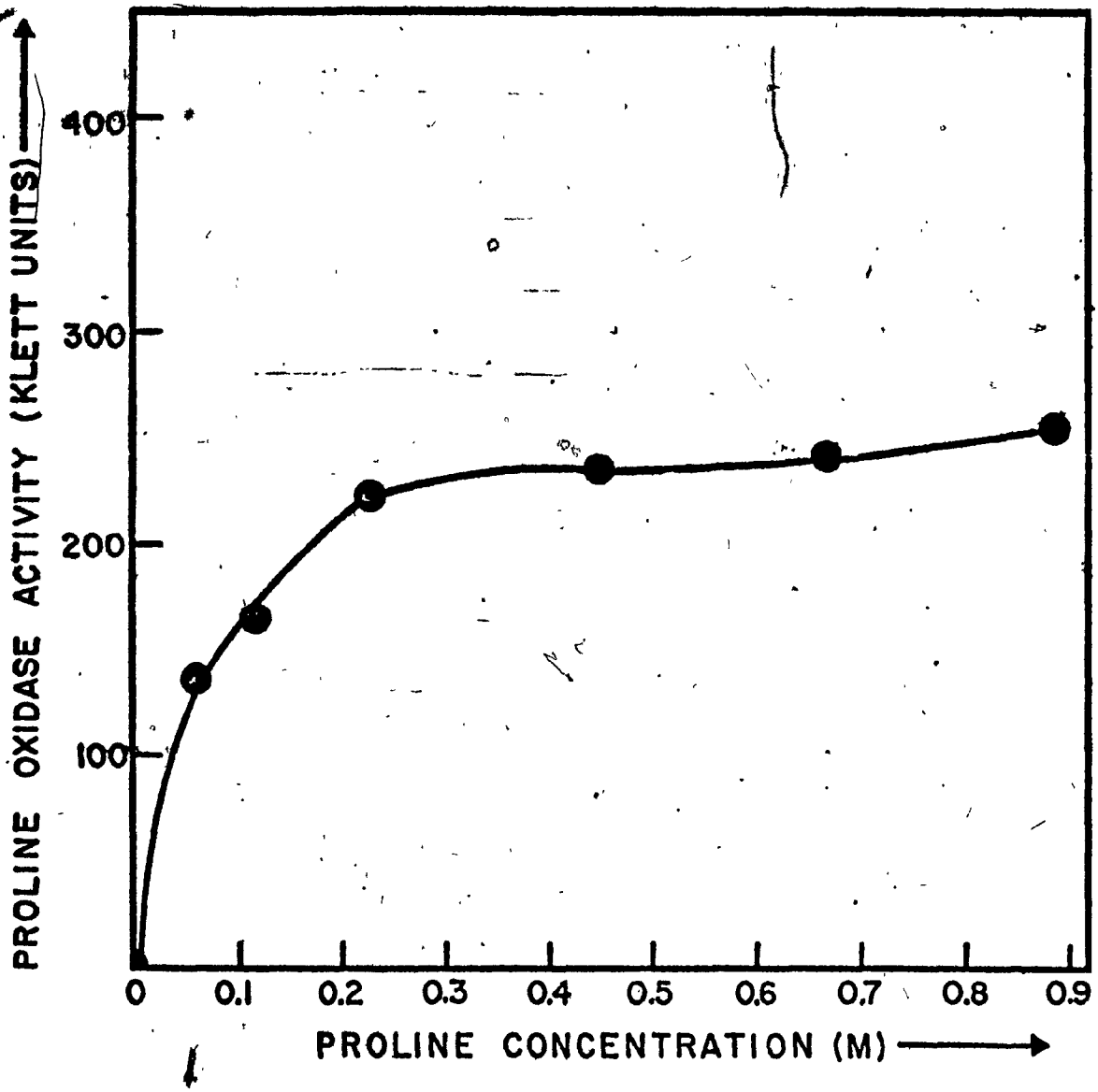
Part 1d

Proline Oxidase Assay on W4977 and MS845-II

After the assay conditions were determined using strain K10, the assay was used to determine the proline oxidase activities in strains W4977 and MS845-II. The two strains were cultured in minimal medium with

Figure 3: Proline Oxidase Assay: Activity versus Proline Concentration

Cells of E. coli K10 were grown in minimal medium with glycerol (0.2%) and proline (200 µg./ml.), harvested and resuspended in buffer to a turbidity of 300 Klett units. 1 ml. of the cell suspension was added to assay mixtures containing 0.055M, 0.11M, 0.22M, 0.44M, 0.66M and 0.88M of proline. The OD of the assays mixtures was determined after a 40 min. incubation period. The OD of the assay mixture incubated without substrate was 61 Klett units. All readings plotted were corrected by this value.


PROLINE OXIDASE ACTIVITY (KLETT UNITS)
400
300
200
100
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
PROLINE CONCENTRATION (M)

glycerol (0.2%) and proline (200 µg./ml.). One ml. aliquot of cell suspension of both strains at a turbidity of 400 Klett units were assayed for their activities within incubation periods of 0, 15, 30 and 45 min. The graph in Fig. 4 shows the absorbance of the assay mixtures as a function of the time of incubation. The graph shows that the OD of the assay mixtures of both strains increased linearly with time of incubation. The gradient of the curve corresponding to MS845-II is 4, while that corresponding to W4977 is 0.83. From these observations, it can be concluded, first that the assay method functioned as well in testing the proline oxidase activity of W4977 and MS845-II as in testing that in K10. Hence, in the following experiments, 1 ml. of cell suspension of OD 400 was the standard amount of cells added to the assay mixture. Secondly, the proline oxidase activity in MS845-II was about 4.8 times higher than that of W4977. Thus, the results obtained here are consistent with the results reported by Deutch and Soffer (1975).

Part 1e

Reproducibility

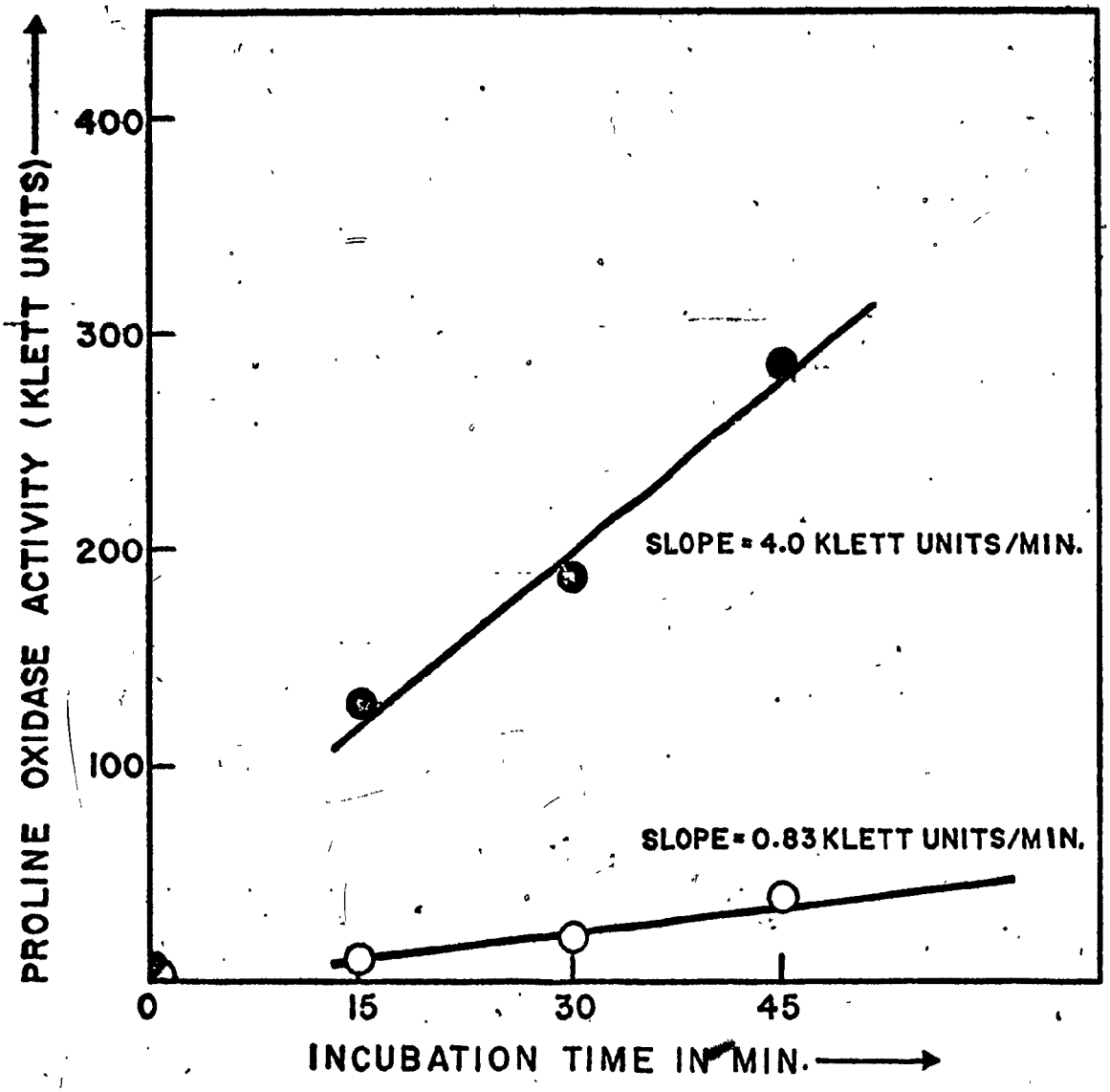
The assay method was intended to be applied to the determination of the levels of proline oxidase in strains with different genotypes. Thus, it was necessary to show that a given strain, when grown in any given medium, would show the same level of proline oxidase as tested by this method. For this purpose, several cultures of W4977 and MS845-II in minimal medium with glycerol (0.2%) and proline (200 µg./ml.) were

