1952

Biotin and Oleic Acid in the Nutrition and Metabolism of Certain Microorganisms.

Emilie Anne Andrews
Louisiana State University and Agricultural & Mechanical College

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BIOTIN AND OLEIC ACID

IN THE NUTRITION AND METABOLISM OF CERTAIN MICROORGANISMS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Agricultural Chemistry and Biochemistry

by

Emilie Anne Andrews
B. S., Southeastern Louisiana College, 1946
M. S., Louisiana State University, 1949
February, 1952
MANUSCRIPT THESES

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ACKNOWLEDGMENTS

The author wishes to express her appreciation to Dr. Virginia H. Williams for her generous share of the work included in this volume and for her consistent encouragement in the development of the problem. Dr. John F. Christman has also kindly contributed valuable direction and experimental assistance. The author wishes to thank Dr. E. A. Fischer for his constant interest throughout this work.
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ABSTRACT

Three hitherto unexplored phases of the relationships of biotin and oleic acid to the metabolism of certain microorganisms have been studied. These phases are: (1) the anomaly between titrimetric and turbidimetric microbiological assays using media containing oleic acid; (2) the phenomenon of oleic acid stimulation of yeast in biotin-free systems; and (3) the relationship of biotin to carbohydrate metabolism in microorganisms.

The various functions of biotin in cellular metabolism have been the source of much interest and quite intensive research for the last ten years. Various workers have shown this vitamin to be necessary for the function of the deaminases of aspartic acid, threonine, and serine, and the decarboxylases of oxalacetic and succinic acids; to function in various tissue decarboxylations and to be related in some manner to oleic acid.

The methods used in phases (1) and (2) were largely those of microbiological assay, utilizing various strains of yeast or Lactobacilli. Phase (3) involved the use of resting cell suspensions and cell-free extracts. The extent of reaction was measured in all cases by the disappearance of glucose. Several methods of glucose analysis were employed.

The turbidimetric-titrimetric disparity which exists in Lactobacillus casei assays of oleic acid was examined under various
conditions: (a) varying the concentration of oleic acid, (b) varying the length of incubation time, (c) including serum albumin or supplying oleic acid in an esterified form, (d) making various substitutions in and additions to the medium, (e) varying the pH of the medium, (f) varying the concentration of glucose per tube, and (g) comparing the effect observed with L. arabinosus. Under most of the conditions tested the apparent biotin contents as measured by turbidities were approximately twice the values as measured by titration. The phenomenon showed some pH sensitivity, the turbidimetric-titrimetric ratios increasing as the pH was raised to 7.0 because of the probable increased toxicity of oleic acid with decreasing acidity. An increase of turbidities as compared with acidities was obtained upon lowering the glucose content of the basal medium in the presence of biotin, not oleic acid. Only the substitution of a high molecular weight ester of oleic acid for the latter compound in the assay medium completely eliminated the anomaly. On the basis of these findings several explanations were offered for the disparity. Upon consideration of the current theories of surface adsorption and lipo-protein effects, it seems most likely that oleic acid is adsorbed upon the bacterial cell membrane and by means of steric effects prevents the utilization of the maximum amount of glucose by the bacterial cell.

That the phenomenon of oleic acid stimulation of microorganisms in biotin-free systems is applicable to yeast was demonstrated with
Saccharomyces cerevisiae Java. Oleic acid was found to be capable of stimulating growth of the organism on a biotin-deficient sucrose medium in the presence of aspartic acid. Synthesis of biotin by cells of the same organism grown in a medium deficient in biotin was shown to occur. Cells of L. casei grown on a biotin deficient medium were also found to contain low concentrations of biotin or its nutritional equivalent. Growth of S. cerevisiae Java did not occur in the absence of biotin if glucose, hydrolyzed sucrose, or fructose were substituted for sucrose as a carbohydrate source.

From the latter observation the third line of research was developed: the relationship of biotin to carbohydrate metabolism. Stimulation of glucose utilization by cells of S. cerevisiae 139 grown on media containing 2 micrograms of biotin per liter was found to be considerably higher than that of cells grown in the presence of 2 x 10^-2 micrograms of biotin per liter. A linkage of biotin to hexokinase function was postulated, but could not be demonstrated with cell-free enzyme preparations.

A continuation of the biotin studies described above should be made on hexokinase resolution. If the enzyme can be resolved by dialysis or the addition of biotin-binding reagents so that its biotin relationship becomes demonstrable, a further contribution will be made to existing knowledge of vitamin function in metabolic pathways.
CHAPTER I

INTRODUCTION AND SELECTED LITERATURE SURVEY

For many investigators, biochemistry has become the study of cyclic processes. These enzymatically-catalyzed cycles, interwoven by direct or indirect linkage, when viewed as a whole become the dynamic framework of life. Individual reactions of each system are enzymatically governed and are constantly in a state of equilibrium with other related reactions. Since no individual enzyme is capable of catalyzing a series of reactions by itself, it becomes necessary to study enzymatic reactions both individually and collectively as a means of understanding the processes of life.

In recent years it has become quite clear that we may justify ourselves in detailed study of the metabolic schemes and mechanisms of microorganisms through the concept of comparative biochemistry. This concept, introduced by Knyvor (1), intimates that the fundamental enzymatic reactions of all cells are the same regardless of cell origin. Again and again metabolic reactions which have been demonstrated to occur in certain microorganisms have later been shown to be applicable, with slight modifications in some cases, to higher plants and animals. This approach to the study of metabolism is by far the easiest since the single cell organism presents a living system conveniently simple for study, as well as being rapidly
multiplying and inexpensive to maintain.

Originally the interests of the bacteriologist lay simply in the cultivation of organisms, usually pathogens, for the purpose of study. The actual growth requirements were considered to be of little importance, though it was frequently quite difficult to maintain the bacteria on the crude media available. Until the advantage of chemically defined media became more obvious, the media were usually changed more or less empirically to make them suitable for the maintenance of growth. Turning to the use of chemically defined media required that a careful study of the nutritional requirements of microorganisms be made. As the nutritional requirements of more microorganisms were determined it began to be obvious that a complementary interrelation must exist between the nutritional requirements of a particular microorganism and its individual synthetic abilities. With the passage of time then, a more general concept of nutrition versus metabolism has developed. This is best conveyed by quoting directly from a review by B. C. J. G. Knight (2).

