STUDIES ON ENZYMATIC ADAPTATION IN MICRO-ORGANISMS

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by

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CHAPTER I

GENERAL INTRODUCTION

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During the past ten years, the importance of the phenomenon of enzymatic adaptation has become more and more apparent. Besides being in itself of considerable interest, it possesses implications for general cellular physiology extending outside of the area in which adaptation was originally studied, that of variations in micro-organisms. Thus, advances in our understanding of genetic mechanisms have already been made through studies of enzymatic adaptation (1). It has been predicted that further knowledge of enzymatic adaptation will throw light on some of the mechanisms of cellular differentiation in animal tissues (2). Arguments have been advanced to link adaptive enzyme formation with detoxication mechanisms and antibody formation in higher animals(3). Elegant techniques have been devised, employing enzymatic adaptation for the study of metabolic pathways in bacteria (4). Finally, with the growing realization that enzymatic adaptation is the expression of a biological phenomenon of fundamental importance, many workers are turning their attention to its use for the study of general mechanisms of enzyme synthesis and of protein synthesis.

This subject has been reviewed several times in the past (5-11), most recently by Stanier (11) in 1951. References to the earliest work are given in the reviews of Karström (5) and Dubos (6).

In view of this, a complete literature survey will not be given here. A short historical account of the work carried out on this phenomenon will be presented, with special attention given to those experiments on which the current views of enzymatic adaptation are based. Past work on the specific problems dealt with in this thesis will be given in separate sections in detail, and a thorough discussion will be given of experiments which relate enzymatic adaptation to protein synthesis. It will be seen subsequently that enzymatic adaptation does involve the synthesis of enzymes, and therefore the synthesis of proteins. Studies of the factors involved in enzymatic adaptation, such as those reported here, are also studies of the factors underlying protein synthesis in the living cell.

Definitions of adaptation

In discussing the phenomenon in micro-organisms, the term "adaptation" has been used in the past to denote reversible changes in these organisms, induced by the introduction of new factors into the environment and not involving alterations of the genes. The term has been applied to various phenomena, some intrinsically, other only superficially related. These include:

1. The development of the ability to grow in the presence of toxic substances through subculture in the presence of sub-lethal amounts of these substances.

2. Changes in virulance of micro-organisms upon passage through a host or upon cultivation in artifical media. The training of micro-organisms to overcome the resistance of prospective hosts.
3. Changes in nutritional requirements induced by training of micro-organisms.
4. The development of the ability to metabolize certain substrates through growth in the presence of these substrates. In some cases, the ability to metabolize a substrate may be gained without cell proliferation or in a medium which will not support growth.

In addition to such phenomena in micro-organisms, it has been found that administration of a given substance to living animals (12) or to embryos (13) may bring about the formation of an enzyme system able to attack this substance or may greatly increase such an enzyme system if already present. This phenomenon has been known for a number of years (5) and interest in it has grown recently with the increase in knowledge of adaptation in micro-organisms. Thus far, however, investigators have directed their attention primarily to the nature of the enzyme systems formed rather than to the mechanisms of formation of such enzyme systems.

The experimental studies and theoretical considerations of Hinshelwood (9) have been devoted to the development of resistance by bacteria to toxic substances. It is his belief that this "training" of bacteria to grow in the presence of toxic substances involves the formation of new enzyme patterns in bacteria. His analyses have shown that

it would be difficult to explain all training phenomena as natural selection. For example, bacteria trained to resist a drug, may lose all resistance upon one passage through a drug-free medium. To account for this by natural selection would require the unlikely assumption that resistant bacteria grew less well in the drug-free medium than non-resistant ones. With the type of experiments, often involving many subcultures, which are necessary in studying the development of resistance to drugs, it is, however, impossible to rule out natural selection as a complicating factor. There have been few demonstrations yet of changes in the enzyme patterns of cells which have become resistant to toxic substances, although recently investigators have begun to give attention to this problem. Yall and Green (14) observed, for example, that in the presence of urea Micrococus pyogenes var. aureus produced a urease the action of which was inhibited by furacin, p-chloromercuri-benzoate, and trivalent arsenicals, but that strains of this organism resistant to furacin produced a urease not affected by the above inhibitors. These authors postulated that the urease normally produced is a sulfhydryl enzyme, whereas the activity of that produced by resistant cells does not depend upon the presence of a sulfhydryl group. Such isolated observations are only hints, however, and much additional work will be required to make clear the relation between the training to toxic substances and other types of adaptation.

Studies of the changes of virulence upon passage through a host are extremely difficult to interpret because of the impossibility of

exactly controlled experiments and the complex nature of the phenomena being studied. Workers on this type of "adaptation" have been limited to describing these changes in the most qualitative manner (15), and it is seldom possible to decide to what extent natural selection is involved.

Training in nutritional requirements is usually linked intimately with natural selection. A well-known example is that of Salmonella typhosa, which, as was shown by Fildes et al. (16), may be trained to grow without added tryptophan through serial cultivation in media containing progressively less and less tryptophan. This, as demonstrated later by Fildes and Whitaker (17), involves the selection of mutants which are able to synthesize their own tryptophan. Such training phenomena have been extremely valuable in studies of metabolic pathways in bacteria, but the difficulties and uncertainties involved in determining to what extent these phenomena involve reversible, genetically stable enzymatic change are obvious.

It is to the adaptation of micro-organisms to utilize "new" substrates that most attention has been given in the past and in the study of which the most valuable knowledge has been gained. In this thesis, the term "enzymatic adaptation" will be used, following the suggestion of Stanier (11), to describe substrate-activated biochemical variations in micro-organisms not involving changes in genotype. A few examples should illustrate this phenomenon.

Bakers' yeast (Saccharomyces cerivisiae), grown on a medium not containing galactose or any substance (e.g. lactose) which will give rise to galactose, is unable to ferment galactose when suspended in its presence. If yeast is grown in the presence of galactose, the cells acquire the ability to ferment galactose. With some strains of yeast, the ability to ferment galactose may be acquired through suspension in a medium containing galactose, under conditions such that no growth occurs - e.g., lack of a nitrogen source, high cell density or short time of incubation. If these adapted cells are then grown in a medium containing no galactose, or suspended in a medium without galactose under conditions such as that an active metabolism may take place, the ability to ferment galactose is quickly lost (10).

The tyrosine decarboxylase system is normally present in small amounts in Strep. faecalis. Growth under the appropriate conditions of temperature and hydrogen ion concentration, and in the presence of tyrosine, brings about a thirty-fold increase in tyrosine decarboxylase activity by the cells of this organism (18).

An interesting example of enzymatic adaptation is that observed in the soil organism isolated by Dubos and Avery (19), which could be trained to utilize the Type III capsular polysaccharide of Pneumococcus as a carbon source for growth through being cultured in a medium containing this polysaccharide. Growth in media containing other capsular polysaccharides, e.g., Type II, did not give this organism the power to utilize the Type III polysaccharide. This finding is an example of one of the most striking facts about enzymatic adaptation, its narrow specificity of induction.

Other adaptive enzyme systems will be mentioned in the course of the historical survey which follows and, where relevant, during the description of the experimental work. One case of the development of an apparently new enzymatic ability by a bacterial population through natural selection will be given here to illustrate the distinction between the latter phenomenon and enzymatic adaptation.

The organism, E. coli mutabile, is ordinarily unable to ferment lactose, but upon repeated subculture in media containing lactose, it gains this ability (20). It has been found (21) that in the non-lactose-fermenting populations of E. coli mutabile normally one cell in every 10^5 has the ability to ferment lactose. In a lactose-containing medium, these cells are encouraged to grow, and after several subcultures may dominate the cell population. This culture, containing mainly lactose-fermenting cells, may then be subcultured several times in a lactose-free medium without loss of activity.

In enzymatic adaptation, as opposed to natural selection, the organism gains its maximal adaptive enzymatic activity after one passage through a medium containing the adaptive substrate, and reverts to its pre-adaptive level of activity after one passage through a medium lacking the substrate. Such a test of reversibility is essential in deciding whether any phenomenon involving growth is adaptive. It has been pointed out, however, (10) that it is dangerous to place too

much reliance on such criteria alone in distinguishing between adaptation and natural selection. The easy loss of an enzymatic activity may be due to a back mutation and selective growth of the old type of organism. The instability of a histidineless strain of E. coli (22, 23) is cited as an example of such a back-mutation. Thus, although it would certainly be impossible to explain all examples of enzymatic adaptation during growth as being due to natural selection, natural selection may not be completely excluded as a complicating factor whenever adaptation takes place during growth. For this reason, many workers have studied those examples of adaptation which take place without cell proliferation and in which natural selection may be ruled out. The development of the galactozymase system of yeast, which, as has already been mentioned, may take place without cell proliferation, has been investigated more than any other case of enzyme formation, and from these studies much of our knowledge of the mechanism of adaptive enzyme formation has been gained.

Historical Survey

The biochemical variations of a bacterial species upon cultivation on growth media of different compositions must have been observed many times during the early history of bacteriology. The credit for first realizing that a substrate-induced enzyme formation was involved is due to Wortmann (24) who, in 1882, demonstrated that certain bacteria produced amylase only when grown in a medium containing starch.

In 1899 Duclaux (25) observed the production of extracellular enzymes by the Aspergili. He noted that these organisms produced proteases and saccharase only when grown in the presence of milk and sucrose respectively.

Dienert, (26) in 1900, was the first to show that the formation of galactozymase in yeast was dependent upon the presence of galactose in the growth medium. He realized the importance of the question of whether or not adaptation was accompanied by growth. Some of his experiments were carried out in suspensions of yeast cells of high enough density to prevent cell proliferation. He concluded that in this case adaptation could take place without proliferation of the cells. His conclusion, after various contradictory findings has more recently been fully confirmed by Spiegelman and Lindegren (27).

In 1912, von Euler and Johannson (28) decided after a study of the rates of adaptation by yeast to galactose fermentation that the process of adaptation is dependent upon the building up of nitrogen-containing materials within the cell.

In 1930, Karström (29) published the results of a systematic study of enzyme formation in bacteria. Using the pentose-fermenting lactic acid bacteria, Betacoccus arabinosus and Lactobacillus pentoaceticus, he devised experiments to determine the manner in which the formation of carbohydrate-splitting enzymes by the bacteria was dependent upon the carbohydrate present in the medium in which the

bacteria were grown. Following these experiments, Karström (5) concluded that each strain of bacteria contained two types of enzymes:

1. Constitutive enzymes: Those which the cells of a micro-organism always form, independently of the composition of the culture medium in which the organism is grown.
2. Adaptive enzymes: Those produced by a micro-organism only when required; that is, those whose formation in a micro-organism is dependent upon adaptation to a specific substrate.

Although Karström's definitions served the purpose of bringing a large number of observed phenomena under one system, it is now realized that his classification of constitutive and adaptive enzymes is not a valid one. Further studies have shown that the distinction between the formation of constitutive and of adaptive enzymes is quantitative and by no means so rigid as Karström supposed.

In one of the earliest discussions of this point, Quastel (30), in 1937, advanced the opinion that - "constitutive and adaptive enzymes do not represent two entirely different classes of enzymes. They represent rather the limits of variability of enzymes in a cell - the constitutive having the least range, and the adaptive the greatest range, of variability under different environmental and nutritional conditions".

Gale (7), as well as Spiegelman (31), has pointed out that certain enzymes, formerly thought absent under certain conditions and

termed adaptive, have been found constitutively present through the use of more exact methods of measuring enzymic activity. Thus, the galactozymase of E. coli is termed an adaptive enzyme but cells of this organism have normally a small galactozymase activity ($Q\text{-}CO_2$ of about 20), which is increased to a $Q\text{-}CO_2$ of about 500 when this organism is grown in the presence of galactose (32). The tyrosine decarboxylase of Strep. faecalis has already been mentioned. The activity of this enzyme undergoes a thirty-fold adaptive increase. It does not increase from a zero level.

In addition, there may be wide variability in these enzymes which are called constitutive in the sense which Karström used. Glucozymase of E. coli, termed a constitutive enzyme, does in fact increase 100% if the organism is grown in the presence of glucose (32). There are only a few known enzymes, for example, those of Pseudomonas pyocyanea, involved in the oxidation of putrescine, cadaverine, and agmatine, which fit into Karström's definition of constitutive enzymes (7). Quastel (30) studied the changes in levels of enzyme activity of the urease, catalase, and fumarase in lysed suspensions of Micrococcus lysodeikticus which took place following changes in composition of the medium on which the intact cells were grown. He found that the presence of glucose in the nutritional medium stimulated the formation of urease and suppressed that of catalase. Urea in the nutritional medium did not stimulate the formation of urease, nor did fumarate stimulate fumarase formation. Because of their variability under dif-

ferent growth conditions, these enzymes could not be classed as constitutive under Karström's scheme, and as they did not vary with their specific substrates, they could not be classed as adaptive. Quastel suggested that enzymes should themselves be considered as metabolites, subject to the same laws as other cell metabolites; he stated that one of the means of action of the specific substrate and of other substances inducing enzyme formation might be through combining with the enzyme and preventing its destruction.

The arbitrary nature of Karström's definition is further illustrated if one attempts to apply it to those enzymes whose substrates are always present, even if in small amounts, in normal cell metabolism. Thus, it would be difficult to classify succinic dehydrogenase as constitutive or adaptive, since traces of succinic acid would be present under almost all conditions and it would not be possible to test if succinic dehydrogenase would disappear on growth in the absence of succinic acid.

Gale (7) has proposed a new classification which brings the observed data into a more logical system. According to his scheme, adaptive enzymes are those whose activity undergoes an increase greater than five-fold through growth of the organism in the presence of the specific substrate; constitutive enzymes are those whose increase in activity is less than 100%. He suggested the term "semi-adaptive" for those enzymes whose activity undergoes a two-to-five fold increase. Such a classification is in accord with the view that the

formation of adaptive and constitutive enzymes differs only in degree. Gale's classification will be employed in the following pages.

Interactions between enzyme-forming systems

The most convincing evidence that the concept of sharply differentiated constitutive and adaptive enzymes is not valid has come through studies of the interactions of enzyme-forming systems. An early observation of this type of interaction was that reported by Katz (33) in 1898, though at the time the full implications of his observation could not, of course, be realized. In following the production of amylase by Penicillium, it was noted that a certain amount of amylase was produced in the absence of any carbohydrate, but that the amount produced was increased in the presence of starch, greatly decreased in the presence of sucrose, and slightly decreased in the presence of lactose and maltose.

The phenomenon of "diauxie", observed by Monod (8) may be due to interactions between enzyme-forming systems. Monod carried out experiments on bacterial growth in media in which the only source of carbon for growth was a mixture of two carbohydrates, one attacked by constitutive, the other by adaptive enzymes. It was observed that bacterial growth took place in two cycles, separated by a pronounced lag period. In each cycle of growth, only one carbohydrate was being utilized. This was explained as due to the inhibitory action of one compound, that attacked by a constitutive enzyme, upon the formation

of the adaptive enzyme attacking the other compound. Only after the first carbohydrate had been exhausted were the enzymes for attacking the second formed, with a consequent lag period after growth at the expense of the first carbohydrate.

In the work of Spiegelman and Dunn (34) on adaptations in yeast are to be found the most clear-cut examples of interaction between enzyme-forming systems. In short-term experiments, in which no cell proliferation took place, the adaptation of yeast to maltose and galactose fermentation was followed. It was observed that if both carbohydrates were present, galactose inhibited the adaptation to maltose fermentation, though the presence of maltose had little effect on the adaptation to galactose fermentation. A yet more striking example of such interaction was given by the effect of adaptation to galactose fermentation on the glucose fermenting system of yeast. The "glucozymase" system in many micro-organisms has long been considered as an established constitutive enzyme system. In the presence of galactose, however, while the galactozymase system was being formed, the ability of the yeast to ferment glucose fell to approximately half its normal value.

Spiegelman and Dunn considered that these interactions were due to competition between the various enzyme-forming systems for endogenous protein or enzyme precursors which could be transformed into enzymes. In the strain of yeast they used, adaptation to maltose

and galactose fermentation could take place in washed cell suspensions without any added source of nitrogen. It was observed (34), however, that the addition of an exogenous nitrogen source, ammonium sulfate, greatly stimulated adaptation, shortened the lag before the appearance of enzymatic activity and increased the amount of enzyme formed. It was thought, then, that if the interactions observed in the absence of an added source of nitrogen were indeed due to competition for nitrogenous enzyme precursors, the addition of a source of nitrogen, which could be used by the yeast to manufacture its proteins, might modify the results of such a competition. This was found to be the case. In the presence of ammonium sulfate, the inhibition of the formation of maltozymase by galactozymase formation was much smaller than in the absence of ammonium sulfate. Also, in the presence of ammonium sulfate, no decrease of glucozymase activity was seen to accompany the formation of galactozymase.

Except for the experiments of Monod (8), mentioned above, little or no work has been carried out on interactions between enzyme-forming systems in bacteria. Spiegelman (2) has pointed out that the interaction of enzyme-forming systems in bacteria amounts almost to a mutual exclusion effect, as in Monod's experiments; in these experiments, even in the presence of an exogenous nitrogen supply, the formation of the enzymes attacking one carbohydrate was completely prevented by the presence of the other carbohydrate. Because of the severity of this exclusion, such interactions in bacteria are more difficult to

study than in yeast. It may be expected, however, that in the future many of the known phenomena of bacterial enzymatic variation will be explained on the basis of a competition for enzyme precursors. Such an explanation has been proposed for the inhibitory effect of nitrate on formic hydrogenlyase formation by E. coli (35), and will be discussed in more detail later. The well known inhibition by glucose of the formation of many enzymes concerned with bacterial amino acid metabolism may be partly due to a competition by glucose and the products of its metabolism for enzyme precursors (7).

The experiments on interactions between enzyme-forming systems support the view that in the cell each enzyme is itself a metabolite whose existence depends upon the relative ease with which the systems forming various enzymes compete for the enzyme precursors. Further support is given this view by the experiments carried out by Spiegelman and his collaborators on the loss of adaptive enzymatic activity by yeast cells in the absence of the adaptive substrate. Some stabilizing action by the substrate on the adaptive enzyme has been proposed (30, 36) to explain the mechanism of action of the substrate. Spiegelman argued that if the loss of enzymatic activity was due to an instability of the enzyme under competitive interaction, the loss of activity might be prevented by conditions that would prevent interaction between enzyme-forming systems; i.e., under those conditions that would prevent adaptive enzyme formation. It was found (37), in agreement with this idea, that the loss of galactozymase activity by

adapted yeast cells did not occur anaerobically. There is no appreciable utilization of endogenous substrates anaerobically by the yeast cell and no energy is liberated. It has been demonstrated (38) that a source of energy is required for adaptation to galactose or maltose fermentation. Aerobically, or anaerobically in the presence of a fermentable substrate other than galactose, galactozymase activity is quickly lost. Sodium azide prevents adaptation (37, 39) as well as assimilatory processes (40, 41), and the incorporation of amino acids into bacterial protein (42). This substance also has the ability to prevent the loss of galactozymase activity in the absence of galactose. Arsenate and 2-4 dinitrophenol, both inhibitors of adaptation (39, 43, 44), are able to prevent the loss of adaptive enzymatic activity in the absence of the substrate. The stabilizing action of these inhibitors of adaptation may be attributed to their ability to stop energy transfers and thus to inhibit the interactions between various enzyme-forming systems.

In some cases, the loss of activity may be due to a simple dilution of adapted cells during growth. This was reported for the nitratase system of E. coli by Wainwright and Pollock (45). It is probable that in many instances both interactions between enzyme-forming systems and dilution of adapted cells operate simultaneously.

The relation of enzymatic adaptation to the general physiological state of the cell

In general, enzymatic adaptation is closely dependent on the physiological state of the cell. Adaptation has never been observed

other than in the intact cell. There has been one report, by Abderhalden (46) in 1926, of adaptation by dried yeast cells, but this work has never been comfirmed, and Abderhalden's finding is probably due to the adaptation of a few viable cells which remained in his dried cell preparation.

Hegarty (47) observed the adaptation to several sugars by Streptococcus lactis, and was able to show that cells taken at the end of the lag period, just before the logarithmic period of growth was reached, had the highest adaptability, as measured by both the shortness of the lag period for adaptation and the ability to adapt to the substrate without proliferation. During the logarithmic period, the adaptability decreased rapidly. The period of the greatest adaptability was also the period of the greatest rate of metabolism per cell.

DeLey and Vandamme (48), however, found that with the semi-adaptive saccharase system in yeast the "adaptability" (method of measurement not stated) was least in young cultures and reached its highest value after the logarithmic phase, during the stationary phase of growth.

The action of cell poisons on adaptive processes reveals the comparative ease with which these processes may be blocked. With many inhibitors, adaptive enzyme formation may be completely inhibited by concentrations which have no action on the adaptive enzyme system once it is formed (11, 10, 39).

Experiments with different inhibitors carried out by Sussman (49) showed that enzymatic adaptation could be blocked selectively without interfering greatly with carbon and nitrogen assimilation. According to Stanier (11) ultraviolet irradiation of an intensity that had no effect on assimilation inhibited adaptation; however, the organism and adaptive system used in this experiment were not stated. Knox (50) has shown that in coliform bacteria, the production of tetrathionase was completely inhibited at a temperature of 41° C., while viability of the cells was not affected.

The relation of enzymatic adaptation to cell proliferation and cell viability

The sensitivity of the adaptive process is further illustrated by the failure of those early experiments in which attempts were made to differentiate between adaptation and cell proliferation by carrying out adaptation while inhibiting growth. Thus, attempts to inhibit growth by heat (51), or by phenols (52, 53) gave complete inhibition of adaptation as well as of growth. Yeast cells killed by alcohols or chloroform could not adapt to galactose fermentation (54) and it is to be expected that a great number of cell poisons that impair viability also inhibit adaptation.

Later studies have shown, however, that in some cases enzymatic adaptation can take place in the absence of active cell division. This fact was established finally for the galactozymase system of yeast by Spiegelman and Lindegren (27), who showed that various earlier conflicting

reports had been due to the failure of the investigators concerned to use pure strains of yeast. Upon survey of a number of different pure strains, it was found that the ability to adapt without growth depended upon the genetic stability of the yeast. Thus, stable diploid cultures could adapt without growth, while unstable haploid cultures could "adapt" only through mutation followed by growth, i.e., through natural selection.

Several other cases of adaptive enzyme formation in the absence of growth are known. These include the maltozymase system of yeast (31), the tetrathionase of B. Parathphosum B. (55), or of the Coliform organism #1433 (56), the formic hydrogenlyase of E. coli (57) and the system in this organism which oxidizes sorbitol (58), as well as others.

Adaptation without cell proliferation can best be demonstrated when no nitrogen source is required for adaptation. This may occur in yeasts, and , less frequently, in bacteria. The adaptation of Pseudomonas species to the oxidation of various aromatic substrates may take place without sources of nitrogen (59). One or two cases of adaptation in the absence of a nitrogen source in E. coli have been reported (44, 60), though most strains of this organism require a source of nitrogen for adaptation. In those instances in which a source of nitrogen is necessary for adaptation, the adaptation may take place in so short a time that no cell proliferation can occur. It has been pointed out by Dubos (16), however, that though enzymatic adaptation may occur without an increase in cell numbers, this does not mean that it occurs without an

increase in cell substance. Most cells, when transferred to a new medium show some enlargement, even without actual division. Their metabolism increases, and new protein is being synthesized.

Up to a few years ago, it was justifiable to believe that only those cells which had the ability to divide could adapt, whether or not division actually took place during adaptation. Recently, however, evidence has appeared which indicates that a distinction may be drawn between the viability of a cell and its ability to adapt. Stephenson and Yudkin (54) measured the total number and the number of viable cells in a population of yeast cells adapting to galactose fermentation, and the effects of ultraviolet irradiation on adaptability and viability. They concluded that it was probably not the viable cells alone which were capable of adaptation. Spiegelman et al. (61) in 1951, observed the effects of X-rays on yeast cells and found it possible to decrease the viability by 99.9% without decreasing the adaptability to galactose or maltose. It was also found that treatment with ultraviolet irradiation and nitrogen mustards caused a greater loss of viability than of adaptability. Billen and Lichstein (62) found that suspensions of E. coli treated with X-rays could synthesize formic hydrogenlyase at a rate greater than was to be expected from the number of viable cells present. Even considering these results, the relationship between the ability of the cell to reproduce and its ability to form adaptive enzymes remains a close one. The nature of this relationship, indeed, is one of the most interesting problems in the study of enzymatic adaptation.

Direct evidence that enzymatic adaptation involves the synthesis of enzymes

More and more direct evidence has been accumulated to show that the increase in enzymic activity observed in adaptation is due to the formation of enzymatic material. The point is not immediately obvious. In many cases of adaptation what is actually observed is a time lag before the appearance of enzymatic activity or before the attainment of maximal activity. This may well be explainable otherwise than through postulating enzyme synthesis. Thus, the lag may be caused:

1. By limited permeability of the cell wall, so that a certain time is required for building up large enough concentrations of the substrate for maximal enzymatic activity.
2. By the time necessary for the formation or the increase of various intermediates and co-factors within the cell.

Fortunately, both these possibilities have been cleared away in those adaptive enzyme systems to which the most study has been given, but they should always be kept in mind in studying any new adaptive enzyme system.

In some cases, it can easily be shown that the adaptive substrate enters the cell immediately. Galactozymase formation is the formation of the enzymes necessary for the fermentation of galactose, but it has been demonstrated that yeast cells, not previously in contact with galactose may possess the ability to oxidize this substrate

without any time lag (63, 64). The formic hydrogenlyase system of E. coli, which splits formic acid into hydrogen and carbon dioxide, is strictly adaptive, but this organism oxidizes formic acid at a steady rate without adaptation.

Evidence against limited permeability being the cause of adaptive phenomena has also been obtained through demonstrations of the activity of the adaptive enzyme in cell-free extracts of adapted cells or in adapted cells whose membranes have been made freely permeable by drying or by treatment with such agents as toluene or acetone. Examples of such evidence will be considered shortly. If a toluene-treated preparation of cells grown in the presence of an adaptive substrate may attack this substrate without a lag, while a similar preparation of cells grown in the absence of the adaptive substrate do not attack this substrate at all, it may be taken as clear evidence that the lag before utilization of the substrate by the unadapted intact cell is not due to permeability factors.

The possibility that the cell wall prevents the substrate from reaching the enzyme may also be avoided when organisms are used in which the enzyme is produced extracellularly. The early studies on adaptation with various molds have already been mentioned. Spiegelman (10) has pointed out the advantage offered by such organisms for the study of enzyme synthesis and suggested that their future use might prove to be of value. The appearance of the

enzymes outside of the cell involves, however, not only the active synthesis but also the active secretion of enzymes; and the occurrence of the latter process might complicate the study of the former.

Exclusion of the second possibility, that of the building up of co-factors and intermediates rather than the formation of new enzymes may be best done through experiments with cell-free extracts in which separation is made between the enzymatic and the non-enzymatic portions of the extract. The first such attempt was made by von Euler and Jansson (53), working with the yeast galactozymase system, in 1927. They concluded that the adaptation observed was apo-enzymatic in nature, but the activities obtained in their preparations were so low as to cast doubts upon their conclusions. Spiegelman and his collaborators (65), in 1947, with similar experiments, confirmed the conclusions of von Euler and Jansson. It was found that though co-factors were necessary for the fermentation of galactose, these were to be found in the boiled extracts of unadapted as well as adapted yeast cells, whereas the apo-enzymatic portion of the system which fermented galactose was found only in adapted cells.

Besides this example, there exist a number of other experiments which show that adaptation does involve the synthesis of an enzyme not present in unadapted cells. Few of the adaptive enzymes have been even partially purified, but few bacterial enzymes of any

kind have been purified to more than a small degree. It is of interest, therefore, to list some of those cases where the activity of the adapted enzyme has been demonstrated in extracts, dried cells, toluene-treated cells, etc., as well as those where the enzyme has been to some extent isolated from other enzymes and from co-factors.

In Karström's (5, 29) experiments with bacterial adaptation to the utilization of various carbohydrates, he was able to confirm the enzymatic activity of the intact cell by demonstrations of the same activities in toluene-treated organisms. Doudoroff et al. (66) demonstrated the enzyme, sucrose phosphorylase, in dry cell preparation of Pseudomonas saccharophila grown on sucrose.

Spiegelman's work on the purification of the galactozymase system has already been mentioned. Recently (67), he and his collaborators extracted, separated, and partly purified by ammonium sulfate fractionation, two enzymes from yeast cells grown in the presence of maltose. Each of these enzymes attacked the α-glucoside linkage, though one would attack maltose and α-phenyl-glucoside, and the other only α-methyl-glucoside. The enzymes, hexokinase and sucrose phosphorylase, have been demonstrated in dry cell preparations of adapted Pseudomonas putrefaciens (68, 69), and amylomaltase in dry cell preparations of adapted E. coli (70, 71, 72).

Stanier and his collaborators found a correlation between the activity of cells of Pseudomonas flourescens grown on various aromatic

substrates and the activity toward the same substrates of these cell dried (73, 74, 75). A soluble enzyme was extracted from creatine-grown cells of Pseudomonas eisenbergii (76) which oxidized creatine to sarcosine. Cohn and Monod (77) have described the extraction and partial purification of the enzyme, β-galactosidase, from cells of E. coli mutabile adapted to the hydrolysis of lactose. Quite recently, the enzyme system, formic hydrogenlyase, was found in dried, adapted cells of E. coli (78) and extracted from fresh adapted cells (79). Similarly, a toluene-water extract of yeast cells grown on α-methyl glucoside was found capable of splitting this glucoside (80).

This evidence indicates that in enzymatic adaptation enzyme synthesis is truly involved. Ideally, the amount of enzyme formed should be measured in extracts of cells rather than in intact cells. In many cases, the amount of enzyme actually present can be measured only in extracts, as destruction of the cell wall may reveal a much greater enzymatic activity than is found in the intact cell (81). Because of the experimental difficulties involved, however, few measurements of adaptive enzyme formation in extracts have been made and most of the information we possess on enzymatic adaptation has been derived from experiments on intact cells.

The mechanism of enzyme synthesis in enzymatic adaptation:

Genetic aspects

The genetic basis of enzymatic adaptation has been worked out most carefully for yeasts by the Lindegrens, Spiegelman, and their col-

laborators (Reviewed in 1). In the life cycle of yeasts, asci which contain spores bearing the haploid number of chromosomes may be formed. By micro-dissection of the asci, separation and separate cultivation of the spores as haploid strains, and recombination of the strains, the above workers were able to elucidate the relation of the genes to enzyme formation. It was found for several adaptive enzymes that not the enzyme itself but the ability to produce the enzyme in response to a specific substrate was inherited. Heredity was determined in some cases by a pair of factors, of which the ability to adapt was the dominant. Thus, these species of yeast were characterized biochemically, not by a specific enzymatic constitution, but rather by the potentiality to form a defined range of enzymes under different growth conditions. Owing to the limited state of knowledge of bacterial genetics, the hereditary basis of enzymatic adaptation in bacteria has not been made as clear as in yeasts. It has been shown in a few cases, however, that bacterial mutations may involve the acquisition of the ability to produce a new adaptive enzyme. This has been demonstrated by Monod and Audureau (83) for the mutabile mutation in E. coli. Klein and Doudoroff (69) showed that such a phenomenon was involved in the mutation of Pseudomonas putrefaciens to utilize glucose. Lederberg (84, 85) has investigated the genetic basis of adaptation to metabolize sugars by E. coli, through studies of genetic recombination in this organism. He has shown that, as in yeasts, the power to adapt to the fermentation of a sugar is genetically dominant.

The role of the substrate

Some experimental work and more speculation have been devoted to understanding the part played by the substrate in inducing adaptive enzyme formation. More than one theory has been put forward. The relationship on which most of Hinshelwood's (9) considerations have been based is the autosynthetic equation,

catalyst + substrate ⟶ more catalyst + products,

Though this equation holds for the building of crystals, for such reactions as the dissociation of calcium carbonate at a calcium oxide interface and for the growth of entire cells in a culture, it is difficult to see how it applies to individual enzymes. It does not appear likely for example that the products of the decomposition of hydrogen peroxide or of formic acid could be used to build up either catalase or formic dehydrogenase (8).

It was the opinion of Dubos (6), following experiments with the organism which hydrolyses the capsular polysaccharide of Type III Pneumococcus, that the formation of the enzyme depends on the utilization of the adaptive substrate; Speigelman et al. (86) showed, however, that maltozymase could be formed in yeast under conditions in which maltose was not utilized. The experiments of Monod et al. (87) to be discussed, also showed that adaptation could take place without utilization of the adaptive substrate.

As the methods for detecting very small amounts of enzymatic activity improved, it became apparent that in many cases adaptation did not involve the appearance of a new enzymatic activity but the increased activity of some enzyme already present in small amounts. Yudkin (36), as well as Quastel (30) postulated that the substrate acted through combining with the enzyme and stabilizing it. If the enzyme in the absence of its substrate were continually being built up and broken down, the stabilizing influence of the substrate would permit the amount of enzyme to increase.

Spiegelman (31), examining this hypothetical mechanism, derived the type of time-activity curve which would be expected from it. He found that this scheme led to a formula which was not experimentally verified. Spiegelman then advanced a hypothesis that the enzyme was stabilized by the substrate and had, in addition, the ability to duplicate itself. From this postulate, a time-activity relationship was derived which was found to hold experimentally for several adaptive systems, the galactozymase, maltozymase, and mellibiozymase systems of yeast and the formic hydrogenlyase system of E. coli. In all these systems, enzymatic adaptation takes place without growth.

An interesting phenomenon, which may be considered an example of adaptation, was observed by Ephrussi and Slonimisky (88). They noted that if bakers' yeast were grown anaerobically, it lost the ability to oxidize glucose, and certain changes took place in its cytochrome system. Synthesis of the normal aerobic cytochrome components, including cyto-

chrome oxidase, could be demonstrated in the presence of oxygen, without cell proliferation. In this case, the response of an enzyme, cytochrome oxidase, to its specific substrate, oxygen, was involved. Of several other hydrogen acceptors tried, none could replace oxygen in inducing the formation of the aerobic cytochrome system. A study of the time-activity curve of this phenomenon revealed a kinetic situation which followed the scheme of Yudkin and Quastel rather than that of Spiegelman.

A type of analysis other than the purely kinetic has been used by Monod and his collaborators (87), in examining the role of the substrate in adaptation. A number of different substances were tested for: (1) their ability to induce the formation of β -galactosidase of E. coli, (2) their ability to be hydrolysed by β -galactosidase, and (3) their affinity for β -galactosidase as measured by their power as competitive inhibitors of its action. It was found that substances having the inductive power were not necessarily substrates of the enzyme, (e.g., melibiose), and the the inductivity of a substance was in general independent of its affinity for the enzyme. In this case, then, it was unlikely that the inductor was acting through the stabilization of an enzyme precursor.

The view of Monod et al. was that the inductor of enzyme formation directs enzyme synthesis through combination with some enzyme precursor, or with the enzyme itself. The experiments on β -galactosidase showed it to be very unlikely that the inductor of adaptation combined

in this case with the enzyme itself, but gave no indication of the exact site of action of the inductor.

Finally, there are several known examples of enzyme formation in the absence of the enzyme's substrate. A continual increase in the total amount of galactozymase in adapted yeast culture growing rapidly in the absence of galactose was observed by Spiegelman and Reiner (89). Pollock (90) found that if cells of Bacillus cereus were exposed to a low concentration of penicillin, washed, and placed in a buffered glucose medium, penicillinase production would take place at a much greater rate than in cells unexposed to penicillin. Wainwright (91) found that cells of E. coli could show considerable nitratase activity if grown in a medium rich in amino acids, even if nitrate were absent.

From these examples, it may be seen that the function of the substrates in inducing adaptive enzyme formation is still far from being understood and remains one of the most important unsolved problems in this field.

Nitrogen sources for enzymatic adaptation - The nature of the protein precursor of the adaptive enzyme

"Enzyme synthesis", in the sense in which it has been discussed, has not been taken to mean the building up of the enzyme from individual amino acids, but rather the transformation of a protein precursor into an active enzyme, or, as experiments on inter-

action between enzyme-forming systems have shown, the transformation of one active enzyme into another. The chemical nature of these transformations is, of course, of the greatest interest, but at present may only be guessed at.