Figure 4: Proline Oxidase Assay on W4977 and MS845-II.

Cells of W4977 and MS845-II were grown in minimal medium with glycerol (0.2%) and proline (200 ug./ml.), harvested and resuspended in buffer to a turbidity of 400 Klett units. Assay mixtures containing 1 ml. of the cell suspension and 0.55M proline were incubated for 0. 15, 30 and 45 min., and the OD was determined. The OD of the assay mixtures is plotted as a function of time of incubation. The zero time absorbance for MS845-II was 75 Klett units and that for W4977 was 74 Klett units. The readings plotted were corrected by their respective zero time absorbances.

● - MS845-II

○ - W4977


PROLINE OXIDASE ACTIVITY (KLETT UNITS)
400
300
200
100
0
15
30
45
INCUBATION TIME IN MIN.
SLOPE = 4.0 KLETT UNITS/MIN.
SLOPE = 0.83 KLETT UNITS/MIN.

assayed for proline oxidase activity. Table 2 presents the results of five assays on each strain. The mean value of proline oxidase activity of MS845-II is 50.8, with a standard deviation of ± 2.3. The corresponding mean value for proline oxidase activity of W4977 is 11.2 ± 1.6. As judged from these low standard deviations in proline oxidase activity of both strains, it can be considered that the proline oxidase assay is very reproducible.

Part 2

The Phenotypic Differences Between W4977 MS845 and the Revertant Porl7

As is described in the Introduction, MS845 was derived from W4977 through nitrosoguanidine mutagenesis. Nitrosoguanidine is a strong mutagen that frequently causes multiple mutations in the E. coli chromosome. Thus the physiological changes in MS845 may be due to several mutations at different loci of its chromosome, rather than to one mutation. Similarly, strain R18 isolated by Soffer by "cycling" MS845 in minimal medium with glycerol (see Materials and Methods), may have gained the parental phenotypes through an accumulation of mutations. In our laboratory, a well-characterized MS845 culture was subjected to ultraviolet irradiation, which induces point mutations, and plated on minimal medium supplemented with glucose (0.2%) and proline (50 µg./ml.). The rationale was that the low level of proline on the plate would not allow a normal growth of MS845 and hence would make possible a selection from MS845 of mutants with lower proline requirement. Porl7 was

TABLE 2

The Activity of Proline Oxidase in Strains W4977 and MS845-II

Experiment	Activity of Proline Oxidase OD/100 Klett units of Cells	
	W4977	MS845-II
1	13	49
2	10	48
3	13	53
4	10	53
5	10	51
mean ± standard deviation	11.2 ± 1.6	50.8 ± 2.3

Cells of strains W4977 and MS845-II were grown in minimal medium supplemented with glycerol (2%) and proline (200 µg./ml.)

isolated in this way. The phenotypic differences between the transferase-negative strains MS845 and MS845-II, the parent W4977 and the revertant Porl7, are shown in the following paragraphs. Since MS845 was lost during the studies, and was replaced by the "low proline-requiring" strain, MS845-II, in the course of cultivation, some assays could only be done on MS845-II.

Part 2a

Differences in Responses of Proline Oxidase Activity in W4977, MS845-II and Porl7 to Proline Induction

Strains W4977, MS845-II and Porl7, grown in minimal medium with glycerol (2%) and proline (200 µg./ml.), were assayed for proline oxidase. As can be seen in Table 3, the proline oxidase level in MS845-II is considerably higher than that of the other two strains. The revertant Porl7 has as low a level of proline oxidase as W4977. This suggests that the inability of MS845 to grow in low proline medium is due to an elevated level of proline oxidase.

The increased activity of proline oxidase in MS845-II could be due either to an increased constitutive expression of the gene or to an increased sensitivity to proline induction. Since the three strains are all auxotrophic for proline, proline oxidase can only be assayed in cells grown in the presence of proline, the inducer of the enzyme (Dendinger and Brill, 1970). The following experiment indicates that strain MS845-II is more sensitive to induction.

Proline-independent derivatives of MS845-II, W4977 and Porl7 were

TABLE 3

Proline Oxidase Activities in Strain W4977 and Its Derivatives

Strain	Addition to Growth Medium	Proline Oxidase
W4977	Proline	11
MS845-II	Proline	51
Por17	Proline	14
W4977/CU4	None	6
	Proline	9
MS845-II/CU4	None	6
	Proline	48
Por17/CU4	None	8
	Proline	9

Strains were grown in minimal medium with glycerol 0.2% proline added at 200 µg./ml. Proline oxidase is expressed as OD/100 K.U. of cells.

constructed by transducing the strains with P1-Cm phage grown on strain CU4, a prototrophic strain of E. coli K12. Proline oxidase assays were then carried out on the transductants (W4977/CU4, MS845-II/CU4 and Porl7/CU4), which had been grown in minimal medium with glycerol (0.2%), with and without proline (200 µg./ml.). The proline oxidase levels of these cultures are shown in Table 3.

As can be seen, MS845-II/CU4, W4977 and Porl7/CU4 made similar low levels of proline oxidase when grown without proline. When grown with proline, strain MS845-II/CU4 had the high level of proline oxidase comparable to strain MS845-II, whereas strains W4977/CU4 made the same low amount of proline oxidase as W4977 and Porl7. It can be concluded, therefore, that in the transferase-negative mutant, MS845-II proline oxidase is produced at a level similar to the wild type, but its enzyme is more highly induced by proline than is that of its parent and its revertant.

Part 2b

Leucyl-, Phenylalanyl-tRNA-Protein Transferase Activity in W4977, MS845-II and Porl7

Strains W4977, MS845-II and Porl7 were assayed for transferase activity by the method described. The results of two separate experiments are shown in Table 4. The data show that a significant amount of radioactive leucine was incorporated into material insoluble in hot TCA in the assay mixtures containing lysates of W4977 and Porl7, while a very low amount of radioactivity was incorporated in the assay mixture containing a lysate of MS845-II. This indicates that W4977 and

TABLE 4

Leucyl-, Phenylalanyl-tRNA-Protein Transferase Activity in W4977, MS845-II and Por17

Lysate	Expt. 1 cpm incorporated	Expt. 2 cpm incorporated
None	47	42
W4977	1279	1715
MS845-II	182	132
Por17	888	900

Transferase activities of the strains were reported as counts of radioactivity incorporated into material insoluble in hot TCA in each assay mixture. ^{14}C-leucyl-tRNA was used as the substrate. The amount of tRNA-bound radioactivity added in Experiment 1 was 16311 cpm, Experiment 2 was 16874 cpm. These values were indicated by a control in each experiment in which no cell extract was added to the assay mixtures and the assay mixture was filtered after an addition of 5% cold TCA.

Porl7 both have significant transferase activity, and MS845-II does not.