When the nutritional question is viewed from the standpoint of metabolism, many of the apparently difficult questions of definition and nomenclature disappear. The substances which an organism takes from its nutrients are used as material for building up the new cells. These cells carry out a complex interwoven series of processes, which is the life of those cells, and consists in taking compounds from the environment and synthesizing other compounds to make new cells. The extent and rate of multiplication of new cells will depend on the efficiency with which the processes of construction are carried out. This efficiency (here
used in the general and not only the thermodynamic sense) will clearly depend partly on the availability of the materials for construction. This will in turn depend upon the rates of utilization and synthesis of the various materials of the enzyme systems whose continued functioning is the life of the cells. The fundamental biochemical processes of cell-life—the essential metabolism of the cells—form the cardinal feature, and certain of these processes are common to the widest variety of cell. Where organisms may differ, however, is in the means whereby the materials for these processes are acquired. But here a sharp metaphysical distinction into "acquired from the environment" and "synthesized by the cell" is not possible. For it is clear that a certain rate of synthesis might be too slow to yield a required substance at the required rate. Effectively then the cell would depend upon an external source of supply to a degree which would be relative to the rate of synthesis of this substance. Hence a given substance, required as a component of one of the essential metabolic processes, might appear in three different roles as a component of the nutrients. It might appear: (1) as an 'essential' nutrient, when its rate of synthesis by the cell was so slow as to be insignificant; (2) as a growth stimulant, when its rate of synthesis was somewhat faster but still slow enough to be a limiting factor; or (3) as a substance not required at all for nutrition, because the cell could synthesize it so fast that it was not a limiting factor in growth. It is the metabolic process which is the essential thing and the compounds used in carrying it out are essential metabolites, i.e., the substrates used for the process, or the substances which form parts (prosthetic groups, etc.) of the enzyme systems which carry out these essential reactions....... What matters is to show how any given substance which affects growth plays its part. And very often it will be found that it has a relation to some essential metabolic processes, the role it plays in nutrition reflecting the mode by which the cell acquired a sufficient quantity of it at a sufficient rate.

As the development of bacterial physiology has proceeded from the use of mixed cultures of organisms and unidentified media to the utilization of pure cultures and chemically defined media, it has been
possible to identify many metabolic products and to create easily reproducible schemes of assay for essential vitamin and amino acid components of the basal medium. The use of these more or less routine procedures has led to many important observations in bacterial metabolism.

From investigations which used proliferating microorganisms to determine metabolic products, bacteriologists turned to the use of incubated washed cell suspensions. These 'resting' cells served as the source of active enzyme systems, stable over a period of hours, which were uncomplicated by the demands of reproduction. This technique was introduced by Quastel and Whetham (3) and, in combination with other standard biochemical methods, has been extensively used. The chief limitations of the technique are the impermeability of the cell wall to certain substrates and the interfering action of other enzymes which may be present.

Within the last fifteen years it has become possible to define the properties of the individual enzymes through the use of cell-free extracts. The wet-crushing mill was developed by Booth and Green in 1938 (4). Cell-free extracts may also be obtained by lysis of cells with toluene or acetone, by crushing them with ground glass, or by alternately freezing and thawing them. Ultrasonic waves are sometimes applied to produce cellular disintegration. No method is applicable to all organisms. Certain enzymatic systems cannot be isolated under any of these conditions since their action apparently is linked in some manner to the intact cellular structure. It is necessary for the
individual in research to select a method which is adaptable to his specific problem.

In order to obtain an acceptable and composite picture of life it is necessary that we examine investigations conducted at all levels: growing cultures, resting cell suspensions, and cell-free extracts. Only by the combination of these approaches can the conclusions which we reach be validated.

Among the problems which have been attacked in the manner previously discussed is that of the role of biotin in cellular metabolism. The various functions of this growth substance have been the source of considerable interest and investigation for the past ten years. During this period certain of its metabolic pathways have been elucidated, but by no means a complete picture has been obtained. Among the interesting (and as yet unsolved) problems which have arisen are those concerning (1) the oleic acid-biotin interrelationship and (2) the apparent connection of biotin to some phase of carbohydrate metabolism. The research with which we are concerned here has three separate, yet interrelated phases. The first is the result of observations made during the study of the nutritional effects of oleic acid on Lactobacillus casei. The second is an attempt to apply the phenomenon of oleic acid stimulation to a different microorganism, Saccharomyces cerevisiae. The third and final section of this dissertation consists of an attempt to demonstrate more clearly the connection of biotin to certain phases of carbohydrate metabolism.
Our knowledge of biotin has developed from the study of three supposedly different factors in three widely separated fields of investigation. In 1901, Middler (5) in Belgium isolated a compound essential to the growth of yeast which he designated as "bligo". This was later found to consist of several fractions of which Blos IIb was used by Kogl and Thunia (6) in Holland to isolate the crystalline methyl ester of the compound which they designated as "bligot".

In 1933 Alliace, Hooper, and Burk (9) reported the isolation of a concentrate from yeast extract and several other sources which was capable of stimulating the respiration of E. coli. Believing this to be a component of respiration they designated it combined biotin. Later in conjunction with du Vigneaud's group (8), he announced the identity of Vitamin H with biotin. This became known as the anti-malnutrition factor or "protective factor". This same factor was designated by Jorgel in Germany as factor A. It was not until 1937 that the factor was isolated in crystalline form.

Du Vigneaud and co-workers (10) isolated a crystalline mono-
methyl ester of biotin from liver and then prepared free crystalline biotin by saponification of the ester with cold alkali. The structure...
of biotin has now been established as 2'-keto-3,4-imidizolido-2,2'-tetrahydro-thiophene valeric acid.

A considerable amount of progress in the knowledge of the metabolic functioning of biotin has been made since its isolation in pure form. It has been shown to be a growth factor for most of the yeasts, many fungi, and large numbers of bacterial species, particularly the Lactobacilli.

Aass, Wright, and Borfman (11) reported that in Torula corniculata the need for biotin could be greatly reduced when aspartic acid was added to the medium. Since aspartic acid could not completely replace the biotin requirement, they concluded that biotin must enter several phases of metabolism, one involving aspartic acid. That biotin-deficient yeast cells could be stimulated to assimilate ammonia by the addition of biotin was shown by Winzler, Burk, and De Vigneaud (12). Stokes, Larsen, and Guinness (13) showed that many lactic acid bacteria require increased amounts of biotin for growth in media lacking in aspartate. Stokes, et al., (14) in their second paper concluded that biotin did not act in the transaminase system, and were unable to demonstrate the involvement of biotin with the deaminases, although they believed that biotin might be involved either in the latter reaction, or in the mechanism concerned with oxalacetic acid formation, or in both.

Evidence that biotin is concerned in the deaminases of aspartic acid, serine, and threonine was presented by Lichstein and Umbreit (15) using cells containing resolved enzyme systems. The process of
resolution which they designated as "aging" consisted of treating the washed cells with molar phosphate buffer for a short period of time. Lichstein and Christman (16), having demonstrated the occurrence of "aging" in cells of several types, concluded that not only biotin but also adenyllic acid was involved in aspartic acid deamination. Although many biological materials would cause stimulation of aged cell preparations at pH 7, at pH 4 the effect was specific for biotin and adenyllic acid, the stimulation being independent and sometimes additive.