Experiments carried out recently have indicated that the changes taking place in protein precursors in adaptive enzyme formation may not be drastic and may sometimes involve the rearrangement of certain groups within the molecule. The experiments of Ephrussi and Slonimsky (88) on adaptive changes in the cytochrome system of yeast are illustrations of this point. Here, the transformation of cytochromes a_1 and b_1 to cytochromes a, b, and c may come about through the shifting of heme groups from one large molecule to another, with a consequent change in spectra and properties. It is true that the cytochromes are electron carriers rather than enzymes, but the process envisaged here would bear a close resemblance to enzyme transformation.

Extremely interesting observations on the protein changes in adaptive enzyme formation have been made by Cohn and Monod (93) and Cohn and Torriani (94). An anti-galactosidase serum was prepared by injecting rabbits with the purified enzyme, β -galactosidase, taken from lactose-grown E. coli mutabile. This anti-serum precipitated completely the β -galactosidase from E. coli mutabile as well as from several other micro-organisms. The precipitated enzyme still

retained all its activity. Unadapted cells contained no β-galactosidase activity, but extracts of unadapted cells contained an antigen which precipitated with the β-galactosidase anti-serum. Studies were carried out in which the adaptive (active) and the non-adaptive (inactive) antigens were measured simultaneously. It was found that only the inactive antigen was present in non-adapted cells, that both the active and inactive antigens were present in the adapted cells, and that in the adapted cells the amount of inactive antigen had decreased. It was concluded that in this case adaptation involved the formation of an active enzyme from a chemically similar but inactive precursor. Considering the difficulty of purifying enzymes, however, the proof of chemical similarity of the precursor to the active enzyme seems subject to considerable doubt.

In recent years, a good deal of attention has been paid to the sources of nitrogen utilized in adaptation and in other enzyme and protein syntheses. It has long been known that in many cases a source of nitrogen is essential for adaptation, and that even when not absolutely necessary, as in galactozymase formation in yeast, an exogenous supply of nitrogen stimulates adaptation (34). Karström (5) found that in the formation of the enzymes fermenting xylose by A. aerogenes an exogenous source of nitrogen, either as ammonium sulfate or as yeast extract, was necessary. Stephenson and Stickland (57) found that in non-proliferating suspensions of E. coli formic hydrogenlyase formation took place only if a tryptic broth were added to the suspension. The problem of the nitrogen supply necessary for formic hydrogenlyase production will be dealt with later at greater length.

Rosenberg (58) found that cells of E. coli adapted to the oxidation of sorbitol only in the presence of certain amino acids which could be metabolized, such as alanine, aspartic, or glutamic acids. Serine, which could be metabolized, did not stimulate adaptation. Nitratase formation by a coliform bacteria was stimulated by a casein hydrolysate or by an amino-acid mixture. The formation of nitratase, even in the absence of the adaptive substrate, nitrate, could take place in this organism in the presence of a mixture of amino acids or of a casein hydrolysate (91).

DeLey and Vandamme (48) observed the effects of various sources of nitrogen upon the semi-adaptive formation of saccharase in brewers' yeast. This enzyme was formed to a certain extent in the absence of any external source of nitrogen. It was found that if ammonium sulfate were supplied as the source of nitrogen, its disappearance could be demonstrated during the formation of the enzyme. Aspartic acid and alanine stimulated adaptation only as much as did ammonium sulfate. Glutamic acid had no stimulating action, and asparagine stimulated the adaption much more strongly than did ammonium sulfate. It is of interest to note here that Melchior, et al. (42) found that asparagine stimulated protein formation in E. coli, as measured by the incorporation of labeled amino acids in the cell protein.

Friedman (95) found that the marine organism, Achromobacter fischeri, could adapt to the utilization of fructose as a carbon source

for growth, only if any one of a large number of amino acids were added to the medium. Especially effective were histidine, glutamic acid, and arginine, which could initiate growth in concentrations as low as 0.01 mg. per 10 ml. of medium.

DeLey and Vandamme (48) assumed that the stimulation of adaptation by the various sources of nitrogen was due to the latter's incorporation into the new enzyme. While studies on the effect of nitrogen sources on enzymatic adaptation will be of the greatest interest if this assumption is true, this important point will only be decided after extensive experiments in which the effect of nitrogenous compounds on adaptation, the assimilation of these nitrogenous compounds, and the degree of their incorporation into the adaptive enzyme are all measured.

The experiments of Hokin (96) on the formation of amylase by pancreas slices should be noted here. He has found that the addition of amino acids stimulated the production of this enzyme, but the amounts of amino acid nitrogen disappearing were far too small to account for the amount of amylase formed in terms of total synthesis from amino acids. It would not appear justified, then, to ascribe the stimulatory action of nitrogen sources on adaptation solely to an incorporation of these sources into the adaptive enzyme without further experimental evidence.

Although detailed studied of the sources of nitrogen involved in adaptation are just beginning, it is probable that such studies will

yield much valuable information for the understanding of the adaptive process. The major part of the work to be reported here was devoted to studying in detail the nitrogen sources essential for the synthesis of formic hydrogenlyase by resting cell suspensions of E. coli. These studies led to an investigation of the energy sources required for formic hydrogenlyase synthesis. Earlier work dealt with the formation of the galactozymase and maltozymase systems of yeast. The experiments carried out on yeast were almost purely descriptive. Some of the nitrogen sources involved in galactozymase formation were explored. In addition, the actions of various inhibitors of maltozymase formation were studied. The work on yeast will be presented first, and following this, a more detailed consideration given to the experiments carried out on formic hydrogenlyase formation.

CHAPTER II

THE GALACTOZYMASE SYSTEM

A. INTRODUCTION

The adaptive formation of the galactozymase system by yeast has already been considered in detail in the General Introduction. No further description of this process, therefore, need be given here. In recent years, the manner in which galactose is fermented by adapted yeast has been studied (97, 98). This fermentation apparently takes place in the following steps:

1. Galactose + ATP $\xrightarrow[Mg^{++} + \text{cysteine}]{\text{galactokinase}}$ galactose-1-phosphate

2. Galactose-1-phosphate $\xrightarrow[\text{uridine-disphosphate-glucose}]{\text{galactowaldenase}}$ glucose-1-phosphate

3. Glucose-1-phosphate fermented by the glycolytic system.

Galactozymase formation, then, involves the formation of at least two apo-enzymes. As the experiments of Spiegelman et al have shown (65), the co-enzyme fraction of the complex is present in the unadapted as well as in the adapted yeast cell.

Experiments with a number of different strains of yeast have shown that the ability to adapt to galactose fermentation may vary

widely from strain to strain (27,31). No galactozymase activity was present originally in the two brands of commercial yeasts which were readily available -- Lallemand's Yeast and Fleischmann's Yeast. Adaptation to galactose fermentation took place slowly; maximum activity was reached only after two days. Means were devised for incubating the yeast with the adaptive substrate during this period of time.

B. EXPERIMENTAL METHODS

Preparation of a standard curve for the measurement of yeast concentrations

It was necessary for these and later experiments to measure the amount of yeast present in suspensions. A standard curve for turbidimetric measurements was constructed in the following manner: A 2% (wet weight) suspension of Fleischmann's Yeast in 0.067 M (M/15) NaH_2PO_4 was diluted with 0.067 M NaH_2PO_4 to give a series of suspensions ranging from 0.04% to 1.0%. The optical densities of these were read on a Fisher Photoelectrometer at a wave-length of 525 mμ. Three 5.0 ml. portions of the 2% suspension were centrifuged, each washed three times with distilled water, and dried to constant weight at 105-110°C. to determine the dry weight of yeast per ml. for each optical density. The standard curve thus obtained is shown in Figure I. A similar curve set up at a different time for Lallemand's yeast did not differ significantly from this one.

Methods of inducing galactozymase formation in yeast.

Fleischmann's bakers' yeast was used in all experiments on galactozymase formation, after preliminary studies had shown this brand to be to a small extent more readily adaptable than Lallemand's yeast.

Yeast cakes were stored in the refrigerator and used within two days of the date received. 1.0 gm. (wet weight) of the yeast cake was washed two or three times with 40 ml. of sterile 0.067M NaH_2PO_4 and suspended in 100 ml. of 0.067M NaH_2PO_4 containing 4.0 gm. of commercial galactose and other substances, to be described.

All incubations were carried out in a room used for soil perfusion experiments and kept constantly at a temperature of 24°C. Two methods were used for aerobic incubation of yeast suspensions. At first, a number of experiments were performed in which the yeast suspension was aerated by being circulated through the soil perfusion apparatus of Audus (99), the soil, of course, being omitted from its usual place in the apparatus. Unless the circulation were sufficiently rapid, the yeast cells settled in the bottom of the U-tube of the apparatus, and in any case, some cells settled within the lower stopper of the cylinder.

In the succeeding experiments of this series a simpler means of incubation was used. The yeast suspension was placed within a gas-washing bottle and kept stirred and aerated by having bubbles of air drawn through it.

While incubating the suspension in one of these ways, samples were taken at various intervals of time, the yeast cells centrifuged and washed twice with 0.067M NaH_2PO_4. The size of the samples taken varied from 2.0 to 5.0 ml., depending upon the activity thought present

in the yeast cells. After washing, the cells were suspended in 2.0 ml. 0.067 M NaH_2PO_4, a part of this suspension was diluted suitably for optical density readings and another part used for the measurement of galactozymase activity.

Sterile precautions

All solutions and apparatus used in these experiments were sterilized by autoclaving; samples were withdrawn using sterilized pipettes. The packaged yeast which was used, however, was almost certainly contaminated to some extent by bacteria or spores. During several of the experiments, samples of the yeast suspension were fixed and stained with methylene blue for microscopic examination. No bacteria were seen in any such preparations. No odor of bacterial putrefaction was noted in any of the experiments.

Measurement of galactozymase activity

0.5 Ml. of the yeast suspension to be tested was pipetted into each of two vessels, one of which contained 0.5 ml. of 20%* purified galactose in the side arm. 0.067 M NaH_2PO_4 was added to the main vessel to a final volume of 3.0 ml. In the center well were place a few drops of water and a small piece of yellow phosphorus to remove all oxygen from the gas in the vessel. The vessels were gassed with 93% N_2-7% CO_2 for 15 minutes and equilibrated until a constant manometric reading showed that all contaminating oxygen had been absorbed. The gassing and subsequent operations were carried out in a bath at 27°C. The

* Per cent concentrations indicate gms. of solute per 100 ml. of solution.

substrate was tipped in, and the evolution of carbon dioxide followed for sixty minutes. In the absence of a substrate, no significant evolution of carbon dioxide was ever observed. Galactozymase activity is expressed as Q-CO_2, the μl. CO_2 evolved from galactose, per hour, per mg. dry weight of yeast.

Reagents: Purification of commercial galactose

The galactose used (Brickman's) was, like other commercial preparations of galactose, contaminated with small amounts of glucose. Unadapted yeast cells evolved an amount of carbon dioxide from this galactose indicating a 4% content of a fermentable carbohydrate, presumably glucose. The purification of galactose was carried out by a modification of the method of Stephenson and Yudkin (54):

100 gm. of bakers' yeast were washed in 800 ml. of distilled water and centrifuged. This process was performed four times. The washed yeast was then suspended in 100 ml. of 20% galactose, made up to 230 ml., left for 10 minutes and centrifuged. Each 100 ml. of the supernatant fluid was treated with 50 gm. of fresh yeast, washed as before, and the process exactly repeated. The final supernatant fluid was heated to boiling and filtered. The filtrate was concentrated down in vacuo, the final syrup taken up in hot 70% ethanol and recrystallized twice from 70% ethanol.

No carbon dioxide was evolved from this purified galactose by unadapted yeast. Because large quantities of galactose were required

as adaptive substrates in the incubation experiments, and because any glucose present would be quickly destroyed in such experiments, unpurified galactose was used for the incubations, and purified galactose only for the measurement of galactozymase activity.

Other reagents

Sodium-l-glutamate and l(+) cysteine hydrochloride were used in these experiments. These, as well as the other reagents used, were dissolved in 0.067 M NaH_2PO_4 and the pH of the resulting solution adjusted to 4.5, unless otherwise stated.

C. EXPERIMENTAL RESULTS

The effects of ammonium phosphate, sodium glutamate, and uridine on galactozymase formation

The results of several preliminary and exploratory experiments are recorded in Table I. These experiments were carried out both in the soil perfusion apparatus and in gas-washing bottles. The effects of adding two different sources of nitrogen, ammonium phosphate and sodium glutamate, are shown. The action of uridine on galactozymase formation was tested because of the importance of uridine-diphosphate-glucose as a co-factor in galactose fermentation.

The following general conclusions may be drawn from these experiments:

1. Ammonium ion exhibited, as shown earlier (34), a definite stimulating action on galactozymase formation in yeast.
2. Sodium glutamate caused a stronger stimulation of adaptation than ammonium ion, in the concentrations used.
3. Uridine had no effect on adaptation.
4. No marked difference was noted when the buffer used was changed from NaH_2PO_4 to KH_2PO_4.

TABLE I

The effects of ammonia, glutamate, and uridine on growth and galactozymase formation in yeast.

Experiment	Time in Hours	Galactozymase activity: ($Q-CO_2$) Incubation medium: Galactose + ---	+ NH_4^+	+ glutamate	+ NH_4^+ + glutamate	+ NH_4^+ + uridine	Degree of growth: mg. dry wt./ml. / initial mg. dry wt./ml. Incubation medium: Galactose + ---	+ NH_4^+	+ glutamate	+ NH_4^+ + glutamate	+ NH_4^+ + uridine
1.	1/2	0	0				1.00	1.00			
	25	7.3	13.7				1.62	1.41			
	49	3.0	9.7				2.03	1.88			
	100	1.5	7.4				2.50	2.34			
2.	1 1/2	0	0				1.00	1.00			
	44	4.0	11.9				0.99	1.50			
	68	4.3	12.3				0.94	1.92			
	90	3.6	2.7				0.85	1.99			
	140	2.0	1.9				0.87	2.08			
3.	2/3			0		-			1.00		1.00
	17 1/2			11.3		9.6			0.97		1.15
	42			25.6		12.7			1.47		1.27
	93			3.7		0.8			2.21		-
4.	20	3.4		5.5			1.27		1.03		
	65	4.9		14.8			1.76		1.81		

Means of incubation: Experiments 1-3, soil-perfusion apparatus. Experiment 4, gas-washing bottle. Other conditions and methods of measurement as given in the text.

Concentrations: 1% (wet weight) yeast, 4% galactose, 0.1% $NH_4H_2PO_4$, 0.01% uridine, 0.17% sodium glutamate. All solutions and suspensions in 0.067 M NaH_2PO_4 in Experiments 1-3. Experiment 4 carried out in 0.067 M KH_2PO_4.

Experiment 3 was carried out with the same lot of yeast used in Experiment 2, after two days' additional storage.

Considering the errors inherent in measuring growth in the soil-perfusion apparatus, it is difficult to draw conclusions concerning the growth which took place during these experiments. Of most interest is the observation that in two out of three experiments in this table, as well as in experiments to be reported later, growth apparently took place in the absence of any external source of nitrogen. This seems to indicate that some nitrogenous reserves were possessed by the yeast cells.

Experiments with other adaptive systems have also shown that glutamate is a readily available source of nitrogen for adaptive enzyme formation (95, 100). (See pages 95 and 99).

The effect of cysteine on galactozymase formation

The results given in Figure II illustrate the effect of cysteine on adaptation. Included in this figure are curves showing the effects of ammonia and sodium glutamate combined. Some stimulation of galactozymase formation was given by cysteine. In the presence of cysteine, the adaptive enzyme system was stabilized; in the course of this experiment the galactozymase activity of the yeast in the medium containing the cysteine did not fall off as it did in the two other media used.

Following this experiment, the effects of two concentrations of cysteine on growth and adaptation were determined. These results are

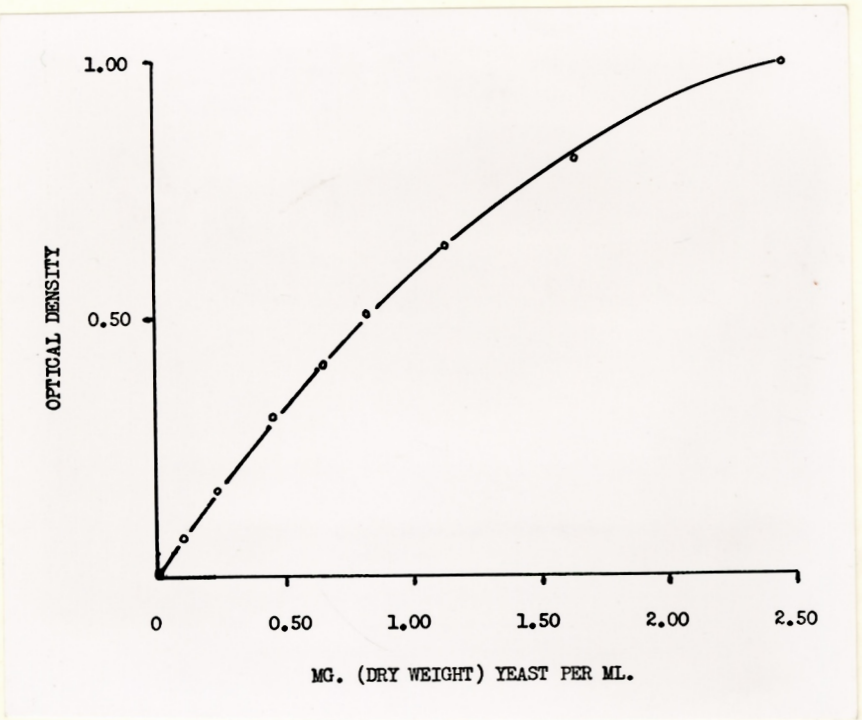


FIGURE I

Standard curve for the determination of yeast concentration.

2.5 mg. per ml., dry weight = 1%, wet weight

Preparation as described in text.

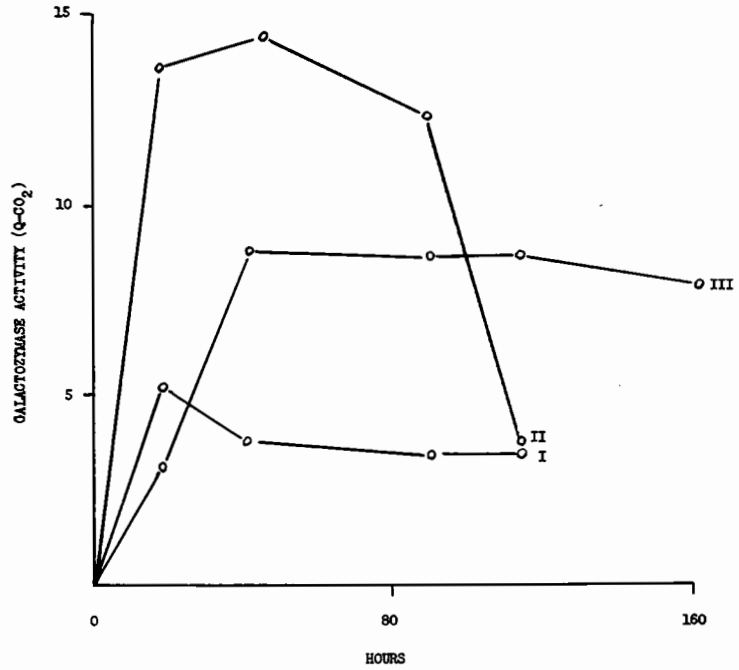


FIGURE II

The effects of glutamate, ammonia, and cysteine on galactozymase formation.

I. Galactose
II. Galactose + glutamate + ammonia
III. Galactose + cysteine

Incubation carried out in gas- washing bottles

Concentrations: 1% (wet weight) yeast, 4% galactose, 1% sodium-glutamate, 0.5% $NH_4H_2PO_4$, 0.12% cysteine hydrochloride. All solutions and suspensions in 0.067 M NaH_2PO_4. Initial pH: 4.5 in I, 5.0 in II and III.

Other conditions and methods of measurement as described in text.

reported in Table II. Cell proliferation was inhibited by cysteine; although both concentrations of cysteine stimulated adaptation, the stimulation was less with the higher concentration. This suggested that cysteine had an inhibitory action on adaptation as well as a stimulatory action, and that at the higher concentration of cysteine the inhibitory action became dominant.

The effect of cysteine and dithioglycerol on the loss of galactozymase activity in the absence of galactose

It was thought possible that some of the stabilizing action of cysteine on galactozymase formation might be connected with an ability to prevent the loss of galactozymase activity of adapted cells in the absence of galactose. This supposition was tested, using cysteine and another sulfhydryl compound, dithioglycerol (British anti-Lewisite). A supply of adapted cells was obtained by incubating 2 gm. (wet weight) of yeast in 200 ml. of a 4% solution of galactose in 0.067 M NaH_2PO_4. 0.17% sodium glutamate was added as a nitrogen source to stimulate adaptation. At the end of 50 hours' incubation, the yeast cells had a galactozymase activity ($Q\text{-}CO_2$) of 11.4. The washed cells were divided into three equal portions and suspended in the following solutions:

1. 50 ml. 0.067 M NaH_2PO_4
2. 50 ml. 0.1% cysteine HCl in 0.067 M NaH_2PO_4
3. 50 ml. 0.1% dithioglycerol in 0.067 M NaH_2PO_4 .

TABLE II

The effects of different concentrations of cysteine on growth and galactozymase formation in yeast.

Time in Hours	Galactozymase activity: ($Q-CO_2$) Incubation medium: Galactose	Galactose + 0.1% cysteine HCl	Galactose + 0.5% cysteine HCl	Degree of growth: mg. dry wt./ml. / initial mg. dry wt./ml. Incubation medium: Galactose	Galactose + 0.1% cysteine HCl	Galactose + 0.5% cysteine HCl
0	0	0	0	1.00	1.00	1.00
47 1/2	2.5	5.1	3.2	1.83	1.23	1.19
73	2.6	10.3	7.8	1.87	1.18	1.22
97	1.3	11.7	6.4	2.04	1.32	1.28
167 1/2	1.2	10.7	5.2	2.30	1.42	1.41
239	3.4	8.6	2.5	2.83	2.18	1.85

Means of incubation: Gas-washing bottles - other conditions and methods of measurement as given in the text.

Concentrations: 1% (wet weight) yeast, 4% galactose, cysteine HCl as shown. All solutions and suspensions in 0.067 M NaH_2PO_4. Initial pH of all solutions, 4.5

These suspensions were incubated in gas-washing bottles for 93 hours, and samples drawn from each at intervals for measurement of the galactozymase activity. The results of this experiment are given in Table III. From these results it may be seen that both cysteine and dithioglycerol had a slight stabilizing action on the adaptive enzyme system in the absence of its substrate.

Under the conditions of incubation employed here, any inhibitor of respiration might be expected to inhibit both the formation and the destruction of the galactozymase system. Quastel and Wheatley have shown (101) that cysteine shifts the aerobic utilization of glucose and fructese by bakers' yeast from an oxidative to a fermentative path. The oxidation of glycerol by bakers' yeast was little affected by cysteine. Thus, cysteine did not act as a general inhibitor of respiratory processes. It is not yet known if its effects on galactozymase formation and destruction may be related to its effects on sugar metabolism.

Other workers have also noted an inhibitory effect by cysteine on the growth of bakers' yeast (102). The results obtained here are too fragmentary to justify any extended considerations on the relation between cysteine's effect on adaptation and on growth.

Because of the long periods of time necessary for adaptation and the low levels of galactozymase activity attained with the available preparations of bakers' yeast, other adaptive systems were sought

TABLE III

The effects of cysteine and dithioglycerol on the loss of galactozymase activity by adapted cells suspended in the absence of galactose.

Time out of contact (hours)	Galactozymase activity ($Q-CO_2$) of cells suspended in: NaH_2PO_4	NaH_2PO_4 + cysteine	NaH_2PO_4 + dithioglycerol
0	11.4	11.4	11.4
20 1/2	4.8	7.1	7.8
44 1/2	2.3	3.7	5.2
92 1/2	1.8	2.0	0.0

Conditions: As given in the text.

in which adaptation would take place in a short time, without concurrent cell proliferation. Such a system, the maltozymase system of bakers' yeast, will be next considered.

Summary and conclusions

The action of various sources of nitrogen on galactozymase formation in a slowly-adapting strain of bakers' yeast has been examined. Some galactozymase was formed in the presence of galactose, even when no exogenous source of nitrogen was present. The addition of a nitrogen source, however, raised the level of enzyme synthesis. As shown already (34), the presence of ammonia stimulated galactozymase formation. A greater stimulation was given by the presence of sodium glutamate.

Cysteine inhibited cell growth, stimulated galactozymase formation, and stabilized the galactozymase system once it was formed. Increasing the concentration of cysteine lowered the stimulation of adaptation given by this amino acid. This suggested that cysteine had an inhibitory as well as a stimulatory effect on adaptation. It was found that both cysteine and dithioglycerol (British anti-Lewisite) prevented in some degree the loss of galactozymase activity by adapted cells suspended in a medium lacking galactose.

CHAPTER III

THE MALTOZYMASE SYSTEM

A. INTRODUCTION

The details of the mechanism of fermentation of maltose and other disaccharides are not so well defined as are those of the fermentation of galactose. The evidence for the adaptive formation of the maltozymase system can best be considered after a brief discussion has been given of the present views of the enzymatic constitution of this system. There is still some uncertainty regarding the enzymes that ferment maltose, and this uncertainty is reflected in the evidence showing that maltose fermentation is an adaptive process.

Two possible mechanisms of maltose fermentation (as well as of the fermentation of other disaccharides) have been considered in the past. According to the first, the so-called Indirect Theory, proposed by Fischer (103), disaccharides are first split to their component sugars, and these sugars are then fermented. According to the second, the so-called Direct Theory, first put forward by Willstätter and his collaborators (104, 105) and later supported by Leibowitz and Hestrin (106, 107), the disaccharide is first attacked, not by a hydrolytic cleavage, but in some other way.

Experimental Evidence for the Indirect and Direct Theories

Fischer (103) noted that yeast cells able to ferment maltose,

sucrose, and lactose possessed maltases, sucrases, and lactases, which could be demonstrated in cell autolysates. It seems certain, and has been accepted by proponents of the Direct Theory (106), that if yeast cells have the power to hydrolyse a disaccharide to two fermentable sugars, some of the disaccharide will be fermented in this way, even if some is attacked by another method.

Willstätter and his collaborators noted that the activity of disaccharases in yeast extracts, prepared by several different methods, was always too low to account for the rate of disaccharide fermentation by Fischer's proposed mechanism. In addition, it was seen that lactose-fermenting yeasts fermented lactose more rapidly than the component sugars, glucose and galactose, separately or together (104, 105).

Leibowitz and Hestrin (106) realized that if it were possible to inhibit the disaccharase activity of an organism without inhibiting the fermentation of the disaccharide, this would constitute proof against the Indirect Theory. To measure the maltase activity of an intact yeast cell was difficult, however, because any glucose formed would be immediately fermented. These workers used the fermantation of α-methyl-glucoside as a means of measuring maltase activity. It was unlikely that α-methyl-glucoside would be fermented without being first hydrolysed. It was known that this substance was fermented by intact yeast cells which contained maltase; the α-glucosidase preparations of yeast available at the time of these experiments hydrolysed both maltose and α-methyl-glucoside. Leibowitz and Hestrin found that

they were able to inhibit the fermentation of α-methyl-glucoside without inhibiting maltose fermentation. At low temperatures and acid pH values the fermentation of α-methyl-glucoside was completely inhibited while maltose was still strongly fermented. Maltose was fermented by the intact cell at pH values at which maltase in cell-free extracts was found inactive. The substrate concentration-activity curves for maltose and α-methyl-glucoside fermentation showed a much greater difference than was to be expected if both substances were being fermented by the same mechanism.

The Direct Theory is supported by the recent demonstrations of non-hydrolytic mechanisms of disaccharide breakdown in bacteria. Thus, the adaptive enzyme, sucrose phosphorylase, found in Pseudomonas sacharophila Doudoroff and in Leuconostoc mesenteroids (66, 108), catalyses the reaction:

Sucrose + $H_3PO_4 \rightleftharpoons$ Glucose-1-phosphate + Fructose,

and the adaptive enzyme, amylomaltase, found in E. coli mutabile (71), catalyses the reaction,

n maltose $\rightleftharpoons$ n glucose + (glucose)n, whereby maltose is transformed into free glucose and a polysaccharide of four to six glucose units. Similar polymerization reactions are known to occur with sucrose in Leuconostoc mesenteroides and in Bacillus subtilis (109). Such mechanisms have not yet been demonstrated, however, for maltose fermentation in yeast.

In addition, the evidence for the Direct Theory is open to serious criticism. Gottschalk has pointed out the danger involved in drawing conclusions from extracts concerning mechanisms operating in the intact cell, and has suggested that the differential effects of temperature and pH on the fermentation of maltose and α-methyl-glucoside might be explained by the limited permeability of the yeast cell (110).

One of the major premises of Leibowitz and Hestrin was the identity of the enzymes splitting maltose and α-methyl-glucoside. Recent experimental work has shown that there may be not one but several α-glucosidases in yeast. The separation by Spiegelman et al. (67) of the two adaptive α-glucosidases, one of which splits maltose and α-phenyl-glucose, the other only α-methyl-glucoside has already been mentioned (See page 25). Hestrin and Lindegren found (80), in a Saccharomyces species, an α-glucosidase which attacked α-methyl-glucoside but no other α-glucosides, including maltose. It is clear that maltase and α-methyl glucosidase are not in all cases identical and that no theory may be based upon the assumption that they are. At present, then, the manner by which yeast ferments maltose is still uncertain.

Evidence that maltozymase formation is an adaptive process

Several workers (111, 112, 113) have observed a lag period before the onset of maltose fermentation by yeast. Yeast cells incubated in the presence of maltose and subsequently washed were seen to increase

in maltozymase activity; a plot of this activity against time of incubation yielded a sigmoid curve, (38).

As already mentioned, adaptively formed α-glucosidases were extracted from yeast cells grown in the presence of maltose (67). Thus, direct evidence exists that yeast can form adaptive enzymes in the presence of maltose, but the foregoing discussion shows that it is not certain that the lag period before maltose fermentation by yeast is taken up with the formation of these specific adaptive enzymes.

It has been noted that the presence of oxygen or the addition of glucose reduced the lag period before the fermentation of maltose and galactose by yeast. When first observed, these facts were explained in terms of the activation of a pre-existing enzyme system (113, 114). Spiegelman, Reiner, and Cohnberg (38) argued that oxygen and glucose act, not by activating enzyme systems already present but by supplying energy for the synthesis of new enzyme systems. They pointed out that glucose or oxygen had little effect in shortening the lag unless the adaptive substrate were also present.

A description of the competition between the formation of the galactozymase and the maltozymase systems has already been given (page 14). The addition of ammonium sulphate greatly increased the level of enzyme activity and reduced the time necessary for the formation

of both these systems. In the presence of ammonium sulphate, the addition of galactose (and the consequent formation of galactozymase) had little effect on maltozymase formation, though in the absence of an external source of nitrogen, galactose strongly inhibited maltozymase formation (34).

By its behavior, then, it appears likely that the fermentation of maltose is an adaptive process, but the evidence for this is not so conclusive as for the galactozymase system. Considering the important theoretical derivations which have been made from the assumption that the maltozymase system is adaptive, further experimental work to decide this point seems warranted.

B. EXPERIMENTAL METHODS

Most of the experiments reported below were carried out in the Warburg manometric apparatus. Except where otherwise stated, the suspension of yeast came into contact with the maltose solution after the vessel had been gassed with 93% N_2 - 7% CO_2 and equilibrated. All experiments were carried out at 27°C. at a pH of 4.5 in 0.067M NaH_2PO_4. The yeast used was normally washed twice with cold 0.067M NaH_2PO_4 before being made up to a 2% or 4% (wet weight) suspension. A few of the experiments were carried out aerobically, in a manner to be described, and the maltozymase activity measured after the cells had been removed from the media in which adaptation had taken place.

The majority of the experimental results obtained are expressed in tabular form. In most experiments there was, strictly speaking, no lag period. A small gas evolution was observed immediately, or within the first five or ten minutes of contact of yeast cells with maltose. A maximum rate of gas evolution was only reached after 120 or more minutes of contact (See Fig. III). Other results are described in terms of the time at which a steady (and maximal) rate of gas evolution was reached, and the final activity, expressed as Q-CO_2, defined as before. The interval between "zero time", the time from which manometric readings were taken, and the time a steady rate was reached will be designated Lag T_2. Lag T_1 represents the time that an evolution of gas

was first observed. In these experiments, Lag T_1 = 0. "Zero time" was usually the time of first contact between yeast and maltose; in some experiments, yeast and maltose were in contact before zero time. In all cases, the meaning of the term will be made clear.

Reagents:

Three commercial brands of maltose - B.D.H., Pfanstiehl, C.P., and Merck Purified Maltose - were used in these experiments. All these brands gave virtually the same results in comparable experiments. To test for the presence of glucose, one brand, Merck Purified Maltose, was incubated with yeast at pH 7.2. At this pH, glucose was fermented strongly by this yeast, but no gas was evolved from maltose. This showed that the maltose was free of contaminating glucose.

Neutral solutions of sugars were used. Many reagents were dissolved in 0.067 M NaH_2PO_4, and the pH of the resulting solutions adjusted to 4.5. Unless otherwise indicated, other solutions were adjusted to pH 4.5 before use. Hexose-diphosphate was supplied as the di-barium salt. This was dissolved in M/6 HCl, the barium precipitated by addition of a stoichiometric amount of Na_2SO_4, the $BaSO_4$ removed by centrifugation, and the supernatant neutralized.

C. EXPERIMENTAL RESULTS

Properties of Lallemand's and Fleischmann's yeast with regard to maltose fermentation

Preliminary experiments showed that fresh Fleischmann's yeast possessed the ability to ferment maltose from the start at a nearly maximum rate. The conditions under which this yeast had been cultivated were not known exactly, but it was thought possible that the maltose-fermenting power was adaptive and would be lost upon incubating the yeast for a time in the absence of any substrate. To test this, air was bubbled through a 2% (wet weight) suspension of Fleischmann's yeast held at room temperature. Samples of this aerated suspension were taken at 17 and 67 hours, and the fermentation of glucose and maltose by these cells followed. The results, given in Figure III, show that aeration in the absence of any substrate produced a profound change in the fermentation of maltose, but had a much smaller effect on the fermentation of glucose. A Lag T_2 of approximately 120 minutes was seen in the maltose fermentation by the cells aerated for 67 hours. For glucose, though the rate of fermentation had been slightly lowered, there was no increase in the lag period. Included in Figure III is a curve showing the fermentation of maltose by a fresh lot of Lallemand's yeast. Here, there was an evident lag period of 110 minutes before the maximal carbon dioxide output was reached. Because fresh Lallemand's yeast was unadapted to maltose

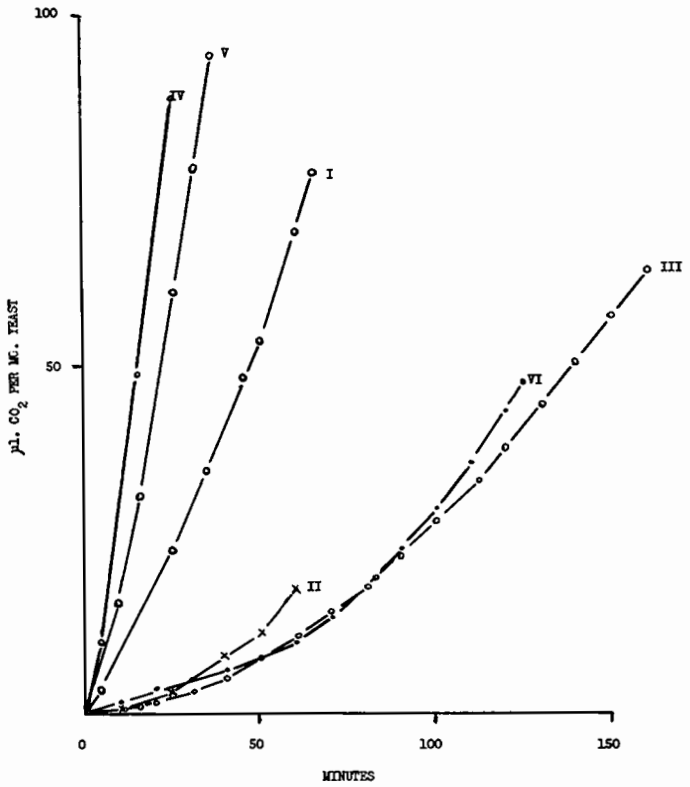


FIGURE III

Maltose and glucose fermentation by Lallemand's and Fleischmann's yeast: The effect of aeration in the absence of a substrate.