Part 2c

Serine Deaminase Activity in W4977, MS845, MS845-II and Porl7

The serine deaminase activities of the strains W4977, MS845, MS845-II and Porl7 are shown in Table 5. The results indicate that MS845 and MS845-II have much elevated levels of serine deaminase when grown without leucine. These levels were about seven to eight times higher than the un-induced enzyme level in W4977. However, the strains are not further induced by leucine. This is in contrast to the parent strain in which serine deaminase activity increased from 0.49 units in cells grown without leucine to 1.54 units in cells grown with leucine. The serine deaminase activity in strain Porl7 is exceedingly low - the level can barely be detected in cells grown without inducer. The level is increased in cells grown with inducer, but even this level is less than the parental un-induced level.

Part 2d

Ability of Strains W4977, MS845, MS845-II and Porl7 to Utilize Maltose and their Susceptibility to Phage Lambda Vir

When streaked on MacConkey maltose plates, W4977 appeared to be reddish pink. Because this appearance is markedly different

TABLE 5

Serine Deaminase Activity in Strains of E. Coli

Strain	μmole Pyruvate Formed/mg. Protein	
	Addition to Growth Medium	
	None	Leucine
W4977	0.49	1.54
MS845	3.81	3.12
MS845-II	4.14	3.25
Por17	~0.10	0.20

The bacterial strains were grown in minimal medium with glucose 0.2% and proline 50 μg./ml. In induction experiments, the media were further supplemented with 50 μg./ml. leucine.

from that of the usual Mal^+ strain, they are referred to as $Mal^{\pm}$. MS845 was totally white on the plate, indicating that it was unable to ferment maltose. This was also true for MS845-II. Strain Porl7 appeared similar to W4977. To show that MS845-II cannot really utilize maltose as sole carbon and energy source, while W4977 and Porl7 can, the three strains were cultured in minimal media with glucose (0.2%) and proline (50 µg./ml.) overnight, and then subcultured in minimal media with proline (50 µg./ml.) and the following additions:

(a) glucose (0.2%),

(b) maltose (0.2%),

(c) glucose (0.02%), and

(d) glucose (0.02%) and maltose (0.2%).

After 12 hours of incubation at 37°C, strain W4977 and Porl7 were found to grow in all the media. Both strains had lower cell density in culture (c) than in other cultures. This was due to the low level of carbon and energy source in the media. MS845-II was found to grow in all media containing glucose, but not in the one with maltose only. Cell density of MS845-II in culture (c) was much lower than that in culture (a). The cell density of culture (c) and culture (d) were approximately the same. These observations indicate that MS845-II, unlike W4977 and Porl7, cannot grow on maltose as the sole source of carbon and energy, and this inability to grow is most probably not

due to a difficulty in adaptation.

It has been reported that the malB gene coding for maltodextrin permease is in the same operon as lamB coding for the receptor proteins for phage lambda (Hofnung, 1974). A mutation in the promoter or operator of this operon, or a mutation in the regulatory gene malT will result in an abnormal expression of both malB and lamB. The consequence will be two-fold:

(i) a loss of the ability to use maltose, and

(ii) resistance to phage lambda vir.

To test if the inability to use maltose in MS845-II is due to such a mutation, strain W4977, MS845-II, Porl7 and two known malB mutants were tested for their susceptibility to phage lambda. Strains cultured in Luria Broth supplemented with maltose (as inducer) were streaked on LB plates. Phage lambda vir were spotted on the streaks and the plates were incubated at 37°C for 12 hours. Strains W4977, MW845-II and Porl7 were found to be susceptible to phage lambda vir as indicated by lack of bacterial growth in the streak where phage lambda vir had been spotted. No inhibitions of bacterial growth were observed on the streaks of cultures of two malB mutants.

The conclusion that the strains are lambda-sensitive was further strengthened by an experiment in which cultures of the five strains were mixed with phage lambda vir and plated over LB plate in soft agar. Confluent lysis was observed in the soft agar containing W4977, MS845-II and

Porl7, indicating that the strains were subjected to lysis by Phage lambda vir. No lysis was seen with the malB mutants. This suggests that MS845-II, W4977 and Porl7 do not have mutations in the regulatory genes of the malB-LamQ operon.

Part 2e

Summary

The above data show that W4977 and Porl7 have similar phenotypes with respect to the four characteristics under study. The transferase-negative mutant(s) is significantly different from them in these four characteristics. Porl7 has been selected as a single step revertant of MS845, selecting for low proline requirement. The fact that the other characteristics have also reverted is convincing evidence that the four characteristics under study are affected by a single gene. As discussed in the description of the strains (Materials and Methods), MS845 has lost the "high proline-requiring" character in the course of cultivation, and its derivative, MS845-II, a form that grows well in minimal medium with glucose (2%) and proline (50 µg./ml.), but is otherwise similar, is the only remaining transferase-negative strain. Thus the isolation of a "low proline-requiring" revertant cannot be repeated. The further experiments required to confirm that all characteristics are influenced by a single pleiotropic gene were carried out using MS845-II as the starting strain.

Part 3

Genetic Study of the *uvrb* Gene

Part 3a

Isolation of a Spontaneous Revertant for "Low Proline-Requiring" Character from MS845-II

Proline oxidase is subject to catabolite repression. This has been confirmed in our studies, as shown in Table 9 in Part 4. Thus when grown with glycerol, MS845-II, the "low proline-requiring" derivative of MS845, was found to have twice as much proline oxidase as when it was grown with glucose. Concomitant with the increase in proline oxidase activity, MS845-II required a higher amount of proline to attain a normal growth in medium with glycerol as the carbon source than in medium with glucose as the carbon source. To select for "low proline-requiring" revertants from MS845-II, the strain, MS845-II, was cultured in Luria Broth until the cell density reached approximately 2×10^8 cells/ml. 0.1 ml. of this culture was plated on minimal medium with glycerol (0.2%) and proline (15 µg./ml.) After five days of incubation at 37°C, approximately 30 small colonies arose from a rather thick background. These colonies were purified on the same type of selection plates. They were found to resemble W4977 on MacConkey maltose plates. One of them, designated as THEK4, was further characterized and the results are shown in Table 6. These results indicate that the "low proline-requiring" revertant has low proline oxidase similar to that of W4977. It has significant transferase activity. Its L-serine deaminase activity is as low as Por17 and the enzyme activity responds to leucine induction as Por17. It is clear, from these data, that this revertant has restored most of the phenotype of W4977. This

TABLE 6

Physiological Characteristics of THEK4

Strain	Transferase	Proline Oxidase	Serine Deaminase		MacConkey Maltose	Growth in Maltose Minimal Medium
			nonind	ind		
THEK4	+	9.5	~0.1	0.2	±	+
Por17	+	14.0	~0.1	0.2	±	+

Proline oxidase is expressed in OD/100 K.U. of cells.

Serine deaminase is expressed in μmole product/mg. protein.

Nonind - no leucine in the medium, ind - with leucine.

strain is most likely to have resulted from one step mutation of a gene rather than an accumulation of several mutations. None of the mutant characteristics other than the proline oxidase level seem to be selected against in minimal medium supplemented with glycerol and a low level of proline. Maltose is not present in the medium. A high level of serine deaminase does not appear to hinder the cells. Nothing is known about the physiological importance of the transferase. In any case, the possibility that all characteristics are selected simultaneously, but by independent mutations, is exceedingly unlikely. The identity in phenotype of this revertant, THEK4, and Por17, as indicated by their levels of serine deaminase activity and proline oxidase activity, implies that these two revertants have very similar genotypes. Thus it seems likely that these two revertants are the results of one step mutation of the transferaseless mutant. Hence, a hypothesis is made here - the four characteristics involved are affected by the expression of one gene. This hypothetical gene is designated wyb. W4977 is regarded as phenotypically Wyb^+ and MS845, as well as MS845-II, are regarded as Wyb^-. The experiments to be described in the following sections were done to verify the hypothesis.

Part 3b

Transduction of wyb

To investigate the expressions of wyb in the cells, attempts were made to transduce the wyb^+ gene from W4977 to MS845-II and the wyb^- gene from MS845-II to W4977.

1. Transductions using MS845-II as Recipient

P1 phages grown on W4977 were added to MS845-II and the culture was plated on minimal medium with maltose (0.2%) and proline (50 µg./ml.), and on glycerol (0.2%) with proline (15 µg./ml.). The rationale of the experiment was that if *wyb*$^+$ really influenced maltose fermentation, then a MS845-II cell, having acquired *wyb*$^+$ through transduction, would be able to grow on the selection plates. The colonies developed on the plates could then be tested for other characteristics under study to see if they were also transduced. According to the same rationale, if the *wyb*$^+$ cells have lower proline oxidase activity, they should be able to grow even on glycerol with lower proline supplements.