Lichstein (17) made the observation that certain systems, both "aged" and cell-free, which could be stimulated before refrigeration by biotin and adenyllic acid together or by yeast extract alone, after refrigeration responded only to the yeast extract. He was led to believe that a preformed biotin-containing coenzyme for aspartic acid deaminase existed in yeast extract and that adenyllic acid was concerned in its formation. Further evidence for the existence of such a coenzyme was presented by Lichstein and Christman (18). By paper strip chromatography they were able to isolate a substance, or substances, from yeast extract which activated the deaminases of aspartic acid, threonine, and serine. The material was shown to be neither biotin nor adenyllic acid as such.

Wright, et al., (19) was able to duplicate the "aging" process in Escherichia coli of Lichstein and Umbreit (15) and to reactivate the aspartic deaminase system by the addition of biotin specifically.
Pilgrim, Axelrod, and Elvehjem (20) in 1942 observed that liver homogenate from biotin-deficient rats had a greatly decreased rate of pyruvate oxidation compared with controls.

That biotin is concerned in oxalacetic acid decarboxylase was demonstrated in four separate laboratories by four different techniques almost simultaneously. Lardy, et al., (21) using a medium deficient in both biotin and aspartic acid demonstrated that the growth of \textit{L. arabinosus} could be stimulated by the addition of oxalacetic acid or bicarbonate ion.

Shive and Rogers (22) obtained results indicating that biotin functions for \textit{E. coli} in the carboxylation of pyruvic to oxalacetic acid by means of inhibition analysis. Similar data for such a biotin function for yeast collected in the same laboratory by Garrison and Babies has never been published.

The resting cell approach was used by Lichstein and Umbreit (23). The cells were grown on complex biotin-containing medium, harvested, and "aged" in molar phosphate buffer. After "aging", the cells lost their ability to produce CO$_2$, and restoration of activity occurred upon the addition of biotin. The action of biotin was specific. It was found possible to substitute oxalacetic or malic acid for aspartic acid. The conclusion was drawn that biotin must be linked in some manner to the coenzyme of oxalacetic acid decarboxylase.

Ochoa, et al., (24) concluded that biotin is concerned in the action of oxalacetic acid decarboxylase either as a structural component
of the enzyme or as a catalyst in its synthesis. The conclusion was reached after producing a biotin deficiency in turkeys and finding that the aforementioned enzyme and the malic dehydrogenase from the deficient cells were much less active than those from normal cells.

The first suggestion that CO₂ transfer might involve a metabolic function of biotin was made by Burk and Winkler (25). Summerson, et al., (26) using biotin-deficient rat liver slices respiring in lactate or pyruvate, found that with the addition of biotin there was increased production of bicarbonate due to increased utilization of lactate. There was a 25 to 35 per cent increase in the rate of disappearance of added lactate in the presence of biotin.

Lardy, Potter, and Burris (27) found that L. arabinosus cells grown on low biotin medium were almost incapable of fixing C¹⁴O₂ into cellular aspartic acid as compared with cells grown with a normal supply of the growth factor. Fixation of CO₂ into cellular aspartate was strongly inhibited by addition of aspartate to the medium. It was suggested that the energy-coupling mechanism which incorporates aspartate into protein might be the driving force to permit the endergonic coupling of CO₂ and pyruvate.

Delwiche (28) discovered a biotin linkage for succinic de-
carboxylase in the metabolism of Propionibacterium pentosaceum. Lichstein (29) showed that the biotin coenzyme is active in the stimulation of partially resolved succinic acid and oxalacetic acid decarboxylases as well as in that of aspartic acid, threonine, and serine deaminases.
Intraperitoneal injection of normal and biotin-deficient rats with NaHCO$_3^{14}$ was performed by MacLeod and Lardy (30). As a result, C$^{14}$ fixation into adenine, guanine, arginine, aspartic acid, citric acid, and bone carbonate occurred to a much larger extent in the control animal than in the biotin deficient animal. In connection with arginine, it appeared possible to the authors that there might be a slower rate of urea formation in the liver of the biotin-deficient rat.

MacLeod, et al., (31) showed that synthesis of citrulline from ornithine by the washed residue of biotin-deficient rat liver homogenates was about 50 per cent less than that of liver homogenates from pair-fed controls. The addition, in vitro, of a heated residue of normal rat liver homogenate increased the rate of synthesis by the deficient liver. Treating biotin-deficient animals with two intraperitoneal injections of 200 micrograms of biotin, 24 hours before sacrificing the animals, restored a normal rate of citrulline synthesis in the liver homogenate preparation. There is a possibility then that the conversion of ornithine to citrulline may be the limiting reaction of the urea cycle which is present in the biotin-deficient rat.

Conclusive evidence that biotin functions as coenzyme in fixation of CO$_2$ was presented by Wesman and Werkman (32). In exchange reactions involving oxalacetate and NaHCO$_3^{13}$O$_3$, the addition of avidin to inactivate biotin prevented the fixation of CO$_2$ present into oxalacetate. Addition of adequate biotin resulted in a return to normal fixation.
Quite recently Broquist and Snell (33) have shown that *L. arabinosus*, *L. casei*, and *Streptococcus faecalis* all require increased amounts of biotin for growth in the absence of aspartic acid, while *L. fermenti* and *Clostridium butyricum* require the same amount of biotin in the presence or absence of aspartate. Since CO₂ was found to stimulate growth of *L. arabinosus* in the presence of oleate, but absence of biotin, and to have a sparing action upon the biotin requirement of this organism in the absence of oleate, it was suggested that the vitamin has a role in this organism in the production of metabolically essential CO₂.

Previous work carried out with biotin-deficient turkeys was followed up in Oehoa's laboratory (34). They adapted cells of *L. arabinosus* by growing them on malate-containing medium. The conclusions were that biotin is necessary for the production of the malic enzyme, but that the role of the growth substance seems to involve the synthesis of the enzyme itself, not that of a prosthetic group.

An involvement for biotin in the carbohydrate metabolism of several strains of *Leuconostoc* was indicated by the data of Carlson and Whiteside-Carlson (35). With three strains of *Leuconostoc* it was found that biotin was required for growth in the media containing invert sugar, glucose, or fructose. In sucrose medium the organisms were free of a biotin requirement to the degree that the disaccharide was utilized via the mechanism resulting in dextran synthesis.

Ajl, et al., (36) indicated that biotin is involved as a respiratory catalyst in the oxidation of succinic acid. Using dialyzed
call-free enzyme preparations of \textit{E. coli}, an increased succinate oxidation was obtained in the presence of biotin which could not be duplicated using malate or fumarate as substrates. No hypothesis was advanced as to the mode of action since slight variations in the biotin structure could be introduced without lowering the activity.

We may then conclude that biotin is necessary for the synthesis of aspartic acid; that it is essential for the action of serine, threonine, and aspartic acid deaminases and probably operates in these reactions in coenzyme form. Biotin is required for the action of succinic and oxalacetic acid decarboxylases and again appears to be linked in some manner to the coenzyme for each reaction. It seems likely that biotin may be generally involved in tissue reactions which bring about fixation of \( \text{CO}_2 \). There are indications that biotin is connected with some phase of carbohydrate metabolism and serves as a respiratory catalyst in the oxidation of succinic acid.