Fleischmann's yeast:
I. Maltose fermentation by fresh yeast
II. Maltose fermentation after 17 hours' aeration.
III. Maltose fermentation after 67 hours' aeration.
IV. Glucose fermentation by fresh yeast.
V. Glucose fermentation after 67 hours' aeration.

Lallemand's yeast:
VI. Maltose fermentation by fresh yeast.

In main vessels: Yeast in 2.7-3.0 ml. 0.067 M NaH_2PO_4. 4.9 mg. (dry weight) yeast/vessel in I, IV and VI; 18.2 mg./vessel in II; 23 mg./vessel in III and V.

In side arms: 0.3 ml. 20% maltose or glucose, tipped in at zero time, after gassing and equilibration.

Temp.: 27° C. Gas: N_2 - CO_2 Final Volume: 3.0 ml. in I and IV, 3.3 ml. in others.

fermentation, it was used in the succeeding experiments.

Aerobic adaptation to maltose fermentation

The increase in maltozymase activity upon aerobic incubation of yeast in the presence of maltose is illustrated in Figure IV. For this incubation, four 25 ml. Erlenmeyer flasks were set up, each containing:

5.0 ml. 4.0% (wet weight) yeast in 0.067M NaH_2PO_4

3.0 ml. 0.067M NaH_2PO_4

2.0 ml. 50% Maltose

Gas above liquid phase: Air.

The flasks were shaken at a temperature of 27°C. A 3.0 ml. sample was taken immediately after addition of the maltose to the yeast, and other 3.0 ml. samples were taken at intervals throughout the experiment, up to 275 minutes after the first contact between yeast and maltose. The samples taken were centrifuged, the yeast washed twice by centrifugation in cold 0.067M NaH_2PO_4 and the volume of the suspension made up finally to 3.0 ml. in 0.067M NaH_2PO_4. 1.0 ml. samples were taken for assay, and 0.5 ml. diluted for turbidity measurements. Measurements of activity were carried out in Warburg vessels in which were placed the washed yeast suspension, 0.067M NaH_2PO_4 to give a final total volume of 3.0 ml., and in the side arm, 0.8 ml. of 50% maltose. After gassing and equilibration, the contents of the side arm were tipped in, and the carbon dioxide output followed for 20 minutes, for a determination of the maltozymase activity. The amount of yeast present at different times, expressed as mg. dry weight/ml. is given under Figure IV.

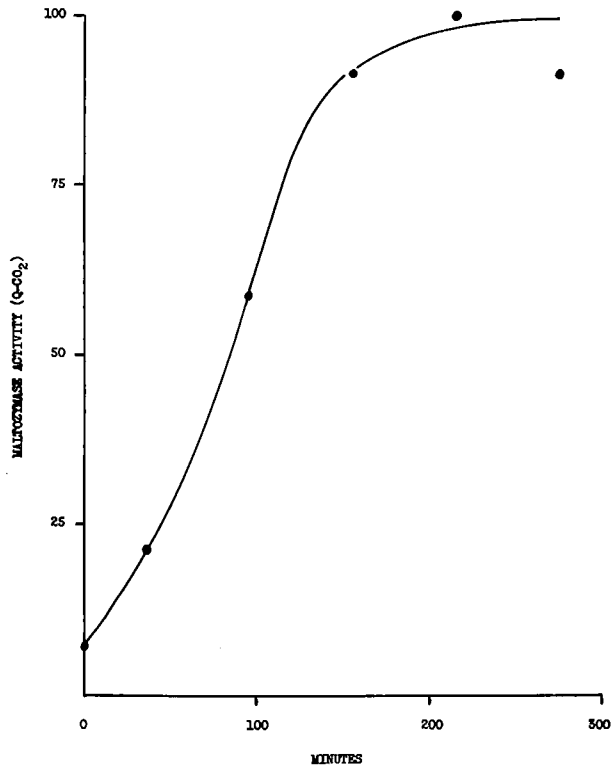


FIGURE IV

Aerobic adaptation to maltose fermentation.

Time (minutes)	Yeast Concentration (mg./ml.)
0	5.7
35	5.9
95	6.0
155	5.6
215	6.2
275	6.2

Conditions as described in text.

The time-activity curve in Figure IV has the sigmoid shape characteristic of several other adaptive curves and found previously for maltozymase as well. From the measurements of the amount of yeast present at different times, it is seen that no growth took place up to 155 minutes, by which time the maximal activity had been reached; and little if any growth took place in the next 120 minutes.

The concentration-activity relationship for maltose fermentation

After fixing on Lallemand's yeast, the relation of substrate concentration to the fermentative activity of this yeast was investigated, to establish the proper concentration of maltose to be used in future experiments. The variation of the final rate of carbon dioxide production with changes in substrate concentration is shown in Figure V. The period, Lag T_2, for each concentration of maltose is given below the figure. The lag varied little in this experiment from the smallest to the greatest maltose concentration used. The maximal activity was reached at the surprisingly high maltose concentration of 13 1/3%. It is not possible to say from this experiment alone if the variation in activity was due to a variation in the amount of adaptive enzyme formed, a variation in activity of the adaptively formed enzyme, or a combination of both. Further experiments were not carried out to decide this point. The concentration of 13 1/3% of maltose was used in most subsequent experiments.

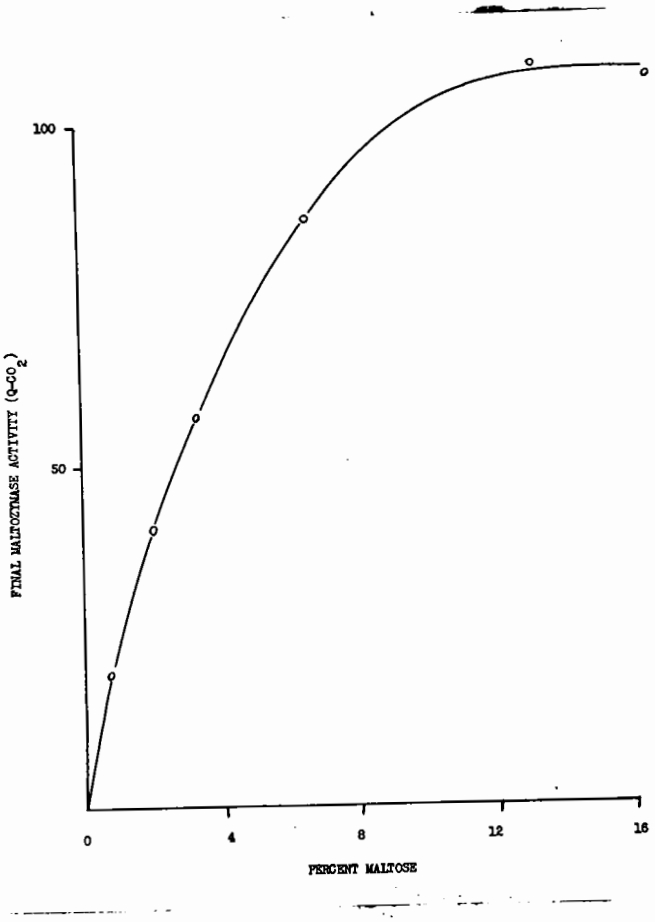


FIGURE V

The effect of maltose concentration on maltose fermentation by unadapted yeast.

Percent Maltose.	Lag T_2, Minutes
2/3	140
2	160
3-1/3	140
6-2/3	140
13-1/3	140
16-2/3	130

Final maltozymase activity as shown.

Conditions: Main Vessels - Maltose in 0.067 M NaH_2PO_4 to give final concentration shown, 0.067 M NaH_2PO_4 to a final volume of 3.0 ml.

Side Arms - 0.5 ml. 4% (wet weight) yeast in 0.067 M NaH_2PO_4, tipped in at zero time after gassing and equilibration.

Other conditions as in Figure III.

The effect of various co-factors on the formation and activity of the maltozymase system

It was of considerable interest to know to what extent, if at all, the lag period observed before the maximum rate of maltose fermentation by unadapted yeast could be accounted for as the time necessary for building up of co-factors essential for fermentation. It is a common experience, for example, that in the fermentation of glucose by yeast, there is a short lag period which disappears if hexose-di-phosphate is added to the medium. The experiments of Spiegelman et al. (65) on the importance of co-factors in galactozymase formation and activity have already been mentioned (See page 24). Such studies, however, have not yet been made on the maltozymase system. In the work to be reported, the effects of possible co-factors, both known and unknown, on maltose fermentation by intact yeast cells, were observed.

In Table IV are presented the results of two experiments in which maltose fermentation was carried out in the presence of different concentrations of hexose diphosphate and sodium pyruvate. In these experiments, the yeast was in contact with the maltose prior to the addition of the sodium pyruvate or hexose diphosphate, and some adaptation must have taken place by the time of this addition. It is seen from the results in Table IV that while both hexose diphosphate and sodium pyruvate had a stimulating effect on carbon dioxide evolution, neither shortened the lag period. The lag period, then, did not represent, even partially, the time necessary for the yeast cell to build

TABLE IV

The effect of sodium pyruvate and hexose diphosphate (HDP) on maltose fermentation by yeast

Experiment	System	Lag T_2, Minutes	Final Q-CO_2
1.	Maltose	120	105
	0.002 M HDP	-	0
	Maltose + 0.002 M HDP	120	127
2.	Maltose	140	43
	0.013 M HDP	-	0
	Maltose + 0.002 M HDP	140	46
	Maltose + 0.013 M HDP	140	52
	0.013 M Pyruvate*		
	Maltose + 0.002 M Pyruvate	140	44
	Maltose + 0.013 M Pyruvate	140	67

* A total of 64 μl. CO_2 was evolved from the pyruvate present.

In main vessels: 0.5 ml. 4% (wet weight) yeast in 0.067 M NaH_2PO_4, 0.067M NaH_2PO_4 to make 3.0 ml. total volume. Maltose: Experiment 1, 1.5 ml. 20% maltose in NaH_2PO_4; Experiment 2, 0.8 ml. 50% maltose.

In side arms: Experiment 1, 0.02 M HDP to give final concentrations as shown; Experiment 2, 0.04 M HDP and Sodium Pyruvate to give final concentrations as shown.

Yeast and maltose were in contact 10 minutes before gassing and equilibration. The contents of the side arm were tipped in at zero time, after gassing and equilibration.

Temp.: 27°C. Gas: N_2 - CO_2

up these substances. It is not possible to determine from this experiment alone whether these substances stimulated maltozymase formation or the activity of the maltozymase formed.

It was thought that if the increase in activity with time, observed in maltose fermentation, were due to the formation of some non-enzymatic co-factor(s), it should be possible to extract this factor(s) from yeast cells which had become adapted to maltose fermentation, and by adding it to cells not previously in contact with maltose, to reduce or eliminate the time lag for maltose fermentation. An experiment was accordingly devised to test this possibility. 20 mg. (wet weight) of yeast cells were adapted to maltose fermentation by 315 minutes anaerobic contact with a buffered maltose solution. These cells were washed once with cold 0.067M NaH_2PO_4, centrifuged, suspended in 2.5 ml. H_2O and heated in a boiling water bath for 10 minutes. The cells were again centrifuged and the supernatant taken. A similar extract was made from 20 mg. of unadapted yeast cells. The results in Table V show the effects on maltose fermentation of these extracts. It is seen that, while the extract of adapted yeast did strongly stimulate carbon dioxide production, it had little greater stimulatory effect than the extract from unadapted yeast. Neither extract caused a shortening of the lag period. This experiment indicates that maltozymase formation does not involve the formation of a non-enzymatic co-factor. The results, however, are not conclusive. The experiment was carried out with intact cells. If adaptation in this case did involve the formation of an essential co-factor, such a co-factor might have been unable to penetrate the cell wall.

TABLE V

The effect of hot water extracts of yeast cells, adapted and unadapted to maltose fermentation, on the fermentation of maltose by unadapted yeast

Experiment	System	Lag, T_2, Minutes'	Final Q-CO_2
1.	Maltose	140	53
	Maltose + 1.5 ml. extract from fresh yeast	160	169
	Maltose + 1.5 ml. extract from adapted yeast	160	182
2.	1.5 ml. extract from fresh yeast		0
	1.5 ml. extract from adapted yeast		0

In main vessels: 0.5 ml. 4% (wet wt.) yeast in 0.067M NaH_2PO_4, 1.0 ml. 20% maltose in 0.067M NaH_2PO_4. Extracts and supernatants as given above; see text for preparation. 0.067M NaH_2PO_4 to a total volume of 3.0 ml. Yeast and maltose were in contact 20 minutes before zero time.

Temp.: 27°C. Gas: N_2 - CO_2

It is impossible to say whether the stimulation of final rate was due to an action on the maltozymase system itself or on its formation. Probably, however, both actions were involved. A stimulation of glucose fermentation by yeast extracts has already been reported (115). In addition, the nitrogenous material contained in the yeast extract would be expected to stimulate maltozymase formation (34).

The evidence obtained in these experiments indicates that the lag period in maltose fermentation by unadapted cells is not the time necessary for the building up of essential co-factors for maltose fermentation. This point may be finally settled, however, only by experiments utilizing cell-free preparations of the enzyme systems involved.

Inhibitors of maltozymase formation: Sulfhydryl compounds

In view of the influence of sulfhydryl compounds on galactozymase formation and disappearance, it was of interest to learn whether such compounds would have any effect on maltozymase formation. Results of experiments showing the effects of cysteine on aerobic and anaerobic maltozymase formation are presented in Figure VI and Table VI. As the results in Table VI show, 0.0067M cysteine decreased the final rate of maltose fermentation by unadapted yeast cells, but had no effect on the fermentation of maltose by adapted cells. The effects observed when concentrations of cysteine higher than 0.0067M were added to unadapted cells were probably also effects on maltozymase formation. This is not certain, however, as experiments were not carried out to determine the action of these higher cysteine concentrations on maltose fermentation by adapted yeast cells.

Sodium thioglycollate caused a slight inhibition of aerobic and anaerobic maltozymase formation. The results of experiments showing these effects are given in Table VII. Even in the presence of 0.1 M thioglycollate, the inhibition of final rate of maltose fermentation by unadapted yeast cells was only 29%. That this represented an inhibition of adaptation was shown by the fact that this concentration of sodium thioglycollate had no effect on maltose fermentation by adapted yeast cells (Experiment 3).

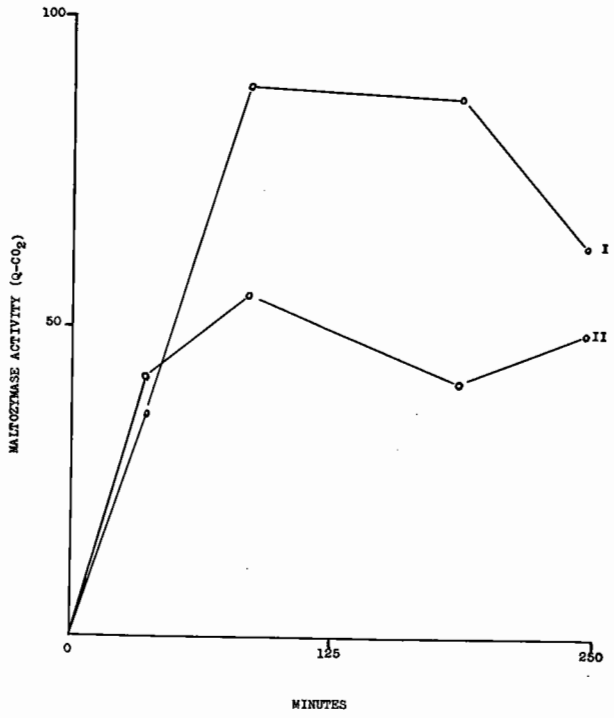


FIGURE VI

The inhibition by cysteine of aerobic maltozymase formation.

I. Maltose
II. Maltose + 0.0067 M cysteine

Conditions as in experiment illustrated in Figure IV. l-cysteine-HCl dissolved in 0.067 M NaH_2PO_4 added to give final concentration shown.

TABLE VI

The effect of different concentrations of cysteine on maltozymase formation

Experiment	System	Lag T_2, Minutes	Final Q-CO_2
1.	Maltose	320	22
	Maltose + 0.00067 M cysteine	320	25
	Maltose + 0.0067 M cysteine	320	16
	Maltose + 0.0134 M cysteine	320	17
	Maltose + 0.027 M cysteine	320	11
	Maltose + 0.038 M cysteine	320	10
		μl CO_2 evolved in 100 minutes	
2.	Maltose	880	
	Maltose + 0.0067 M cysteine	980	

In main vessels: Experiment 1, unadapted yeast, 20 mg. (wet weight) per vessel. Experiment 2, yeast adapted by 250 minutes aerobic exposure to maltose (conditions as in experiment represented in Curve I, Figure VI), 20 mg. (wet weight) per vessel.

Cysteine HCl in 0.067M NaH_2PO_4 to give final concentration shown, 0.067M NaH_2PO_4 to a final volume of 3.0 ml.

Side arms: 0.8 ml. 50% maltose, tipped in after gassing and equilibration, at zero time. Yellow phosphorous in center well in Experiment 1.

Temp.: 27°C. Gas: N_2 - CO_2

The effects of glutathione and sodium diethyldithiocarbamate on aerobic maltozymase formation were also tested. From the results presented in Table VIII, it is seen that neither of these substances had any effect.

In comparing the results presented in Figure VI and Table VI, it is seen that 0.0067 M cysteine had a rather greater inhibitory effect on aerobic than on anaerobic maltozymase formation. The data available on

TABLE VII

The effect of sodium thioglycollate on maltozymase formation

Experiment	System	Q-CO_2 after 3 hrs. aerobic incubation at 29° C.	
1.	Maltose	50	
	Maltose + 0.01 M sodium thioglycollate	40	
		Lag T_2, minutes	Final Q-CO_2
2.	Maltose	120	119
	Maltose + 0.02 M sodium thioglycollate	120	104
	Maltose + 0.1 M sodium thioglycollate	140	85
		μl. CO_2 evolved in 60 minutes	
3.	Maltose	196	
	Maltose + 0.1 M thioglycollate	200	

Experiment 1: In 125 ml. Erlenmeyer flask, 8.0 ml. 4% (wet weight) yeast in 0.067M NaH_2PO_4, 4.0 ml. 50% maltose, 2.0 ml. 0.1 M thioglycollate, 0.067M NaH_2PO_4 to 20 ml. Methods of measurement as described in the text.

Experiment 2: Main vessels: 0.8 ml. 50% maltose, 0.2 M sodium thioglycollate in 0.067M NaH_2PO_4 to give concentrations shown above. 0.067M NaH_2PO_4 to a final volume of 3.0 ml.
Side arms: 0.5 ml. 4% yeast in 0.067M NaH_2PO_4, tipped in after gassing and equilibration at zero time.

Experiment 3: Conditions as in Experiment 2, except that 0.5 ml. 1.3% washed, adapted yeast in 0.067M NaH_2PO_4 was tipped in at zero time. Adapted yeast was from the "maltose" vessel of Experiment 2.

Temp.: 27°C. Gas: N_2 - CO_2

this phenomenon, however, are insufficient to warrant a prolonged discussion. The fact that cysteine and thioglycollate inhibited both aerobic and anaerobic maltozymase formation shows that their action cannot be attributed to an effect on a respiratory process alone.

TABLE VIII

The effects of diethyldithiocarbamate (DDC) and glutathione (GSH) on maltozymase formation (incubation experiment)

System	Maltozymase activity ($Q-CO_2$) after minutes 0	30	130	210
Maltose	4.2	37	57	61
Maltose - DDC	3.8	28	61	59
Maltose - GSH	6.7	35	58	69

Conditions: Aerobic incubation in Erlenmeyer flasks.
10.0 ml. 4% yeast in 0.067M NaH_2PO_4
4.0 ml. 50% maltose
4.0 ml. 0.05 M glutathione or diethyldithiocarbamate in 0.067M NaH_2PO_4
0.067M NaH_2PO_4 to 20.0 ml.

Methods of measurement as described in text.

Temp.: 27°C.

The action of atabrine and riboflavine on maltozymase formation

It is known that agents, such as sodium azide and 2-4 dinitrophenol, which uncouple phosphorylation from the general metabolism of the cell, also inhibit adaptation (39, 44). Because atabrine is known to uncouple phosphorylation from oxidation in kidney particles (116), experiments were carried out to determine whether this substance had any effect on maltozymase formation.

The experiments reported in Table IX show that an inhibition of adaptation by atabrine did in fact take place. In the experiment in which aerobic incubation in the presence of atabrine was carried out for three hours, the yeast cells were found to be stained yellow; this color could not be removed from the cells by washing. It was possible that the adsorbed atabrine was still inhibiting the fermentation of maltose in the washed cells, rather than that an inhibition of adaptation had taken place. As Experiment 3 of this table showed, however, atabrine did not inhibit maltose fermentation by adapted yeast cells.

It was also found that the effect of atabrine could be partially reversed by the structurally similar compound, riboflavine. The results of an experiment showing this reversal are presented in Table X. Because of the low solubility of riboflavine, solutions of much greater concentration than those given in Table X could not be used; and it was only possible to obtain a slight reversal of the atabrine inhibition. Attempts were not made, therefore, to obtain a complete reversal of the action of atabrine.

The action of α-methyl glucoside on maltozymase formation

Leibowitz and Hestrin (106) observed that α-methyl glucoside inhibited the fermentation of maltose by yeast if it were added during the lag period, but not if it were added after the maximal rate had been reached. Although these workers were not concerned with adaptation in their experiments, this finding indicated that what they had observed was in reality an inhibition of maltozymase formation. One experiment was

TABLE IX

The effect of atabrine on maltozymase formation

Experiment	System	Maltozymase activity (Q-CO_2) after 3 hours' aerobic incubation at 29°C.	
1.	Maltose	50	
	Maltose + 0.0116 M atabrine	25	
		Lag T2 Minutes	Final Q-CO_2
2.	Maltose	110	112
	Maltose + 0.0001 M atabrine	110	101
	Maltose + 0.001 M atabrine	110	95
	Maltose + 0.01 M atabrine	90	61.6
		μl. CO_2/hr.	
3.	Maltose; Fermentation of adapted yeast		
	Before adding 0.01 M atabrine	524	
	After adding 0.01 M atabrine	517	

Conditions: Experiment 1: As in Table VII, with changes as shown.

Experiment 2: Main vessels: 0.8 ml. 50% maltose Atabrine dihydrochloride in 0.067M NaH_2PO_4 to give final concentration as shown. NaH_2PO_4 to a final volume of 3.0 ml.

Side arms: 0.5 ml. 4% yeast (wet weight) in 0.067M NaH_2PO_4, tipped in after gassing and equilibration, at zero time.

Experiment 3: Main vessels: 0.5 ml. 4% yeast in 0.067M NaH_2PO_4, 0.8 ml. 50% maltose, 0.067M NaH_2PO_4 to a final volume of 3.0 ml.

Side arms: 0.6 ml. of 0.05M atabrine di-HCl in 0.067M NaH_2PO_4. Vessels gassed and equilibrated. Contents of side arm tipped in after 95 minutes of contact between yeast and maltose when a steady rate of CO_2 evolution had been reached.

Experiments 2 and 3:- Temp.: 27°C. Gas: N_2 - CO_2

TABLE X

The reversal of the atabrine inhibition of maltozymase formation by riboflavine

System	Lag T_2, Minutes	Final Q-CO_2
Maltose	140	56
Maltose + 0.0001 M riboflavine	140	57
Maltose + 0.01 M atabrine	160	43
Maltose + 0.01 M atabrine + 0.0001 M riboflavine	160	49

Conditions: Main vessels: 0.8 ml. 50% maltose, atabrine and riboflavine in 0.067M NaH_2PO_4 to give final concentrations shown, 0.067M NaH_2PO_4 to a total volume of 3.0 ml.

Side arms: 0.5 ml. 4% (wet weight) yeast in 0.067M NaH_2PO_4, tipped in at zero time after gassing and equilibration.

Temp.: 27°C. Gas: N_2 - CO_2

carried out to confirm this observation, using a different method of following maltozymase formation. A comparison was made between the maltozymase activity of yeast incubated with maltose and yeast incubated with maltose plus α-methyl glucoside. The conditions of incubations were those given in Table VII. Solid α-methyl glucoside, to give a final concentration of 0.05 M, was dissolved in a buffered maltose medium before the addition of the yeast. At the end of three hours' aerobic incubation, the yeast incubated with maltose alone had a maltozymase activity (Q-CO_2) of 50, while that incubated with maltose + α-methyl glucoside had a Q-CO_2 of 35.

The effect of steroids on maltozymase formation

In view of the growing interest in the action of hormones on enzyme systems, the action of such substances on maltozymase formation was investigated. The synthetic hormone, diethyl stilbestrol, has been found to act as a hydrogen carrier for dehydrogenase systems (117). The natural hormone, Δ -4-androstenedione, strongly inhibits the anaerobic oxidation of α -glycerophosphate by yeast powder (118). In Figure VII, A and B, are shown the effects of alcoholic solutions of these steroids on maltose fermentation by unadapted and adapted yeast cells respectively. Although these steroids inhibited almost completely the fermentation of maltose by unadapted yeast, they had no, or considerably less, effect on maltose fermentation of adapted yeast. Thus, comparing the final rates of carbon dioxide evolution, it is seen that stilbestrol caused a 97% inhibition of fermentation by unadapted but only a 69% inhibition of fermentation by adapted yeast. The comparative figures for Δ -4-androstene-dione were 89% and 4% respectively. The break in carbon dioxide evolution in Figure VII B, was due to the change in vapor pressure when the alcoholic solution of the steroid was tipped into the aqueous solution in the main vessel. The great stimulation of maltose fermentation caused by ethanol should be noted. A separate experiment on the fermentation of a 2% glucose solution by yeast at pH 4.5 showed that there was no stimulation by the concentration of ethanol (6 1/3%) used in these experiments. The rates of carbon dioxide evolution from glucose were 791 μl./hr. in the absence and 775 μl./hr. in the presence of ethanol. When the alcohol was tipped into the yeast-maltose

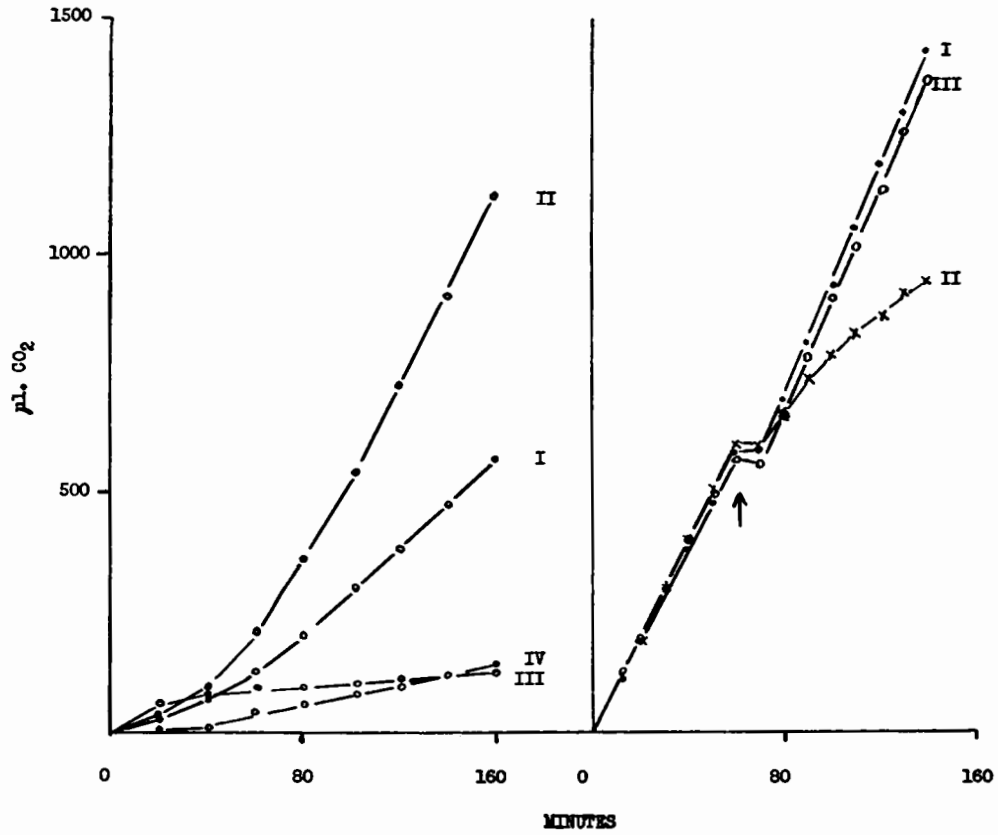


FIGURE VII

A B

A. The effect of diethyl stilbestrol and Δ -4 androstenedione on maltose fermentation by unadapted yeast.

I. Maltose
II. Maltose + ethanol
III. Maltose + ethanol + diethyl stilbestrol
IV. Maltose + ethanol + Δ -4 androstenedione.

Conditions: Main vessels: 0.8 ml. 50% maltose, 0.2 ml. 95% ethanol or 0.2 ml. 1% solutions of steroids as shown, in 95% ethanol, 0.067 M NaH_2PO_4 to a final volume of 3.0 ml.

Side arms: 0.5 ml. 4% (wet weight) yeast in 0.067 M NaH_2PO_4, tipped in at zero time, after gassing and equilibration.

FIGURE VII (Cont'd)

B. The effect of diethyl stilbestrol and Δ -4 androstenedione on maltose fermentation by adapted yeast.

I. Maltose + ethanol
II. Maltose + ethanol + diethyl stilbestrol
III. Maltose + ethanol + Δ -4 androstenedione.

Conditions: Main vessels: 0.8 ml. 50% maltose, 0.5 ml. 4% (wet weight) yeast in 0.067 M NaH_2PO_4, 0.067 M NaH_2PO_4 to a final volume of 3.0 ml.

Side arms: 0.2 ml. 95% ethanol or 0.2 ml. 1% solutions of steroids as shown, tipped in after gassing and equilibration, when a final rate of CO_2 evolution had been reached (Arrow.)

Yeast and maltose were in contact 50 minutes before zero time indicated.

In both experiments: Temp.: 27° C. Gas: N_2-CO_2

solution after fermentation had taken place for some time, there was little effect on the fermentation. This would normally indicate that the action of ethanol was one on maltozymase formation rather than on maltose fermentation. It might be argued, however, that the action of ethanol was one on maltose fermentation and that some ethanol had distilled into the main vessel from the side arm and had already produced a maximal amount of stimulation before the bulk of the ethanol was tipped in. Comparison of the carbon dioxide evolution in vessel I, Figure VII B with that of another vessel, prepared in the same way but with ethanol omitted from the side arm, indicated that a small stimulation of fermentation had come about in this way, but that it was insufficient to account for the stimulation observed in unadapted yeast. Thus, before tipping in the ethanol, the rate of carbon dioxide evolution was 603 μ l./hr. for vessel I, and 545 μ l./hr. for a comparable period for the vessel without ethanol in the side arm. A further indication that the observed stimulation was one of adaptation rather than fermentation comes from a comparison of Curves I and II of Figure VII A. If the stimulation were merely one of fermentation, it would be expected that the ratio, μ l CO_2 evolved by II/μ l. CO_2 evolved by I would be constant throughout the experiment, while if the stimulation were one of adaptation, the ratio should increase with time. The latter was found to be the case. The ratio increased from a value of 1.39 at 20 minutes to 2.02 at 160 minutes. It is possible that some stimulation of fermentation was involved here, but the main effect appears to have been one of adaptation.

The effect of steroids in aqueous solution on adaptation was also observed.

Two methods were used in preparing saturated aqueous solutions of these steroids. In the first (Experiment 1 and 3, Table XI), 0.1 ml. of 1% stilbestrol and 0.2 ml. of 1% Δ-4-androstenedione in 95% ethanol were added to 5 ml. H_2O and the solution heated in a boiling water bath 30 minutes to drive off the ethanol. The solutions were cooled and filtered. In the second method (Experiment 2, Table XI), 5 mg. of each steroid was heated in 10 ml. H_2O, the hot solutions filtered, cooled, and refiltered. As on cooling of the hot filtered solutions, a precipitate was seen to form, the filtrates were taken to be saturated solutions of the steroids.

The results presented in Table XI show that in the much lower concentrations of steroids involved here, no inhibition of adaptation took place. Rather, a slight stimulation of adaptation was observed. The magnitude of this effect, especially in the case of Δ-4-androstenedione, was much less than the inhibitory effect of alcoholic steroid solutions. The manner by which these steroids influenced adaptation is still unknown.

Summary and conclusions

The fermentation of maltose by two commercial brands of bakers' yeast, Fleischmann's and Lallemand's, has been studied. Lallemand's yeast fermented maltose at a maximal rate only after an induction period of two hours or longer. Fleischmann's yeast fermented maltose at a maximal rate

TABLE XI

The action of aqueous solutions of diethylstilbestrol and Δ -4-androstenedione on maltozymase formation

Experiment	System	Lag T_2, Minutes	Final Q-CO_2
1.	Maltose	120	85
	Maltose + diethylstilbestrol	120	104
	Maltose + Δ -4-androstenedione	120	106
2.	Maltose	100	71
	Maltose + diethylstilbestrol	120	100
	Maltose + Δ -4-androstenedione	100	75
3.	Rate of Fermentation of Adapted Yeast (Q-CO2)	Before Adding	After Adding
	Diethylstilbestrol	127	137
	Δ -4-androstenedione	140	142

Conditions: Experiments 1 and 2: Main vessel: 0.8 ml. 50% maltose, 0.6 ml. saturated aqueous steroid solutions, 0.067M NaH_2PO_4 to a final volume of 3.0 ml. Side arm: 0.5 ml. 4% (wet wt.) yeast in 0.067M NaH_2PO_4, tipped in after gassing and equilibration at zero time.

Experiment 3: Yeast and maltose in contact 170 minutes before tipping in steroid solutions from side arm. Concentrations as in Experiments 1 and 2.

Yellow phosphorus in all center wells.

Temp.: 27°C. Gas: N_2 - CO_2

with little or no induction period. After a period of aerobic incubation in the absence of any substrate, however, Fleischman's yeast exhibited a prolonged induction period in the fermentation of maltose. Lallemand's yeast was used for the remainder of the experiments reported in this chapter.

Sodium pyruvate and hexose di-phosphate both increased the final rate of maltose fermentation without decreasing the lag period before a final rate was reached. Hot water extracts of adapted and unadapted yeast cells both stimulated the final rate of fermentation to approximately the same extent. Neither shortened the lag period. The lag period, then, probably did not involve the formation of either hexose-diphosphate, sodium pyruvate or substances extractable by hot water from adapted yeast.

It was found that both cysteine and sodium thioglycollate inhibited maltozymase formation. The other thiol compounds tested, glutathione and sodium diethyldithiocarbamate, were without effect. The inhibitory effects were noted in aerobic as well as anaerobic adaptation. The action of these inhibitors, then, could not have been solely on a respiratory process.

The previously reported (106) inhibition of maltozymase formation by α-methyl-glucoside was confirmed by another method.

Atabrine, which is known to uncouple phosphorylation from oxidation in kidney particles was found to inhibit both aerobic and

anaerobic maltozymase formation. This inhibition was to some extent reversible by the structurally similar compound, riboflavine.

Ethanolic solutions of diethyl stilbestrol and Δ -4-androstenedione strongly inhibited maltozymase formation. The former compound also inhibited maltose fermentation by adapted cells, though to a lesser extent than it inhibited maltozymase formation. Ethyl alcohol itself strongly stimulated maltozymase formation. In aqueous solutions, diethylstilbestrol and Δ -4-androstenedione gave a slight stimulation to maltozymase formation.

CHAPTER IV

THE FORMIC HYDROGENLYASE SYSTEM

A. INTRODUCTION

Historical Survey

Since a detailed historical review of the earliest work on formic hydrogenlyase may be found in the review by Stephenson (119), this work will be discussed only briefly here. The first demonstration of bacterial production of molecular hydrogen from formic acid was that of Hoppe-Seyler (120) in 1876. He found that mixed cultures of bacteria obtained from mud decomposed calcium formate with the production of carbon dioxide and hydrogen. In 1901, Pakes and Jollyman (121) showed that all organisms which produced hydrogen in the fermentation of sugars also produced hydrogen and carbon dioxide from formic acid. The conversion of glucose to formic acid in E. coli was demonstrated in the same year by Harden (122). In 1932, Stephenson and Stickland (123) took up the study of the bacterial enzymes which liberated molecular hydrogen from formic acid and other substances. They proposed the name "hydrogenlyase" for enzymes liberating molecular hydrogen, to distinguish them from dehydrogenases, which transfer hydrogen from a substrate to an acceptor. These workers were the first to demonstrate the adaptive formation of formic hydrogenlyase (123). It was produced by growing cultures of E. coli only when formic

acid or substances giving rise to formic acid were present in the growth medium.