Mal$^+$ transductants of MS845-II were obtained on the selection plates six days after the transduction. Three were tested for the other phenotypes. However, they were found to have serine deaminase and proline oxidase at high levels comparable to MS845-II. They also showed negative transferase activity (data not shown). Thus, it seems that only the Mal$^+$ character has been transduced in these MS845-II/W4977 strains.

MS845-II was transduced to maltose-positive by M. Schwartz of the Institute Pasteur, using a phage ø80d*malA*. This strain, THEK7, was tested for other characteristics in our laboratory and its characteristics are shown in Table 7. It is clear that THEK7 has the phenotype of strain MS845-II for all characteristics under study, except for maltose utilization.

The MS845-II culture transduced by W4977 phages showed a thick background growth on the glycerol-proline plates. This is because growth of MS845-II was only retarded but not completely inhibited in this medium.

TABLE 7

Physiological Characteristics of Strains THEK5, 6 and 7

Strain	Transferase	Proline Oxidase	Serine Deaminase		MacConkey Maltose	Growth in Maltose Minimum Medium
			nonind	ind		
THEK5	-	52	4.8	5.7	±	+
THEK6	-	45	4.6	4.7	±	+
THEK7	-	45	3.9	2.9	±	+
MS845-II	-	51	4.1	3.3	-	-

Proline oxidase is expressed in OD/100 K.U. of cells

Serine deaminase is expressed in μmole product/mg. protein.

After five days of incubation, as many colonies developed on the control plates as on the transductant plates. These were more likely to be spontaneous revertants than transductants.

2. Transductions with MS845-II as Donor

After encountering the above difficulties, attempts were made to transduce W4977 to wyb⁻ by phage grown on MS845-II. However, it proved difficult to grow phage on MS845-II. When P1-Cm phage and a MS845-II culture were cross-streaked on a chloramphenicol LB plate, no MS845-II lysogens appeared along the streak after two days of incubation, a duration which is usually long enough for colonies of lysogenic bacteria to develop. Instead, minute colonies started to appear on both the "cross streak" and the control streak which had not been exposed to phage. These small colonies were most probably chloramphenicol-resistant derivatives of MS845-II. It seems that the chloramphenicol method of making lysates cannot be applied to this strain. Preparation of phage by the plate lysate method (Miller, 1972) was also attempted, but was unsuccessful. These facts may indicate that MS845-II is not able to support normal replication of P1 phages. The same conclusion is reached from the fact that in the transductions of MS845-II and W4977 to proline independence by phages grown on CU4, only three transductants were obtained when MS845-II was the recipient, while W4977 gave as many as 200 transductants.

Part 3c

Selection of U.V.-induced Mal⁺ Revertants of MS845-II

In an attempt to select Mal⁺ revertants, MS845-II grown on minimal medium with glucose (0.2%) and proline (50 µg./ml.) was irradiated with

ultraviolet light. A portion of the irradiated cells was plated immediately on minimal medium with maltose(0.2%)and proline(50 µg./ml.) Another portion of the irradiated cells was cultured in minimal medium with glucose(0.2%)and proline(50 µg./ml.) for 12 hrs. and then plated on the same type of maltose selection plates. Cells plated immediately after irradiation gave rise to very small slow-growing colonies which were found to be maltose-negative on MacConkey plates. Those plated after an intermediate cultivation in glucose-supplemented liquid medium gave only slightly larger colonies even after one week incubation at 37°C. Among these colonies, two were found to be maltose^{+} on MacConkey plates. They were isolated and designated as THEK5 and 6. The phenotypes of the two strains were determined and are shown in Table 7. The results indicate that THEK5 and 6 have the phenotypes of MS845-II for all characteristics under study except maltose-utilization. Thus THEK5 and 6 are not wyb^{+} but do ferment maltose. This result may be due to a new ultraviolet induced mutation, resulting in a by-pass of the effect of the wyb mutation on the maltose-utilizing ability. If this were so, the effect of the wyb^{-} on the other characteristics would not be affected.

Part 3d

Transduction with Porl and THEK5 as Recipient Strains

In the process of characterizing THEK5 and 6, their reaction to phage infection was also tested. It was found that both strains could be lysed by P1-Cm, could establish lysogeny with the phage, and could be transduced readily to proline independence by the phages grown on CU4. In other words, the behaviour of the strains toward phage infection was

similar to that of prototrophic E. coli. Because of these observations, Porl, another wyb$^+$ strain having low proline oxidase activity and derived from MS845, was also tested and found to support P1-Cm growth.

Strain Porl was then transduced to maltose positive by phage grown on W4977. Eighteen transductants to Maltose positive were able to grow with maltose as carbon and energy source, but had high serine deaminase. Two were tested for transferase activity and found to be transferase negative. Thus these Mal$^+$ transductants, like MS845-II/W4977 are probable wyb$^-$ but have acquired somehow the maltose fermenting ability.

Since THEK5 was proved to be a much better recipient strain for transduction with P1-Cm than MS845-II, an attempt was made to transduce THEK5 to "low-proline-requiring" with phages grown on W4977 with selection on minimal medium with glycerol (0.2%) and proline (15 µg./ml.). THEK5 itself grew slowly on the selection plate and formed a thick background as had MS845-II. After six days of incubation, colonies developed on both the control plates and the transductant plates, with about twice as many on the transductant plates. This relatively small difference between the transductant plates and the controls makes it impossible to decide whether there are real transductants. The colonies were therefore not examined further.

Part 3e

A Trial of Several Media for Selecting Wyb$^+$ or Wyb$^-$ Phenotypes

The failure of the preceding experiments was probably due to the fact that the medium supplemented with glycerol and proline (15 µg./ml.) does not select sufficiently against Wyb$^-$. A rather unsuccessful search

for a better selection medium, which would support the growth of the strains of one phenotype and not that of the other, was carried out. The media which have been tried and the rationale for trying them are listed as follows:

1. Minimal Medium (Ammonium Sulfate Omitted) with Proline (2 mg./ml.) as Carbon, Energy and Nitrogen Source

Strains with high proline oxidase (Wyb^-) might be able to metabolize proline effectively and be able to grow in medium with proline as the sole source of carbon, energy and nitrogen. The strains with low proline oxidase (Wyb^+) might have difficulty growing in this medium. Hence, this medium might be selective for the Wyb^- phenotype. However, it was found that W4977 and all derivatives tested were unable to grow with proline as the sole carbon, energy and nitrogen source.

2. Minimal Medium with Glycerol (2%) and Proline Amide (1 mg./ml.)

A few analogs of proline are known to have inhibitory effects on E. coli (Shive and Skinner, 1963). Proline amide is an analog of proline and thus there is a possibility that it can be a substrate of proline oxidase (see Appendix II). It was hoped that a strain with high proline oxidase (Wyb^-) could metabolize the proline amide supplied in the medium and hence its growth would not be inhibited, while a strain with low proline oxidase (Wyb^+) might be inhibited. Since the toxic effects of proline analogs are usually reversed by proline, MS845-II/CU4 and W4977/CU4, the proline independent transductants of MS845-II and W4977 were tested for their ability to grow on medium with proline amide, but without proline. It was found that proline amide was not toxic even to strain W4977/CU4.

3. Minimal Medium with Glucose (0.2%) Proline (50 µg./ml.) and Serine Methyl Ester (1 mg./ml.)

According to the same rationale, serine methyl ester was added to the medium to see if it could inhibit the growth of strains with low serine deaminase (Wyb^+), but not the strains with high serine deaminase (Wyb^-). However, serine methyl ester did not interfere with the growth of either strain.

4. Minimal Medium (Ammonium Sulfate Omitted) with L-serine (2 mg./ml.), Proline (1 mg./ml.), Isoleucine (50 µg./ml.) and Valine (50 µg./ml.)

L-serine is toxic to E. coli strain K10 and most of its derivatives because it inhibits L-threonine deaminase. The toxic effect can be relieved when isoleucine and valine are also present in the medium. In such medium, a strain with high L-serine deaminase (Wyb^-) might make use of the L-serine as a carbon, energy and nitrogen source. If the strain also has high proline oxidase activity, then the high concentration of proline in the medium may enable the strain to grow still better. Thus this medium may favour the growth of Wyb^- strains and not that of Wyb^+ strains. It was found that W4977 grew very poorly on this medium. MS845-II grew better, but Porl7 was unable to grow on the medium. Thus this medium does differentiate W4977 and MS845-II, but the difference in growth pattern is very small. However, this medium does differentiate MS845-II and Porl7 to a significant extent.