The biotin-oleic acid relationship will be discussed in the following section.

\textbf{Oleic Acid in Metabolism.}

As early as 1909 Fleming (37) observed that \textit{Corynebacterium acnes} grew best in a nutrient agar to which 1 to 5 per cent oleic acid had been added.

Almost thirty years later Cohen, et al., (38) found that certain strains of \textit{G. diphtheriae} would not grow in the presence of
all known growth factors unless additional materials from serum, milk, or commercial casein were added. One of the active materials was isolated, purified, and shown to be oleic acid.

Oleic acid in concentrations of 0.3 to 0.6 mg./10 ml. medium gave good growth in the presence of saponin for *Kynipsialothrix rhusiopathiae* (39). If saponin were omitted, as much as 0.2 mg./10 ml. was inhibitory.

Both the fungus *Fityresporum ovale* (40) and the anaerobic bacterium *C. tetani* (41, 42) require oleic acid. The optimal concentration, however, varies from 100 mg./10 ml. medium with the former to 0.01 mg./10 ml. for the latter.

Bauernfeind, et al., (43) in 1942 first discovered the stimulation by lipoidal substances which may occur in the course of assays for pantothenic acid and riboflavin. In the same year Strong and Carpenter (44) discovered the effect of fatty acids on the growth of *L. casei* and worked out a method for eliminating this effect in riboflavin assays.

In 1945 it was noted by Williams and Fieger (45) that lipoidal stimulation of *L. casei* also occurs in microbiological assays for biotin. They were unable to demonstrate synthesis of biotin in tubes showing high acid and cell production in the presence of basal medium and rice oil without additions of pure biotin. In the same year Williams (46) noted a similar effect of lipoidal substances upon the *L. arabinosus* assay for biotin.
Hodson (47) reported that oleic and aspartic acids would not completely replace biotin in biotin assay by the cholineless *Neurospora crassa* under the conditions used in his laboratory. The addition, however, of oleic acid and Tween 80 alone or in combination with aspartic acid produced a slight growth response in the absence of biotin and some stimulation in its presence.

Studies of the phenomenon of oleic acid stimulation of *L. casei* were begun by Williams and Pieger (48) in 1946. Their findings were as follows:

1) The pH of the medium, length of incubation period, temperature, and concentration of oleic acid added were found to affect growth. Optimal conditions for growth were at pH 5.5, incubation period of 96 to 120 hours, incubation temperature of 37°, and concentration of 400 micrograms of oleic acid per tube.

2) Turbidimetric assay showed a much higher biotin equivalence than did titrimetric measurements.

3) A number of fatty acids were tested, of which linoleic, lauric, and myristic acids were found to be strongly inhibiting. Elaidic acid, the trans isomer of oleic, was found to show greater stimulation than oleic acid.
4) Stimulation in the absence of one vitamin was obtained only in the case of biotin.

Further studies of lipid stimulation of *L. casei* by Williams and Finger (49) led to the following results:

1) *L. casei* stimulation by lipids occurred when casein hydrolysate was replaced by either amino acids, or peroxide treated components, and upon the addition of avidin sufficient to bind 1000 micromicrograms of biotin.

2) Of synthetic detergents examined for stimulatory effect non-ionic detergents proved generally stimulatory. The most stimulatory of all detergents tested were the oleates.

3) Oxidation-reduction potentials determined on cultures containing standard biotin, oleic acid, or a non-ionic detergent corroborated acidimetric and titrimetric data.

4) It was postulated on the basis of experimental data that biotin functions as a cell permeability factor and can be replaced by the proper lipids.

Kodieck and Worden (50) demonstrated the inhibitory action of oleic, linoleic, and linolenic acids upon the growth of *L. helveticus* and several other gram positive bacteria. The inhibition was reversed by certain surface active agents. It was suggested that
evidence pointed to a physicochemical explanation for such behavior. In an extension of this work (51), the depression of acid production by certain unsaturated fatty acids was shown to increase with unsaturation. It could be reversed by other surface-active agents which were capable of forming molecular associations with the acids. A physicochemical mechanism for the variable effect of various acids on different microorganisms was further elaborated upon.

Williams, Broquist, and Snell (52) studied the phenomenon of oleic acid stimulation in connection with various Lactobacilli. They learned that:

1) Oleic, linoleic, or a combined source of these materials was required by several of the strains. The even-numbered, saturated fatty acids C_6 to C_18 were completely inactive.

2) For L. bulgaricus oleic acid, although essential, was exceptionally toxic. The addition of certain inactive, water soluble esters of oleic acid served as an excellent non-toxic source.

3) Although most of the lactic organisms do not require oleic acid on a complete medium, it becomes essential in the absence of biotin.

4) Avidin does not nullify the action of oleic acid for the organisms tested.
5) It was hypothesized that biotin functions in the synthesis of oleic acid.

In the laboratory of Dubos (53) investigations of the antigenicity of tubercle bacilli grown in the presence of Tween 80, a synthetic ester of oleic acid, led to the formulation of an hypothesis concerning the absorption of such materials on the bacterial surface. At a slightly later date, a study of the inhibitory effect of lipase on the tubercle bacillus grown in media containing fatty acid esters (54) caused the workers to conclude that fatty acids and their esters were utilized by microorganisms by two somewhat different pathways. Abundant growth of *Microoccus* from oleic, linoleic, and linolenic acids was obtained by Dubos (55). He suggested that long chain fatty acids could affect the growth of different microbial species through different metabolic channels. In order to study the mechanisms of these metabolic reactions he suggested that it is necessary to use fatty acids under conditions where they cannot manifest their toxic properties.

Wattchings and Boggiano (56) reported that a stimulation for certain strains of *Lactobacilli* was produced by the addition of sodium oleate to medium containing yeast extract. They also noted the toxic effects of some concentrations.

Axelrod, *et al.*, (57) isolated a vaccenic acid fraction which was active in producing growth of *L. casei*, was slightly less potent for *L. arabinosus*, and was inactive for *S. hemolyticus* and *S. cerevisiae*. The chemical nature of the fat-soluble substances with
biotin activity in human plasma have been investigated by Axelrod, Hitz, and Hofmann (58). They found that the aforementioned biotin-like activity was due to the presence of oleic, linoleic and arachidonic acids. A synergistic activity was demonstrated between these acids and such saturated fatty acids as stearic and palmitic.

A fat- and ether-soluble fraction in plasma which could substitute for biotin in the growth of lactic organisms was discovered by Trager (59). He later reported (60) that the intramuscular injection of a fat soluble material from horse plasma reduced the severity of dermatitis in chicks which had been fed egg-white. Although this substance was almost as effective as injected biotin, the injection of oleic acid alone was ineffective. He concluded that perhaps fatty acids in a combined form were necessary as a substitute for biotin in higher species.