In studies on the decomposition of sugars by various bacteria, aerobically in liquid cultures, it was noted that the production of hydrogen by way of formic acid was inhibited by increased atmospheric pressure (122, 124-126). The breakdown of formic acid was also inhibited by molecular hydrogen (123). These facts suggested that the reaction liberating hydrogen was reversible. This was verified by Woods (127), who in 1936 showed that the reaction;

$$HCOOH \rightleftharpoons H_2 + CO_2,$$

could be used by bacteria either for the breakdown or for the synthesis of formic acid. If the evolved carbon dioxide is absorbed in alkali, the above reaction may proceed to completion. This method is used for the manometric measurement of formic hydrogenlyase activity.

The enzyme system is produced by other bacteria of the Colon-Aerogenes group as well as by E. coli, and is the most important if not the only mechanism whereby molecular hydrogen may be liberated by these bacteria. Under certain growth conditions a few strains of E. coli and one of A. aerogenes have been found to produce small amounts of hydrogen from glucose but none from formate (119, 128, 129). Glucose, however, is degraded to formate in these organisms. It has been demonstrated recently (130) that glucose may activate formic hydrogenlyase. It appears likely that when hydrogen evolution from glucose but not from formate alone was observed, the amount of formic hydrogenlyase present

was too small to produce a measurable amount of hydrogen from formate unless the enzyme(s) were stimulated by the presence of glucose. Thus, there seem to be no grounds for postulating a glucose hydrogenlyase distinct from formic hydrogenlyase.

In the Clostridia, hydrogen may be formed from pyruvate without passage through formate as an intermediate. (Reviewed in 131). Some of the properties of the hydrogen-producing system in Clostridia will be discussed later.

Using resting cell suspensions of E. coli grown in the presence of formate, Stephenson and Stickland (123) measured the effects of changes of hydrogen-ion concentration, of various salts, and of inhibitors on the formic hydrogenlyase system. It was found that in intact cells this system possessed a sharp pH-activity curve with a maximum activity approximately at pH 7. The enzyme system was exceedingly sensitive to a number of poisons, including toluene, fluoride, urethane, carbon monoxide, and cyanide. The findings of Waring and Werkman (132), to be discussed later, suggest strongly that some iron-containing component is essential to this system. This also is suggested by the nature of some of the inhibitors of the system.

Recently, Lichstein and Boyd (78) demonstrated formic hydrogenlyase activity in adapted cells of Aerobacter aerogenes dried in vacuo. Gest and Gibbs (79) have prepared a cell-free extract from adapted cells of E. coli by grinding these organisms with "Alumina A 301".

This extract produced hydrogen and carbon dioxide from formic acid. A similar extract has been reported by Hughes (133). The maximal formic hydrogenlyase activity of the extract prepared by Gest and Gibbs occured at pH 6.1, whereas at pH 7.1, approximately the value for maximal activity by the intact cells, the extract was virtually inactive (79).

It was found that the divalent metal-complexing agent, α-α'-dipyridyl, inhibited the formic hydrogenlyase activity of this preparation. The inhibition was partially reversed by the addition of Mn^{++}, and completely reversed by the addition of Fe^{++}. This reversal was considered by Gest and Gibbs to indicate the importance of iron to formic hydrogenlyase activity. Interpretation is difficult in this case, however, because of the possibility that Fe^{++} relieved the α-α'-dipyridyl inhibition simply by combining with it in solution and removing it from its site of action on the enzyme system.

The question of the separate existence of the formic hydrogenlyase system

Since the discovery of the formic hydrogenlyase system, the following question has had to be faced: Is this reaction due to a single enzyme or to the combined action of two enzymes? Hydrogen could be liberated from formic acid by a single splitting reaction, catalysed by one enzyme;

$$HCOOH \rightleftharpoons H_2 + CO_2 ,$$

or it could be liberated by the combined action of the enzymes,

hydrogenase and formic dehydrogenase, both present in most, if not all bacterial cells which liberate hydrogen from formic acid. Thus:

$$HCOOH + A \underset{\text{formic dehydrogenase}}{\rightleftharpoons} CO_2 + AH_2$$

$$AH_2 \underset{\text{hydrogenase}}{\rightleftharpoons} A + H_2$$

$$HCOOH \rightleftharpoons H_2 + CO_2$$

where A represents an intracellular hydrogen acceptor or system of hydrogen carriers.

The importance of this problem was early realized and investigated by Stephenson and Stickland (123, 134). They pointed out that the hypothesis that two enzymes rather than one are involved would be disproved if an organism were found possessing formic hydrogenlyase activity but lacking either hydrogenase or formic dehydrogenase. It was their further postulate, though obviously a less valid one, that the existence of an organism possessing the two latter enzymes but lacking formic hydrogenlyase would also constitute proof against the two-enzyme theory.

A survey was made in which several bacterial species were tested for the presence of the three enzymes concerned (123). Four strains of Bact. lactis aerogenes (Aerobacter aerogenes) lacking hydrogenase but possessing formic hydrogenlyase were found. One strain of Bact. dispar (Shigella dispar) was found which possessed both hydrogenase and formic dehydrogenase but lacked formic hydrogenlyase.

In addition, it was noted that E. coli grown on plain broth possessed both hydrogenase and formic dehydrogenase but lacked formic hydrogenlyase. On the basis of these results, Stephenson and Stickland concluded that formic hydrogenlyase activity was, in fact, due to a single enzyme and not to a combination of two enzymes.

This conclusion has since been repeatedly questioned. Ordal and Halvorson (135), in a survey similar to that conducted by Stephenson and Stickland, established the presence of hydrogenase in three strains of Aerobacter aerogenes and two strains of Aerobacter cloacae; they found, as well, that all strains of E. coli which produced hydrogen from formate possessed both hydrogenase and formic dehydrogenase. Because both hydrogenase and formic dehydrogenase could be present in unadapted cells, which would not liberate hydrogen from formate, they suggested that a third factor, essential for electron conduction between hydrogenase and formic dehydrogenase, was necessary for formic hydrogenlyase activity. In the equations given above, this factor would be represented by A, the acceptor or acceptor system.

Waring and Werkman (132) studied the effects of iron deficiency in the growth medium on various enzyme systems of Aerobacter indologenes. They found that iron-deficient cells had markedly lower formic dehydrogenase, and formic hydrogenlyase activities than normal cells. These workers also believed that an intermediate carrier between the hydrogenase and formic dehydrogenase systems was essential for formic hydrogenlyase activity. They proposed that this electron carrier

contained functional iron. In support of this view, it was noted that the formic hydrogenlyase system was rather more sensitive to the effects of iron depletion than either hydrogenase or formic dehydrogenase. Cells grown under moderate conditions of iron deficiency were found to have small amounts of hydrogenase and formic dehydrogenase activities but to be completely lacking in formic hydrogenlyase activity.

In studies on the enzyme systems of nitrogen-deficient cells of E. coli, DeLey (136) found a parallelism between the activity-cellular nitrogen content curves of formic hydrogenlyase and hydrogenase, for different degrees of nitrogen depletion.

The striking similarities between the nitrogen sources required for the production of hydrogenase and formic hydrogenlyase found by Billen and Lichstein (100, 137)(described on page 99) for one strain of E. coli also indicates that one system may be dependent upon the other.

Detailed studies of the role of iron in the formic hydrogenlyase system have not yet been made. Iron is apparently of importance in the system in Clostridia by which hydrogen is liberated from pyruvate without passage through formate (131). This system is inhibited by carbon monoxide (138) and the carbon monoxide-inhibition is reversed by light (139). A study of the effectiveness of reversal by various wave-lengths of light showed a steady increase of light adsorption by the carbon monoxide complex from 650 to 360 mμ. In this system a

hemoprotein is apparently not involved, but comparable studies have not yet been made on the formic hydrogenlyase system.

Hobermann and Rittenberg (140) have pointed out that the conclusions of Stephenson and Stickland were based largely on the absence of hydrogenase in Aerobacter aerogenes strains, as measured by the failure of methylene blue and nitrate to act as acceptors for molecular hydrogen. This test for hydrogenase activity, however, is by no means infallible. Hobermann and Rittenberg studied the exchange reaction,

$$D_2 + H_2O \rightleftharpoons D_2O + H_2,$$

which may be used as a measure of hydrogenase activity. They found that one strain of Aerobacter aerogenes was unable to reduce methylene blue with molecular hydrogen unless a trace of fumarate were added as a carrier, but still contained the hydrogenase system as measured by the exchange reaction. Thus, the evidence based on the distribution of enzymes, considered conclusive by Stephenson and Stickland, would seem at present to be highly questionable.

If the adaptive formic hydrogenlyase system is, in fact, the result of combination of the two non-adaptive enzymes, hydrogenase and formic dehydrogenase, then adaptation here may well involve the formation of an intermediate electron carrier or of a system for transporting electrons between hydrogenase and formic dehydrogenase. The whole problem of the constitution of the formic hydrogenlyase system may be expected to be solved only through the use of active cell-free extracts and the isolation of the component parts of the system.

Coenzymes of the formic hydrogenlyase system

Recently, more and more evidence has been accumulated to indicate the importance of co-factors in the formic hydrogenlyase system. Considering the possibility that an electron carrier may be involved as part of this system, the nature of these co-factors takes on added interest.

Lascelles (130) discovered that the formic hydrogenlyase activity of adapted suspensions of E. coli fell off markedly on storage, and that this loss was increased by dilution of the cells. Instead of evolving hydrogen at a maximal rate from the start, the stored cells exhibited a lag period of twenty minutes or more before a steady rate was reached. The activity could be restored by the addition of small quantities (0.001 M) of glucose or other fermentable sugars to the cells at the same time that the formate was added, or by preincubation of the cells with glucose before the addition of formate. With such additions, the lag period was eliminated. Other substances were not found as effective as the fermentable sugars. Of the compounds tested, adenylic acid, in the relatively high concentration of 0.0025 M gave the strongest stimulation.

Lichstein and Boyd (141) have recently reported experiments with a biotineless mutant strain of E. coli. This organism could not grow unless either biotin or oleic acid were added to the growth medium. Cells grown in the presence of biotin, in a medium which ordinarily

stimulated formic hydrogenlyase production, were practically lacking in formic hydrogenlyase activity. Cells grown in the same medium, with oleic acid replacing biotin, had a strong formic hydrogenlyase activity, and the addition of oleic acid to biotin-grown cells raised their level of formic hydrogenlyase activity almost to that of the oleic acid-grown cells. A similar, though not so marked effect was noted on the formic dehydrogenase activity of these cells. Other fatty acids (78) were found capable of stimulating the formic hydrogenlyase activity, though none were as active as oleic acid. Aqueous extracts of yeast and liver had an even stronger stimulating action than oleic acid. This observation confirms that made earlier by Sevag, Henry and Richardson (142) of the stimulation of formic hydrogenlyase activity by yeast extract. Only preliminary experiments have been carried out to determine the chemical nature of the responsible factors in yeast and liver extracts (78). With the growth of interest in this system, it may be expected that in the near future the exact nature of the co-factors involved in formic hydrogenlyase activity will be made clear.

The adaptive formation of formic hydrogenlyase

Following the discovery by Stephenson and Stickland (123) of the adaptive nature of the formic hydrogenlyase system, Yudkin (129) investigated in detail the factors underlying the formation of enzymes liberating molecular hydrogen from formate, glycerol, and glucose in several bacterial species (*E. coli*, *A. aerogenes*, *A. cloacae*, *B. freundii*). Cultures were grown under various conditions, and the

enzymatic activities of the washed cells determined. Some variation was found in the degree to which different factors operated on enzyme formation in these species, but the following general conclusions were reached:

1. Aeration had an inhibitory effect on the formation of hydrogenlyase though it did not destroy the enzyme system once it was formed.
2. The presence of formate or some substance giving rise to formate was essential to the formation of hydrogenlyase.*
3. Except in one species, A. aerogenes, little or no hydrogenlyase was produced by bacteria grown in a glucose-ammonia-mineral salts medium. Some constituent(s) of the broth used (in this case, tryptic digest of caseinogin) was essential to hydrogenlyase formation.

Yudkin also considered the question of whether adaptation or natural selection were involved in hydrogenlyase formation. He found that cells of B. freundii, subcultured several times in the presence of formate and possessing formic hydrogenlyase activity, lost this activity following one subculture in plain broth. He considered this evidence that the hydrogenlyase activity had not been acquired by natural selection.

Stephenson and Stickland (57) investigated the question of natural selection in more detail. In experiments carried out in the Barcroft manometric apparatus, as well as in culture flasks, it was found that cells of E. coli could attain a maximal amount of formic

* - In broth, a small amount of hydrogenlyase was formed in the absence of any added formate or formate-precursor, because formic acid was produced during amino-acid metabolism. (See page 161)

hydrogenlyase activity in the relatively short time of one to two hours. Careful measurements showed that during this time no increase in cell numbers occurred. This finding, together with the observation that sodium formate had no influence upon the growth of young cultures of E. coli, was taken as conclusive evidence that natural selection was not involved in this adaptation.

Although hydrogenlyase formation could take place without cell proliferation, it did not take place in these experiments unless tryptic broth as well as sodium formate were added to the cells. Thus, the enzyme was not synthesized unless the cells were in a medium in which they could grow, even though growth did not necessarily take place while the enzyme was being formed.

A plot of formic hydrogenlyase activity against time showed the sigmoid shape characteristics of the formation of other adaptive enzymes (31, 57).

A detailed study of the nitrogen sources involved in the production of bacterial enzymes concerned with formate and hydrogen metabolism was recently made by Billen and Lichstein (100, 137) using the "Texas" strain of E. coli. This organism, like most strains of E. coli, could be grown on a simple glucose-ammonia-mineral salts medium (basic medium), but organisms so grown possessed neither hydrogenase nor formic hydrogenlyase activity. If tryptone or casein hydrolysate were added to the basic medium, both enzymes were produced during growth.

Casein hydrolysate could be replaced by a synthetic mixture of the seventeen amino acids in the hydrolysate. Detailed investigations were made of the amino acids used. A mixture of seven (glutamate, methionine, tryosine, cystine, serine, arginine, and alanine) were found able virtually to replace the seventeen amino acids for hydrogenase production, and a mixture of six (glutamate, methionine, tyrosine, cystine, lysine, and valine) could do the same for formic hydrogenlyase production. Individually, both glutamate and methionine were almost equally efficient in stimulating hydrogenase production, and the other amino acids less so. With formic hydrogenlyase production, only glutamate gave any appreciable stimulus when used alone. In the presence of glucose and glutamate this organism could synthesize formic hydrogenlyase and hydrogenase without cell division taking place. Although cells grown on the basic medium possessed formic dehydrogenase activity, some stimulation of formic dehydrogenase production was given by the addition of a casein hydrolysate. Individually, glutamate, methionine, and tyrosine stimulated formic dehydrogenase formation. The stimulation by glutamate could take place in non-proliferating cell suspensions.

The papers of Billen and Lichstein appeared at the time the writer was carrying out preliminary experiments using different amino acids as nitrogen sources for formic hydrogenlyase synthesis. The main portion of the work to be reported here deals with the effect of various sources of nitrogen on the <u>time</u> required for the appearance of formic

hydrogenlyase activity as well as on the amount of activity finally attained. Previous studies of the nitrogenous factors essential for formic hydrogenlyase formation have been carried out with growing cells and have been aimed at discovering the effect of these factors on the final amount of enzyme synthesized. In growing cells, however, so many synthetic reactions are taking place that it becomes impossible to determine the extent to which substances added at the beginning of growth go to make up products found at the end of growth. In the experiments reported here, adaptation was accompanied by little or no cell proliferation. Under such conditions it seemed that much valuable information concerning the function of nitrogenous compounds in adaptation could be gained through a systematic study of factors influencing the length of time elapsing before the appearance of enzymatic activity.

Observations made during the course of these studies led to a series of experiments on the energy sources necessary for formic hydrogenlyase formation, a subject not examined up to this time. Work on the nature of the energy sources was carried out using both synthetic and non-synthetic sources of nitrogen for formic hydrogenlyase synthesis. A full account of these experiments will be given only after a description of the development of a simplified nitrogen source for the stimulation of this adaptive process.

B. EXPERIMENTAL METHODS

The strain of *E. coli* used in the experiments on formic hydrogenlyase formation was one isolated from human faeces. For most experiments these bacteria were grown on agar plates of the following composition: 0.1% yeast extract, 0.5% peptone, 0.5% NaCl, 1.5% agar, in tap water (Nutrient agar). The following precautions were taken to insure the use of pure cultures of bacteria in each experiment: One agar plate was inoculated with a single colony of bacteria. After growth had occurred, two or more plates were thickly inoculated from this plate, using a bent glass rod to spread the bacteria.

For some experiments, it was necessary to use bacteria possessing formic hydrogenlyase activity. For this, the bacteria were grown in glucose or formate broth. The broth used was of the same composition as the medium described above, except that the agar was omitted and 1% glucose or sodium formate included as substrates for adaptation. Growth took place at 37°C. in all cases. The time of growth is given with the description of individual experiments.

The bacteria were taken up from the agar plates in distilled water, centrifuged, and washed twice with distilled water. Bacteria grown in broth were centrifuged down and washed twice with distilled

water. The washed bacteria were suspended in distilled water and, with the aid of a standard curve for the turbidimetric determination of bacterial concentration, the suspension was diluted to contain 3.0 mg. dry weight of bacteria per ml. This concentration of bacteria was used in most of the experiments reported here.

The standard curve for these experiments was made up in a manner similar to that used for preparing a yeast standard curve. The optical densities of dilutions of a thick suspension of bacteria in distilled water were read in the colorimeter at 525 mμ, and samples of the thick suspension dried at 105-110°C. The term, "milligram of bacteria" will be used to refer to dry weight.

In the majority of experiments, adaptation was followed throughout in the Warburg apparatus. All vessels contained 0.033 M sodium phosphate buffer, usually of pH 7.4. Sodium formate, sodium pyruvate, or glucose were added as adaptive substrates.* The final volume in the vessels was 3.0 ml. In the side arms were placed 0.5 ml. of the bacterial suspension. A roll of filter paper and 0.2 ml. of 20% KOH were placed in the center well to absorb evolved carbon dioxide. Experiments were carried out at 37.5°C ± 0.5°C. After gassing with nitrogen and equilibrating for five to ten minutes, the bacteria were tipped into the main vessel. In experiments with unadapted cells, no hydrogen evolution was observed for at least thirty minutes after tipping in the bacteria.

* - Formic acid is formed from pyruvate as well as glucose by E. coli, (92, 131)

Measurements of the formic hydrogenlyase activity of adapted cells were done by a similar procedure: After gassing and equilibration, the bacteria were tipped into a buffered formate solution. In a few cases, which will be noted, adaptation was carried out in the Warburg vessel, and substances whose effect on formic hydrogenlyase activity were to be tested tipped in after a steady rate of hydrogen evolution had been reached.

Most of the experimental results obtained are expressed numerically, in terms of two lag periods and a final constant hydrogenlyase activity ($Q\text{-}H_2$). In the experiments carried out here, no hydrogen evolution was noted for at least thirty minutes. Following the first appearance of hydrogen, a period of from thirty to fifty minutes elapsed before a steady rate of hydrogen evolution was reached by the bacteria. The period of the first evolution of hydrogen is designated Lag T_1. The period at which a steady rate of hydrogen evolution was reached is designated Lag T_2. Measurements of both Lag T_1 and Lag T_2 are usually given to the nearest ten minutes. Because of the experimental errors involved in determining these quantities a more precise expression of them is not justified. Final rates are expressed as $Q\text{-}H_2$ = μl. H_2 evolved/mg. dry wt./hr. In those experiments where the rate fell off through pH changes, the time of the beginning of the steepest portion of the curve is given as Lag T_2. In those cases where no hydrogen evolution was observed in the time of the experiment, the Lag T_1 period is given simply as greater than the time of observation, e.g. $>$ 180 minutes,

and the final Q-H_2 is given as zero. Where some hydrogen was evolved, but the evolution was not followed until a constant rate was reached, Lag T_2 is given as the time at which the maximal rate of hydrogen evolution began, and designated (M). In such a case, the maximal Q-H_2 observed is given as the final activity.

Lag T_1 shows the time of the first appearance of enzymatic activity, Lag T_2 the time at which a maximal amount of activity was reached, and the final Q-H_2 indicates the actual amount of enzyme synthesized. The final Q-H_2 is not, of course, a measurement of the amount of enzyme synthesized, but a figure which may be used in comparing the amounts synthesized in the same experiment under different conditions, providing that adequate controls are made of the factors which can influence the activity of the enzyme once it is formed.

In studies on bacterial growth, and in some studies of adaptive enzyme formation, a different method has been employed for the measurement of the lag period than that given here. When the logarithm of the number of bacteria present at any time divided by the number originally present is plotted against time, the lag is determined by extrapolating the steepest (the "logarithmic") portion of the curve to the abscissa (9, 143). When adaptive enzyme formation is followed manometrically and the output or uptake of gas is plotted against time, the lag is estimated by extrapolating the steepest portion of the curve to the time axis (144). What is measured by such a method is a period between the

time of the first appearance of cell growth or enzymatic activity and the time when a maximal rate of growth or activity is reached. This value of the lag period is dependent upon the final rate attained. A few measurements of the lag period were made in this way; these measurements were convenient and in many cases could be used in comparing the times necessary for enzyme formation. In Tables XII, XXI, and XXIV, the lag as just defined is given (denoted "Lag"), together with the periods Lag T_1 and Lag T_2. In Figure X these three values of the lag are illustrated as well, so that a comparison may be made between the two methods of measurement. Except in these, and a few other cases where only the "Lag" was measured, the time necessary for enzyme formation will be expressed in terms of Lag T_1 and Lag T_2.

Usually, when bacteria were grown on glucose or formate broth, the washed cells evolved hydrogen from formate at a steady rate from the start. In a few cases there was a small lag period before the maximal rate of hydrogen production was reached. In such cases, the constant rate of hydrogen evolution was used in determining $Q\text{-}H_2$ values. In all experiments with adapted cells, the interval of time during which the constant rate was measured will be given.

Other methods used for incubating cells of E. coli with adaptive substrates need only brief description, which will be given when the results obtained by these methods are presented.

Preparation of a synthetic amino-acid medium

Following the observation of the effect of a casein hydrolysate on adaptation, (see p.124), synthetic amino-acid media were made up so that each Warburg vessel would contain the amounts of amino acids present in 1 ml. of a 5% solution of casein hydrolysate. Following the example of Billen and Lichstein (100, 137), the data of Williamson (145) for the amino-acid content of casein from cow's milk were used as a guide to the composition of such a medium. The composition of a casein hydrolysate, the concentration of l-amino acids in a 5% hydrolysate, and the milligrams of each amino acid contained in 1.0 ml. of such a hydrolysate are given below.

Amino acid	% in casein	% in a 5% hydrolysate	mg. in 1.0 ml. of 5% hydrolysate
tyrosine	5.5	0.275	2.75
alanine	2.3	0.115	1.15
glycine	0.4	0.02	0.2
proline	8.1	0.405	4.05
glutamic acid	21.9	1.095	10.95
aspartic acid	4.2	0.21	2.1
serine	5.0	0.25	2.5
cystine	0.4	0.02	0.2
arginine	3.9	0.195	1.95
phenylalanine	5.5	0.275	2.75
leucine	14.4	0.72	7.2
isoleucine	5.2	0.26	2.6
histidine	2.0	0.1	1.0
lysine	6.0	0.3	3.0
threonine	4.6	0.23	2.3
methionine	3.1	0.155	1.55
tryptophan	1.3	0.065	0.65
valine	5.3	0.265	2.65

In addition, norleucine and hydroxyproline, as described below, were added to the synthetic mixture.

For convenient use of this medium, four amino-acid solutions, of the following composition, were prepared:

No. 1. 4 mg. glycine, 46 mg. dl-alanine, 81 mg. l-proline, 100 mg. dl-serine, 92 mg. dl-threonine, 4 mg. l-hydroxy proline, in 2.0 ml. H_2O.

No. 2. 230 mg. sodium l-glutamate, 62 mg. dl-methionine, in 2.0 ml. H_2O.

No. 3. 47.2 mg. l-arginine HCl, 75.2 mg. l-lysine HCl, 13 mg. l-tryptophan, 24.8 mg. l-histidine HCl, in 2.0 ml. H_2O.

No. 4. 10 mg. l-tyrosine, 84 mg. dl-aspartic acid, 1 mg. l-cystine, 144 mg. l-leucine, 104 mg. dl-isoleucine, 15 mg. dl-norleucine, 110 mg. dl-phenylalanine, 106 mg. dl-valine, in 10.0 ml. H_2O.

0.1 ml. of solutions 1, 2, and 3, and 0.5 ml. of solution 4 were normally added to each Warburg vessel. It should be noted that where only the dl-amino acid was available, an amount was added which gave the same quantity of the l-form as found in 1.0 ml. of the 5% hydrolysate.

Except where otherwise stated, these amino-acid solutions, as well as all other acid or alkaline solutions, were neutralized with M NaOH or HCl, using phenol red indicator.

Preparation of hydrolysates of gelatin and tryptone

0.9 gm. of gelatin or bacto-tryptone were refluxed in 20 ml. of 1:1 HCl overnight. The biuret reaction was used as a test for the completion of hydrolysis. The hydrolysed solution was evaporated in vacuo at 80°C. and the solid material remaining dissolved in water, filtered and neutralized with M NaOH. The final concentration of NaCl in the Warburg vessel, resulting from addition of the hydrolysates will be given with each experiment described.

Determination of nitrate and nitrite

Nitrate was estimated by the phenol disulphonic acid reagent, nitrite by the Griess - Ilosvay reagent (see reference 145a). The writer is indebted to Mr. W. Brown of this institute for carrying out these estimations.

Techniques of paper chromatography

In the one chromatographic experiment reported here, the amino acid mixtures were spotted on paper. The chromatograph was developed in a solvent of the following composition:

60 ml. ethanol
20 ml. butanol
7 ml. conc. NH_4OH
13 ml. H_2O

After the solvent had ascended overnight, the paper was dried, sprayed with 0.1% ninhydrin in water-saturated butanol, re-dried and heated at 105-110°C for a few minutes to enable the reaction between ninhydrin and the amino acids to take place.

C. EXPERIMENTAL RESULTS

PART I

Introductory Experiments: The Use of Synthetic and Non-synthetic Media as Nitrogen Sources for Formic Hydrogenlyase Formation.

Washed suspensions of E. coli grown on nutrient agar had no ability to produce hydrogen gas from sodium formate. If bacto-tryptone or bacto-peptone* were added to the formate medium, the bacteria could produce hydrogen from formate after a lag of 60-70 minutes. These agar-grown cells, however, were able to oxidize formate from the start at a maximal rate. These facts are illustrated in Figure VIII which represents experiments carried out with two separate bacterial suspensions. No endogenous production of hydrogen was ever observed in unadapted or adapted bacteria, nor were unadapted bacteria ever found to liberate hydrogen from formate, tryptone, or peptone, alone. It may be seen from Figure VIII that the lag period elapsing before the production of hydrogen from formate could not have been due to a low permeability of the cell, as formate was immediately oxidized by these organisms. Both bacto-tryptone and bacto-peptone served almost equally well to stimulate adaptation (Table XII). As bacto-peptone was more readily available, it

* Difco Bacto-tryptone and Bacto-peptone were used throughout these experiments.

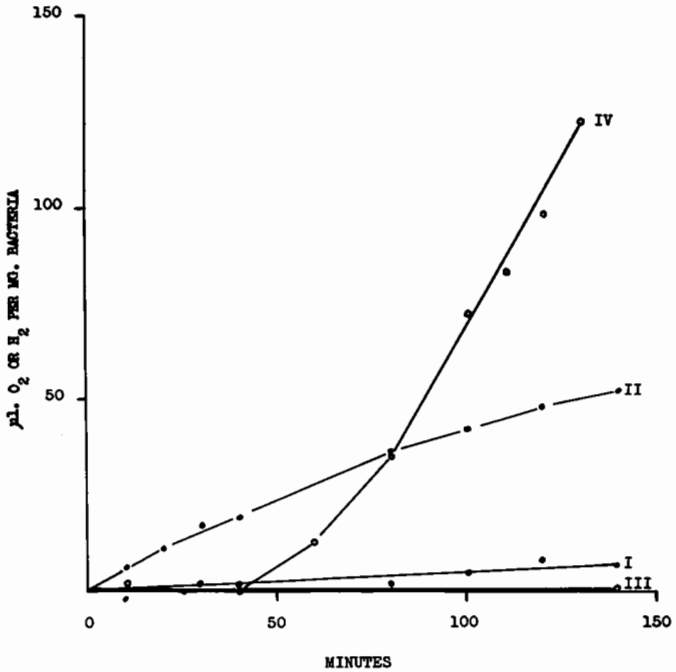


FIGURE VIII

Oxygen consumption and hydrogen evolution by unddapted bacteria in the presence of formate.

A comparison of the ability of unadapted bacteria to oxidize and to liberate hydrogen from formate.

Oxygen consumption:

I. Endogenous
II. Formate

Hydrogen evolution:

III. Formate
IV. Formate + tryptone

Bacteria: Grown overnight on nutrient agar; 1.3 mg. bacteria/vessel for I and II, 1.5 mg./vessel for III and IV.

Concentrations: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, 1% tryptone.

In I and II, bacteria were in main vessels, H_2O or formate were tipped in at zero time, after equilibration. In III and IV, bacteria (0.5 ml.) were tipped in from the side arm at zero time after gassing and equilibration.

Temp.: 37° C. Gas: Air, I and II; N_2, III and IV. Filter paper and KOH in all center wells.

TABLE XII

Comparison of the formate-tryptone and formate-peptone systems: The effects of varying concentrations of tryptone and peptone

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Lag, minutes "Lag"	Final Q-H_2
1.	Formate + 1% tryptone	50	120	76	84
	Formate + 1% peptone	30	80	64	50
2.	Formate + 0.5% tryptone	80	120	104	50
	Formate + 1% tryptone	80	140	102	116
3.	Formate + 0.1% peptone	100	120	120	13
	Formate + 1.0% peptone	-	80	52	129
	Formate + 2% peptone	-	70	42	390

Bacteria: Grown overnight on nutrient agar; 1.5 mg. bacteria/vessel in experiments 1 and 2; 1.4 mg./vessel in Experiment 3. Bacteria tipped in from side arm after gassing and equilibration, at zero time.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, other final concentrations as shown.

Temp: 37°C. Gas: N_2 Final volume: 3.0 ml.

Filter paper and 0.2 ml. 20% KOH in center wells.

was used in most of the experiments reported here. The effects of changes of tryptone and peptone concentrations on the stimulation of hydrogen production from formate by unadapted bacteria are also given in Table XII. A small effect on the lag periods and a much greater one on the final rate were found with increasing concentrations of tryptone and peptone.

Interpretation of the effects of different concentrations of these complex materials is made difficult by the fact that such natural materials may have a stimulatory effect on hydrogen production by adapted cells. This was found true for yeast and liver extracts by Lichstein and Boyd (78). Peptone was also found to stimulate hydrogen production by adapted cells (Table XIII) and though tryptone was not tested for this property, it almost certainly had it as well.

In Table XIV are shown the effects of changes in the initial formate concentration on adaptation in the formate-peptone system. The initial concentration of formate was lowered in this experiment to test the possibility that high concentrations of formate inhibited adaptation. As may be seen, when the formate concentration was lowered to 1/10 that normally used, no adaptation occurred.

Following the demonstration that bacto-tryptone was able to stimulate formic hydrogenlyase production, experiments were performed to determine whether the action of bacto-tryptone could be ascribed to the

TABLE XIII

The effect of peptone on formic hydrogenlyase activity:

Experiment	System	$Q-H_2$	Per cent. stimulation by peptone
1.	Formate, pH 7.4	88	
	Formate + peptone, pH 7.4	356	305
2.	Formate, pH 7.4	336	
	Formate + peptone, pH 7.4	510	52
	Formate, pH 6.8	369	
	Formate + peptone, pH 6.8	650	76

Bacteria: Experiment 1, grown overnight in formate broth, 1 mg. bacteria/vessel.
Experiment 2, grown 22 hours in glucose broth, 1.5 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH as shown. 0.067 M formate, 1% peptone.

H_2 evolution measured from 0 to 30 minutes in Experiment 1, from 20 to 50 minutes in Experiment 2.

Other conditions as in Table XII.

TABLE XIV

Hydrogenlyase formation in the formate-peptone system at different initial formate concentrations

System	Lag, minutes T_1	T_2	Final Q-H_2
0.067 M Formate + peptone	45	90	51
0.0067 M Formate + peptone	>180		0

Bacteria: Grown 12 1/2 hours on nutrient agar; 1.5 mg. bacteria/vessel.

In main vessel: 0.033 M phosphate buffer, pH 7.4, 1% peptone, formate as shown.

Other conditions as in Table XII.

amino acids it contained. It was found that after acid hydrolysis, tryptone still retained some ability to stimulate adaptation. Though the stimulating power of the hydrolysate was less than that of the unhydrolysed tryptone, the loss of this power could be partially ascribed to the NaCl present in the neutralized hydrolysate. These facts are summarized in Table XV.

It was found in a separate experiment that NaCl had no effect on formic hydrogenlyase activity. When adaptation was carried out in a formate-tryptone medium in a Warburg vessel and NaCl to a final concentration of 0.2 M tipped into the adapted bacteria, the Q-H_2 values before and after adding the NaCl were 88 and 94 respectively.

Next, seventeen different amino acids were tested separately for their ability to stimulate formic hydrogenlyase formation. In no

TABLE XV

The use of acid hydrolysates of tryptone, gelatin, and casein as nitrogen sources for formic hydrogenlyase formation.

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Lag, minutes "Lag"	Final Q-H_2
1.	Formate + 1% tryptone	50	120		84
	Formate + 1.5% tryptone hydrolysate, 0.15 M in NaCl	60	140 (M)		46
	Formate + 1% tryptone + 0.15 M NaCl	40	140 (M)		22
	Formate + 1% tryptone + 1.5% tryptone hydrolysate	70	140 (M)		286
2.	Formate + 1% tryptone	-	-	74	89
	Formate + 1.7% casein hydrolysate, 0.17 M in NaCl	-	-	320	16
3.	Formate + 1% tryptone	-	-	66	98
	Formate + 1.5% gelatin hydrolysate, 0.11 M in NaCl	-	-	>255	0
	Formate + 1% tryptone + 0.11 M NaCl	-	-	90	49
	Formate + 1% tryptone + 1.5% gelatin hydrolysate	-	-	84	214
4.	Formate + 1% peptone	-	-	52	129
	Formate + 1% peptone + 1.5% gelatin hydrolysate	-	-	68	314

Bacteria: Grown overnight on nutrient agar; 1.5 mg. bacteria/vessel in Experiments 1-3, 1.4 mg./vessel in Experiment 4.

In main vessel: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, other final concentrations as shown.

The same lot of gelatin hydrolysate was used in Experiments 3 and 4

Other conditions as in Table XII

case did adaptation take place (Table XVI). In addition, acid hydrolysates of casein and gelatin, containing little besides amino acids, had practically no power to stimulate formic hydrogenlyase formation in the presence of formate alone (Table XV). An interesting observation made at this time is also recorded in Table XV. A hydrolysate of gelatin, which could not stimulate adaptation in the presence of formate alone, increased the rate of hydrogen production in the formate-tryptone system. Further experimental work was carried out on this phenomenon, but a discussion of its significance will be deferred till later. Control experiments showed that no hydrogen was evolved by bacteria grown on nutrient agar from gelatin or tryptone hydrolysates alone. From the above results, it was seen that some factor was essential, or very important, for adaptation in the presence of formate alone, which was contained in peptone, tryptone, and tryptone hydrolysate but which was not an amino acid.