Part 3f

Transduction with Porl7 as the Recipient

Because the medium with serine, proline, isoleucine and valine appeared at least somewhat selective, an attempt was made to transduce

Porl7 with phages grown on THEK5 selecting for growth on the above medium. No colonies were found on the transductant plates in the first eight days of incubation. After that, tiny colonies appeared on the transductant plates. These colonies were isolated, tested for serine deaminase and proline oxidase activities, and were found to have as low a level of serine deaminase and proline oxidase as Porl7. Thus they are most probably *wyb*$^+$ strains that are resistant to serine toxicity. It can be concluded that the transduction of Porl7 to *wyb*$^-$ was also unsuccessful.

Part 3g

Evaluation of the Methods of Phage Production and Transduction

To be sure that the failure of the transduction of the *wyb* gene was not due to faulty phage techniques, other transduction experiments with the same phages and by the same methods were carried out. Phages P1-Cm grown on W4977 and on THEK5 were used to transduce strain CU1008, carrying an *ilvA* deletion and incapable of isoleucine biosynthesis, to isoleucine independence. In both experiments, about 200 colonies appeared on each of the transductant plates of minimal medium, with no amino acids supplement, three days after the transduction, while no colonies appeared on the control plates. Thus, the methods of phage production and transduction seemed to be working well.

Part 3h

Summary

The result of the work described in this section can be summarized

as follows: from MS845-II, a revertant, THEK4, with a low proline requirement was isolated. This revertant was identical to Porl7 in the four characteristics studied. This strongly indicates that the four characteristics are under the influence of one gene. On the other hand, none of the Mal^+ derivatives of MS845-II isolated for the Mal^+ character appeared to be Wyb^+. These Mal^+ strains include the transductants MS845-II/W4977 and THEK7, and two ultraviolet-induced Mal^+ mutants of MS845-II. All these strains were very similar to MS845-II except for the maltose character. In addition, Porl, a derivative of MS845-II, when transduced with phages grown on W4977, also gave rise to transductants with no transferase activity and high serine deaminase activity. It is thus possible for a strain to regain the ability to use maltose while maintaining the wyb$^-$ genotype. The various attempts to derive Wyb^- strain from Wyb^+ by transduction were not successful. It is clear and unfortunate that none of the transduction experiments in transducing either Wyb^+ to Wyb^- or Wyb^- to Wyb^+ were successful. That the transduction methods and the phages preparation were not at fault was demonstrated by a successful transduction of another marker, an ilvA deletion, with the same phages and the methods used for the wyb study.

Part 4

Regulation of Proline Oxidase in Strains W4977 and MS845-II

Part 4a

Test of Leucine and Phenylalanine as Possible Inducers of Proline Oxidase

It is clear that the parental character of serine deaminase is altered in MS845 (Table 5, Part 2c). In our laboratory, another amino

acid degradation enzyme, threonine deaminase, which is inducible by leucine, was also found to be affected to a very slight extent in the transferase-negative mutant (1.68 units in MS845-II as compared to 1.49 units in W4977). It seemed possible, therefore, that proline oxidase which degrades proline and the activity of which is greatly affected by the transferase mutation, may also be a leucine-inducible enzyme. To examine this, and to see whether phenylalanine, the other amino acid transferred by the transferase, is an inducer of proline oxidase, the following experiments were carried out: E. coli strain K10 was grown in minimal medium with glycerol (0.2%) in the conditions noted in Table 8. Strain W4977 which has a much lower proline oxidase than K10, was also tested for the possible induction of proline oxidase. The strain was grown in minimal medium with glycerol (0.2%) proline (200 µg./ml.) in the conditions noted in Table 8. The results of proline oxidase assays on these cultures shown in Table 8 indicate that proline oxidase is not induced by either leucine or phenylalanine, but is inducible by proline in strain K10. The addition of either leucine or phenylalanine with proline does not increase the induction due to proline. There is much less enzyme activity in W4977 than in K10 when proline is present. This low level of proline oxidase in W4977 is also not inducible by leucine and phenylalanine. Thus, it can be concluded that proline oxidase is different from serine deaminase with respect to induction by leucine.

Part 4b

Catabolite Repression of Proline Oxidase in Strains W4977 and MS845-II

Strains W4977 and MS845-II were tested to see if their levels of

TABLE 8

Regulation of Proline Oxidase in Glycerol-grown Cultures of Strains W4977 and K10

Experiment	Strain	Addition to Growth Medium	Proline Oxidase*
1	K10	0	7
		Proline	63
		Leucine, ile, val*	9
		Proline, leucine, ile, val	61
2	K10	0	10
		Proline	93
		Phenylalanine	13
		Proline, phenylalanine	87
3	W4977	Proline	11
		Proline, phenylalanine	10
		Proline, leucine, ile, val	10

Leucine added at 100 µg./ml., with isoleucine and valine 50 µg./ml.; phenylalanine, 100 µg./ml.; proline, 50 µg./ml. (K10) or 200 µg./ml. (W4977); proline oxidase is expressed in OD/100 K.U. of cells.

*In Expt. 1, isoleucine and valine were added to relieve the inhibitory effect of leucine on the growth of cells.

proline oxidase were subject to catabolite repression by glucose. The two strains were cultured in minimal medium with glycerol (0.2%) and proline (200 µg./ml.) with and without 0.2% glucose. The results of proline oxidase assays on these cultures are shown in Table 9. The data indicate that the proline oxidase activity of MS845-II cultured in the presence of glucose is half as much as that in cells cultured without glucose. Thus the enzyme of MS845-II is subject to catabolite repression. The low level of proline oxidase activity in W4977 does not seem to be altered by the presence of glucose. If the enzyme was subject to catabolite repression to the same extent as that of MS845-II, the assay method used here would have detected the change. It can be concluded that the proline oxidase of W4977 is not subject to catabolite repression by glucose, but the enzyme in MS845-II is.

TABLE 9

Catabolite Repression of Proline Oxidase by Glucose in Strains MS845-II and W4977

Strain	Activity of Proline Oxidase	
	No glucose	0.2% glucose
W4977	15	14
MS845-II	43	22

Strains were grown in minimal medium with glycerol (0.2%), proline (200 µg./ml.), with or without glucose (0.2%). Proline oxidase activity is expressed as OD/100 K.U. of cells.

DISCUSSION

The main phenomenon described in this thesis is the following: a strain (MS845) deficient in the enzyme leucyl-, phenylalanyl-tRNA-protein transferase has been found to differ from its transferase-containing parent (W4977) in four physiological unrelated metabolic activities. From the transferase-deficient strain, two independent revertants (THEK4, Porl7) have been isolated. These revertants show essentially the same phenotype as the transferase-containing parent. From this it is concluded that the four metabolic activities are influenced by a single pleiotropic gene.

These changes in metabolic characteristics are:

1. Transferase Activity

The parent strain W4977 has a detectable amount of transferase activity. The mutant MS845-II shows an insignificant amount of enzyme activity, while both revertants, Porl7 and THEK4 have regained transferase activity (Table 4).

2. L-serine Deaminase Activity

The level and the inducibility by leucine of serine deaminase in W4977 is about the same as that of other *E. coli* strains studied in our laboratory. Strain MS845 had seven times as much serine deaminase activity as W4977, but the level of activity did not change in a culture supplemented with leucine. Both Porl7 and THEK4 have very low, though detectable, serine deaminase activity (approximately one-fifth of that

of W4977). Their levels of serine deaminase are about two-fold increased in the presence of leucine (Table 5).

3. Proline Oxidase Activity

Strain W4977 has a low level of proline oxidase activity. MS845-II has 4-5 times as much proline oxidase activity as W4977 when both are cultured in glycerol medium supplemented with proline. The revertants have the same level of enzyme activity as W4977 (Table 3).

4. Ability to Utilize Maltose as the Sole Carbon and Energy Source

W4977 can grow on maltose as sole carbon and energy source. It appears pinkish when streaked on a MacConkey maltose plate. MS845 could not grow on maltose and appeared white on a MacConkey maltose plate. Porl7 and THEK4 regained the ability to utilize maltose as the sole source of carbon and energy. They appear similar to W4977 on a MacConkey maltose plate.

As judged from the experimental data, it is clear that Porl7 and THEK4, isolated separately, have almost identical phenotypes for the four characteristics concerned. Thus, it is very likely that the two strains have very similar genotypes for the four metabolic activities under study. This, together with the fact that both revertants were selected in medium which favoured only the reversion to low proline oxidase activity, but not the reversion of other three phenotypes, suggests that these revertants regained the parental phenotypes through a one-step mutation rather than through an accumulation of several mutations. The gene that is thought to be responsible for the changes in phenotypes is called wyb.