Potter and Klvehjem (61) noted that although either oleic acid or aspartic acid spares biotin, neither can replace the other in the absence of biotin. They concluded that biotin might accordingly participate in the synthesis of oleic as well as aspartic acid.

Demonstration of the biotin replacement effect of unsaturated fatty acids for Cl. sporogenes was reported to be much facilitated by the use of the Tweens (62). Oleic, linoleic, vaccenic, and ricinoleic acids were effective in this function. Klaidic was slightly less active than oleic, and trans-vaccenic slightly less than the cis form.
The usual L. casei medium was found to be unproductive for a strain of cecal Lactobacilli isolated from rats fed a highly purified diet (63). Oleic acid proved to be necessary. The organisms would not grow in the absence of oleate when all vitamins were supplied.

Guirard, Snell, and Williams (64) noted the effect of acetate as a stimulant for early growth in lactic acid bacteria. A probable role for acetate in the synthesis of various lipids was suggested by the ability of numerous compounds of this class to duplicate in some degree the effect of acetate.

Twenty-eight strains of Lactobacilli previously reported not to grow in media of known composition were studied by Kitay and Snell (65). All of these except two required oleic acid or other unsaturated fatty acids for growth.

Broquist and Snell (66) noted that oleic, linoleic, and linolenic acids when detoxified were equally effective in promoting growth of G. putrificus and S. faecalis. Saturated fatty acids were shown to maintain the same synergism toward unsaturated fatty acids when the latter had been detoxified.

The effect of positional- and stereo-isomerism on the biotin-like activity of octadecenoic acids was studied by Greenburg, et al., (67), using L. arabinosus. Wide variations in activity of the trans forms were obtained though only minor differences occurred among the cis forms.

Carlson, Whiteside-Carlson, and Kospetos (68) studied the ability of various fatty acids to substitute for biotin in the
growth-production of Leuconostoc. With all strains tested oleic and lauric acids in the form of their esters, Tween 80 and 20 respectively, produced a higher level of growth in sucrose medium than that obtained with biotin. Half-maximal to maximal growth was obtained for these strains when the esters were substituted for biotin in glucose and fructose media. The stearate and palmitate esters were less active than oleate and laurate. In sucrose media their ability to substitute for biotin paralleled the dextran-synthesizing capacities of the organism. They could not substitute for biotin in media containing glucose and fructose.

Thus it becomes clear that oleic acid has been demonstrated to be an essential growth factor for various microorganisms. It possesses a definite interrelationship with biotin and the hypothesis has been advanced that biotin functions in the synthesis of oleic acid. The inhibitory action of the acid at higher concentrations is believed to be either a steric or lipo-protein effect.
CHAPTER II

EXPERIMENTAL METHODS

A. Materials Used.

1. Microorganisms.

The organisms used were *Lactobacillus casei* 7469, *Lactobacillus arabinosus* 17-5, *Saccharomyces cerevisiae* Java 4125, and *Saccharomyces fragilis* 8644, all obtained from the American Type Culture Collection, and *Saccharomyces cerevisiae* 139 obtained from Hoffman-LaRoche. For the isolation of hexokinase, compressed brewer's yeast was obtained from Anheuser Busch Incorporated.

2. Natural Products.

Oleic acid, obtained from the Hormel Foundation, had an iodine value of 90.83, as compared with the theoretical of 89.87. The adenosine triphosphate (di-barium salt) bought from either Nutritional Biochemicals Corporation or Sigma biochemicals was claimed to be 90 per cent ATP. Vitamin-free casein hydrolysate was obtained from Nutritional Biochemicals Corporation.

The adenylic acid (adenosine-5-phosphoric acid) was kindly furnished by the Ernst Bischoff Company.

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B. Microbiological Assays for Biotin or Biotin Substitutes.

1. Lactobacillus casei.

The microbiological method used was adapted from the original procedure of Shull, Hutchings, and Peterson (69). Stock cultures of *L. casei* were carried as stabs on agar (1 per cent yeast extract, 1 per cent glucose, and 1.5 per cent agar) and were transferred every two weeks. Inocula for use in tests were prepared by inoculating the stock cultures into tubes of sterile broth (1 per cent yeast extract, 1 per cent glucose, and 0.6 per cent sodium acetate) and incubating for 24 hours at 37°. The cells were washed once with 10 ml. of sterile water or physiological saline, and resuspended in 4 ml. of water. One milliliter of the resulting suspension was added to 85 ml. of sterile water and 0.1 ml. of this final dilution was used for inoculation of a single assay vessel.

A series of biotin standards to obtain a standard curve was set up in triplicate with each assay, and concentrations used were 0, 200, 400, 600, 800, and 1000 micromicrograms of biotin per tube. A typical standard curve is given in Figure 1.

The composition of the biotin-free medium is given in Table 1. Under ordinary conditions the pH was adjusted to 5.8. Materials to be tested and the standard biotin solutions were placed in separate test tubes and the volume of each made to 5 ml. with distilled water. Five milliliters of the double strength basal medium were then added to each assay tube, and the tubes capped and autoclaved for fifteen minutes at 121° (15 pounds pressure). After cooling, the tubes were
FIGURE 1

TYPICAL STANDARD GROWTH RESPONSE
OF LACTOBACILLUS CASEI
TABLE I
COMPOSITION OF A BASAL MEDIUM FOR LACTOBACILLUS CASEI

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, hydrolyzed (100 ml. vitamin-free hydrolysate)</td>
<td>10.0 g.</td>
</tr>
<tr>
<td>D-Glucose, anhydrous</td>
<td>40.0 g.</td>
</tr>
<tr>
<td>Sodium acetate trihydrate</td>
<td>66.0 g.</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>400 mg.</td>
</tr>
<tr>
<td>L-Tryptophane</td>
<td>200 mg.</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>400 mg.</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.0 g.</td>
</tr>
<tr>
<td>Potassium hydrogen phosphate</td>
<td>1.0 g.</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>400 mg.</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>20 mg.</td>
</tr>
<tr>
<td>Ferrous sulfate heptahydrate</td>
<td>20 mg.</td>
</tr>
<tr>
<td>Manganese sulfate tetrahydrate</td>
<td>20 mg.</td>
</tr>
<tr>
<td>Adenine sulfate</td>
<td>20 mg.</td>
</tr>
<tr>
<td>Guanine hydrochloride</td>
<td>20 mg.</td>
</tr>
<tr>
<td>Xanthine</td>
<td>20 mg.</td>
</tr>
<tr>
<td>Uracil</td>
<td>20 mg.</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>2000 µg.</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>2000 µg.</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>2000 µg.</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>2000 µg.</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>4000 µg.</td>
</tr>
</tbody>
</table>
inoculated and incubated at 37° for 48 to 96 hours. Upon completion of the incubation period the mixtures were diluted with 10 ml. of distilled water and equal volumes of each solution were taken for analysis by titration and turbidimetry. Titrations to pH 7 against 0.1 N alkali were made with a Beckman pH meter. Turbidities were determined by reading against a distilled water blank in a Beckman spectrophotometer at wave length 6000 å and slit width 0.3 mm. When only the acidimetric values were desired titrations were made on the undiluted samples.