While this work was going on, the attention of the writer was drawn to the experiments of Billen and Lichstein (100, 137). Experiments to be reported, confirming and extending some of their work, revealed that a much higher level of formic hydrogenlyase activity could be reached by using glucose or pyruvate as a source of formate than by using formate alone. Preliminary experiments also showed that fewer factors were needed for formic hydrogenlyase formation in the presence of glucose or pyruvate than in the presence of formate. For this reason, glucose was used as a source of the adaptive substrate in most

TABLE XVI

The inability of individual amino acids to bring about formic hydrogenlyase formation in the presence of formate

Amino acid	Final concentration (Molar)	Lag T_1 greater than
glycine	0.1	240 minutes
dl-serine	0.1	240 minutes
l-proline	0.1	240 minutes
l-cysteine	0.1	240 minutes
dl-methionine	0.1	455 minutes
dl-alanine	0.1	355 minutes
l-leucine	0.1	355 minutes
dl-norleucine	0.05	355 minutes
l-lysine	0.1	355 minutes
dl-threonine	0.1	355 minutes
l-tyrosine	0.00125	355 minutes
dl-phenylalanine	0.04	355 minutes
dl-valine	0.1	180 minutes
dl-aspartate	0.1	240 minutes
l-hydroxyproline	0.1	240 minutes
l-tryptophan	0.1	140 minutes
l-glutamate	0.1	290 minutes

Bacteria: Grown overnight on nutrient agar; 1.5 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, amino acids as shown.

Other conditions as in Table XII.

of the experiments directed toward finding a completely synthetic medium which would stimulate formic hydrogenlyase formation to the same extent as more complex materials of unknown composition. Some experiments were subsequently carried out in which formic hydrogenlyase was synthesized by E. coli using a synthetic medium in the presence of formate alone. This was made possible by the findings on (1) The sources of nitrogen needed for formic hydrogenlyase synthesis in the presence of glucose or pyruvate, and (2) The sources of energy used in formic hydrogenlyase synthesis. This work will be reported after Part III, in which the sources of energy required in formic hydrogenlyase formation are discussed.

Formic hydrogenlyase formation in the glucose-glutamate system without growth

Several experiments were carried out to determine whether or not formic hydrogenlyase could be formed without cell proliferation in the presence of glucose and glutamate, by the strain of E. coli used, under the conditions employed by Billen and Lichstein with their "Texas" strain of E. coli. Incubation medium of the same composition as that used by these workers were made up; the exact conditions of their incubations, however, were not stated in their report. A six-hour aerobic incubation with glucose-glutamate medium (see Table XVII) in Erlenmeyer flasks continuously shaken at 38° C., induced no formic hydrogenlyase activity in the exposed cells. Considering the known inhibitory effect of oxygen on formic hydrogenlyase formation (129),

this result is not surprising. Subsequent incubations were carried out in test tubes at 37° C. The tubes were not agitated during the incubation, but there was no appreciable settling of bacteria in the time of the experiment. At the end of the incubation period, samples of the contents of each tube were diluted for turbidity measurements, the remainder of the tubes' contents centrifuged, suspended once in water, recentrifuged, suspended in 1.5 ml. of water, and 0.5 ml. pipetted into the side arm of a Warburg vessel for measurement of formic hydrogenlyase activity. A sample of the remaining suspension of washed bacteria was diluted for turbidity measurements to determine the amount of bacteria in the vessels.

The results of the experiments performed are presented in Table XVII. The appearance of formic hydrogenlyase activity took place only in the glucose-glutamate system. In this system, as in the formate-tryptone system, NaCl inhibited formic hydrogenlyase formation. According to the figures on cell density, no significant cell proliferation, as measured turbidimetrically, took place in those cell suspensions developing formic hydrogenlyase activity.

The results of Experiments 2 and 3 of this table are given in more detail in Figure IX. It can be seen from these results that cells grown five hours on nutrient agar produced more formic hydrogenlyase activity upon incubation with glutamate than cells grown eighteen hours. In addition, the older cells, after adaptation, showed a lag

TABLE XVII

The use of glutamate as a nitrogen source for formic hydrogenlyase formation

Experiment	Age of culture used	Time of incubation	System	Cell density (mg./ml.) beginning of incubation	Cell density (mg./ml.) end of incubation	Q-H_2 at end of incubation
1.	14 hours	6 hours	glucose + NaCl	0.36	--	0
			glucose + glutamate + NaCl	0.36	0.36	13
			formate + NaCl	0.36	--	0
			formate + glutamate + NaCl	0.36	0.36	0
			glucose	--	--	0
			glucose + glutamate	0.36	0.36	41
			formate	--	--	0
			formate + glutamate	0.36	0.37	0
2.	18 hours	13 hours	glucose	1.03	1.30	0
			glucose + glutamate	1.01	0.95	20
3.	5 hours	13 hours	glucose	0.32	0.54	0
			glucose + glutamate	0.32	0.34	35
			formate	0.32	0.30	0
			formate + glutamate	0.32	0.25	0
			glutamate	0.32	0.27	0

Conditions: 1.0 ml. 2% glucose or sodium formate; 2.0 ml. 3% sodium l-glutamate; 0.1 ml. 0.01 M $MgSO_4$ in all tubes; bacteria as shown; H_2O to 10.1 ml. 1.0 ml. 10% NaCl.

Incubations as described in text.

Measurement of formic hydrogenlyase:

In side arms: 0.5 ml. bacteria.

In main vessels: 0.033M phosphate buffer, pH 7.4, 0.067M formate. H_2 evolution measured for 30-60 minute intervals.

Other conditions as in Table XII.

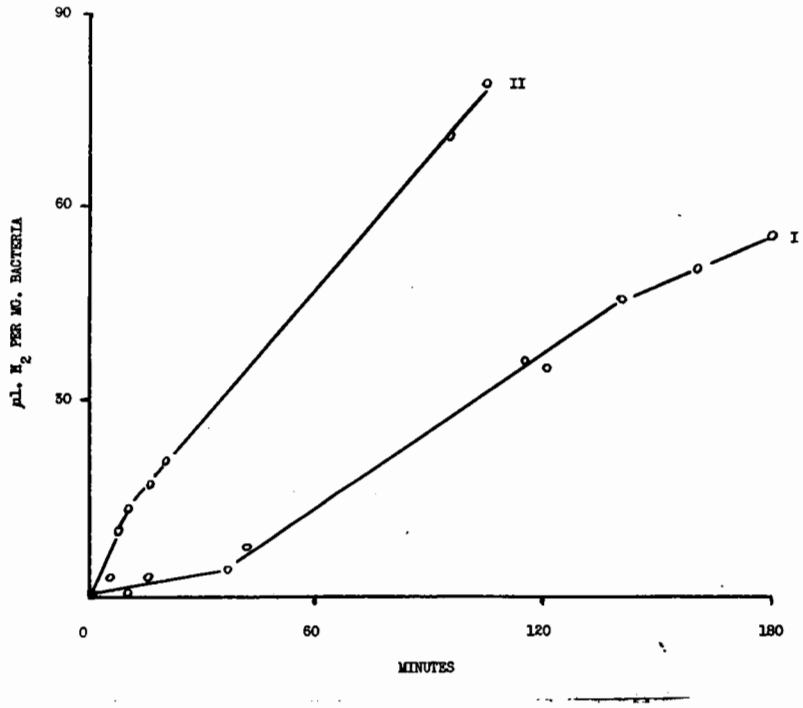


FIGURE IX

The effect of age of culture on adaptability in a glucose-glutamate medium.

Hydrogen production from formate after 13 hours' incubation in a glucose-glutamate medium.

I. 18 hour culture of bacteria.
II. 5 hour culture of bacteria.

Conditions: See Table XVII, Experiments 2 and 3, and text.

period of about thirty minutes before the production of hydrogen, while the five hour cells exhibited no such lag. In further experiments in which the amino acid requirements for formic hydrogenlyase formation were determined, cells grown five to seven hours in nutrient agar were used.

Formic hydrogenlyase formation in the glucose-glutamate system, the pyruvate-glutamate system, and in non-synthetic systems

When it had been found that glutamate alone could stimulate formic hydrogenlyase production in the presence of glucose , the time necessary for the formation of this enzyme system was examined. In Figure X are shown the results of an experiment in which the effectiveness of glutamate and of peptone as nitrogen sources for formic hydrogenlyase production were compared. It is apparent that there is a marked difference between the effects of peptone and of glutamate. The final Q-H_2 obtained in the presence of peptone was 1478; that obtained in the presence of glutamate, 156. This difference is far too great to be accounted for completely by the known stimulating effect of peptone on formic hydrogenlyase activity. In addition, the lag periods in the presence of peptone were 40 and 80 minutes, while in the presence of glutamate, they were 90 and 120 minutes. It was believed likely that time was required for the bacteria to synthesize substances from glutamate essential for the formation of the adaptive enzyme and that these substances were already present in peptone. When pyruvate

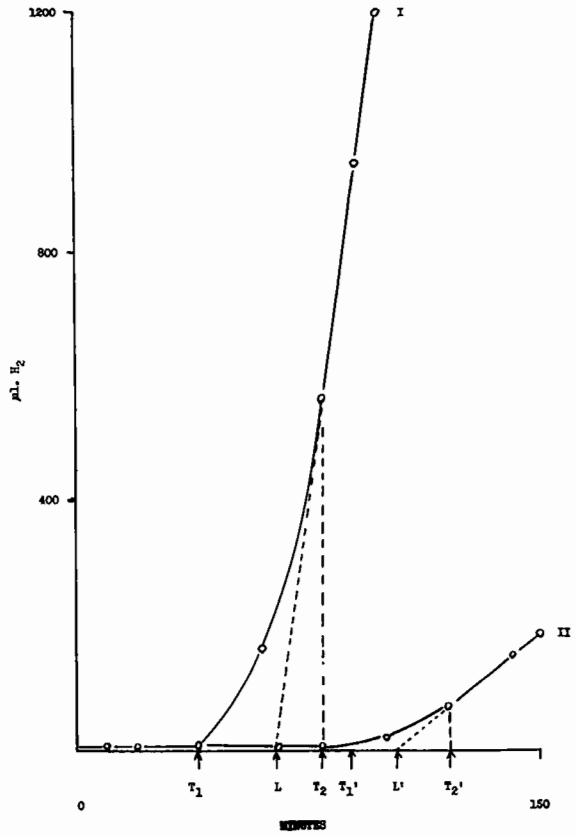


FIGURE X

Hydrogen production in glucose-peptone and glucose-glutamate systems.

I. Glucose + peptone
II. Glucose + glutamate

T_1, T_2, and L = $LagT_1$, $LagT_2$ and "Lag" for the glucose-peptone system.

T_1', T_2', and L' = The same values for the glucose-glutamate system.

Bacteria: Grown 5 hours on nutrient agar, 1.5 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.04 M l-glutamate, 1% peptone.

Other conditions as in Table XII.

was used as the source of adaptive substrate, the comparison of glutamate and peptone revealed a situation similar to that found when glucose was used (Table XVIII). It has already been mentioned (page 116) that with formate alone, glutamate gave no stimulation of hydrogen production by unadapted bacteria, in an experiment lasting 290 minutes.

TABLE XVIII

The comparative effects of glutamate and peptone on formic hydrogenlyase formation in the presence of pyruvate

	Lag, minutes T_1	T_2	Final Q-H_2
Pyruvate + 1% peptone	60	120	2305
Pyruvate + 0.04 M glutamate	160	170 (M)	62

Bacteria: Grown 5 hours on nutrient agar; 0.7 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M pyruvate, peptone and l-glutamate as shown.

Other conditions as in Table XII.

Non-synthetic media as stimulators of formic hydrogenlyase formation in the presence of pyruvate: peptone, casein hydrolysate, and gelatin hydrolysate

The effect of protein hydrolysates on formic hydrogenlyase production in the presence of pyruvate was examined to determine to what extent the stimulation of adaptation by peptone was due to the amino acids it contained. It has been found (100) that a casein hydrolysate can replace tryptone as a nitrogen source for formic

hydrogenlyase synthesis during growth in a medium containing glucose, but the periods of time required for this synthesis, using each source of nitrogen, have not been previously measured.

The results of experiments carried out in the Warburg apparatus, revealed that in the presence of pyruvate, the action of peptone could be virtually duplicated, both in respect to lag periods and final activity, by either casein hydrolysate or gelatin hydrolysate. These results are given in Table XIX.

The hydrolysate of gelatin used in earlier experiments contained humin formed in the process of acid digestion, which was removed when the hydrolysate was boiled with adsorbent charcoal and filtered. As may be seen from the results of Experiment 1 of Table XIX, this charcoal treatment removed some inhibitor either of adaptation or of formic hydrogenlyase activity. The nature of the inhibitor, however, was not investigated further.

Tryptophan was absent from both hydrolysates, since this amino acid is not present in gelatin, and is destroyed by acid hydrolysis. As the results of Experiments 2 and 3 of this table show, the addition of tryptophan to the pyruvate-casein hydrolysate system brought about a slight shortening of the lag periods and increase in the final rate of hydrogen production. The results of later experiments (see Table XXVI) have shown that a mixture of amino acids containing tryptophan has no effect on the formic hydrogenlyase activity of adapted

TABLE XIX

The effects of casein and gelatin hydrolysates on formic hydrogenlyase formation in the presence of pyruvate.

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1.	Pyruvate + 1% peptone	60	80 (M)	755
	Pyruvate + 0.7% gelatin hydrolysate	60	80 (M)	243
	Pyruvate + 0.7% charcoal-treated gelatin hydrolysate	40	80 (M)	589
2.	Pyruvate + 1% peptone	50	110	1230
	Pyruvate + 1.67% casein hydrolysate	70	110	970
	Pyruvate + 1.67% casein hydrolysate + 0.001 M tryptophan	50	100	1082
3.	Pyruvate + 1% peptone	80	109 (M)	764
	Pyruvate + 1.67% casein hydrolysate	70	99	846
	Pyruvate + 1.67% casein hydrolysate + 0.001 M tryptophan	60	99	1160
	Pyruvate + 1.67% casein hydrolysate + 0.001 M tryptophan + 0.067 M NaCl	60	99	732

Bacteria: Grown overnight on nutrient agar; 1.68 mg. bacteria/vessel in Experiment 1, 1.2 mg./vessel in Experiment 2, 1.4 mg./vessel in Experiment 3.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M pyruvate, 0.043 M NaCl from casein hydrolysate, other final concentrations as shown.

Other conditions as in Table XII.

cells, so that the action of tryptophan on the final rate was probably due to an effect on adaptation. It is also seen that NaCl in this system had but little effect on adaptation. Only a small decrease in final rate and no change in the lag periods were noted when the amount of NaCl present was more than doubled.

A completely synthetic medium for the stimulation of formic hydrogenlyase production

Once it had been found that acid hydrolysates of casein or gelatin were as efficient as peptone in promoting formic hydrogenlyase production in the presence of pyruvate, attempts were made to replace these hydrolysates with a mixture of amino acids. At first those six amino acids found most effective by Billen and Lichstein (100) for stimulating formic hydrogenlyase production were used. The results of an experiment in which the effects of these amino acids were tested are given in Table XX. Following the example of the above authors, the amino acids were tested in pairs, as well as all together. Though all but one of the pairs of amino acids tested served as a source of nitrogen for formic hydrogenlyase production, none brought about the production of this enzyme in as short a time as did peptone.

Next, to determine if the effect of casein hydrolysate were due solely to the amino acids contained therein, a mixture of all the amino acids found in casein hydrolysate, with the addition of tryptophan, nor-leucine and hydroxyproline was used. The exact composition of such

TABLE XX

The effects of cystine, tyrosine, lysine, valine, glutamate, and methionine on formic hydrogenlyase formation in the presence of pyruvate

System	Lag, minutes T_1	T_2	Final Q-H_2
Pyruvate + peptone	70	120 (M)	1258
Pyruvate + cystine + tyrosine	>120	-	0
Pyruvate + lysine + valine	140	160	23
Pyruvate + glutamate + methionine	120	180 (M)	76
Pyruvate + six amino acids above	160	180	35

Bacteria: Grown 5 1/2 hours on nutrient agar; 1.1 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M pyruvate, 1% peptone.

Final concentration of amino acids in pairs: 0.00033 M l-cystine, 0.002 M l-tyrosine, 0.02 M l-lysine, 0.02 M dl-valine, 0.04 M sodium l-glutamate, 0.04 M dl-methionine. Final concentration of amino acids in a mixture of six: One half that used in pairs.

Other conditions as in Table XII.

an amino-acid mixture has already been described (page 107). For this experiment, and for most subsequent experiments on the nitrogen sources necessary for formic hydrogenlyase formation, glucose instead of pyruvate was used as a source of the adaptive substrate. The lag periods in the presence of glucose were generally shorter than those in the presence of pyruvate. With glucose, experiments could more conveniently be followed until a steady rate of gas evolution was attained. Previous experiments had indicated that in the presence of glucose or pyruvate, the nitrogen sources needed for formic hydrogenlyase formation were the same. This was fully confirmed in later experiments (see Table XXV).

The results presented in Figure XI show the comparative effects of peptone and a synthetic mixture of amino acids as nitrogen sources for formic hydrogenlyase production in the presence of glucose. In this experiment the lag periods were approximately equal, though the final rate in the glucose-peptone system was slightly higher than that in the glucose-amino acid system. In addition to the controls shown in this experiment, it should be noted again that these bacteria were never found able to produce hydrogen from peptone alone.

The comparative effects of increasing the concentration of the nitrogen source, using peptone, a complete amino acid mixture, and glutamate alone, are presented in Table XXI. Increasing the concentration of each nitrogen source increased the final rate of hydrogen evolution; the lag periods changed little in the range of concentrations

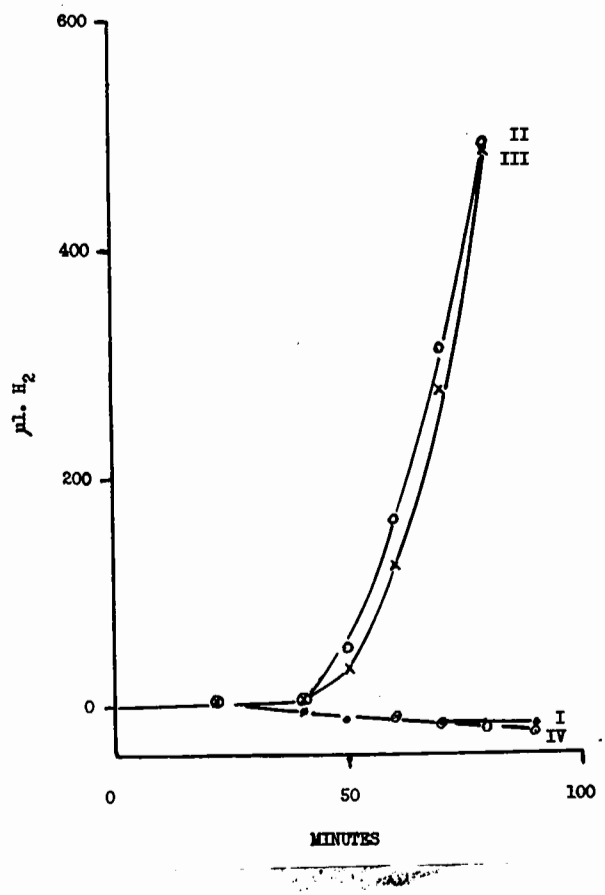


FIGURE XI

Hydrogen production in glucose-peptone and glucose-amino acid systems

I. Glucose
II. Glucose + 20 amino acids
III. Glucose + peptone
IV. 20 Amino acids

Bacteria: Grown 5 hours on nutrient agar, 1.5 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 1% peptone, amino acids as on page 107 .

Other conditions as in Table XII.

TABLE XXI

The effect of varying concentrations of amino acids and peptone on formic hydrogenlyase formation in the presence of glucose:

Experiment	System	Lag, minutes T_1	T_2	"Lag"	Final Q-H_2
1.	Glucose	>70	-	>70	0
	Glucose + 0.5% peptone	50	60 (M)	50 (M)	470
	Glucose + 1% peptone	30	60 (M)	48 (M)	857
	Glucose + 2% peptone	30	60	44	1351
	Glucose + 5% peptone	30	60	41	2120
	Glucose + 10% peptone	30	60	42	2305
2.	Glucose + 1/2 normal^{20} amino acids' concentration	40	80	52	486
	Glucose + normal^{20} amino acids' concentration	40	80	53	694
	Glucose + 1.8 x normal^{20} amino acids' concentration	50	80	65	876
3.	Glucose + 0.001 M glutamate			140	164
	Glucose + 0.01 M glutamate			137	208
	Glucose + 0.05 M glutamate			115	272
	Glucose + 0.1 M glutamate			117	294

Bacteria: Grown overnight for Experiment 1, for 6 hours for Experiments 2 and 3, on nutrient agar; 1.33 mg. bacteria/vessel in Experiment 1, 1.5 mg./vessel in Experiment 2, 1.43 mg./vessel in Experiment 3.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, peptone and l-glutamate as shown. Normal concentrations of all 20 amino acids as given on page 107.

Other conditions as in Table XII.

used. Because of the known stimulating effect of peptone on formic hydrogenlyase activity, it cannot be concluded whether the increased final rate of hydrogen evolution found in Experiment 1 was due to increased enzyme formation or to a stimulatory effect on enzyme activity. Later experiments have shown, however, that the amino acids mixture as well as glutamate alone had no effect on the formic hydrogenlyase activity of adapted cells (Table XXVI). It may be concluded that the increased rate observed with increasing concentrations of pure amino acids and of glutamate is due to increased formic hydrogenlyase synthesis.

Summary and Conclusions:

In the presence of formate, washed cells of E. coli synthesized the enzyme system, formic hydrogenlyase, if peptone or tryptone were added to the medium in which the cells were suspended. If instead of peptone or tryptone, a casein hydrolysate, a gelatin hydrolysate, or any one amino acid were added, the bacteria could synthesize little or no formic hydrogenlyase.

In the presence of glucose or pyruvate, each of which is broken down to yield formate, formic hydrogenlyase was formed almost as rapidly in the presence of a casein or gelatin hydrolysate as in the presence of peptone. Casein hydrolysate could be replaced by a synthetic mixture of amino acids.

In the presence of glucose or pyruvate, formic hydrogenlyase formation took place if only sodium glutamate were added to the medium in which the cells were suspended. The time for the production of formic hydrogenlyase, however, was approximately twice as long if only glutamate were added as if peptone were added.

In the presence of glucose, with peptone, glutamate, and with a complete amino acid mixture, raising the concentration of the source of nitrogen increased the final rate of hydrogen evolution. Little change in lag periods was noted over the range of concentrations of each nitrogen source tested. For glutamate, and for the complete amino acid mixture, the increased rate could be ascribed to an increased enzyme synthesis. Since peptone stimulates formic hydrogenlyase activity as well as formic hydrogenlyase formation, the interpretation of the effects of increased peptone concentration was less clear.

PART II

The Determination of a Simplified Nitrogen Source for Formic Hydrogenlyase Formation

The determination of the amino acids whose presence is most important for formic hydrogenlyase formation.

Once it was established that a synthetic amino-acid mixture could serve as a nitrogen source for formic hydrogenlyase formation to approximately the same extent as peptone or as a protein hydrolysate, experiments were devised to determine which of the twenty amino acids were most important to formic hydrogenlyase formation.

Before the start of these experiments, it was found separately that the prescence of histidine was extremely important to this adaptive process. Omission of this amino acid from the complete mixture increased both lag periods by 60 minutes or longer, and greatly lowered the final rate of hydrogen production.

A preliminary experiment (Table XXII) showed that of the groups into which the amino acids had been arbitrarily divided, only the second, that containing glutamate and methionine, could be omitted without markedly increasing the lag periods. The following plan was devised

for investigating the importance of the individual amino acids: In a series of experiments, the effects on the lag periods and the final rate of omitting each of the twenty amino acids were observed.

TABLE XXII

The effect of omission of groups of amino acids on formic hydrogenlyase formation.

System	Lag, minutes T_1	T_2	Final Q-H_2
Glucose + all amino acids	40	80	686
Glucose + all amino acids but Group I	60	110	351
Glucose + all amino acids but Group II	40	80	775
Glucose + all amino acids but lysine, arginine, and tryptophan	60	100	656
Glucose + all amino acids but Group IV	60	123 (M)	359

Bacteria: Grown 7 hours on nutrient agar, 1.5 mg. bacteria/vessel.
In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, concentrations of amino acids and composition of groups as given on page 107.

Other conditions as in Table XII.

In these experiments, groups of amino acids were also left out, so that the effect of omitting each amino acid in the group could be compared with that of omitting the group as a whole. The results of this series of experiments are presented in Table XXIII; the amino acids found most important are underlined. Experiment 6 of this table is illustrated in Figure XII. From the results given in this figure, it is seen that the increased lags and decreased final rate observed when the four amino acids, aspartate, cystine, tyrosine, and phenylalanine, were

TABLE XXIII

Formic hydrogenlyase formation in a complete amino acid medium: The effect of omitting separately each amino acid and of omitting groups of amino acids.

Experiment	1			2			3			4			5			6			7		
Amino acid omitted	Lag T_1	T_2	$Q-H_2$	Lag T_1	T_2	$Q-H_2$	Lag T_1	T_2	$Q-H_2$	Lag T_1	T_2	$Q-H_2$	Lag T_1	T_2	$Q-H_2$	Lag T_1	T_2	$Q-H_2$	Lag T_1	T_2	$Q-H_2$
None	40	80	694				40	80	788	50	90	828	40	80	810	40	75	810	40	75	875
glycine							50	110	662	50	90	872									
alanine							40	80	774	50	90	854									
proline				40	80	706	40	90	653												
hydroxy proline							40	80	738	50	90	854									
serine				50	100(M)	715	50	110	608												
threonine				40	80	729	40	80	696												
lysine	50	90	786	40	80	862															
arginine	>120	-	0	>120	-	0				60	110	808							60	115(M)	658
histidine																					
tryptophan	50	80	727	50	80	905															
glutamate										50	90	800									
methionine										50	90	800									
leucine													50	105	405				50	90	331
isoleucine													40	80	698						
norleucine													40	90	808						
aspartate																70	110	440	100	115(M)	16
tyrosine																50	80	765			
phenylalanine																40	75	769			
valine													50	90	796						
cystine																40	75	840			
Group omitted																					
No. 1							60	110	424												
No. 2										50	90	762									
arginine, lysine, tryptophan	70	100	540	--	100(M)	490															
leucine, isoleucine, norleucine, valine													50	105	372						
aspartate, cystine, tyrosine, phenylalanine																60	110	492			

TABLE XXIII (Cont'd)

Bacteria: Grown 5-7 hours on nutrient agar; 1.5 mg. bacteria/vessel in all experiments but 4, 1.0 mg./vessel in Experiment 4.

In main vessles: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose. Amino acid concentrations and composition of groups as given on page 107.

Lag periods expressed in minutes. Q-H_2 represents final value.

Other conditions as in Table XII.

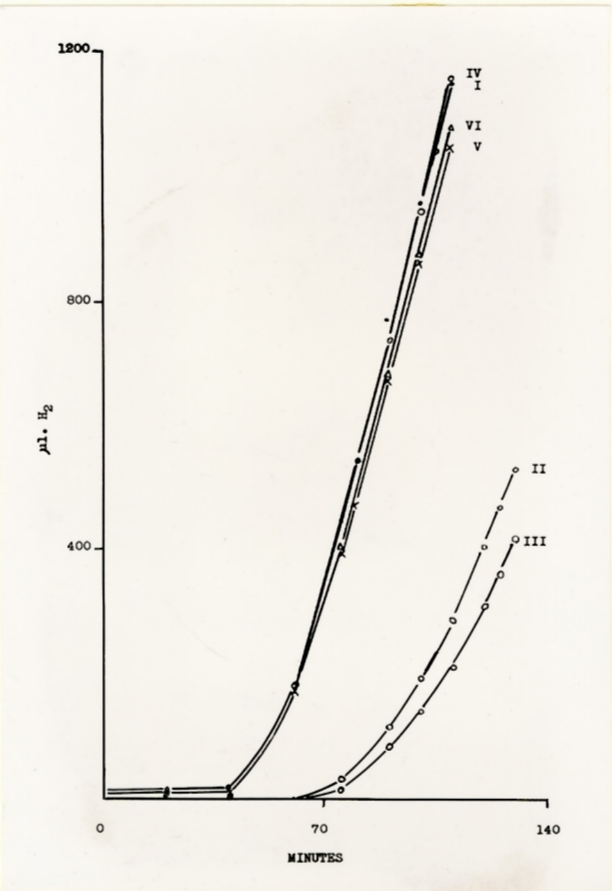


FIGURE XII

The effects of omission of aspartate, cystine, tyrosine, and phenylalanine on formic hydrogenlyase formation in a glucose- twenty amino acid system.

Glucose +

I. All twenty amino acids.
II. All twenty amino acids but aspartate, cystine, tyrosine, and phenylalanine.
III. All twenty amino acids but aspartate
IV. All twenty amino acids but cystine
V. All twenty amino acids but tyrosine
VI. All twenty amino acids but phenylalanine.

Conditions: See Experiment 6, Table XXIII.

omitted could be attributed almost completely to the omission of aspartate. In using this method, there was the danger that one amino acid could replace another; thus, the importance of each might not be realized when each was omitted separately. The result of some experiments carried out to check this possibility are given in Table XXIV. From these it was seen that the amino acids found important by the effect of their omission could replace the entire group in which they occurred. For example, those amino acids found important in the first three groups could virtually replace these groups. Proline had not been found important, but it was tried in this simplified system to determine whether in its absence from the complete amino acid medium it had been replaced by hydroxyproline. Experiment 2 of Table XXIV shows the results on formic hydrogenlyase formation of a combination of those five amino acids found most important, serine, histidine, arginine, leucine, and aspartate. It may be seen that with these five only slightly longer lag periods and lower final rates were observed than with all twenty amino acids. From the results of this experiment, as well as from the data in Tables XXII and XXIII, it is seen that the omission of glutamate had little effect on adaptation, even though glutamate alone had been found a better stimulator of adaptation than any individual amino acid tried.

An experiment showing the effects of omitting in turn each amino acid from a simplified mixture of the five amino acids given above plus glutamate is reported in Table XXV. If the results in this

TABLE XXIV

Formic hydrogenlyase formation with simplified amino acid mixtures

Experiment	System	Lag, minutes T_1	T_2	"Lag"	Final $Q-H_2$
1.	Glucose + all amino acids	40	80	56	810
	Glucose + glutamate, histidine, arginine, proline, and serine	80	120	103	228
	Glucose + glutamate, histidine, arginine, proline, serine, and Group IV	50	90	60	676
	Glucose + glutamate, histidine, arginine, serine, and Group IV	50	90	60	736
2.	Glucose + all amino acids	40	75	53	875
	Glucose + serine, arginine, leucine, aspartate, and histidine	50	80	63	558
	Glucose + serine, arginine, leucine, aspartate, histidine, and glutamate	50	80	62	697
3.	Pyruvate + serine, arginine, leucine, aspartate, and histidine	70	100	78	598

Bacteria: Grown 5-6 hours on nutrient agar; 1.5 mg./vessel in Experiment 1 and 2, 1.45 mg./vessel in Experiment 3.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.067 M pyruvate, composition of Group IV as given on page 107. Individual amino acids in same concentrations as in complete mixture. See page 107 and Table XXVII (normal concentrations).

Other conditions as in Table XII.

table are compared with those in Table XXIII, it is seen that the omission of leucine and serine from this simplified medium brought about more marked increases in the lag periods and decreases in the final rates than the omission of the same amino acids from the mixture of twenty amino acids. This suggest that some replacement of leucine and serine by other amino acids in the complete mixture did take place.

TABLE XXV

The effect on formic hydrogenlyase formation of omitting each of a simplified mixture of amino acids.

System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
Glucose + all six amino acids below	50	95	682
Glucose + all six amino acids below but arginine	60	110	758
Glucose + all six amino acids below but aspartate	70	120	406
Glucose + all six amino acids below but leucine	80	120	92
Glucose + all six amino acids below but serine	70	120	398
Glucose + all six amino acids below but histidine	80	120	108
Glucose + all six amino acids below but glutamate	50	90	624

Bacteria: Grown 6 hours on nutrient agar; 1.5 mg. bacteria/vessel.
In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose. Amino acids: arginine, aspartate, leucine, serine, histidine, and glutamate in concentrations as given in Table XXVII (normal concentrations).

Other conditions as in Table XII.

The action of amino acids upon the formic hydrogenlyase activity of adapted bacteria.

To interpret the results of these experiments, it was essential to know if the amino acids had any effect on the formic hydrogenlyase activity of E. coli once it was formed. It was possible, for example, that the low final rate observed when histidine was omitted was due to a stimulating action of this amino acid upon formic hydrogenlyase activity rather than on formic hydrogenlyase formation. In Table XXVI are shown the results of experiments in which the effects of various single amino acids and of amino acid mixtures on the formic hydrogenlyase activity of adapted cells were measured. These experiments were carried out at pH 6.8. Although the foregoing experiments were carried out at an initial pH of 7.4, the formation of organic acids from glucose caused the pH to fall to approximately 6.4 at the end of 120 minutes, (see Table LI). Most of the hydrogen evolution took place at an intermediate pH value, and for this reason, a pH of 6.8 rather than of 7.4 was chosen for these measurements. As the results show, neither glutamate alone, the five amino acids found most effective individually, a mixture of these five, nor a mixture of all twenty amino acids had any significant effect on the activity of the adaptive enzyme once formed. It was concluded that the final rates observed in the presence of the amino acids could be considered as indicative of the amount of adaptive enzyme formed.

TABLE XXVI

The effect of amino acids on formic hydrogenlyase activity:

Experiment	System	Q-H_2
1.	Formate	526
	Formate + serine	563
	Formate + arginine	545
	Formate + leucine	541
	Formate + histidine	517
	Formate + aspartate	488
	Formate + 5 amino acids above	532
	Formate + 20 amino acids	538
2.	Formate	428
	Formate + 0.2 M glutamate	432

Bacteria: Grown 20-22 hours on glucose broth; 1.5 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 6.8, 0.067 M formate, amino acid concentrations as given in page 107 and Table XXVII (normal concentrations), sodium l-glutamate as shown.

H_2 evolution measure from 0 to 33 minutes in Experiment 1, from 10 to 40 minutes in Experiment 2.

Other conditions as in Table XII.

The minimal effective concentration of each of the five most important amino acids.

Following the demonstration that formic hydrogenlyase formation could be stimulated to virtually the same extent by a mixture of five amino acids as by a mixture of twenty, a series of experiments was carried out to determine the smallest concentration of each of

these five amino acids which would bring about a maximal stimulation of adaptation. Glucose was used as a source of the adaptive substrate in these experiments, and the concentration of each of the amino acids was varied in turn, while the concentrations of the other four were held constant. The results of this series of experiments are presented in Table XXVII. In this table, the minimal effective concentrations found are underlined.

Histidine brought about a striking stimulation of adaptation in concentrations as low as 2×10^{-5} M, and the degree of stimulation was increased only slightly by raising the histidine concentration above this level. Arginine was also effective in a low concentration, 3.7×10^{-4} M, but this amino acid was not as important to adaptation as any of the other four. The concentrations of serine, aspartate and leucine could not be lowered beyond 10^{-2} M without bringing about considerable increases in the lag periods and decreases in the final rates. It should also be noted that very little effect was obtained by increasing the concentrations of the amino acids over those normally used, i.e., those given on page 107 and in Table XXVII.

After the minimal effective concentrations of each amino acid had been determined separately, it was found that a mixture in which each amino acid was present in minimal effective concentration served almost as well as a nitrogen source for adaptation as a mixture in which each amino acid was present in the normal concentrations previously used, (Table XXVIII).

TABLE XXVII

The effect of varying the concentration of each of the five essential amino acids on formic hydrogenlyase formation.

System: Glucose, five amino acids

Experiment	Amino acid varied	Concentration mg./vessel	Concentration molar	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1.	Serine	0	0	70	110	333
		0.5	0.0016	60	120	415
		5.0	0.016	50	80	663
2.	Serine	0	0	70	130	458
		2.5	0.0079	50	90	622
		5.0	0.016	50	90	616
		10.0	0.032	40	90	612
3.	Histidine	0	0	70	100	149
		0.00236	0.0000041	70	120	160
		0.0118	0.0000205	50	100	598
		0.059	0.000103	50	80	600
		0.118	0.000205	50	80	637
		1.18	0.00205	50	80	672
		3.54	0.00615	50	80	634
4.	Histidine	0.0	0	95	100	51
		0.0047	0.0000082	60	115	309
		00.0083	0.000014	50	80	382
		0.0118	0.0000205	50	80	545
		1.18	0.00205	50	80	621
5.	Arginine	0.0	0.0	60	100	583
		0.0236	0.0000373	70	100	510
		0.236	0.000373	50	90	649
		2.36	0.00373	50	90	640
6.	Arginine	0.0	0.0	60	110	568
		0.0236	0.0000373	70	105	476
		0.118	0.000187	50	80	576
		2.36	0.00373	50	80	631
		7.08	0.0112	50	90	648

TABLE XXVII (Cont'd)

Experiment	Amino acid varied	Concentration mg./vessel	Concentration molar	Lag, minutes T_1	Lag, minutes T_2		Final Q-H_2
7.	Leucine	0.0	0.0	80	100		128
		0.072	0.000183	60	100	(M)	168
		0.72	0.00183	50	100		479
		7.2	0.0183	50	90		640
		14.4	0.0366	50	90		683
8.	Leucine	0.0	0.0	80	110		115
		0.72	0.00183	60	100		387
		3.6	0.0092	50	90		550
		7.2	0.0183	50	80		631
9.	Aspartate	0.042	0.000105	70	120		488
		4.2	0.0105	50	80		621
10.	Aspartate	0.0	0.0	80	110		333
		0.084	0.000211	80	110		333
		0.21	0.00053	80	120		298
		0.42	0.00105	80	110		503
		4.2	0.0105	50	80		636
		8.4	0.0211	50	80		650
11.	Aspartate	0.0	0.0	80	110		443
		0.84	0.00211	60	120		413
		2.52	0.0064	50	90		465
		4.2	0.0105	50	90		692

Bacteria: Grown 5-7 hours on nutrient agar, 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose. The five amino acids in the following normal concentrations: 5.0 mg./vessel dl-serine, 1.18 mg./vessel l-histidine HCl, 2.36 mg./vessel l-arginine HCl, 7.2 mg./vessel l-leucine, 4.2 mg./vessel dl-aspartic acid. Varied concentrations as shown.