Other experiments designed to further confirm the existence of the wyb gene were attempted. Mal^+ mutants isolated from MS845-II after

ultraviolet treatment were found to retain the phenotype of MS845-II for all the characteristics concerned except for the maltose-utilizing character. Mal$^+$ transductants of MS845-II and Porl also retained the phenotype of the wyb$^-$ parent for the other three characteristics. Thus, none of these experiments can confirm that the four characteristics under study are under the influence of one gene, though they do not disprove it. The nature of these strains will be discussed later. In the following paragraphs, the pleiotropic effect of the wyb gene will be interpreted further with the results obtained in this work.

If the four metabolic characteristics are really affected by one gene, what might be the mechanism by which the effects of this gene are transmitted? Various hypotheses may be suggested, as follows, though all of these are at present entirely speculative. The diagrams illustrating these hypothese are shown in Fig. 5 and Fig. 6.

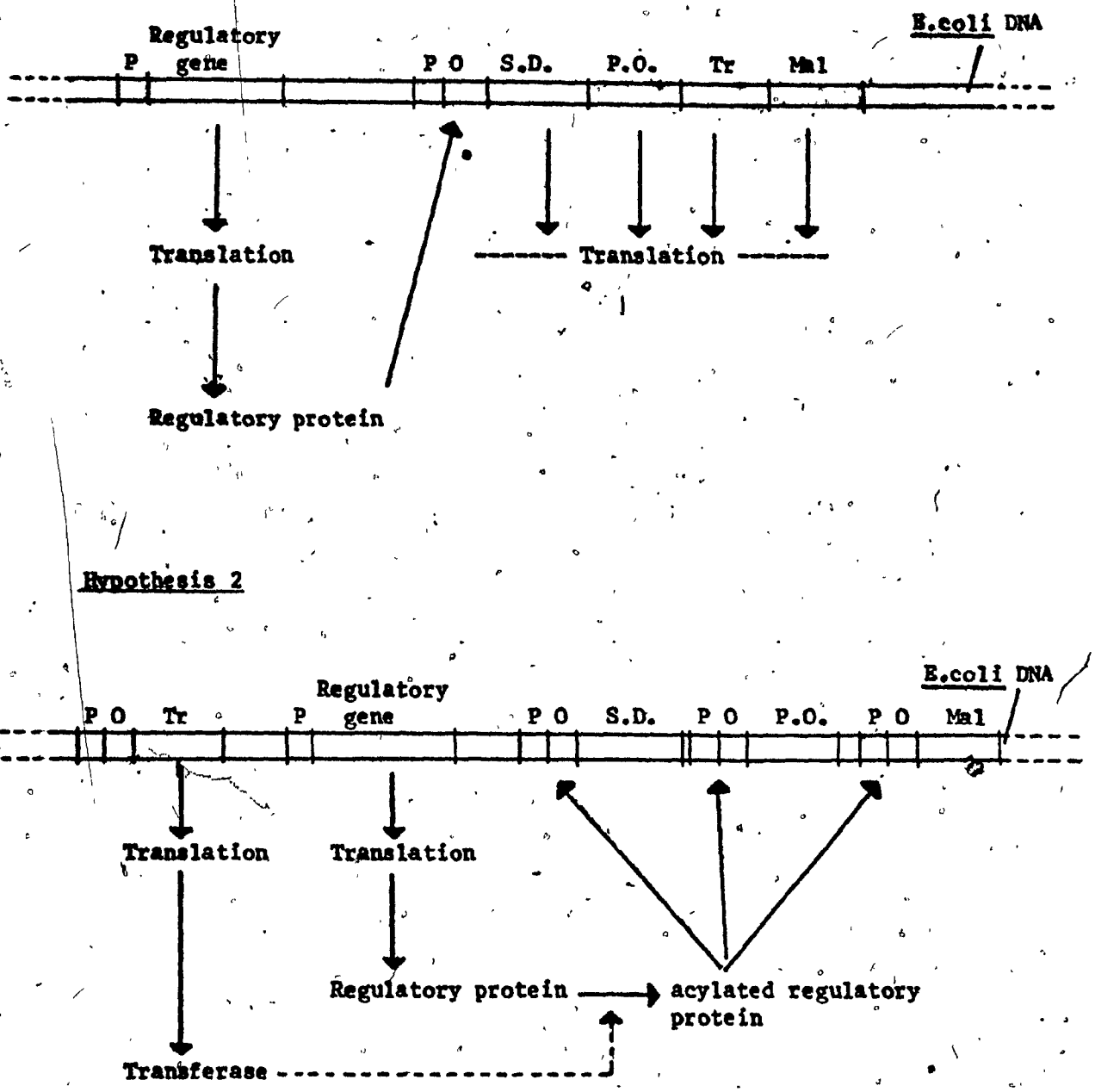
Hypothesis 1: Regulation of Gene Expression by a Common Factor

The several genes responsible for the phenotypic changes in MS845 may all be situated in the same operon. Then a mutation of the regulator, the promoter or the operator would cause an abormal expression of all the genes in the operon. A mutation of the first gene to be transcribed could also affect the expression of all other genes (polar mutation). Alternatively, the genes responsible for the phenotypes might be at different loci but they might all be affected by a common regulatory protein. A mutation of the gene specifying this protein might then result in changes in the four metabolic characteristics.

Hypothesis 2: Modification of Regulatory Proteins by Transferase

The substrates which accept the amino acids from transferase may

Figure 5: Diagramatic Illustration of Hypothesis 1 and 2.



Abbreviation :

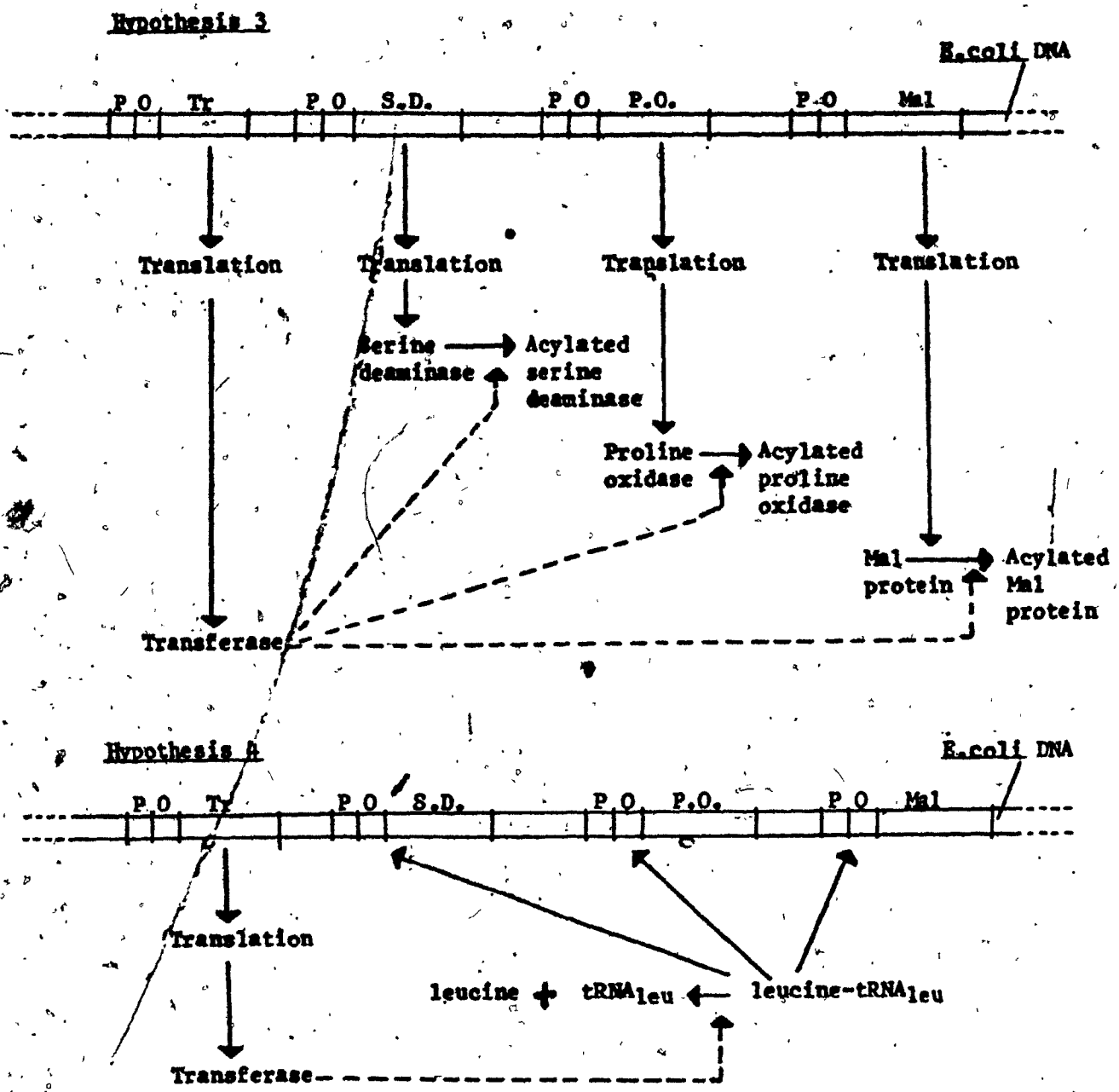
P - Promoter O - Operator

S.D. - structural gene of serine deaminase.