2. *Saccharomyces fragilis.*

The following assay procedure is based on that of Snell, et al. (70). Stock cultures were carried on agar slants (1 per cent glucose, 1 per cent yeast extract, 2 per cent agar, pH 5.0) and subcultured in tubes containing approximately 8 ml. of glucose medium (1 per cent yeast extract, 1 per cent tryptone, 0.5 per cent monopotassium phosphate, and 1 per cent glucose) or of a similar medium to which one

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>para-Amino benzoic acid</td>
<td>200 μg.</td>
</tr>
<tr>
<td>Folic acid</td>
<td>50 μg.</td>
</tr>
<tr>
<td>Water</td>
<td>to 1000 ml.</td>
</tr>
</tbody>
</table>

TABLE I (Continued)

COMPOSITION OF A BASAL MEDIUM FOR *LACTOBACILLUS CASEI*

para-Amino benzoic acid......................................................................... 200 μg.
Folic acid.................................................................................................. 50 μg.
Water........................................................................................................ to 1000 ml.
drop of sterile 20 per cent sucrose had been added in lieu of the
glucose. After 18 hour incubation at 30°, the subcultures were washed
three times with 10 ml. of distilled water and suspended in an equal
volume of water. One tenth of a milliliter of a one to one hundred
dilution of the above suspension was sufficient for the inoculation
of each 100 ml. of medium.

A standard biotin curve accompanied each assay. The con-
centrations used were 0, 10⁻⁵, 10⁻⁴, 10⁻³, 10⁻², 10⁻¹, and 10⁻²
micrograms of biotin per tube. A typical standard curve is given in
Figure 2.

The composition of the basal medium is given in Table II.
For optimal growth, adjustment to pH 3.0 was necessary. The entire
quantity of medium was sterilized and then inoculated. The solutions
to be tested and the standard biotin solutions were placed in Pyrex
test tubes (25 x 150 mm.) and made to a total volume of 2 ml. The
tubes were plugged and sterilized for fifteen minutes at 121°. The
previously inoculated medium was added in 5 ml. aliquots to each
tube and all the tubes were incubated at 35°. When small quantities of
growth began to appear in the tubes containing no biotin, the incu-
bation was stopped.

After incubation the tubes were well shaken to suspend all
the cells, and the turbidities were read against a distilled water
blank in the Beckman spectrophotometer at wave length 6000 Å and
slit width 0.3 mm.
FIGURE 2

TYPICAL STANDARD GROWTH RESPONSE
OF SACCHAROMYCES FRAGILIS
### Table II

**An Assay Medium for Biotin Using Saccharomyces**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>0.4 g</td>
</tr>
<tr>
<td>DL-Aspartic acid</td>
<td>0.2 g</td>
</tr>
<tr>
<td>DL-Tryptophane</td>
<td>20 mg</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>20 mg</td>
</tr>
<tr>
<td>beta-Alanine</td>
<td>1 mg</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.8 mg</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>0.7 mg</td>
</tr>
<tr>
<td>Manganese sulfate</td>
<td>0.7 mg</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>Cupric sulfate pentahydrate</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Sodium molybdate dihydrate</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>400 μg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2 μg</td>
</tr>
<tr>
<td>Inositol</td>
<td>2 mg</td>
</tr>
<tr>
<td>Para-Aminobenzoic acid</td>
<td>200 μg</td>
</tr>
<tr>
<td>Niacin</td>
<td>400 μg</td>
</tr>
</tbody>
</table>
TABLE II (Continued)

AN ASSAY MEDIUM FOR BIOTIN USING SACCHAROMYCES

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>400 μg.</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>200 μg.</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>400 μg.</td>
</tr>
<tr>
<td>Water</td>
<td>to 1000 ml.</td>
</tr>
</tbody>
</table>

C. Isolation of Hexokinase.

Hexokinase was isolated according to the general procedure of Berger, et al. (71), although purification was not carried to the final point of crystallization. In detail the method used was as follows:

Five pounds of brewer's yeast were warmed to 37° over a two hour period. One hundred and sixty milliliters of toluene were well mixed with the cells, and the mixture was permitted to liquefy partially at 37° for forty-five minutes. At the end of this period, 104 ml. of 50 per cent glucose were added and the mixture stirred for an additional forty minutes. Then 2.3 kg. of crushed ice and 52 ml. of 50 per cent glucose were added. The mixture stood for 18 hours at 6°.

The suspension was then centrifuged in 250 ml. bottles at 3000 rpm. A second centrifugation was generally necessary to produce a sufficiently clear supernatant. The supernatant stood overnight at 0 to 6°.
The protein of the crude extract was precipitated by the addition of an equal volume of 95 per cent alcohol. During this addition the temperature was never allowed to rise above 5°. After standing for one hour, the suspension was centrifuged in the cold, and the resulting precipitate was suspended in 400 ml. of 1 per cent glucose and stirred overnight in the cold.

All remaining suspended protein was centrifuged down, and the pH of the supernatant adjusted to 4.7 by the addition of approximately 12 ml. of 0.1 M acetic acid. A slushy precipitate was removed by centrifugation. The addition of 2 to 3 ml. of 1.5 M sodium hydroxide raised the pH to 5.6, and 210 ml. of alcohol were added. (This brought the alcoholic content to slightly less than the 29 per cent specified by Berger and coworkers for the original 20 pounds of yeast. It appears, however, to be necessary to lower the concentration slightly for a smaller quantity of yeast.) A rather large gray precipitate was discarded. The addition of 245 ml. more of alcohol caused precipitation of a rather small and exceptionally gummy material. The latter was dissolved in 80 ml. of 1 per cent glucose. Seventy-five milliliters of 0.1 M acetate buffer, pH 5.4, were added to the glucose solution and the protein again fractionated between 25 and 48 per cent alcohol. The resulting fraction was dissolved in 40 ml. of 1 per cent glucose and an equal quantity of 0.02 M acetate buffer, pH 5.4, containing 1 per cent glucose was added.

The enzyme was then adsorbed at 0° on 40 ml. of aluminum hydroxide gel. The mixture was stirred and left to stand for 30
minutes. The aluminum hydroxide was then centrifuged out, washed with three 100-ml. portions of 0.01 M acetate buffer, pH 5.4, and extracted with three 40-ml. portions of 0.01 M phosphate buffer, pH 7.15. These extracts were combined and used for assay.