Minimal effective concentrations are underlined.

Other conditions as in Table XII.

TABLE XXVIII

Formic hydrogenlyase formation with minimal effective concentrations of all amino acids

System	Lag, minutes T_1	T_2	Final $Q-H_2$
Glucose + five amino acids (normal concentration)	40	85	672
Glucose + five amino acids (minimal concentration)	40	75	472

Bacteria: Grown 5 1/2 hours on nutrient agar, 1.5 mg. bacteria/vessel.
In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, normal and minimal effective concentrations of amino acids as given in Table XXVII.

Other conditions as in Table XII

The degree of cell proliferation during adaptation

It has already been mentioned that bacterial cells suspended in the presence of glucose and glutamate could develop formic hydrogenlyase activity without growth. In the previous experiments, carried out completely in the Warburg apparatus, it was not possible to determine whether growth had occurred by turbidimetric measurements. When glucose was used as a source of the adaptive substrate and the initial pH was 7.4, after prolonged shaking the bacterial cells in the vessels became clotted in long strings. A number of the cells were seen to adhere to the walls of the vessel. Although the cells in this condition were

still able to bring about a rapid evolution of hydrogen, measurements of the turbidity at the end of the experiment would, of course, have been meaningless.

It was discovered, however, that if incubation were carried out at a lower initial pH, the cells did not become clotted during the experiment, and the optical density of the contents of the Warburg vessel at the end of the experiment gave a measure of the extent to which growth had taken place. Some of the other effects of the initial pH upon adaptation will be dealt with in detail later.

The experiments to be reported were carried out using 0.5 ml. of a 0.2 M phosphate buffer, pH 5.7, in each Warburg vessel. Additions of the neutralized amino acid solutions brought the initial pH to 6.1. In some of these experiments the effects on growth and adaptation of omitting histidine and serine were observed. The minimal effective concentration of histidine (2×10^{-5} M) was used in these experiments, since the presence of this small amount of neutral solution could have no effect on the pH. In the experiment in which the effects of omitting serine were tested, this amino acid was dissolved in distilled water and the solution was not neutralized further. In the presence or absence of serine, the initial pH was 6.1.

Three or more Warburg vessels containing media of identical composition and the same amount of bacteria were set up; hydrogen evolution from these was followed in the usual manner, and at varying

periods of time each vessel was removed from the bath. The grease was carefully wiped from the vessel, the paper removed from the center well, and the center well blotted with dry filter paper. After pouring off the contents, the vessel was washed out with two 3.0 ml. portions of water; a small rubber spatula was used to dislodge any bacteria adhering to the walls. The contents of the vessel and the washings were made up to a final volume of 10.0 ml. and the optical density of this suspension read. The time elapsing between removal of a pair of vessels from the bath and reading the optical density of their diluted contents was about ten minutes. In Table XXIX are shown the results of a number of such experiments. The figures for hydrogen evolution in each experiment represent that of the vessel shaken for the longest time. Experiment 1 of this table was a control experiment. Within experimental error, a buffered suspension of bacteria in the presence of glucose exhibited no change in turbidity on being shaken for up to 90 minutes in Warburg vessels. In the experiments with 5-6 hour cultures of bacteria in the presence of glucose and the five most important amino acids, a small amount of growth did occur in one hour. The increase in turbidity with time was slight, but measurable, and occurred in all cases where these five amino acids were present. When histidine was omitted (Experiments 2 and 3) a small increase in turbidity was noted; it is impossible to say from the results of these experiments whether or not growth occurred in the absence of histidine. In the absence of serine, a small amount of growth appears to have taken place.

TABLE XXIX

Measurement of growth during formic hydrogenlyase formation in manometric experiments

Experiment	Age of culture	System		Time in minutes 0	10	20	30	35	40	50	60	70	80	90	100	110	120	130	210
1.	5 hours	Glucose	μl.H_2	0										0					
			mg. bact./vessel	1.50			1.60				1.51			1.55					
2.	$5\frac{5}{4}$ hours	Glucose + 5 amino acids	μl.H_2	0	9	8	18		72	159	268	387	487	575					
			mg. bact./vessel	1.50			1.50				1.77			1.83					
		Glucose + 4 amino acids (-histidine)	μl.H_2	0	7	9	16		40	79	124	179	240	303					
			mg. bact./vessel	1.50			1.44				1.68			1.59					
3.	5 hours	Glucose + 5 amino acids	μl.H_2	0	14	14	18		37	78	126	-	233	320					
			mg. bact./vessel	1.50			1.65				1.71			1.83					
		Glucose + 4 amino acids (-histidine)	μl.H_2	0	7	6	7		24	54	90	-	172	207					
			mg. bact./vessel	1.50			1.47				1.65			1.62					
4.	5 hours	Glucose + 5 amino acids	μl.H_2	0	14	11	23		74	141	248	360	465	554					
			mg. bact./vessel	1.50			1.65				1.74			1.86					
		Glucose - 4 amino acids (-serine)	μl.H_2	0	8	3	3		12	33	65	117	174	237					
			mg. bact./vessel	1.50			1.62				1.65			1.71					
5.	5 hours	Glucose + 5 amino acids	μl.H_2	0	10	10	14		40	97	175	268	372	482	554	-	700	760	1098
			mg. bact./vessel	1.50			1.63				1.80			1.92			1.90		2.00

TABLE XXIX (Cont'd)

Experiment	Age of culture	System		Time in minutes 0	10	20	30	35	40	50	60	70	80	90	100	110	120	130	210
6.	13 2/3 hours	Glucose + 5 amino acids	μl.H_2	0	9	9	9	25	68	160	259	375	485	563					
			mg. bact./vessel	1.55				1.45			1.50			1.65					
7.	16 1/3 hours	Glucose + 5 amino acids	μl.H_2	0	11	7	11	28	65	156	265								
			mg. bact./vessel	1.53				1.56			1.59								

Bacteria: As shown, all grown on nutrient agar.

In main vessels: 0.033 M phosphate buffer, pH 5.7, 0.033 M glucose, amino acids as given in Table XXVII (normal concentrations).

Growth measured as described in text.

Other conditions as in Table XII.

The 5-6 hour cultures used in these experiments were in a period of active growth at the time they were harvested. Experiments were carried out to determine to what extent adaptation was accompanied by growth in bacterial cultures which had grown to a maximal extent on agar plates. For experiments 6 and 7 of Table XXIX such cultures were used. With these, though adaptation took place in approximately the same period of time as in the younger cultures, there was no growth for 60 minutes, by which time the maximal rate of hydrogen evolution had been reached. It was concluded from this set of experiments, that though the medium and conditions of incubation made growth possible, adaptation could occur without growth taking place, and was, to that extent, independent of growth.

A simple calculation shows that even though growth sometimes occurred during adaptation it would be impossible to account for the appearance of formic hydrogenlyase activity by postulating the growth of a few cells possessing this activity. The maximal $Q\text{-}H_2$ observed in the experiments reported in Table XXIX was about 400. At the beginning of the experiments, no hydrogen evolution was observed. If this apparent inactivity represented a low activity, e.g., a $Q\text{-}H_2$ of 10, a forty-fold increase in the number of bacteria between zero time and 60 minutes would be required to bring about the final rate of hydrogen evolution observed. When any growth did take place in this time, an increase of 10-15% at most was actually observed. The appearance of formic hydrogenlyase activity, therefore, could not have been due to the growth of adapted cells.

In these experiments, only the total cell mass was measured. It should be noted that Stephenson and Stickland (57) also followed the increase in the number of viable cells with the development of formic hydrogenlyase activity. They found by this method as well as by a method of total counts that formic hydrogenlyase synthesis was not dependent upon cell proliferation.

Two other aspects of Table XXIX deserve comment. First, at a lower pH there was a decrease in the lag periods. In these experiments, the lag periods (T_1 and T_2) for the glucose-five amino acid system were only 30 and 50 minutes, as compared with lags of 40 and 80 minutes at an initial pH of 7.4. Second, at the lower pH value, the omission of histidine did not bring about an extension of the lag period, though it did cause a decrease in the final rate of hydrogen evolution. Both these phenomena were studied in detail, and the results of these studies will be given in a later section.

5-7 Hour cultures of bacteria had been used in the experiments establishing a simplified amino acid medium which would act as a nitrogen source for adaptation, as well as in the experiments in which the minimal effective concentrations of the amino acids in this medium were determined. This was done because of the greater adaptability found in such cultures when glutamate alone was used as a source of nitrogen. It was noted during the experiments given in Table XXIX, however, that with a mixture of the five most important amino acids, the adaptability was practically as great with 13-16 hour cultures

as with 5-7 hour cultures. For this reason, both types of cultures were used in the succeeding experiments.

Summary and conclusions

A mixture of five amino acids; serine, histidine, arginine, leucine, and aspartate, was found capable of replacing a mixture of twenty amino acids as a nitrogen source for formic hydrogenlyase formation by resting cells of E. coli. The smallest concentration of each amino acid which was able to give a maximal effect was determined. These concentrations were:

dl-serine	7.9×10^{-3} M
l-histidine	2×10^{-5} M
l-arginine	3.7×10^{-4} M
l-leucine	1.8×10^{-2} M
dl-aspartate	1×10^{-2} M

It was shown that though this simplified medium supported growth, formic hydrogenlyase could be synthesized without growth taking place. In older cultures of E. coli adaptation took place without growth, while in younger cultures, a small amount of growth usually accompanied adaptation. Calculations showed that it would be impossible to account for the amount of enzyme formed by any scheme involving the proliferation of a small fraction of the cells already possessing formic hydrogenlyase activity.

PART III

Energy Sources for Formic Hydrogenlyase Synthesis

The effect of aspartate and fumarate on formic hydrogenlyase formation

It was early found (Table XV) that a gelatin hydrolysate could not stimulate adaptation in the presence of formate alone. When added to the formate-tryptone or formate-peptone system, however, the hydrolysate greatly increased the final rate of hydrogen production. Following this observation, experiments were carried out to determine if this property of the gelatin hydrolysate could be ascribed to a single amino acid. The results of these experiments, given in Table XXX, show that of seventeen amino acids tested, some inhibited hydrogen production, and only one, aspartate, stimulated hydrogen production in the formate-peptone system. The inhibitory effects were not examined further.

The enzyme, aspartase, which catalyses the reaction:

$$\text{Aspartate} \rightleftharpoons \text{Fumarate} + NH_3 ,$$

has long been known to occur in *E. coli*, and was, in fact, first demonstrated in that organism, (146). It was thought possible that the stimulatory action of aspartate was due to the fumarate derived

TABLE XXX

The effect of individual amino acids on hydrogen production in the formate-peptone system

Experiment	Amino Acid Added	Concentration (Molar)	Lag, minutes T_1	Lag, minutes T_2	Lag, minutes "Lag"	Final Q-H_2
1.	None	-			52	130
	dl-alanine	0.1			72	72
	dl-methionine	0.1			76	83
	glycine	0.1			64	70
	dl-serine	0.1			66	83
2.	None	-	40	85		71
	glycine	0.01	40	85		63
	l-proline	0.01	40	85		80
	dl-aspartate	0.1	50	100		215
	l-leucine	0.06	60	85		56
	l-lysine	0.1	60	85		37
	dl-phenylalanine	0.03	60	85		33
	dl-threonine	0.1	60	85		35
	l-tryptophan	0.02	60	85		41
	l-glutamate	0.1	40	85		36
3.	None	-	60	120		26
	dl-aspartate	0.1	60	100		113
	dl-valine	0.1	60	100		23
	l-tyrosine	0.001	60	120		41
	l-hydroxyproline	0.1	60	120		31
	dl-norleucine	.03	60	120		35
	dl-isoleucine	.06	60	120		33
4.	None	-			56	76
	l-tyrosine	0.0025			56	75

Bacteria: Grown overnight on nutrient agar; 1.4 mg. bacteria/vessel in Experiment 1, 1.5 mg./vessel in Experiments 2, 3 and 4.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067M formate, 1% peptone, additions of amino acids as shown.

Other conditions as in Table XII.

from it, and an experiment was carried out in which the effects of fumarate and aspartate on hydrogen production in the formate-peptone system were compared. In this experiment (Table XXXI) fumarate was found to give a stronger stimulation of hydrogen production than an equal concentration of dl-aspartate.

TABLE XXXI

The comparative effects of aspartate and fumarate on hydrogen production in the formate-peptone system.

System	Lag, minutes T_1	T_2	Final $Q-H_2$
Formate + peptone	60	80	50
Formate + peptone + aspartate	60	110	187
Formate + peptone + fumarate	60	100	198

(In separate experiments, no H_2 was evolved from peptone + fumarate or peptone + aspartate media. See also Table XXXIII)

Bacteria: Grown overnight on nutrient agar; 1.5 mg. bacteria/vessel

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, 1% peptone, 0.1 M dl-aspartate, 0.1 M fumarate.

Other conditions as in Table XII.

The action of fumarate was then investigated further. The results of two experiments on the effects of different concentrations of fumarate on (1) hydrogen evolution by unadapted bacteria in the formate-peptone system, and (2) hydrogen evolution from formate by adapted bacteria, are reported in Table XXXII. It is seen that all concentrations

TABLE XXXII

The effect of different concentrations of fumarate on hydrogen production by unadapted and adapted bacteria: The effect of succinate on hydrogen production by unadapted bacteria

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1. Unadapted Bacteria	Formate + peptone	40	80	70
	Formate + peptone + 0.001 M fumarate	40	80	115
	Formate + peptone + 0.01 M fumarate	40	80	368
	Formate + peptone + 0.1 M fumarate	60	100	311
	Formate + peptone + 0.2 M fumarate	80	140(M)	120
	Formate + peptone + 0.1 M succinate	60	80	28
2. Unadapted Bacteria	Formate + peptone	70	120	19
	Formate + peptone + 0.01 M succinate	70	110	15
3. Adapted Bacteria	Formate			315
	Formate + 0.001 M fumarate			296
	Formate + 0.01 M fumarate			208
	Formate + 0.2 M fumarate			0

Bacteria: Experiment 1, grown overnight on nutrient agar; Experiment 2, grown 6 hours on nutrient agar; Experiment 3, grown 22 hours in glucose broth. 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067M formate, 1% peptone, sodium fumarate and sodium succinate as shown.

H_2 evolution in Experiment 3 measured from 10 to 30 minutes.

Other conditions as in Table XII.

of fumarate tested, below 0.2 M, stimulated the hydrogen production of unadapted bacteria but inhibited the hydrogen production by adapted bacteria. Since concentrations of fumarate which did not stimulate or which inhibited hydrogen evolution by adapted cells stimulated hydrogen evolution by unadapted cells, it was concluded that fumarate acted on unadapted cells to stimulate formic hydrogenlyase synthesis.

It is known that fumarate acts as an acceptor of molecular hydrogen through the enzyme, hydrogenase (119); thus fumarate would decrease the amount of hydrogen evolved by adapted cells. A comparison of the results of Experiments 1 and 3 of Table XXXII indicates that with increasing concentrations, the inhibitory action of fumarate on hydrogen production outweighed its stimulatory action on formic hydrogenlyase formation.

It is also seen from the results given in Table XXXII that succinate had no stimulatory effect on formic hydrogenlyase formation. The inhibition observed with the higher succinate concentration used was possibly due to an osmotic effect.

These facts suggested that fumarate stimulated adaptation by acting as a hydrogen acceptor. It has long been known that E. coli can activate this molecule to accept hydrogen from a donator (147). There is direct evidence for the anaerobic oxidation of a number of substances by fumarate in E. coli. These include dl-lactate, acetate, glycerol, dl-glyceraldehyde, l-glutamate, pyruvate, acetoacetate, butyrate, and glucose (148, 149). A large number of other substances may almost

certainly be oxidized by fumarate in this organism. Thus, in the presence of formate and peptone, fumarate could function as an acceptor of hydrogen from compounds (e.g., glutamate) present in peptone.

The hypothesis that fumarate was acting as a hydrogen acceptor was tested further. As stated above, glycerol may be oxidized anaerobically by fumarate in E. coli. The overall reaction is:

$$\text{Gycerol} + 2 \text{ fumarate} = \text{pyruvate} + 2 \text{ succinate} \quad (150)$$

The addition of fumarate, then, should permit glycerol to act as a source of the adaptive substrate, and ultimately as a source of molecular hydrogen. It is seen from the results presented in Table XXXIII that only in the presence of added fumarate was hydrogen evolved from a glycerol-peptone medium.

These results were further evidence that fumarate was serving as a hydrogen acceptor. In a transfer of hydrogen from a donor to fumarate, as in other oxidative processes, energy is released. It was thought that fumarate stimulated adaptation because of the energy supplied. That adaptation takes place in a formate-peptone system, without the addition of fumarate, may be explained by the fact that some fumarate is formed from the aspartate present in the peptone. Before further evidence is given on this scheme, experiments carried out by Quastel and Wooldridge (150) on bacterial growth should be mentioned.

These workers found that the addition of formate to a synthetic

TABLE XXXIII

The ability of fumarate to stimulate hydrogen production in the glycerol-peptone system

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1.	Fumarate + peptone	>180		0
2.	Glycerol + peptone	>180		0
	Glycerol + peptone + fumarate	130	160	118
3.	Glycerol + peptone	>200		0
	Glycerol + peptone + fumarate	160	170	52

Bacteria: Grown on nutrient agar; Experiment 1, overnight culture, 1.5 mg. bacteria/vessel; Experiment 2, 5 hour culture, 0.7 mg./vessel; Experiment 3, 8 hour culture, 1.5 mg./vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067M glycerol, 1% peptone, 0.01 M sodium fumarate.

Other conditions as in Table XII.

medium in which fumarate and lactate were the only other sources of carbon, greatly increased the anaerobic growth of E. coli. In a fumarate-lactate system, energy was supplied for anaerobic growth by the oxidation:

Fumarate + lactate = Succinate + pyruvate + 14 Cal.

The addition of formate, however, presented the cells with a much greater source of energy. The oxidation of formate yields practically as much energy as does the oxidation of hydrogen:

Formate + fumarate = CO_2 + succinate + 28 Cal.

Formate could not be used as a carbon source for growth by these organisms. Its stimulating action was attributed entirely to the great amount of energy supplied for growth by its oxidation.

Although the substance(s) donating hydrogen to fumarate in the experiments reported here was not known, the possibility that it was formate was of interest because of the energy which the oxidation of formate supplies. Krebs (149) found that suspensions of E. coli could catalyse the anaerobic oxidation of formate by fumarate if the bacteria had been grown in a formate-glucose broth, and possessed formic hydrogenlyase, but not if they had been grown aerobically and lacked this enzyme.

A direct examination of the ability of the unadapted bacteria used in these experiments to catalyse the anaerobic oxidation of formate by fumarate was not carried out. Considering the evidence offered by Krebs, it would appear unlikely that this oxidation took place. (It should be noted in this connection that the organisms used in the experiments of Quastel and Wooldridge were cultured in tryptic broth and probably possessed a small amount of formic hydrogenlyase activity).

That adapted cells of E. coli may bring about the anaerobic oxidation of formate by fumarate was indicated by the results of the experiment illustrated in Figure XIII. In this experiment, the effect of fumarate on hydrogen evolution from formate by adapted cells in the presence and absence of peptone was measured. The initial pH of each vessel was 7.4. The final pH values are given under Figure XIII.

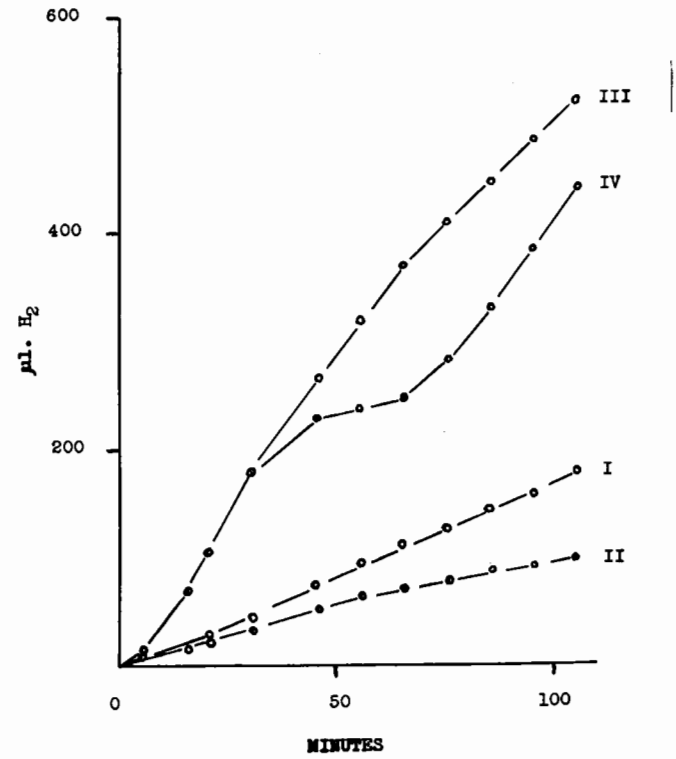


FIGURE XIII

The effect of fumarate on hydrogen production from formate by adapted bacteria in the presence and absence of peptone.

		Final pH
I.	Formate	8.1
II.	Formate + fumarate	8.6
III.	Formate + peptone	8.2
IV.	Formate + fumarate + peptone	8.6

Bacteria: Grown overnight in formate broth; 1 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, 0.01 M sodium fumarate, 1% peptone.

Other conditions as in Table XII.

Comparing the results shown in Curves I and II, it is seen that the amount of hydrogen evolved was greater in the presence of formate alone than in the presence of formate plus fumarate, but that the final pH in the presence of formate plus fumarate was higher. Destruction of sodium formate, either by the action of formic hydrogenlyase or by oxidation would raise the pH of the medium. These results indicate that in the presence of fumarate, oxidative destruction of sodium formate took place.

Comparing the results given in curves III and IV of Figure XIII, an especially interesting effect of fumarate is seen. The stimulation of formic hydrogenlyase activity by peptone has already been mentioned (Table XIII). When both peptone and fumarate were added to formate, after an early falling off of activity there was an increased rate of hydrogen evolution. The time at which this increased rate began was approximately that at which unadapted cells, incubated, in the presence of formate and peptone, begin to evolve hydrogen. The breakdown of formate by formic hydrogenlyase yields little energy. It seems possible that in the presence of peptone fumarate stimulated the adapted cells to produce further hydrogenlyase by serving as a source of energy.

After it had been discovered that aspartate was one of the five amino acids most important for formic hydrogenlyase formation in the presence of glucose, experiments were carried out to determine

whether fumarate had the same action as aspartate. It was found (Table XXXIV) that fumarate could replace aspartate. Succinate was quite inactive in this respect. These findings suggested that the ability of fumarate to stimulate adaptation in this system was connected with its action as a hydrogen acceptor. Further evidence on this point will be considered later.

The addition of fumarate to the glucose-glutamate system brought about an inhibition of formic-hydrogenlyase formation. This was first shown in experiments carried out in the Warburg apparatus. As may be seen from the results given in Table XXXV, both fumarate and aspartate increased the lag periods and lowered the final rate of hydrogen production. Fumarate is known to inhibit hydrogen production by adapted cells (Table XXXII); it was unlikely, however, that this inhibition would account for the increased lag periods. Furthermore, the inhibition of hydrogen evolution observed in Table XXXV was much greater than that caused by the same concentration of fumarate on adapted cells. It seemed probable, therefore, that fumarate was inhibiting enzyme synthesis rather than enzyme activity.

To be certain that the inhibition observed was one of adaptation rather than of hydrogen evolution, another type of experiment was devised. Unadapted cells of E. coli were suspended anaerobically in the presence of glucose and sodium l-glutamate, with and without the addition of sodium fumarate, and the formic hydrogenlyase activity of the washed

TABLE XXXIV

The ability of fumarate to replace aspartate in the glucose-five amino acid system

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1.	Glucose + all five amino acids	50	80	636
	Glucose + all five amino acids but aspartate	80	110	333
	Glucose + fumarate + all five amino acids but aspartate	60	85	580
	Glucose + fumarate + all five amino acids	50	80	703
2.	Glucose + all five amino acids	50	90	692
	Glucose + all five amino acids but aspartate	70	120	443
	Glucose + fumarate + all five amino acids but aspartate	50	80	634
	Glucose + succinate + all five amino acids but aspartate	70	120	483
3.	Glucose + all five amino acids	50	90	616
	Glucose + all five amino acids but aspartate	70	130	528
	Glucose + 0.002 M fumarate + all five amino acids but aspartate	60	130	624
	Glucose + 0.01M fumarate + all five amino acids but aspartate	50	80	573
	Glucose + 0.02 M fumarate + all five amino acids but aspartate	50	80	596

Bacteria: Grown 5-6 hours on nutrient agar; 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.01 M sodium succinate, 0.01 M sodium fumarate, except as otherwise shown; amino acids as in Table XXVII (normal concentrations).

Other conditions as in Table XII.

cells determined after different times of incubation. The exact composition of the media used is given in Table XXXVI. Eight test tubes were prepared, four containing glucose and glutamate, four containing glucose, glutamate, and fumarate. The tubes were made anaerobic by being gassed for fifteen minutes with nitrogen after the addition of the bacteria, and were tightly stoppered immediately after gassing. Incubation was carried out at 37°C. The vibration of the bath in which the tubes were suspended kept the bacteria from settling. At 140 and 240 minute intervals after the first contact of the bacteria with the medium, duplicate tubes of the glucose-glutamate medium and duplicate tubes of the glucose-glutamate-fumarate medium were removed and their contents centrifuged in the cold. The clot of bacteria in the bottom of the centrifuge tube was washed once with cold water and recentrifuged. The bacteria in each centrifuge tube were finally suspended in 2.0 ml. H_2O and 0.5 ml. of the suspension taken for turbidity measurements. 1.0 ml. of each bacteria suspension was added to the main compartment of a Warburg vessel, together with water and 0.033 M phosphate buffer of pH 6.8. After gassing and equilibration 0.2 ml. of M formate was tipped in from the side arm and the evolution of hydrogen followed for 40-45 minutes, (For other conditions, see Table XII). The results in Table XXXVI show clearly that in the glucose-glutamate system, fumarate inhibited formic hydrogenlyase formation.

At the end of Experiment 3 of Table XXXV, the contents of the vessels were centrifuged and a 0.02 ml. aliquot of each supernatant

TABLE XXXV

The inhibition of hydrogen production in the glucose-glutamate system by fumarate and aspartate.

Experiment	System	Lag, minutes T_1	T_2	Final $Q-H_2$
1.	Glucose + glutamate	90	120	156
	Glucose + glutamate + fumarate	120	140	23
2.	Glucose + glutamate	80	140	202
	Glucose + glutamate + fumarate	110	150(M)	44
	Glucose + glutamate + aspartate	100	150(M)	80
3.	Glucose + glutamate	90	120(M)	176
	Glucose + glutamate + fumarate	100	110(M)	29

Bacteria: Grown 5-6 hours on nutrient agar, 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.01 M sodium fumarate, 0.01 M dl-aspartate; 0.04 M sodium l-glutamate in Experiment 1, 0.023 M glutamate in Experiments 2 and 3.

Other conditions as in Table XII.

TABLE XXXVI

The inhibition of formic hydrogenlyase formation in the glucose-glutamate system by fumarate:

System	Formic hydrogenlyase activity, $(Q\text{-}H_2)^{*}$, after minutes 140	240
Glucose + glutamate	51	89
Glucose + glutamate + fumarate	9	36

Bacteria: Grown 6 hours on nutrient agar, 3 mg./ml. in suspension. In test tubes, 0.6 ml. 0.5 M, glucose, 1.5 ml. 0.2 M phosphate buffer, pH 7.4, 34.5 mg. sodium l-glutamate, 15.2 mg. sodium fumarate, 1.5 ml. bacteria, water to 9.0 ml. Incubation and measurement of formic hydrogenlyase activity as described in the text.

* Values of $Q\text{-}H_2$ are the mean values of duplicate determination.

spotted on paper and made to ascend by capillary action in an ethanol-butanol-ammonia mixture. For comparison, 0.02 ml. of a sodium l-glutamate solution in a final concentration of 0.023 M was treated in the same way. The papers were sprayed with ninhydrin and a rough estimate of the amount of glutamate and aspartate present, made.

A small amount of aspartate was formed in the glucose-glutamate medium by the bacteria, regardless of the presence of fumarate. It may be seen, from the semi-quantitative results given in Table XXXVII that the presence of fumarate caused a decrease in the amount of glutamate present at the end of the experiment.

TABLE XXXVII

The relative amounts of glutamate and aspartate present after Experiment 3, Table XXXV.

Chromatographed	Relative intensity of spot of Glutamate	Aspartate
Glutamate	+++	-
Glutamate + glucose, after contact with bacteria	++	(+)
Glutamate + glucose + fumarate, after contact with bacteria	+	(+)

Conditions: See text.

Other Hydrogen Acceptors

Following the demonstration that fumarate stimulated adaptation in the formate-peptone system, experiments were carried out to study the action of other hydrogen acceptors on adaptation in this system. Nitrate has long been known to act as a hydrogen acceptor in E. coli (see 148). As the results illustrated in Figure XIV show, 0.01 M sodium nitrate brought about a marked stimulation of hydrogen production by unadapted cells in the formate-peptone system. The data presented in Table XXXVIII show, furthermore, that this stimulation could not have been due to a stimulation of formic hydrogenlyase activity; 0.01 M nitrate inhibited the hydrogen evolution by adapted cells, and with 0.1 M nitrate, this inhibition was complete. It was concluded from these findings that nitrate acted as a stimulator of formic hydrogenlyase formation in the formate-peptone system. Since nitrate is also known

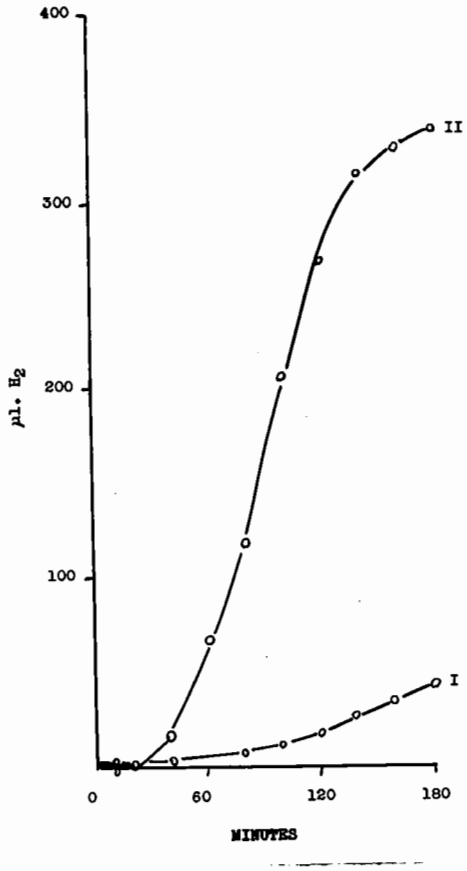


FIGURE XIV

The stimulation of hydrogen production by nitrate in a formate-peptone system.

I. Formate + peptone
II. Formate + peptone + nitrate

H_2 was not evolved from peptone + nitrate.

Bacteria: Grown 5 hours on nutrient agar; 1.1 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, 0.01 M $NaNO_3$, 1% peptone.

Other conditions as in Table XII.

TABLE XXXVIII

The effect of nitrate on the hydrogenlyase activity of adapted cells.

System	$Q\text{-}H_2$
Formate	342
Formate + 0.01 M $NaNO_3$	199
Formate + 0.1 M $NaNO_3$	0

Bacteria: Grown 12 hours in glucose broth, 1.5 mg./vessel

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, $NaNO_3$ as shown.

H_2 evolution measured from 10 to 40 minutes, other conditions as in Table XII.

to act as an acceptor of molecular hydrogen (119), the mechanism of the nitrate inhibition of hydrogenlyase activity was probably the same as that of the inhibition by fumarate.

Following this observation, a paper by Billen appeared (35), in which it was reported that nitrate inhibited the synthesis of formic hydrogenlyase in cultures of E. coli grown in the presence of glucose and an amino acid mixture. Experiments measuring the action of nitrate on adaptation in the formate-peptone, the glucose-peptone and pyruvate-peptone systems as well as in the glucose-amino acid systems are reported in Table XXXIX. Nitrate failed to replace aspartate in the glucose-five amino acid system. A complete inhibition of hydrogen evolution by nitrate was noted in this system, both in the presence and

TABLE XXXIX

The effect of nitrate on hydrogen production in formate-peptone, pyruvate-peptone, glucose-peptone, and glucose amino acids systems:

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final $Q-H_2$
1.	Formate + peptone	100	100	19
	Formate + peptone + nitrate	30	70	271
	Pyruvate + peptone	80	135	845
	Pyruvate + peptone + nitrate	80	80	52
	Glucose + peptone	30	70 (M)	1084
	Glucose + peptone + nitrate	80	100	75
	Glucose + nitrate	>150	-	0
	Peptone + nitrate *	>160	-	0
2.	Glucose + five amino acids	50	80	592
	Glucose + five amino acids + nitrate	>125	-	0
	Glucose + four amino acids	60	110	384
	Glucose + four amino acids + nitrate	>125	-	0

* - carried out in a separate experiment.

Bacteria: Grown 5-6 hours on nutrient agar, 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate and pyruvate, 0.033 M glucose, 1% peptone, 0.01 M $NaNO_3$, amino acids as given in Table XXVII (normal concentrations), aspartate omitted in "four amino acids".

Other conditions as in Table XII.

the absence of aspartate. Although nitrate stimulated hydrogen production in the formate-peptone system, it caused a strong inhibition of hydrogen production in the glucose-peptone and pyruvate-peptone systems. Nitrate and nitrite were tested for qualitatively at the end of Experiment 1. It was found that nitrate had completely disappeared from all vessels to which it had been added; of the only two vessels tested, that containing formate and paptone, and that containing formate, peptone and nitrate, the former contained a trace of nitrite at the end of the experiment and the latter a much larger amount.

The inhibition of hydrogen production in the presence of glucose and pyruvate seemed far too great to be accounted for as due only to an inhibition of formic hydrogenlyase activity. Most probably, the phenomenon observed here was the same as that observed by Billen in growing cells. Further experimental work would be required to establish definitely the extent to which enzyme formation rather than enzyme activity was inhibited.

It will be seen from the results presented in the following section that nitrate also stimulated adaptation in a formate-amino acid medium.

It was believed that nitrate, like fumarate, stimulated adaptation in the formate-peptone system by acting as a hydrogen acceptor and liberating energy. Nitrate was shown to be reduced anaerobically in the formate-peptone system. The substance(s) oxidized by nitrate is, however, still unknown.

It should be mentioned that suspensions of E. coli, grown in a manner which would induce no formic hydrogenlyase formation, were found capable of bringing about the anaerobic oxidation of formate by nitrate (91). It is possible that this oxidation took place in the experiments reported here, and that the large amount of energy released became available for adaptive enzyme synthesis.

The inhibition of adaptation by nitrate when glucose or pyruvate was used as a source of formate is still not understood. Some possible explanations, however, may be considered.

If formate is oxidized by nitrate, then, when glucose or pyruvate is used as a source of formate, and an excess of formate is no longer present, the oxidation of formate might be expected to inhibit adaptation by lowering the concentration of the adaptive substrate.