P.O. - structural gene of proline oxidase.

Tr - structural gene of transferase. Mal - structural gene of protein involved in maltose utilization.

Figure 6: Diagramatic Illustration of Hypothesis 3 and 4.



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P - Promoter O - Operator

S.D. - structural gene of serine deaminase.

P.O. - structural gene of proline oxidase.

Tr - structural gene of transferase.

Mal - structural gene of protein involved in maltose utilization.

be regulatory proteins which control the expression of the genes governing the proline oxidase activity, serine deaminase activity and the ability to utilize maltose. Then, if a mutation in MS845 causes a loss in activity of transferase, one or several regulatory protein(s) might not be acylated. They might then not function properly and this might result in an abnormal expression of the genes for proline oxidase, serine deaminase and maltose utilization.

Hypothesis 3: Transferase Modifies the Enzymes Themselves

The enzymes proline oxidase, serine deaminase and one or more enzymes involved in maltose utilization might all be acceptors in the transferase reaction. An absence of aminoacylation of these proteins might then maintain the conformation of these proteins in such a state that they either are hyperactive or inactive.

Hypothesis 4: Leucyl-tRNA as a Signal

The concentration of leucine or leucyl-tRNA in the cell might regulate the synthesis of amino acid-degrading enzymes and the enzyme(s) involved in maltose utilization. A change in concentration of these substances due to the absence of transferase activity in the mutant might cause the change in activity of the enzymes.

None of the hypotheses listed above was directly tested in this work, nor is this an exhaustive list of possible hypotheses. Still the experimental results allow some discussions of the hypotheses.

Let us consider first the increased inducibility of proline oxidase in strain MS845. The levels of proline oxidase in the proline independent strains W4977/CU4, MS845-II/CU4 and Por17/CU4 when grown without the inducer, proline, were found to be the same (Table 3).

When the cells were grown with proline, proline oxidase activity was more elevated in MS845-II/CU4 than in either Por17/CU4 and W4977/CU4. This indicates that the mutation in MS845-II actually caused a change in the response to proline induction. This observation makes unlikely Hypothesis 3 that the enzyme proline oxidase is a substrate of the transferase, the activity of the enzyme varying in the aminoacylated and nonaminoacylated forms. According to Hypothesis 3, the reason that MS845-II has four to five-fold more enzyme activity than W4977 is because the enzyme molecules are not aminoacylated in MS845-II. The difference in enzyme activities should also be seen in cells grown without inducer, but it is not. If transferase does play a direct role in the regulation of proline induction, it is more likely that the acceptor of the transferase is a regulatory protein, i.e., Hypothesis 2. For convenience to the discussion here, the regulatory protein involved is taken to be an aporepressor, though it can also be an apoinducer or a regulatory protein functionally similar to the cyclic AMP receptor protein. This regulatory protein may bind equally well to the operator whether aminoacylated or not. In this case, in the absence of proline, proline oxidase will be synthesized at the same rate in the parent and the mutant. However, the nonaminoacylated and aminoacylated regulatory proteins may differ in the conformation they assume on binding with proline. The nonaminoacylated regulatory protein may undergo an abnormal conformational change on binding with a proline molecule, such that it cannot bind to the operator of the proline oxidase operon. Then, in the presence of proline, the mutant strain in which the regulatory protein would be nonaminoacylated, would overproduce proline oxidase.

The observations on proline oxidase also do not agree too well with Hypothesis 1, which states that the gene (wyb) responsible for the changes in the proline oxidase phenotype in MS845, shares the same operon with the genes responsible for other physiological changes. It seems unlikely that the structural genes for the four activities could lie in a single operon. An operator mutation can only turn the operon on and off. But the wyb mutation increases serine deaminase activity without increasing proline oxidase activity and, at the same time, causes a loss of transferase activity. How this could be the function of the action of a single operon is not obvious. It is unlikely that the operon is composed of regulatory genes (for serine deaminase and proline oxidase) and structural gene (for transferase). No such example has ever been described.

It should be noted here that strain W4977 differs very much from prototrophic E. coli K10 in proline oxidase expression. Strain W4977 is rather unresponsive to proline induction as compared to strain K10. Its enzyme is also not much subject to catabolite repression. Thus, the mutation(s) in MS845-II may cause, somehow, a "by-pass" of a mutation(s) present in W4977 and responsible for the phenotype of proline oxidase in the strain.

L-serine deaminase activity was found to be about seven-fold higher in MS845 than in W4977. But the three fold increase in activity by leucine induction in W4977 was not observed in MS845. Porl7 and THEK4 all show exceedingly low serine deaminase activity, but the activity is inducible by leucine (Table 5). The fact that serine deaminase activity is so much lower in strain Porl7 than in strain W4977 seems to

exclude the possibility that Por17 is an absolute revertant of strain MS845, since, if it were an absolute revertant, it would have a phenotype exactly identical to W4977. It may be that, as is most frequently the case, the reversion takes place at a different base pair than the original mutation. However, because strain MS845 was isolated with nitrosoguanidine, it may contain several mutations. Por17 would be a revertant for only one of them. Then, even if the mutation in Por17 exactly reversed the mutation forming MS845, the allele might be expressed differently due to the additional mutations in MS845.

The increase of serine deaminase activity and the loss in inducibility by leucine can be explained in terms of leucyl-tRNA as regulatory signal (Hypothesis 4). The level of leucyl-tRNA may be controlled by the activity of transferase which functions to deacylate the leucyl-tRNA in cells. In the transferase-deficient strain, MS845, no discharging of leucyl-tRNA by the enzyme can take place. An accumulation of leucyl-tRNA may be the cause for elevated serine deaminase activity in the cells. The concentration of leucyl-tRNA may be so high that serine deaminase is fully induced. Thus, further addition of leucine would then make little difference to the rate of synthesis of serine deaminase. The hypothesis made here is based on the fact that quite a few aminoacyl-tRNA are known to have regulatory effect on certain biosynthetic enzymes (Brenchley and Williams, 1975). In Salmonella typhimurium, an increase in concentration of exogenous leucine resulted in an increased leucinylation of leucine-accepting tRNA. Correlated with this is an increase in the levels of branched-chain biosynthetic enzymes in cells grown with leucine (Freundlich et al, 1971). Thus, there is some support for the idea

that the level of leucyl-tRNA may have a role in regulating the synthesis of serine deaminase. The fact that leucine does not induce proline oxidase makes this hypothesis less likely (Table 8)..

The defect of MS845 in maltose utilization has not been worked out at this time.. Strains W4977, MS845 and Porl7 can ferment glucose. This indicates that the enzymes in the pathway for glycolysis are functioning well in these strains. The inability of MS845 to utilize maltose as carbon and energy source may be due to some defects specific to the maltose utilizing system - the maltodextrin permease, amylomaltase and maltodextrin phosphorylase. No attempt to investigate this has been made. The ultraviolet-induced Mal^+ mutant, THEK5 and THEK6 were found to be similar to their parent, MS845-II, in the other three characteristics under study. The occurrence of these mutants may be the result of a mutation at a gene other than the wyb gene. Suppose Hypothesis 2 was correct. The inability of MS845-II to use maltose would then be due to the fact that the regulatory protein of the maltose operon could not be acylated. The nonacylated regulatory protein would be unable to bind to its operator, and the operon could not be transcribed. Then a further mutation at the operator or promoter of this operon might allow a normal transcription of the operon, even when the regulatory protein is not normally bound to its site of action.