An active aluminum hydroxide gel was obtained by dissolving 33.1 g. of aluminum sulfate octadecahydrate in 600 ml. of water and adding 140 ml. of concentrated ammonium hydroxide with stirring. This was diluted to one liter, centrifuged, and the supernatant discarded. The residue was washed with distilled water until 100 ml. of supernatant titrated 0.2 ml. or less of 0.1 N sulfuric acid to the methyl red end point. The precipitate was then suspended in 250 ml. of water and activated further by heating at 100° over steam for four hours.

D. Enzyme Assays.

Hexokinase assays were carried out at 30° in 0.1 M phosphate buffer, pH 7.15. Although some variation in the concentration of materials was made from time to time, assay tubes generally contained the following ingredients:

0.2 ml. of 0.4 M magnesium chloride
0.6 ml. of 0.1 M phosphate buffer, pH 7.15
1.0 ml. of glucose, 1 mg./ml.
0.1 ml. of hexokinase preparation
0.2 ml. of additions.

With the exception of the enzyme preparation, all materials were combined and equilibrated in the water bath for ten minutes. After
addition of the hormone, tubes were removed at specified time
intervals and inactivated by heating for one minute in a boiling water
bath. Aliquots were then removed for glucose analysis.

E. Glucose Determinations.

Several methods for the determination of glucose were used.


Reagents: Ten grams of anthrone (9-keto-10-dihydroanthracene) were
dissolved in one liter of 95 per cent sulfuric acid.

Four or five milliliters of the solutions to be determined
were measured into a test tube of 19 to 25 mm. diameter and 8 or 10
ml. of the reagent were added. The solutions were at once thoroughly
mixed by swirling. After 10 minutes or more the color was measured
in an Evelyn photometric colorimeter against a blank with filter
620 m\. Glucose standards were used with each series of unknowns.
The range of the method was 2 to 200 micrograms.

2. Felix and Balarce (73).

Reagents: Carbonate-cyanide—Eight grams of anhydrous sodium carbonate
were dissolved in 40 to 50 ml. of water. To this solution 15 ml. of
freshly prepared 1 per cent sodium cyanide were added and the whole
diluted to 500 ml.

Ferricyanide—Four grams of potassium ferricyanide were
dissolved in 996 ml. of water.
Ferric Iron—Twenty grams of gum ghatti or of gum arabic were wrapped in cheesecloth and suspended in one liter of water for 24 hrs. A mixture of 5 g. anhydrous ferric sulfate, 75 ml. of 85 per cent phosphoric acid, and 100 ml. of water were then combined with the gum solution. After mixing, 15 ml. of 1 per cent potassium permanganate were slowly added to destroy the reducing materials present in the gum. The solution was allowed to stand for several days.

Procedure: Two milliliters of 0.4 per cent potassium ferricyanide and 1 ml. of carbonate-cyanide reagent were added to the 4 ml. sample in a colorimeter tube. After mixing the tube contents, all samples and standards were heated for eight minutes in boiling water. The solutions were cooled for two minutes before the addition of 5 ml. of ferric iron reagent. Dilution to 25 ml. with distilled water was made before reading in an Evelyn colorimeter using a 515 mμ filter.

The problem presented by partial precipitation of the colored complex made it necessary at times to add the ferric iron and water immediately before reading. The range of the method was 10 to 100 micrograms.

3. Nelson (74).

Reagents: Copper Reagent A—Twenty-five grams of anhydrous sodium carbonate, 25 g. of Rochelle salt, 20 g. of sodium bicarbonate, and 200 g. of anhydrous sodium sulfate were dissolved in approximately 800 ml. of water and diluted to a liter. When necessary the reagent was filtered, and any sediment forming later was also removed by filtration without injury to the reagent.
**Copper Reagent B.**—A 15 per cent solution of cupric sulfate, pentahydrate was prepared. To this was added 1 or 2 drops of concentrated sulfuric acid per liter.

**Arsenazo-molybdate Color Reagent**—Twenty-five grams of ammonium molybdate were dissolved in 450 ml. of distilled water, and 2 ml. of concentrated sulfuric acid were combined with it. Three grams of disodium acid arsenate heptahydrate dissolved in 25 ml. of water were added. The entire solution was placed in the incubator at 37° for 48 hrs., and then stored in a glass-stoppered brown bottle.

**Procedure:** One milliliter of supernatant from the zinc sulfate-barium hydroxide precipitation described in section F was pipetted into a Folin-Wu blood sugar tube, graduated at 25 ml. One milliliter of a mixture of 25 parts copper reagent A to 1 part copper reagent B was added to each. Solutions were mixed and heated for 20 minutes in a boiling water bath. At the end of this period the tubes were cooled in cold water and 1 ml. of Arsenazo-molybdate reagent was added to each. After mixing and the evolution of CO₂ were complete, the tubes were diluted to 25 ml. and read in the Evelyn photoelectric colorimeter at 515 mμ. Suitable standards and a blank were included with each assay. The method appeared to be most sensitive for the range 20 to 100 micrograms of glucose.

**F. Removal of Interferences in Glucose Analysis.**

Somogyi in 1945 (75) suggested a method for removal of the reducing substances which give interference in the determination of blood sugar.
Reagents: Zinc sulfate, 5 per cent.

Barium hydroxide, 0.3 N.

Ten milliliters of zinc sulfate solution were diluted with approximately 100 ml. of water and a drop of 1 per cent phenolphthalein was added. Barium hydroxide was added dropwise until the pink of the phenolphthalein persisted for one minute. Since it was desired that equal volumes of these solutions should be empirically equivalent, the necessary dilution was made on the basis of the titration. Eight milliliters of zinc sulfate solution were added to each 5 ml. of incubation mixture in 25 ml. volumetric flasks to bring about protein denaturation. The addition of an equal quantity of barium hydroxide caused the precipitation of such phosphorylated intermediates as might produce interference and removed the excess sulfate ion. After mixing, the flask contents were centrifuged to remove the precipitated protein and barium salts.

A discussion of the general experimental methods used in this research project has been presented. The following topics have been included: materials used, two microbiological assay methods for biotin, the isolation of hexokinase, the procedure for enzyme incubation, three methods for glucose analysis, and a mode of removing interfering substances in glucose analysis.
CHAPTER III

EXPERIMENTS AND DISCUSSION OF RESULTS

The problem under consideration has three separate, yet interrelated phases. The first of these is the result of observations made during the study of the nutritional effects of oleic acid stimulation on L. casei. The second is concerned with the application of the phenomenon of oleic acid stimulation to a different microorganism, Saccharomyces cerevisiae. The third section consists of an attempt to demonstrate more clearly the connection of biotin with certain phases of carbohydrate metabolism. Each of these divisions will be discussed separately.