Billen (35) suggested that the inhibitory action of nitrate on formic hydrogenlyase formation was due to a competition between enzyme-forming systems. Thus, the stimulation by nitrate of nitratase formation would bring about the utilization of nitrogenous material which would otherwise be used in the building up of the formic hydrogenlyase system. In support of this view, he pointed out that increasing concentrations of the nitrogen source (casein hydrolysate) antagonized the action of nitrate. He did not, however, measure the changes of nitratase activity induced by nitrate in the organism he used.

The inhibitory action of nitrate could also be attributed to nitrite formation. Nitrite has been found to inhibit bacterial growth (148), and would be expected to block adaptation as well.

If the nitrate inhibition of adaptation in the presence of glucose or pyruvate took place in the first manner proposed here, the stimulation of adaptation in the formate-peptone system is not puzzling. If other mechanisms were involved, then it would appear that the stimulatory action given by nitrate in the presence of an excess of formate outweighs any inhibitory action it might have on adaptation.

The action of other substances which might act as hydrogen acceptors was also examined. Table XL presents the results of an experiment in which the effects of potassium ferricyanide and methylene blue on hydrogen production by unadapted cells in the formate-peptone and glucose-five amino acid systems were observed. Ferricyanide had no effect, and it is not known if it was reduced in this experiment. Methylene blue was reduced; the solutions containing methylene blue had become completely decolorized by or before twenty-five minutes of contact with the bacteria. Some inhibition of hydrogen production was evident in the formate-peptone system, and a complete inhibition in the glucose-five amino acid system. Since methylene blue is known to be an inhibitor of adaptation as well as a hydrogen acceptor (151), no further experiments were carried out with this substance.

TABLE XL

The effects of ferricyanide and methylene blue on hydrogen production in the formate-peptone and glucose-five amino acid systems:

System	Lag, minutes T_1	T_2	Final Q-H_2
Glucose + five amino acids	70	100 (M)	498
Glucose + five amino acids + ferricyanide	70	100 (M)	625
Glucose + five amino acids + methylene blue	>120	-	0
Formate + peptone	-	45	312
Formate + peptone + ferricyanide	-	60	322
Formate + peptone + methylene blue	-	60	230

Bacteria: Grown 15 hours on nutrient agar; 1.5 mg. bacteria/vessel

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.067 M formate, 1.67% peptone, 0.001 M $K_3Fe(CN)6$, 0.0033% methylene blue chloride, amino acids as given in Table XXVII (normal concentrations).

The effect of ammonia on formic hydrogenlyase synthesis

Support for the scheme set forward to explain the influence of hydrogen acceptors on formic hydrogenlyase synthesis has been obtained through experiments on the action of ammonia on formic hydrogenlyase formation in various systems. The results of four experiments given in Table XLI show that in the formate-peptone system, high concentrations of ammonia completely inhibited formic hydrogenlyase formation (See also Table XLII and XLIII). To some extent, this inhibition may have been caused by the high osmotic pressure of the ammonia solution, but since 0.1 M Na_2SO_4 inhibited adaptation to a smaller extent than

TABLE XLI

The effect of ammonia on formic hydrogenlyase formation

Experiment	System	Lag, minutes T_1	T_2	Final $Q\text{-}H_2$
1a. Unadapted bacteria	Formate + tryptone	40	80	106
	Formate + tryptone + 0.01 M $(NH_4)_2SO_4$	60	120 (M)	92
	Formate + tryptone + 0.1 M $(NH_4)_2SO_4$	>130	-	0
b. Adapted bacteria	Before adding 0.1 M $(NH_4)_2SO_4$		-	160
	After adding 0.1 M $(NH_4)_2SO_4$		-	118
2a. Unadapted bacteria	Formate + tryptone	80	140	116
	Formate + tryptone + 0.1 M Na_2SO_4	120	180	33
b. Adapted bacteria	Before adding 0.1 M Na_2SO_4		-	92
	After adding 0.1 M Na_2SO_4		-	96
3. Unadapted bacteria	Formate + peptone	30	50	97
	Formate + peptone + 0.1 M NH_4Cl	>80	-	0
4. Adapted bacteria	Formate		-	342
	Formate + 0.1 M NH_4Cl		-	362
5. Unadapted bacteria	Glucose + five amino acids	50	80	592
	Glucose + five amino acids + 0.001 M NH_4Cl	40	80	626
	Glucose + five amino acids + 0.01 M NH_4Cl	50	80	660
	Glucose + five amino acids + 0.1 M NH_4Cl	50	80	774
6. Unadapted bacteria	Glucose + five amino acids	50	90	706
	Glucose + five amino acids + 0.1 M NH_4Cl	50	90	908

Bacteria: Grown 12 hours in glucose broth for Experiment 4, on nutrient agar for other experiments. Experiments 1 and 2, overnight cultures; Experiments 3,5, and 6, 5-6 hour cultures; 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.067 M formate, 1% peptone or tryptone, amino acids as given in Table XXVII (normal concentrations), other concentrations as shown.

In Experiments 1 and 2, adaptation carried out in the Warburg vessel in formate-tryptone media. H_2 evolution measured 20 minutes before and 20 minutes after tipping in $(NH_4)_2SO_4$ or Na_2SO_4.

H_2 evolution measured from 10-40 minutes in Experiment 4.

Other conditions as in Table XII.

0.1 M $(NH_4)_2SO_4$, part of the inhibition by ammonia must have been due to some other cause. Later experiments, also reported in Table XLI, showed that high concentrations of ammonia had no inhibitory effect on adaptation in the glucose-five amino acid system, but rather a slight stimulatory effect.

In Experiment 1 of this table, a slight reduction in the rate of hydrogen production was observed when ammonia was added to adapted cells. This was probably due, not to an inhibition of formic hydrogenlyase activity but to the fact that at the time of addition of ammonia the formic hydrogenlyase activity was falling because of the increasing alkalinity caused by the decomposition of sodium formate (See also Experiment 4).

The following mechanism of action was postulated for the ammonia inhibition of adaptation in the formate-peptone system: In all systems, energy is necessary for the synthesis of formic hydrogenlyase. In the formate-peptone system, fumarate, formed from the aspartate present in the peptone, serves as a hydrogen acceptor, thus supplying energy. High concentrations of ammonia shift the equilibrium,

$$\text{Aspartate} \rightleftharpoons \text{fumarate} + NH_3 ,$$

to the left, removing the essential source of energy from the system. If this scheme were correct, the addition of fumarate would be expected to cause some reversal of the ammonia inhibition. Such a reversal was found to take place (Table XLII). This, however, is not conclusive

TABLE XLII

The action of fumarate on the ammonia inhibition of adaptation in the formate-peptone system

System	Lag, minutes T_1	T_2	Final $Q-H_2$
Formate + peptone	60	80	50
Formate + peptone + fumarate	60	100	187
Formate + peptone + NH_4Cl	>180		0
Formate + peptone + NH_4Cl + fumarate	120	160 (M)	58

Bacteria: Grown overnight on nutrient agar; 1.5 mg. bacteria/vessel

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, 1% peptone, 0.1 M NH_4Cl and sodium fumarate.

Other conditions as in Table XII.

proof of the above scheme. Even if ammonia acts in another way than that pictured, the addition of fumarate would tie up ammonia in the form of aspartate, and remove it from its site of action.

It was further realized that if the mechanism pictured above were correct, addition of another source of energy which could replace fumarate, e.g. nitrate, should reverse the inhibition caused by ammonia. This possibility was tested, and, as the results given in Table XLIII show, the ammonia inhibition of adaptation in the formate-peptone system was to some extent reversed by nitrate.

The experiments on the action of ammonia on adaptation in the formate-peptone system confirmed the necessity of an energy source for

TABLE XLIII

Reversal of the ammonia inhibition of formic hydrogenlyase formation in the formate-peptone system by nitrate.

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final $Q\text{-}H_2$	Percent Inhibition of Final $Q\text{-}H_2$
1.	Formate + peptone	30	50	56	-
	Formate + peptone + $NaNO_3$	30	60	368	-
	Formate + peptone + NH_4Cl	>80	-	0	100
	Formate + peptone + NH_4Cl + $NaNO_3$	40	40	97	74
2.	Formate + peptone	30	70	100	-
	Formate + peptone + $NaNO_3$	30	70	368	-
	Formate + peptone + NH_4Cl	>120	-	0	100
	Formate + peptone + NH_4Cl + $NaNO_3$	30	70	110	70

Bacteria: Grown 5 hours on nutrient agar; 1.5 mg. bacteria/vessel in Experiment 1, 1.45 mg./vessel in Experiment 2.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, 1.67% peptone, 0.01 M $NaNO_3$, 0.1 M NH_4Cl.

Other conditions as in Table XII.

formic hydrogenlyase synthesis. It appears, then, that the chief role of the aspartic acid contained in the peptone, is to serve as a hydrogen acceptor, providing energy for adaptation. Whether some of the aspartate present also serves as a nitrogen source for the synthesis of the adaptive enzyme is not yet known. This may only be decided by experiments employing synthetic media for the stimulation of formic hydrogenlyase synthesis.

If glucose is present, energy for adaptation is supplied by glycolysis. A supply of energy is available, whether or not aspartate can function as a hydrogen acceptor. A complete inhibition of adaptation by ammonia is not to be expected in this system.

The function of the aspartate in the glucose-five amino acid system remains uncertain. There is a strong possibility that it acts as a hydrogen acceptor, following transformation to fumarate, and supplies energy for adaptation in addition to the energy produced by glycolysis. The fact that fumarate replaces aspartate is consistent with this theory. The minimum effective concentration of dl-aspartate is relatively high (0.01 M); it is very unlikely that enough free ammonia would be present to synthesize this amount of aspartate from fumarate. The stimulation of adaptation caused by the addition of 0.01 M fumarate to a system composed of glucose, serine, histidine, arginine, and leucine was considerably less than that caused by the addition of the same amount of fumarate to the formate-peptone system. In the former case, only a shortening of the lag period and a little increase in final rate was noted.

Since a considerable source of energy is already present (through glycolysis), the addition of another might be expected to produce only a moderate effect on adaptation.

The fact that ammonia had no inhibitory effect on adaptation in the glucose-five amino acid system seemed at first to indicate that aspartate was not acting as a hydrogen acceptor in this system. If it were, then the addition of ammonia should cause some inhibition of adaptation by preventing the conversion of aspartate to fumarate. The possibility was considered, however, that ammonia was acting as a stimulator of glycolysis, as well as in the way postulated above. It is already known that ammonia stimulates glycolysis in the brain (152). If it had a similar action on these bacteria, then the extra energy produced by the stimulation of glycolysis could conceivably balance out the loss of energy caused by the inhibition of aspartate's action as a hydrogen acceptor. It was found that approximately a 50% stimulation of glycolysis was caused by 0.1 M NH_4Cl (Table XLV). Thus, there is no strong objection to the idea that aspartate acts in the presence of glucose chiefly as a hydrogen acceptor, supplying energy for adaptation. Further experimental work will be necessary to establish definitely the role of aspartic acid in the adaptive systems studied here.

The action of fumarate on adaptation in the glucose-glutamate system is still not understood. Glutamate is not one of the five most important amino acids, and it is possible that fumarate acts by preventing

the conversion of glutamate to other amino acids essential for formic hydrogenlyase synthesis. It has been noted (Table XXXVII) that in the presence of fumarate, the amount of glutamate present decreases.

It was thought possible that in some way fumarate was removing ammonia from glutamate or from amino acids formed from glutamate. For this reason, the effects of the addition of ammonia on the fumarate inhibition were investigated. Little or no effect was seen (Table XLIV). (The experiment reported here was not followed for a long enough period to make a comparison of all final rates possible).

TABLE XLIV

The effect of ammonia on the fumarate inhibition of formic hydrogenlyase formation in the glucose-glutamate system.

System	Lag, minutes T_1	T_2	Final Q-H_2
Glucose + glutamate	90	120	129
Glucose + glutamate + NH_4Cl	80	140	104
Glucose + glutamate + fumarate	140	150 (M)	84
Glucose + glutamate + fumarate + NH_4Cl	120	140 (M)	34

Bacteria: Grown 16 hours on nutrient agar; 1.5 mg. bacteria/vessel

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.023 M sodium l-glutamate, 0.01 M fumarate, 0.1 M NH_4Cl.

Other conditions as in Table XII.

Factors influencing glycolysis in E. coli

In many of the experiments reported here, the adaptive substrate, formate, was supplied by the glycolytic breakdown of glucose or pyruvate. Glycolysis not only supplies the adaptive substrate but also serves as the chief if not the only source of energy for formic hydrogenlyase formation. The production of organic acids in glycolysis lowers the pH of the medium; effects of pH changes on adaptation will be discussed in detail in Part V.

Fowler (153) has reported that for one strain of E. coli, transfer from an aerobic to an anaerobic growth medium, with glucose as the only carbon source in each, brought about a lag before the beginning of anaerobic growth. There was also a lag before the fermentation of glucose began, and there were some indications that this lag period was due to the formation of an adaptive enzyme system.

With the bacteria used in the experiments reported here, however, no such delay in the anaerobic utilization of glucose was observed. The experiment given in Figure XV illustrates this point. After gassing the Warburg vessels with 93% N_2-7% CO_2, the bacteria were tipped from the side arm into a phosphate-bicarbonate medium, with glucose and other substances present as shown. Acid production was measured as carbon-dioxide evolution. In the presence of glucose, carbon dioxide was evolved immediately at a maximal rate. A point of interest is the great stimulation of carbon dioxide evolution caused by peptone. Stimulation

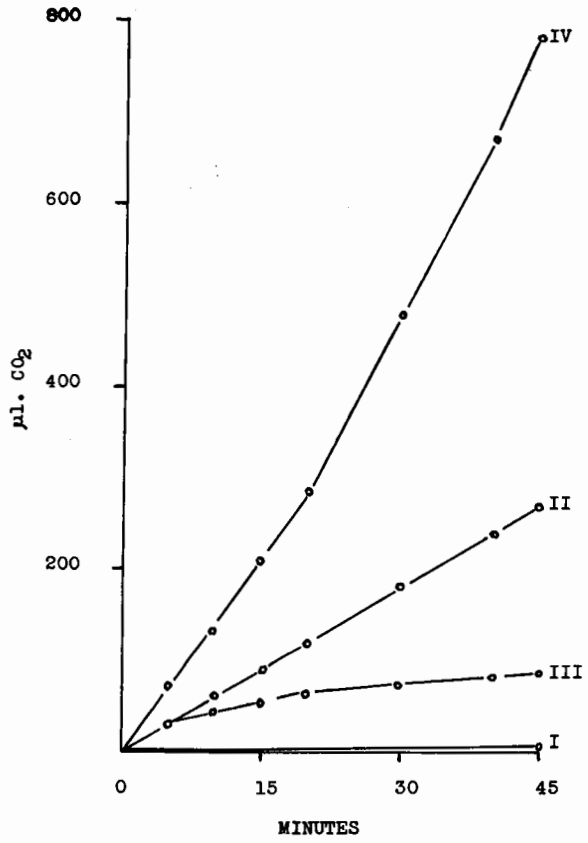


FIGURE XV

The effect of peptone on glycolysis in E. coli.

Phosphate-Bicarbonate buffer +

I. ----
II. Glucose
III. Peptone
IV. Glucose peptone

Bacteria: Grown 6 hours on nutrient agar; 1.5 mg. bacteria/vessel.

In main vessels: 0.0025 M phosphate buffer, pH 7.6, 0.025 M $NaHCO_3$, 0.033 M glucose, 1% peptone.

Temp.: 37° C. Gas: N_2 - CO_2. Bacteria were tipped in after gassing and equilibration, five minutes before the zero time represented in this figure. In the presence of glucose, CO_2 evolution began immediately.

of fermentation and glycolysis by complex natural products has been reported by several authors (115, 154). The fact that peptone acts as a stimulator of glycolysis as well as of formic hydrogenlyase activity introduces a further complicating factor when peptone is used in the presence of glucose as a nitrogen source for adaptation.

The increased rate of gas evolution illustrated in Curve IV in the latter part of the experiment is thought due to the evolution of hydrogen by the formic hydrogenlyase system formed during the first part of the experiment.

A stimulation by peptone of the acid production from pyruvate by these bacteria was noted. Some acid was produced from the amino acids alone; the stimulation given to acid production by the amino acids in the presence of glucose was virtually an additive one. These observations, as well as observations on the effect of ammonia on glycolysis, are recorded in Table XLV.

Summary and conclusions

Both aspartate and fumarate (which may be derived from aspartate) stimulated formic hydrogenlyase synthesis in a formate-peptone medium. It is known that cells of E. coli may activate these substances to serve as hydrogen acceptors. An indication that fumarate was acting in this manner was the observation that it stimulated formic hydrogenlyase formation in a glycerol-peptone system, presumably through the oxidation

TABLE XLV

The effect of peptone amino acids and ammonia on glycolysis and the breakdown of pyruvate.

Experiment	System	Q-CO_2
1.	Pyruvate	197
	Peptone	85
	Pyruvate + peptone	520
2.	Glucose	270
	Amino acids	110
	Glucose + amino acids	370
3.	Glucose	266
	l-histidine	3
	Glucose + l-histidine	272
4.	Glucose	207
	NH_4Cl	0
	Glucose + NH_4Cl	319

Bacteria: Grown 5-6 hours on nutrient agar; 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.0025 M sodium phosphate buffer, pH 7.6, 0.025 M $NaHCO_3$, 0.033 M glucose, 0.067 M pyruvate, 1% peptone, 0.1 M NH_4Cl. Amino acids as given on page 107, but with d-histidine instead of l-histidine present, 0.002 M l-histidine.

Zero time 5 minutes after tipping in bacteria from the side arm. CO_2 evolution measured for 40 minutes.

Temp.: 37°C. Gas: N_2-CO_2

of glycerol to pyruvate. Nitrate also stimulated formic hydrogenlyase synthesis in a formate-peptone medium. The reduction of nitrate to nitrite could be demonstrated.

It was proposed that fumarate and nitrate, as hydrogen acceptors, stimulated adaptation by supplying energy for the adaptive process. That adaptation takes place in a formate-peptone system without added fumarate was attributed to the fact that fumarate was formed from aspartate present in the peptone.

It was found, in support of this scheme, that 0.1 M NH_4Cl or $(NH_4)_2SO_4$, which would tend to prevent the transformation of aspartate to fumarate, completely inhibited adaptation in the formate-peptone system. In a glucose-five amino acid system, where energy was supplied by glycolysis, high concentrations of ammonia had no inhibitory effect. The ammonia inhibition of adaptation in the formate-peptone system could be partially reversed by the addition of nitrate, which could supply the necessary energy for adaptation.

In the glucose-five amino acid system, aspartate, one of the amino acids, could be replaced by fumarate. The possibility was considered that aspartate stimulated adaptation by supplying fumarate and thereby a source of energy for adaptation in addition to that supplied by glycolysis. The experimental evidence was seen to be consistent with this hypothesis, though it could not be regarded as proven.

In agreement with an earlier report, experiments indicated that nitrate inhibited formic hydrogenlyase synthesis in a glucose-five amino acid, a glucose-peptone, and a pyruvate-peptone system. Possible mechanisms of this inhibition and the relation of this inhibition to the stimulation of adaptation by nitrate in the formate-peptone system were considered.

Fumarate was found to inhibit formic hydrogenlyase synthesis in a glucose-glutamate medium. The mechanism of this inhibition is unknown.

Some of the factors affecting glycolysis by E. coli were studied. Peptone strongly stimulated glycolysis and the anaerobic breakdown of pyruvate, while amino acids had little or no effect on glycolysis. It was found that 0.1 M NH_4Cl stimulated glycolysis. The relation of this observation to the observed effects of ammonia on adaptation was discussed.

PART IV

Formic Hydrogenlyase Synthesis in the Presence of Formate Alone: The Use of Completely Synthetic Media

In the previous sections, those amino acids most important for formic hydrogenlyase synthesis in the presence of glucose or pyruvate were determined, and a study was made of the means by which energy is supplied for this adaptive process. Following these experiments, attempts were made to carry out adaptation in the presence of formate alone, using a synthetic medium which would supply both a nitrogen source and a source of energy for adaptation.

The results of a number of these experiments are reported in Table XLVI. It is seen that with the synthetic medium, the lag periods were considerably longer than those normally observed in the formate-peptone system or in the glucose-five amino acid system. (See the results here and also those in Table XII *et seq.*)

In some of the experiments reported in this table, acetate and alanine were added. It has long been known that acetate is formed during the fermentation of glucose by *E. coli* (155). Pyruvate is also formed (156), and might serve as a carbon source for the

TABLE XLVI

Formic hydrogenlyase synthesis in the presence of formate and completely synthetic media:

Experiment	System	Lag T_1	T_2	Final Q-H_2
1.	Formate + five amino acids, pH 7.4	>140	-	0
	Formate + five amino acids, pH 6.1	>140	-	0
	Glucose + five amino acids, pH 7.4	50	90	692
2.	Formate + five amino acids + nitrate, pH 7.4	110	110	12
	Formate + five amino acids + nitrate + acetate, pH 7.4	110	110	20
	Glucose + five amino acids, pH 7.4	50	80	650
3.	Formate + five amino acids + alanine + nitrate, pH 7.4	80	80	19
	Formate + five amino acids + alanine + nitrate, pH 6.8	122	122 (M)	36
	Formate + five amino acids + alanine + fumarate, pH 7.4	105	122 (M)	11
	Glucose + five amino acids, pH 7.4 (minimum concentration)	50	80	497
4.	Formate + five amino acids + alanine, pH 7.4	140	200	44
	Formate + five amino acids + alanine + fumarate, pH 7.4	125	222 (M)	90

Bacteria: Grown on nutrient agar
Experiment 1, 5 hour culture; Experiment 2, 15 1/2 hour culture; Experiment 3, 15 hour culture; Experiment 4, 15 hour culture, 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH as shown, 0.067 M formate, 0.033 M glucose, 0.01 M $NaNO_3$, 0.01 M sodium fumarate, 0.067 M sodium acetate. Amino acids as given in page 107 and Table XXVII (normal concentrations except where otherwise specified).

Other conditions as in Table XII.

synthesis of alanine. It thus seemed possible that these substances were important to formic hydrogenlyase synthesis but that it had not been found necessary to add them in the previous experiments when glucose was present. The addition of these substances, however, produced little if any effect on formic hydrogenlyase formation in the system being studied.

From the results given in Table XLVI, it may be seen that both nitrate and fumarate had an enhancing effect on formic hydrogenlyase formation. The results of another experiment on the effect of nitrate on adaptation in a synthetic medium are presented in Table XLVII. Here, the bacterial suspension was incubated 220 minutes in Warburg vessels under the conditions given in the table, the cells washed, as previously described, after the incubation period and their hydrogenlyase activity determined. It is seen that in the presence of formate, with a synthetic medium as with peptone, nitrate caused a definite stimulation of formic hydrogenlyase formation.

The reason that longer lag periods were observed with the formate-amino acid system than with the other systems studied is still unknown. In comparing adaptation in the formate-amino acid system with adaptation in the glucose-amino acid system, the fall in pH due to glycolysis in the latter system must be taken into account. Insufficient experimental work has yet been carried out to make a decision on the role of pH in these systems possible. (See Part V).

TABLE XLVII

The effect of nitrate on formic hydrogenlyase formation in a formate-amino acid medium:

System	Formic hydrogenlyase activity ($Q-H_2$) after 220 minutes, anaerobic incubation
Formate + five amino acids + alanine	4
Formate + five amino acids + alanine + nitrate	33

Incubation carried out in Warburg vessels.

Bacteria: Grown 14 hours on nutrient agar; 3 mg. bacteria/vessel.
In main vessels: 0.067 M phosphate buffer, pH 7.4, 0.067 M formate, 0.01 M $NaNO_3$, amino acids as in page 107 and Table XXVII (normal concentrations).

Activity of washed bacteria measured at pH 6.8; formate to a final concentration of 0.067 M tipped in, after gassing and equilibration, 5 minutes before zero time. H_2 evolution measured from 0 to 50 minutes.

Other conditions as in Table XII.

The differences between adaptation in the formate-amino acid system and the formate-peptone system cannot be attributed to pH effects. Some of the earlier considerations on the possible importance of co-factors should be recalled here (pages 95-96). There is evidence that a co-factor (s) for the formic hydrogenlyase system exists in yeast extracts and liver extracts (78). Similarly, the stimulating action of peptone in formic hydrogenlyase activity (Table XIII) may indicate the presence of a co-factor(s) in this substance. It is conceivable that time is required for the synthesis in a formate-amino acid system

of a co-factor already present in peptone, but this possibility can only be settled by further experimental work.

Summary and conclusions

Formic hydrogenlyase synthesis was studied in the presence of formate alone and, instead of peptone, different completely synthetic media. The media used were those which, it was thought, would supply both the amino acids and the energy necessary for adaptation.

Both fumarate and nitrate were found to stimulate adaptation in the synthetic system as they did in the formate-peptone system.

In all cases, the time required for enzyme synthesis was considerably longer than that observed in the formate-peptone system or in the glucose-five amino acid system. Possible explanations for this finding, such as the influence of pH and the activating effect of possible co-factors contained in peptone, were considered, though the evidence available was insufficient for any definite conclusions.

PART V

The Effect of pH on Formic Hydrogenlyase Formation

The effect of the pH of the medium upon the amount of formic hydrogenlyase produced by growing cultures of E. coli was investigated by Gale and Epps (157). This enzyme is described as one "whose potential activity (i.e., the amount synthesized) increases as the pH during growth deviates from the pH of optimum activity, the loss in activity.....caused by the deviation from the pH of optimum activity being compensated by an increased enzyme formation, so that the effective activity is approximately constant throughout the whole pH range of growth."

In the studies reported here, most attention was given to the effects of pH on the lag periods. Stephenson has shown that formic hydrogenlyase activity varies markedly with changes in pH (119). Experiments carried out with the strain of E. coli used in these experiments showed that the pH-activity curve for adapted organisms is very similar to that found by Stephenson. It may be seen from the results of the three experiments reported in Table XLVIII that maximum formic hydrogenlyase activity occurred approximately at pH 7.0, and that the

activity fell off below this pH and above pH 7.5. Because of the variance of activity with pH, the final rates of hydrogen evolution in the experiment carried out here would be dependent both upon the amount of enzyme synthesized and on the pH of the medium. It was not possible to draw conclusions from these experiments concerning the amounts of enzyme synthesized at various pH values, but only on the time required for enzyme synthesis.

TABLE XLVIII

The effect of pH on formic hydrogenlyase activity

Experiment	pH	Activity ($Q\text{-}H_2$)
1.	5.4	12
	5.9	12
	6.4	126
	6.8	156
	7.0	238
	7.5	288
	7.9	218
2.	7.0	540
	7.4	456
3.	7.0	284
	7.4	318

Bacteria: Grown on glucose broth. Experiment 1, 19 hour culture; Experiment 2, 12 hour culture; Experiment 3, 22 hour culture; 1.5 mg. bacteria/vessel in each experiment.

In main vessels: 0.067 M formate, 0.033 M phosphate buffer, initial pH as shown. pH values shown are those obtained by the mixture of 0.5 ml. of 0.2 M buffers with 0.2 ml. of M formate.

H_2 evolution measured from 0 to 20 minutes in Experiment 1, from 10 to 40 minutes in Experiments 2 and 3.

Other conditions as in Table XII.

The formate-peptone system

Results reported in Table XLIX show that an initial pH of 7.4 is roughly optimal for formic hydrogenlyase synthesis in this system. Raising the pH to 7.9 increased the lag periods and lowered the final rate of hydrogen evolution. At an initial pH of 6.1, no hydrogen was evolved in the time of the experiment. Gale and Epps (157) noted that formate was most toxic to bacterial growth at pH values below 6.3. The toxicity of formate at this pH may explain the failure of bacteria to adapt at pH 6.1 in the presence of 0.067 M formate. That adaptation occurred at pH values of 6.1 or less when glucose was used as a source of formate may be explained by the fact that in such a case the formate concentration was much lower than when formate alone was present.

The glucose-peptone and glucose-five amino acid systems

In the presence of glucose, with peptone as a nitrogen source, it was found that at an initial pH of 5.8 the lag periods were reduced to approximately one-half their values at pH 7.4; at pH values below 5.8 there was no further reduction of the lag periods, (Table L). The initial pH values given in this table were those obtained by mixing 0.5 ml. of 0.2 M phosphate buffers of different values with 0.5 ml. of 6% peptone. Separate tubes were made up containing the concentrations of peptone and buffer found in each vessel and the pH values of these measured to obtain the initial pH in the Warburg vessels.

TABLE XLIX

Hydrogenlyase formation in the formate-peptone system at different initial pH values

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1.	Formate + peptone, pH 7.4	40	70	322
	Formate + peptone, pH 6.1	>210	-	0
2.	Formate + peptone, pH 7.4	50	80	51
	Formate + peptone, pH 7.9	80	100	25

Bacteria: Grown on nutrient agar. Experiment 1, 6 hour culture, 1.5 mg. bacteria/vessel, Experiment 2, 12 1/2 hour culture, 1.5 mg./vessel.

In main vessels: 1.67% peptone, Experiment 1; 1% peptone, Experiment 2. 0.067 M formate, 0.033 M phosphate buffer, initial pH as shown.

TABLE L

The effect of pH on formic hydrogenlyase formation in the glucose-peptone and glucose-amino acid systems.

Experiment	System	Initial pH	Lag, minutes T_1	Lag, minutes T_2
1.	Glucose + peptone	7.4	40	70
		6.9	30	60
		5.8	20	40
2.	Glucose + peptone	5.8	20	40
		5.4	20	50
		5.1	30	50
3.	Glucose + five amino acids	7.8	45	80
		7.4	40	80
		6.3	30	45
		5.6	30	60

Bacteria: Grown on nutrient agar; Experiment 1, 5 1/2 hour culture, Experiment 2, 6 hour culture, Experiment 3, 5 hour culture, 1.5 mg. bacteria/vessel in all.

In main vessels: 0.033 M phosphate buffer, pH as indicated (see text), 1% peptone, 0.033 M glucose, amino acids as given in Table XXVII (normal concentrations).

Other conditions as in Table XII.

For comparison, the effects of changes of pH on adaptation in the glucose-five amino acid system are also shown in this table (Experiment 3). It is seen that the effects of lowering the pH in this system are very similar to those observed in the glucose-peptone system.

After the observation had been made that at an acid pH there was little effect on the lag period when histidine was omitted from the mixture of the five most important amino acids (Table XXIX), a series of experiments was carried out to determine the effects of omitting each amino acid from this mixture at four different pH values. These experiments are reported in Table LI. For this series of experiments, four buffers were used: 0.2 M Na_2HPO_4, 0.2 M phosphate buffers of pH 7.4 and 5.7, and 0.2 M NaH_2PO_4. In each experiment, four control vessels, containing all five amino acids at each pH value were set up, together with four vessels with one amino acid omitted. The solutions of amino acids which were to be present throughout were made neutral to phenol red. The amino acid solution, the effect of whose omission was to be tested, was adjusted to a pH of approximately 6 before being added to the two control vessels containing buffers of lower pH, and to a pH of approximately 7.4 before being added to the two control vessels containing buffers of higher pH. For each vessel, a duplicate solution was made up in a test tube, only the bacteria being omitted, and the initial pH of the solution in the vessel thus determined. For most of these experiments, the final pH was measured as well. Final pH values are given in Table LI, together with the time elapsing between tipping in the bacteria and removal of the vessels for determination of the final pH.

The experiments reported here revealed the fact that for all amino acids except arginine, omission brought about a considerable

TABLE LI

The effect on formic hydrogenlyase formation of omitting each amino acid at different pH values in the glucose-five amino acid system.

Experiment	Amino acid omitted	Initial pH	5 amino acids Lag, minutes T_1	T_2	Final $Q\text{-}H_2$	Final pH*	4 amino acids Lag, minutes T_1	T_2	Final $Q\text{-}H_2$	Final pH*
1.	serine	8.1	-	-	-	-	110	140(M)	192	6.8(150)
		8.0	60	100	470	6.6(150)	-	-	-	-
		7.5	60	100	490	6.5(150)	80	130	208	6.7(150)
		6.1	35	50	396	5.4(120)	40	60	252	5.6(120)
		5.6	35	50	301	5.1(120)	40	60	206	5.1(120)
2.	histidine	8.0	-	-	-	-	170	180	24	-
		7.9	70	100	614	-	-	-	-	-
		7.4	60	100	648	-	160	160	32	-
		6.0	30	50	453	-	-	-	-	-
		5.9	-	-	-	-	40	60	190	-
		5.5	30	50	320	-	-	-	-	-
		5.3	-	-	-	-	50	50	44	-
3.	arginine	8.0	60	100	563	6.5(160)	100	140	134	6.5(160)
		7.55	55	100	680	6.5(130)	60	110	553	6.5(130)
		6.1	40	50	478	5.4(130)	45	80	411	5.4(130)
		5.7	-	-	-	-	45	80	197	5.2(160)
4.	leucine	7.9	130	160	185	6.5(190)	150	170(M)	24	6.6(190)
		7.5	60	90	601	6.5(190)	80	130	111	6.1(190)
		6.0	30	60	429	5.3(120)	40	60	121	5.3(120)
		5.5	40	60	247	5.0(120)	40	70	71	4.9(120)
5.	aspartate	8.0	60	110	562	6.6(180)	90	160	430	6.7(180)
		7.5	60	90	563	6.6(180)	75	160	457	6.7(180)
		6.1	30	45	434	5.5(120)	40	60	294	5.6(120)
		5.7	35	50	347	5.3(120)	40	70	151	4.6(120)

Bacteria: Grown on nutrient agar; Experiment 1, 16 hour culture, Experiment 2, 13 hour culture, Experiment 3, 7 hour culture, Experiment 4, 13 hour culture, Experiment 5, 12 hour culture; 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, initial pH as shown. Method of measuring initial pH as given in the test. 0.033 M glucose, amino acids as indicated, concentrations as given in Table XXVII (normal concentration).

Other conditions as in Table XII.

* The figures in parentheses by the final pH values indicate the duration of the experiment (minutes).

lengthening of the lag periods only at neutral or slightly alkaline pH values (7.4-8.0). At slightly acid pH values (5.6-6.1), no, or comparatively little, increase in the lag period was found to take place on omission of the amino acid in question. For all amino acids, however, omission did cause a decrease in the final rate of hydrogen evolution. It has been noted that none of these amino acids had any stimulatory effect on the formic hydrogenlyase activity of adapted cells. The fall of final activity following their omission could not, therefore, be ascribed to the removal of a stimulating substance.

It was also possible, that the decrease in final rate of hydrogen evolution was caused by a decreased buffering power of the medium on removal of the amino acid and a consequent greater fall of pH due to glycolysis. It is already known and has been shown for these bacteria as well (Table XLVIII) that if the pH is reduced from a neutral value a decrease in formic hydrogenlyase activity ensues. The measurements of pH at the end of these experiments showed, however, that in general the final pH was the same for solutions of the same initial pH, regardless of the presence or absence of any one amino acid. The final pH of solutions from which histidine was missing was not measured in this series of experiments, but the buffering power of this amino acid, in the concentrations used, was no greater than that of the other amino acids. Furthermore, histidine was found maximally effective in a concentration of 2×10^{-5} M, at which concentration its buffering power is negligible.

These findings are of importance for a proper interpretation of the significance of the lag periods in formic hydrogenlyase synthesis. Considering them, it would appear unlikely that the lag periods increase when one of the five important amino acids is omitted at a neutral pH because time is necessary for the synthesis of the omitted amino acid. More detailed interpretation of the effects of pH changes are as yet not possible.

Summary and conclusions:

In the formate-peptone system, at pH values above 7.4 the lag periods before the onset of hydrogen evolution and before the maximal rate of hydrogen evolution was reached were longer than those at pH 7.4; at a pH of 6.1 no formic hydrogenlyase was formed.

In the presence of glucose and either peptone or a mixture of five amino acids, the lag periods were approximately half as long at an initial pH of 6.1 as at an initial pH of 7.4. At pH values below 6.1 no further decrease in the lag periods was observed.

At a pH of 7.4 or 7.9, omission of each of the five most important amino acids, separately, caused an increase in the lag periods. At a pH of 5.6 or 6.1, each amino acid, with the exception of arginine, could be omitted, so long as the other four amino acids were present, with little or no consequent increase in lag periods. If an amino acid was omitted at one of the lower pH values, even when there was no increased lag period, there was a decreased rate of hydrogen evolution, indicating a lowered amount of enzyme synthesis.