The existence of this class of mutations is formally identical to the isolation of "up-promoter" mutants in a Cya^- strain of E. coli. A Cya^- strain with a defect in the synthesis of adenylcyclase, cannot form cyclic AMP. Without cyclic AMP, the "CRP-dependent" operons, including those coding for a number of carbohydrate-metabolizing enzymes,

cannot be transcribed. As a result, the Cya⁻ strain cannot ferment a number of sugars. From the Cya⁻ strain, have been isolated mutants which are still cya⁻ but can ferment a given sugar (e.g., lactose), which the parent cannot ferment (Perlman, 1969; Hopkins, 1975). This Lac⁺ strain arises by a second mutation in the lactose operon, such that the operon is expressed in the absence of cyclic AMP? Thus THEK5 and THEK6 may contain a second mutation which allows them to utilize maltose even in the absence of transferase.

While the mutants THEK5 and THEK6 may be understood in this way, the occurrence of the Mal⁺ transductants THEK7, MS845-II/W4977 and Porl/W4977 is more difficult to explain. Since all these transductants are transferase-negative and have high serine deaminase activity, it is clear that no wyb⁺ gene has been transduced. The isolations of MS845-II/W4977 and Porl/W4977 capable of growing with maltose as the sole carbon and energy source mean that some genetic material derived from W4977, when incorporated to the genome of MS845-II and Porl, enable the strains to utilize maltose. But, at this time, which fragment of chromosome in W4977 is responsible for the conversion of MS845-II and Porl to Mal⁺ is not known. The fact that phage ϕ80malA can transduce MS845-II to Mal⁺ suggests strongly that MS845-II carries a mutation in the malA region (74 minutes). However, it is not excluded that a nearby gene is involved. In any case, this mutation is different from the wyb mutation. An understanding of these genetic data would be aided by more information on the nature of the corresponding physiological defects.

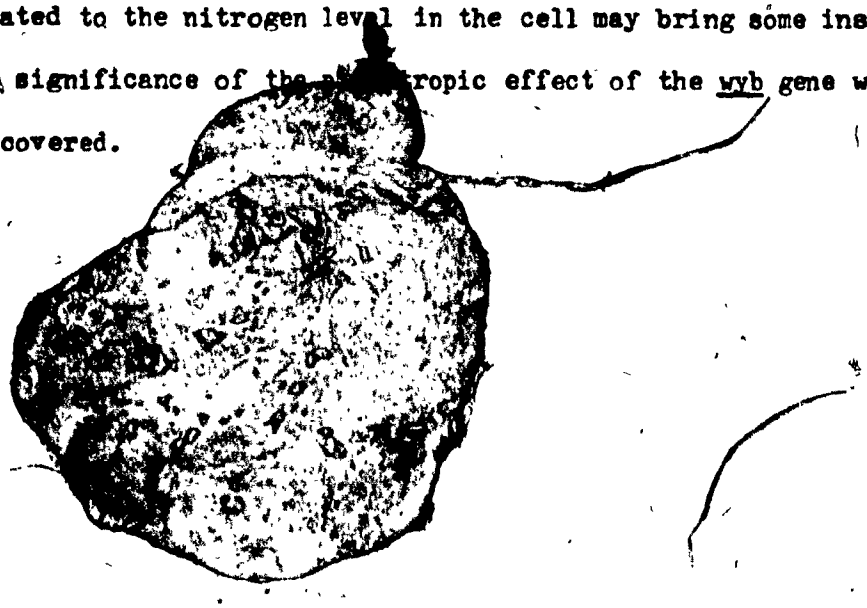
The transduction of the wyb gene described here have proved impossible, largely due to the lack of a good selective medium. One

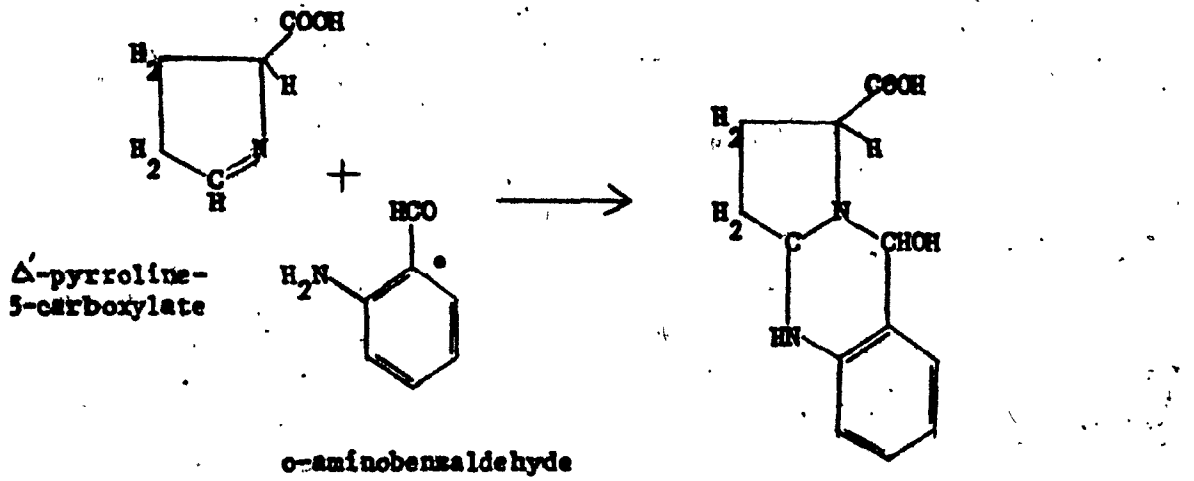
possible explanation of why no wyb transductant has ever been obtained is that the expression of the wyb, as stated in the hypotheses, may not directly determine the phenotypes of the metabolic activities. A wyb gene product may be a regulatory protein functioning to control the expression of other operons or may be the transferase enzyme responsible for post-translational modifications of other proteins. In the transduction process, time is required for the transduced wyb to express itself and a duration of time may be required for the wyb gene product to exert its effects before the transduced cells can show the phenotypes to be transduced. The transduced cells in the intermediate state may have high mortality in a medium that does not favour their original phenotypes. Another approach to transduction that does not require a selection based on the differences between the Wyb^+ and the Wyb^- is suggested in the following paragraph.

The gene causing a loss of transferase has been reported to be located between 45 min. and 54 min. of the E. coli chromosome (Soffer, 1974). One could use P1 phages grown on a transferase-negative strain to transduce any strain with unrelated mutations in the 45 min. to 54 min. range, selecting recombinants of those mutations, then screening for inability to ferment maltose or poor ability to grown on "low-proline" medium as unselected marker. If maltose-negative transductants or "high proline-requiring" transductants are found, the extent of cotransduction with any gene in this region would indicate the location of the wyb gene. All maltose negative transductants or all "high proline-requiring" transductants could then be tested for the other characteristics under study and the extent to which they are cotransduced could

then be determined. If the characteristics are cotransducible, but do not show a 100% cotransduction, this probably indicates that the changes in phenotypes in MS845 are due to mutations of several neighbouring genes. This is quite possible, because nitrosoguanidine is known to induce mutations primary at the replication point of the chromosome, which leads to clustering of induced mutation (Cerdá - Olmedo et al, 1968). If the characteristics are always cotransduced, then the metabolic activities would indeed be affected by one gene.

The evidence in this thesis suggests that four diverse metabolic activities are regulated in common. The physiological significance of this is not obvious. The closest relation is between L-serine deaminase and proline oxidase, both of which degrade amino acids to produce nitrogen and both of which increase in activity in the wyb⁻ strain. Perhaps then all the metabolic activities studied here form a (or a part of a) regulatory system involved in adjusting the level of nitrogen in the cells. Then an investigation of how the transferase activity is related to the nitrogen level in the cell may bring some insight as to the significance of the pleiotropic effect of the wyb gene which we discovered.



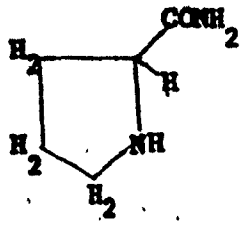


Δ'-pyrroline-5-carboxylate

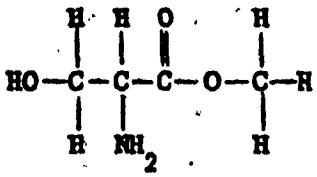
o-aminobenzaldehyde

derivative of hydroquinazolium

Appendix I : Chemical reaction between Δ'-pyrroline-5-carboxylate and ö-aminobenzaldehyde



proline amide



serine methyl ester

Appendix II : Structures of proline amide and serine methyl ester

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