PART VI

Inhibitors of Formic Hydrogenlyase Formation

Inhibitors of adaptation in the formate-tryptone system:

2-4 Dinitrophenol and chloromycetin

Before a synthetic amino acid medium for formic hydrogenlyase formation had been developed and while peptone and tryptone were being used as sources of nitrogen, substances known to be inhibitors of other adaptive processes were tested as possible inhibitors of formic hydrogenlyase formation.

It is known from the work of Spiegelman (39) and Monod (44) that 2-4 dinitrophenol inhibits adaptive enzyme formation in yeast and in bacteria. The results of an experiment in which the effect of this substance on formic hydrogenlyase formation was measured are reported in Table LII. It may be seen from these results that in a 10^{-4} M concentration, 2-4 dinitrophenol had a strong inhibitory effect on the final rate of hydrogen production when added to unadapted cells in the presence of formate and tryptone, but had little or no effect when added to adapted cells evolving hydrogen at a steady rate. This showed that 2-4 dinitrophenol acted as an inhibitor of formic hydrogenlyase formation. Further experiments with this substance were not performed.

TABLE LII

The inhibition of formic hydrogenlyase formation by 2-4 dinitrophenol

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1. Unadapted bacteria	Formate + tryptone	60	140	33
	Formate + tryptone + 10^{-4} M 2-4 dinitrophenol	80	140	12
2. Adapted bacteria	Before adding 10^{-4} M 2-4 dinitrophenol	-		36
	After adding 10^{-4} M 2-4 dinitrophenol	-		31

Bacteria: Grown overnight on nutrient agar; 3.0 mg. bacteria/vessel.
In main vessels: 0.033M phosphate buffer, pH 7.4, 0.067M formate, 1% tryptone.

In Experiment 1, bacteria were tipped in from the side arm at zero time, 2-4 dinitrophenol as shown was in the main vessel. In Experiment 2, bacteria were in the main vessel and H_2 evolution was followed until a steady rate was reached. Then 2-4 dinitrophenol, as shown, was tipped in from the side arm. Q-H_2 values given were measured in the 20 minutes before and the 20 minutes after tipping in the 2-4 dinitrophenol.

Other conditions as in Table XII.

Chloromycetin was found by Hahn and Wisseman (60) completely to inhibit adaptation to lactose oxidation by *E. coli*. In the strain of *E. coli* employed by these investigators, adaptation apparently took place without the addition of a source of nitrogen. It was found for the formic hydrogenlyase system as well that chloromycetin, in a concentration of 50 µgm./ml. (the concentration used by Hahn and Wisseman), completely inhibited adaptation, without affecting hydrogen production by adapted cells (Table LIII).

TABLE LIII

The effect of chloromycetin on formic hydrogenlyase formation

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1. Unadapted bacteria	Formate + tryptone	60	140	33
	Tryptone + chloromycetin	>180	0	0
	Formate + tryptone + chloromycetin	>180	0	0
2. Adapted bacteria	Formate		-	281
	Formate + chloromycetin		-	264

Bacteria: Experiment 1, grown overnight on nutrient agar, 3 mg. bacteria/vessel. Experiment 2, grown 8 hours in glucose broth, 1.5 mg. bacteria/vessel.
In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.067 M formate, 1% tryptone, 50 µgm./ml. chloromycetin.

H_2 evolution in Experiment 2 measured from 0 to 30 minutes.

Other conditions as in Table XII.

Inhibition of formic hydrogenlyase formation in the glucose-five amino acid system.

The development of a simplified amino acid mixture as a nitrogen source for formic hydrogenlyase formation made it possible to study in detail the action of some inhibitors of adaptation. The results obtained indicated that some of these inhibitors interfered with reactions involving specific amino acids. It will be seen that such information could not have been gained when a medium of unknown composition was used as a nitrogen source.

D-histidine and histamine

Because of the importance of l-histidine in formic hydrogenlyase formation, experiments were performed with substances thought likely to interfere in some way with the assimilation or metabolism of this amino acid. Two substances to be tested for this property were d-histidine and histamine. It is known that many bacteria have the ability to form histamine by decarboxylating histidine (158). The enzyme, histidine decarboxylase, is adaptive, and requires for its formation not only the presence of histidine, but also an acid growth medium and a temperature lower than 37°C. Since growth on agar plates took place at a neutral pH and at 37°C, it is unlikely that histidine decarboxylase was present in the bacteria used. In any case, as the results of experiment 1 of Table LIV show, histamine was unable to replace l-histidine as a source of nitrogen for formic-hydrogenlyase formation. It was also found, and is shown in Table LIV, that d-histidine could not substitute for l-histidine.

Experiments in which the inhibitory effects of d-histidine and histamine on formic hydrogenlyase formation were measured are reported in Tables LV and LVI. Here, the effect of these substances on adaptation in the glucose-five amino acid system was tested in the presence of two concentrations of l-histidine, 2×10^{-3} M and 2×10^{-5} M. Only a slightly greater amount of formic hydrogenlyase was formed in the presence of the higher concentration of histidine and the lag periods

TABLE LIV

The inability of d-histidine and histamine to replace l-histidine in formic hydrogenlyase formation.

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1.	Glucose + all twenty amino acids	40	70 (M)	712
	Glucose + all twenty amino acids but l-histidine	>90	-	0
	Glucose + all twenty amino acids but l-histidine + d-histidine	>90	-	0
2.	Glucose + all twenty amino acids	40	60 (M)	692
	Glucose + all twenty amino acids but l-histidine	95	95 (M)	21
	Glucose + all twenty amino acids but l-histidine + 2 x 10^{-3} M histamine	95	95 (M)	16
	Glucose + all twenty amino acids but l-histidine + 2 x 10^{-4} M histamine	95	95 (M)	21

Bacteria: Grown 5 hours on nutrient agar, 1.5 mg. bacteria/vessel in both experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, amino acids as given on page 107, 0.002 M d-histidine, histamine as shown.

Other conditions as in Table XII.

at the two histidine concentrations were approximately identical. The lower concentration of histidine was used when first investigating the effects of histamine and other possible inhibitors, because if any competition between histidine and the molecule being tested were taking place, the inhibitor would be expected to have a more marked effect at the lower histidine concentration.

It was found that adaptation was inhibited by histamine in concentrations of 0.0017 or 0.01 M when 2×10^{-5} M l-histidine was present in the amino acid mixture, but that no inhibition took place when the concentration of histidine was raised to approximate that of the histamine. The reversal of inhibition by higher concentrations of histidine was taken to indicate that histamine was acting in some way as an antagonist of histidine.

The inhibition due to histamine, however, was only a moderate one, and was no greater with 0.01 M than with 0.0017 M histamine. It was thought that the inhibition by histamine might be dependent upon the pH of the medium and that a greater effect of histamine would be found at pH values other than 7.4. In Table LVII are shown the results of an experiment testing this possibility. The buffers used in this experiment are given in the table. The initial pH value of the solution in each Warburg vessel was measured as in the experiments reported in Table LI , in solutions made up separately. It may be seen from the data in Table LVII that only at pH 7.4 was there any effect on adaptation by histamine. Because no greater inhibitory effect of histamine than

TABLE LV

The inhibition of formic hydrogenlyase formation by histamine and the reversal of this inhibition by histidine

Experiment	System	Lag, minutes T_1	T_2	Final $Q\text{-}H_2$
1.	Glucose + four amino acids + 2×10^{-5} M histidine	50	80	557
	Glucose + four amino acids + 2×10^{-3} M histidine	50	90	646
	Glucose + four amino acids + 2×10^{-5} M histidine + 1.7×10^{-3} M histamine	60	100	440
	Glucose + four amino acids + 2×10^{-3} M histidine + 1.7×10^{-3} M histamine	50	90	708
2.	Glucose + four amino acids + 2×10^{-5} M histidine	40	80	567
	Glucose + four amino acids + 10^{-2} M histidine	50	80	747
	Glucose + four amino acids + 2×10^{-5} M histidine + 10^{-2} M histamine *	60	115	552
	Glucose + four amino acids + 10^{-2} M histidine + 10^{-2} M histamine	50	80	755

Bacteria: Grown 5-6 hours on nutrient agar, 1.5 mg. bacteria/vessel in both experiments.
In main vessels: 0.033 M phosphate buffer, pH 7.4 0.033 M glucose. Four amino acids: dl-serine, l-arginine, l-leucine, and dl-aspartate as given in Table XXVII (normal concentrations), l-histidine and histamine as shown.

Other conditions as in Table XII.

* This concentration of histamine had no effect on the formic hydrogenlyase activity of adapted bacteria: $Q\text{-}H_2$, formate = 540; $Q\text{-}H_2$, formate + 0.01 M histamine = 544. See Table LXII, Experiment 2, for conditions.

TABLE LVI

The effects of d-histidine on formic hydrogenlyase formation

Experiment	System	Lag, minutes T_1	T_2	Final $Q\text{-}H_2$
1.	Glucose + four amino acids + 2×10^{-3} M l-histidine	50	80	692
	Glucose + four amino acids + 2×10^{-3} M l-histidine + 2×10^{-3} M d-histidine	50	80	668
2.	Glucose + four amino acids + 2×10^{-5} M l-histidine	50	80	545
	Glucose + four amino acids + 2×10^{-5} M l-histidine + 2×10^{-3} M d-histidine	55	115	472

Bacteria: Grown 5 hours on nutrient agar, 1.5 mg. bacteria/vessel in both experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, four amino acids as given in Table LV, l-histidine and d-histidine as shown.

Other conditions as in Table XII.

TABLE LVII

The influence of pH upon the inhibition by histamine of formic hydrogenlyase formation.

Initial pH	System: Glucose + four amino acids + 2×10^{-5} M histidine — Lag, minutes T_1	T_2	System: Glucose + four amino acids + 2×10^{-5} M histidine + 10^{-2} M histamine — Lag, minutes T_1	T_2
7.8	60	120		
7.7			70	120
7.4	50	80	60	120
6.4			20	50
6.3	30	50		
6.1			20	50
5.6	40	70		

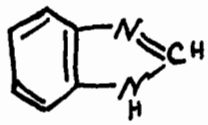
Bacteria: Grown 6 hours on nutrient agar, 1.5 mg. bacteria/vessel

In main vessels: Buffers: 0.033 M Na_2HPO_4, 0.033 M phosphate buffers of pH 7.4 and 5.7, 0.033 M NaH_2PO_4. Initial pH values as shown (see text). Four amino acids as in Table LV. l-histidine and histamine as shown.

Other conditions as in Table XII.

that reported, could be attained, further studies of the histamine inhibition were not carried out.

The results reported in Table LVI show that d-histidine had no effect on adaptation, when present in the same concentration as l-histidine, and only a slight inhibitory effect when present in a concentration one hundred times as great. No further studies were made of the action of this substance on formic hydrogenlyase formation.

Benzimidazole 

The action of benzimidazole on formic hydrogenlyase formation was investigated because of the possibility that this substance, containing the imidazole nucleus, might act as an antagonist to histidine. It was found that in 0.003 M to 0.0033 M concentrations, benzimidazole inhibited adaptation without inhibiting formic hydrogenlyase activity. At a benzimidazole concentration of 0.01 M, formic hydrogenlyase activity was also inhibited. These facts are presented in Table LVII2. From these and later experimental results it will be seen that the degree of inhibition of adaptation by benzimidazole varied with different bacterial cultures.

When the effect of pH upon inhibition by benzimidazole was tested, it was found that a considerable degree of inhibition by benzimidazole took place over in the range, pH 5.6-7.9, (Table LVIII).

Increasing the histidine concentration brought about no reversal of inhibition by benzimidazole, (Table LVIIA). In this experiment, with the histidine concentration of $2.\times 10^{-5}$ M, the lags were longer and the final rate lower than was usual in this system. The results showed, nevertheless, that there was a definite inhibition by benzimidazole at the lower histidine concentration and that there was no reversal of this inhibition by higher concentrations of histidine. It seemed unlikely, therefore, that benzimidazole was acting as an antagonist of histidine.

TABLE LVII*a*

The effect of benzimidazole on formic hydrogenlyase formation in the glucose-five amino acid system

Experiment	System	Lag, minutes T_1	T_2	Final $Q\text{-}H_2$
1. Unadapted bacteria	Glucose + four amino acids + 2×10^{-5} M histidine	50	80	531
	Glucose + four amino acids + 2×10^{-5} M histidine + 2×10^{-3} M benzimidazole	120	180	131
2. Unadapted bacteria	Glucose + four amino acids + 2×10^{-5} M histidine	80	110	148
	Glucose + four amino acids + 2×10^{-3} M histidine	50	80	536
	Glucose + four amino acids + 2×10^{-5} M histidine + 3.3×10^{-3} M benzimidazole	100	140 (M)	90
	Glucose + four amino acids + 2×10^{-3} M histidine + 3.3×10^{-3} M benzimidazole	>160		0
2a. Adapted bacteria	Before adding 3.3×10^{-3} M benzimidazole		-	188
	After adding 3.3×10^{-3} M benzimidazole		-	181
3. Adapted bacteria	Formate, pH 6.8		-	491
	Formate, pH 6.8 + 1×10^{-3} M benzimidazole		-	491
	Formate, pH 6.8 + 3×10^{-3} M benzimidazole		-	489
4. Adapted bacteria	Formate, pH 6.8		-	405
	Formate, pH 6.8 + 1×10^{-2} M benzimidazole		-	240

TABLE LVIIa (Cont'd)

Bacteria: Experiment 1, 7 hour culture on nutrient agar, Experiments 2 and 2 a, 5 1/2 hour culture on nutrient agar, Experiment 3, 8 hour culture in glucose broth, Experiment 4, 12 hour culture in glucose broth; 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4 unless otherwise indicated, 0.033 M glucose, 0.067M formate four amino acids as given in Table LV, l-histidine and benzimidazole as shown.

In Experiment 2a., adaptation was carried out in the Warburg vessel in a glucose-five amino acid medium. H_2 evolution was measured 20 minutes before and 50 minutes after tipping in benzimidazole.

H_2 evolution measured from 10 to 40 minutes in Experiment 3, from 10 to 40 minutes in Experiment 4.

Other conditions as in Table XII.

TABLE LVIII

The influence of pH upon the inhibition by benzimidazole of formic hydrogenlyase formation.

Initial pH	System: Glucose + five amino acids — Lag, minutes T_1	T_2	System: Glucose + five amino acids + 3.3×10^{-3} M benzimidazole — Lag, minutes T_1	T_2
7.8	45	80	90	140
7.4	40	80	90	140
6.3	30	45	80	100
5.6	30	60	70	100

Bacteria: Grown 5 hours on nutrient agar, 1.5 mg. bacteria/vessel

In main vessels: Buffers as in Table LVII. Initial pH as shown. 0.033 M glucose, five amino acids as in Table XXVII (normal concentrations). Benzimidazole as shown.

Other conditions as in Table XII.

Subsequent experiments revealed that the inhibition of formic hydrogenlyase formation by benzimidazole could be counteracted by amino acids other than histidine. After it had been found that benzimidazole inhibited adaptation in the simplified amino acid medium, its effect on adaptation was tested using peptone as a source of nitrogen. It was found that there was virtually no inhibition of adaptation by benzimidazole in the glucose-peptone system. Further experiments revealed that there was no inhibition by benzimidazole when casein hydrolysate or a synthetic mixture of twenty amino acids were used as nitrogen sources for formic hydrogenlyase formation. The results of these experiments are presented in Table LIX; in

TABLE LIX

The effect of benzimidazole on formic hydrogenlyase formation in the glucose-peptone, glucose-casein hydrolysate, and the glucose-five and glucose-twenty amino acid systems.

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H2
1.	Glucose + peptone	50	90	1138
	Glucose + peptone + 0.0033 M benzimidazole	50	90	922
2.	Glucose + casein hydrolysate	60	90	692
	Glucose + casein hydrolysate + 0.0033 M benzimidazole	70	100	600
3.	Glucose + five amino acids	50	90	651
	Glucose + five amino acids + 0.0033 M benzimidazole	80	130	584
	Glucose + twenty amino acids	40	90	810
	Glucose + twenty amino acids + 0.0033 M benzimidazole	50	90	876

Bacteria: Grown 5-6 hours on nutrient agar, 1.5 mg. bacteria/vessel in all experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 1% peptone, 1.67% casein hydrolysate, amino acids as given on page 107 and Table XXVII, benzimidazole as shown.

Other conditions as in Table XII.

experiment 3 of this table the effects of benzimidazole on adaptation in the glucose-five amino acid system and the glucose-twenty amino acid system were compared, with the results shown.

These observations were taken to indicate that these three sources of nitrogen contained some antagonist(s) to the action of benzimidazole, and the last observation showed that this antagonist(s)

must be an amino acid(s). Accordingly, experiments were carried out to determine precisely which amino acid(s) was involved. A preliminary experiment, using the four groups into which the amino acids had been arbitrarily divided (page/07), showed that two or more amino acids were involved in counteracting the inhibitory effect of benzimidazole (Table LX). The groups found effective were that containing lysine and tryptophan (No. 3) and that containing glycine, alanine, proline, hydroxyproline, and threonine (No. 1). These amino acids were then tested separately, (Table LXI). It was known from earlier work that the addition of other amino acids to the five most important ones produced but little effect on adaptation. In these preliminary experiments, therefore, a control series was not carried out to test the action of each amino acid or group of amino acids on adaptation in the glucose-five amino acid system in the absence of any inhibitor. It may be seen from the results in Table LXI that only two amino acids had any significant effect in reversing the inhibition by benzimidazole. These were tryptophan and alanine.

It was found that in combination tryptophan and alanine could virtually reverse the inhibition of adaptation caused by benzimidazole (Figure XVI). The interpretation of this phenomenon is not yet known. It is suggested, however, that tryptophan and alanine are both essential to formic hydrogenlyase formation, but that they can be formed in a sufficient rate from the five most important amino acids to make their separate addition unnecessary. It is possible that benzimidazole

TABLE LX

The reversal of the benzimidazole inhibition of formic hydrogenlyase formation by different groups of amino acids.

System	Lag, minutes T_1	T_2	Final Q-H_2
Glucose + five amino acids	45	90	715
Glucose + five amino acids + 0.0033 M benzimidazole	100	140	300
Glucose + five amino acids + 0.0033 M benzimidazole + amino acid group No. 1	70	130	724
Glucose + five amino acids + 0.0033 M benzimidazole + amino acid group No. 2	100	140	176
Glucose + five amino acids + 0.0033 M benzimidazole + amino acid group No. 3	50	100	829
Glucose + five amino acids + 0.0033 M benzimidazole + amino acid group No. 4	100	140	324

Bacteria: Grown 6 hours on nutrient agar, 1.5 mg. bacteria/vessel

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, five amino acids as given in Table XXVII (normal concentrations), groups of amino acids as on page 107, omitting those amino acids in the five amino acid mixture; benzimidazole as shown.

Other conditions as in Table XII.

TABLE LXI

The ability of individual amino acids in Groups 1 and 3 to reverse the benzimidazole inhibition of formic hydrogenlyase formation in the glucose-five amino acid system.

System	Lag, minutes T_1	T_2	Final Q-H_2
Glucose + five amino acids	60	100	331
Glucose + five amino acids + 0.003 M benzimidazole	90	120	278
Glucose + five amino acids + 0.003 M benzimidazole + glycine	90	130	212
Glucose + five amino acids + 0.003 M benzimidazole + proline	90	120	294
Glucose + five amino acids + 0.003 M benzimidazole + alanine	60	110	319
Glucose + five amino acids + 0.003 M benzimidazole + hydroxyproline	90	120	282
Glucose + five amino acids + 0.003 M benzimidazole + threonine*	-	-	-
Glucose + five amino acids + 0.003 M benzimidazole + lysine	90	130	224
Glucose + five amino acids + 0.003 M benzimidazole + tryptophan	60	110	462

* - Lost; in a separate experiment, threonine gave no reversal of benzimidazole inhibition.

Bacteria: Grown 5 hours on nutrient agar, 1.5 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, five amino acids as given in Table XXVII (normal concentrations), individual amino acids in same concentrations as in complete mixture (see page 107), benzimidazole as shown.

Other conditions as in Table XII.

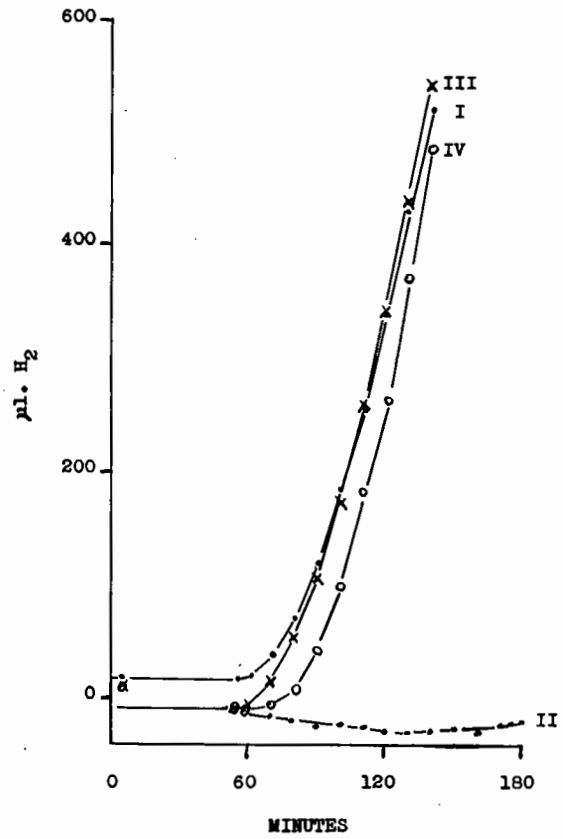


FIGURE XVI

Reversal of the benzimidazole inhibition of formic hydrogenlyase synthesis by tryptophan and alanine.

I. Glucose + five amino acids
II. Glucose + five amino acids + benzimidazole
III. Glucose + five amino acids + tryptophan + alanine
IV. Glucose + five amino acids + benzimidazole + tryptophan + alanine.

Bacteria: Grown 14 hours on nutrient agar; 1.5 mg. bacteria/vessel.

In main vessels: 0.033 M phosphate buffer, pH 7.4, 0.033 M glucose, 0.003 M benzimidazole, amino acids as in Table XXVII (normal concentrations), tryptophan and alanine as in Table .

Other conditions as in Table XII.

interferes with the synthesis of these two amino acids, so that its action can be reversed if the amino acids are added to the medium.

Benzimidazole is known to inhibit bacterial growth by acting as a purine antagonist. Its inhibitory effects on growth may be reversed competitively by adenine or guanine (159). In its inhibition of adaptation, it seems to act rather as an amino acid antagonist. The effects of purines on the benzimidazole inhibition of adaptation were not investigated in this work.

Other inhibitors

The anti-histaminic compounds, neoantergan and chlorprophen-pyridamine maleate (1-parachlorophenyl-1-(2-pyridyl)-3-dimethylamino propane maleate) were both found to inhibit formic hydrogenlyase synthesis. No attempt was made, however, to find whether these substances acted as histidine antagonists. The drug, Rimifon (isonicotinyl hydrazide), recently discovered to be of value in tuberculosis therapy (160), had no effect on formic hydrogenlyase synthesis. These facts are presented in Table LXII.

TABLE LXII

The effect of neo-antergan, chlorprophenpyridamine maleate, and Rimifon on formic hydrogenlyaze synthesis.

Experiment	System	Lag, minutes T_1	Lag, minutes T_2	Final Q-H_2
1. Unadapted bacteria	Glucose + five amino acids	50	80	531
	Glucose + five amino acids + neo-antergan	80	155	466
	Glucose + five amino acids + chlorprophenpyridamine maleate	70	155	760
	Glucose + five amino acids + Rimifon	50	80	556
2. Adapted bacteria	Formate	-		540
	Formate + neo-antergan	-		542
	Formate + chlorprophenpyridamine maleate	-		566

Bacteria: Experiment 1, grown 7 hours on nutrient agar, Experiment 2, grown 12 hours in glucose broth, 1.5 mg. bacteria/vessel in both experiments.

In main vessels: 0.033 M phosphate buffer, pH 7.4 in Experiment 1, pH 6.8 in Experiment 2, 0.033 M glucose; amino acids as in Table LVII, Experiment 1, 1 mg./vessel of Rimifon and of inhibitors, 0.067 M formate.

H_2 evolution measured from 10 to 40 minutes.

Other conditions as in Table XII.

Summary and conclusions

2-4 Dinitrophenol and chloromycetin, which are known to inhibit the formation of various adaptive enzymes, also inhibited the synthesis of formic hydrogenlyase in the presence of formate and tryptone.

In the glucose-five amino acid system, histamine in 0.0017-0.01 M concentration inhibited adaptation when l-histidine was present in a minimal effective concentration, but had no effect on adaptation when the l-histidine concentration was approximately the same as that of histamine. This indicated that histamine inhibited adaptation by acting as an antagonist to l-histidine. In a similar manner it was shown that d-histidine behaves to a small extent as an antagonist of l-histidine.

Benzimidazole also inhibited adaptation in the glucose-five amino acid system. The inhibition by benzimidazole was approximately the same at a minimal effective concentration as at all other concentrations investigated of histidine. It was unlikely, therefore, that benzimidazole acted as a histidine antagonist. The inhibitory action of benzimidazole could be partially reversed by either alanine or tryptophan, and almost completely reversed by these two amino acids in combination. It was suggested from their finding that benzimidazole may prevent the synthesis of these two amino acids from the other amino acids present.

Two anti-histaminic substances, neo-antergan, and chlorprophen-pyridamine maleate, inhibited formic hydrogenlyase synthesis. The mechanism of this inhibition was not studied.

PART VII

Discussion

During the presentation of the experimental results, the implications of the findings reported were discussed. Only a general consideration of the salient points treated in this work, therefore, need be given here.

In this work two lines of enquiry were stressed. The first of these dealt with the nitrogen sources necessary for formic hydrogenlyase synthesis, the second with the mechanisms by which energy is supplied for this synthesis. The two problems were not independent. An adequate examination of the energetic basis of formic hydrogenlyase formation could only be made after a simplified medium which would support formic hydrogenlyase formation had been devised.

A detailed study of the effect of amino acids on the time necessary for formic hydrogenlyase formation in resting cell suspensions was undertaken because it was believed that such a study would reveal more of the nature of the nitrogen sources required for formic hydrogenlyase formation than could a study in which only the final amount of formic hydrogenlyase produced after a long period of growth

was measured. This supposition was shown to be fully justified. Billen and Lichstein (100) found that l-glutamate alone could replace a complete amino acid mixture in stimulating formic hydrogenlyase production in growing cultures of E. coli. It was also found with the bacteria used here that glutamate alone could serve as a nitrogen source for this adaptive process, but there was an important difference between its action and that of a complete amino acid mixture. The time required for the synthesis of the formic hydrogenlyase system in the presence of glutamate alone was considerably greater than the time required if a more complete mixture of amino acids was present. Further, despite the activity of glutamate when tested alone, when a determination was made of those amino acids most important in stimulating formic hydrogenlyase synthesis, glutamate was not among them.

It is possible that glutamate was found important, not so much because it was used in formic hydrogenlyase synthesis but because it served as a source of those amino acids used in this adaptive process. If this is true, it might explain why more time was required for formic hydrogenlyase synthesis in the presence of glutamate, than in the presence of a more complete amino acid mixture. The extra time could be taken up in the synthesis from glutamate of those amino acids most important in formic hydrogenlyase synthesis.

A comparison may be made between those amino acids found most important for formic hydrogenlyase synthesis by the bacteria used here,

in resting cell suspensions: serine, histidine, arginine, leucine, and aspartate, and those found important by Billen and Lichstein (100) in growing cell suspensions of their "Texas" strain of E. coli: glutamate, methionine, tyrosine, cystine, lysine, and valine. It is seen that the two methods of testing give a completely different picture of those amino acids most important in the synthesis of this adaptive enzyme system. Because the strains of bacteria used were different, a more extended comparison of these nitrogen sources does not appear justified.

Even with the method used here, employing resting cell suspensions of bacteria, some of the amino acids important for formic hydrogenlyase formation may escape notice. This would occur if the required amounts of these amino acids are relatively small and if they are easily synthesized from the other amino acids present. The fact that the benzimidazole inhibition was reversible by tryptophan and alanine indicates that both these amino acids may be important in formic hydrogenlyase synthesis, although they were not found so by the method of testing first employed.

It must be realized that although it is necessary to add amino acids to cells of E. coli for the synthesis of formic hydrogenlyase, this is not because the organism is itself unable to synthesize these amino acids. E. coli may grow in a simple medium consisting of glucose, ammonia, and inorganic salts. It can, therefore, synthesize all amino acids. It seems that unless the necessary amino acids are present in relatively high concentrations, no enzyme synthesis can

take place. The minimal effective concentrations of the amino acids added in these studies must have been many times as great as the concentrations of amino acids present in a bacterial culture using ammonia as its sole source of nitrogen.

Certain amino acids have been spoken of as being of importance for formic hydrogenlyase synthesis. Although this may seem to imply that these amino acids are used for the synthesis of the enzyme, there is as yet no direct evidence on the manner in which the amino acids act. It is not known if the amino acids are incorporated more or less directly into the molecules of the formic-hydrogenlyase system, if their presence stimulates enzyme synthesis without their utilization being involved, or if some other mechanism is in operation. Investigations which would decide conclusively between these possibilities would be difficult at present because the formic hydrogenlyase system has not yet been purified.

These studies have indicated the importance of an energy source for formic hydrogenlyase synthesis. In the presence of formate alone, this source of energy is provided by an anaerobic transfer of hydrogen from a donor to an acceptor. The results have indicated that fumarate, derived from aspartate, is the hydrogen acceptor when the synthesis of formic hydrogenlyase takes place in the presence of formate and peptone. When either glucose or pyruvate serves as a source of formate, the energy may be supplied through the anaerobic breakdown of these compounds. There is some evidence, not yet conclusive, that

fumarate and aspartate, as hydrogen acceptors, may supply additional energy for adaptation in the presence of glucose and pyruvate.

The experiments on inhibitors of formic hydrogenlyase synthesis in the simplified medium have introduced a new method into the study of enzymatic adaptation. It has long been known that enzymatic adaptation is sensitive to very many cell poisons. The mechanism of action of such substances, however, has seldom been closely explored. Thus, sodium azide and 2-4 dinitrophenol inhibit enzymatic adaptation by cutting off the essential energy supply. They act in a general, rather than a specific way, at a site which may be functionally far removed from the active site of enzyme synthesis. The fact that these agents inhibit adaptation only confirms the fact that energy is needed for adaptation.

In the few experiments performed here, it was seen that inhibitors such as histamine and benzimidazole may inhibit formic hydrogenlyase synthesis by inhibiting the utilization of amino acids or the synthesis of essential amino acids from those present in the synthetic medium. It is to be expected that future experiments on the effect of substances structurally similar to amino acids will yield valuable information regarding the role of amino acids in enzymatic adaptation.

It seems in place to give here a résumé of the present state of knowledge of enzymatic adaptation and some prediction of the future advances which may be expected in the understanding of this phenomenon. Work carried out up to the present has led to a description and an understanding of some of the general factors involved in adaptive enzyme formation. It has been shown that enzymatic adaptation is a synthetic process, requiring energy. In many cases, enzymatic adaptation has been demonstrated to involve the synthesis of new enzymatic material. Evidence has been advanced to show that the new enzymatic material is taken from a pool of enzyme-precursor material, suitable for the synthesis of many different enzymes. The fact has been established that the power to synthesize adaptive enzymes is under genetic control.

Thus, the general nature of enzymatic adaptation has been mapped out. In the future it is to be expected that more specific properties of this phenomenon will be studied. The aspects of enzymatic adaptation of the greatest interest and those on which a considerable amount of future work is to be expected are, in the writer's opinion:

1. The mechanism whereby the substrate induces adaptive enzyme formation.
2. The role of nucleic acids in adaptive enzyme formation. That nucleic acids are of importance is indicated by the genetic studies; but only a little direct evidence on this point has thus far appeared (161, 162).

3. The nature of the transformation of an inactive precursor into an active enzyme.
4. The mechanisms whereby external sources of nitrogen influence adaptive enzyme formation.
5. The mode of action of specific rather than general inhibitors on the adaptive process.

It is readily seen how wide are the implications of these fields of study. Each is concerned with the intimate mechanism of intracellular protein and enzyme synthesis. Knowledge of the problems presented here, gained by a study of adaptive enzyme formation should give an insight into mechanisms of protein synthesis, both in micro-organisms and in higher animals. In addition, it is to be expected that further knowledge of protein synthesis, gained by other methods of enquiry, will throw light on the mechanisms of adaptive enzyme formation.

CLAIMS TO ORIGINAL RESEARCH

The Galactozymase System.

1. Sodium l-glutamate stimulated galactozymase formation in a slowly-adapting strain of bakers' yeast. The stimulation given by glutamate was greater than that given by an equal concentration of $NH_4H_2PO_4$.

2. 0.1% l-cysteine HCl stimulated galactozymase formation. A smaller stimulation was given by 0.5% cysteine HCl. Cysteine stabilized the galactozymase system once it was formed.

3. Both cysteine and dithioglycerol inhibited the loss of galactozymase activity by adapted cells suspended aerobically in the absence of galactose.

The Maltozymase System.

1. The sulfhydryl compounds, l-cysteine and sodium thioglycollate, inhibited aerobic and anaerobic maltozymase formation in bakers' yeast.

2. Similar effects were produced by atabrine. The atabrine inhibition was, to a small extent, reversed by riboflavine.

3. Alcoholic solutions of diethyl stilbestrol and Δ-4-androstenedione strongly inhibited anaerobic maltozymase formation. In addition, diethyl stilbestrol in alcoholic solution inhibited maltose fermentation by adapted yeast. In aqueous solution, these steroids had no inhibitory effect, but rather caused a slight stimulation of adaptation.

4. Ethyl alcohol itself was found to stimulate maltozymase formation.

The Formic Hydrogenlyase System.

1. The amino acids most important to formic hydrogenlyase formation in non-proliferating cells of E. coli were determined. These amino acids, and the smallest concentration of each which gave a maximal effect, were:

dl-serine	7.9×10^{-3} M
l-histidine	2×10^{-5} M
l-arginine	3.7×10^{-4} M
l-leucine	1.8×10^{-2} M
dl-aspartic acid	1×10^{-2} M

It was possible to replace aspartate with an equal concentration of fumarate.

A mixture of these five amino acids was found almost as effective in stimulating adaptation as a mixture of twenty amino acids, peptone, or casein or gelatin hydrolysates.

2. When formic hydrogenlyase synthesis took place in the presence of sodium formate and peptone, the addition of aspartate, fumarate, and nitrate brought about a stimulation of adaptation.

3. In the presence of glycerol and peptone, the addition of fumarate stimulated formic hydrogenlyase formation. It was proposed that fumarate, aspartate, and nitrate, which are known to act as hydrogen acceptors in E. coli, stimulate formic hydrogenlyase formation by supplying energy for the adaptive process.

4. 0.1 M NH_4Cl or $(NH_4)_2SO_4$ completely inhibited adaptation in the presence of formate and peptone, but had no effect on adaptation when glucose was used as a source of the adaptive substrate, formate. The inhibition by ammonia in the formate-peptone system could be partly reversed by the addition of 0.01 M $NaNO_3$.

5. When formic hydrogenlyase formation took place in the presence of glucose and glutamate, the addition of aspartate or fumarate inhibited adaptation.

6. 0.1 M NH_4Cl was found to stimulate anaerobic glycolysis in E. coli.

7. When glucose was used as a source of the adaptive substrate, a reduction of the pH of the medium from 7.4 to 5.6-6.1 reduced the time necessary for the synthesis of formic hydrogenlyase.

8. At a neutral pH, omission of any of the five amino acids given above increased the time required for enzyme formation and decreased the final amount of enzyme formed. At pH values of 5.6-6.1, little increase in the time necessary for enzyme formation was observed following the omission of serine, histidine, leucine, or aspartate. If arginine were omitted at the lower pH values, the lag periods were increased. Omission of any of the five amino acids decreased the final amount of enzymatic activity.

9. 2-4 Dinitrophenol and Chloromycetin, which are known to inhibit the formation of other adaptive enzyme systems, also inhibited the formation of the formic hydrogenlyase system.

10. In a glucose-amino acid medium in the presence of 2×10^{-5} M histidine,

d-histidine and histamine inhibited adaptation. If the l-histidine concentration were increased to 2×10^{-3} M, d-histidine and histamine caused no inhibition of adaptation. These substances, then, inhibited adaptation by acting as antagonists to l-histidine.

11. Benzimidazole inhibited adaptation in the presence of glucose and the five amino acids given above. This inhibition was partly reversed if either tryptophan or alanine were added and completely reversed if both amino acids were added.

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