A. The Turbidimetric-Titrimetric Disparity in Oleic Acid Stimulation of Lactobacillus Casei.

It has been generally assumed that equal validity may be assigned to the two microbiological assay procedures most widely employed: the titration of acid and the measurement of turbidity of cell suspensions. In 1946, however, it was reported by Williams and Fiser (48) that there was lack of agreement between titration and turbidity in L. casei assays of biotin-free media containing oleic acid. The point seemed of sufficient importance to require further investigation.
The anomaly has been examined from several standpoints:

1. Varying the concentration of oleic acid,
2. Varying the length of incubation time,
3. Including serum albumin, or supplying oleic acid in an esterified form,
4. Making various substitutions in and additions to the medium,
5. Varying the pH of the medium,
6. Varying the concentration of glucose per tube, and
7. Comparing the effect observed with \( Lc. \) \( \text{arabinosus} \).

**General Procedure.**

In all cases triplicate tubes were analyzed for each dilution of test substance; turbidity, titration measurements, and where necessary, glucose determinations were made on aliquots from the same tube. Except where otherwise stated the pH of the biotin-free medium was adjusted to 5.8 and the tubes were incubated for 72 hours. That the high turbidities observed throughout most of this investigation were due to high cell production is supported by (a) the agreement of turbidities with cell volume measurements (48), (b) the agreement of turbidities with plate counts of viable cells, and (c) the normal appearance of cells on microscopic examination.
Since turbidimetric and titrimetric values obtained by microbiological assay have been assumed to be directly comparable in value, the ratio of the apparent biotin content by turbidimetric assay to the apparent biotin content by titrimetric assay theoretically should be 1.0. The turbidimetric-titrimetric ratios so frequently referred to in the following pages are based on the above concept. The method of calculation becomes obvious through examination of the data in Table V.

1. Variation in the concentration of oleic acid.

The oleic acid content per tube was varied from 10 to 4,000 micrograms, and biotin-free tubes were compared with those containing 500 micromicrograms of biotin in addition to oleic acid. The results are illustrated in Figure 3, where apparent biotin content as determined from the growth in the oleic acid tubes is plotted against the logarithm of the concentration of oleic acid per tube. From these curves it may be seen that both acid and cell production were stimulated by oleic acid with and without biotin, but cell production much more so. This disparity is obvious over a wide range of concentration, agreement of titration and turbidity data being approached only at very low concentrations of oleic acid in biotin-free tubes.

2. Variation in the length of incubation time.

Tubes containing 25, 50, and 100 micrograms of oleic acid were inoculated and incubated for 24, 48, 72, and 120 hours. To a duplicate set of tubes 400 micromicrograms of biotin were added per
Figure 3

Apparent Biotin Content by Titrmetric and Turbidimetric Methods with Varying Oleic Acid Concentrations
tube. The highest ratios of turbidimetric to titrimetric data were obtained at 48 hours. After that period of time, fairly constant ratios averaging about 1.96 were found, the higher concentrations of oleic acid tending to produce higher ratios. The presence of biotin along with the oleic acid had very little effect on the disagreement between the two methods of assay.

3. Inclusion of serum albumin or use of esterified oleic acid.

It has been shown that the inhibiting effect of oleic acid on the early growth of L. casei can be counteracted by the addition of sterile biotin-free albumin to the medium or by adding oleic acid in the form of an ester (76). The effect of these two variations was consequently studied from the standpoint of turbidity and titration analyses. Tubes containing either 25 or 50 micrograms of oleic acid were inoculated with and without albumin. Several concentrations of Nopalcol 6-0 gave values very close to the theoretical 1.0. In the case of the ester, the ratios of approximately 1.0 were achieved by high acid production paralleling the high turbidity, instead of a reduction in turbidity to match low acid production, as the case might have been. The acidity in the Nopalcol 6-0 tubes reached the amount theoretically possible to obtain from the glucose content of the medium. The ratios obtained in the presence of albumin were brought about by high cell growth and low acidity. These data are shown in Table III.
TABLE III

TURBIDIMETRIC-TITRIMETRIC RATIOS OBTAINED WITH VARIOUS CONCENTRATIONS OF OLEIC ACID, OLEIC ACID PLUS ALBUMIN, AND NOPALCOL 6-0

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Turbidimetric-Titrmetric Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per Tube</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td>50</td>
<td>1.90</td>
</tr>
<tr>
<td>100</td>
<td>1.96</td>
</tr>
<tr>
<td>200</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

4. Alterations in the medium.

A great number of substances were tested in the attempt to reduce the high ratios obtained to the theoretical value of 1.0. Although certain inhibitions and stimulations were noted in some cases, the effects operated uniformly on both acid and cell production, and the usual abnormal ratios were obtained. The changes were as follows: (1) each of the following substances was substituted in turn for the glucose of the basal medium--maltose, fructose, galactose, lactose, mannose, sucrose, xylose, arabinose, and mannitol; (2) small
amounts of the following amino acids were added singly to the casein hydrolysate medium—alanine, serine, methionine, threonine, lysine, arginine, phenylalanine, histidine, proline, isoleucine, valine, leucine, tyrosine, cysteine, glutamic acid, aspartic acid, glycine, and tryptophane; and (3) the following miscellaneous substances were added—stearic acid, ascorbic acid, oxalacetic acid, adenosine triphosphate, glucose-1-phosphate, hexose diphosphate, iodoacetic acid, and sodium fluoride.

5. Variation in pH of medium.

The effect of variation in pH on the turbidity-titration ratios was studied in the following manner: The usual medium was made and divided into seven portions. Each portion was adjusted to a different pH, the range being 4.0 to 7.0 by increments of 0.5 pH unit. Biotin standards and tubes containing several concentrations of oleic acid and Nopalsel 6-0 were set up for each pH. At pH values of 4.0 and 4.5 there was no measurable growth, and at pH 5.0 growth was so low in the presence of oleic acid that the values obtained were not reliable. Data for the other pH values are given in Table IV. It is obvious that for oleic acid there is a rise in the ratio with increase in pH. The effect however in the presence of Nopalsel 6-0 is relatively slight. The increasing ratios obtained at the higher pH values for oleic acid probably reflect the increasing toxicity of oleic acid with decreasing acidity. The infinite values obtained at pH 7.0 are due to a complete lack of acid production in the presence of some cell growth.
### TABLE IV

**EFFECT OF pH ON TURBIDIMETRIC-TITRIMETRIC RATIOS**

**OBTAINED WITH OLEIC ACID AND NOPALCOL 6-O**

<table>
<thead>
<tr>
<th>Concentration Per Tube</th>
<th>Turbidimetric-Titrmetric Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td><strong>μg</strong></td>
<td></td>
</tr>
<tr>
<td>NOPALCOL 6-O</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.86</td>
</tr>
<tr>
<td>100</td>
<td>0.92</td>
</tr>
<tr>
<td>200</td>
<td>0.87</td>
</tr>
<tr>
<td>OLEIC ACID</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>---</td>
</tr>
<tr>
<td>100</td>
<td>---</td>
</tr>
<tr>
<td>200</td>
<td>---</td>
</tr>
</tbody>
</table>

The ratios for NOPALCOL 6-O are somewhat higher at pH 6.5 and 7.0, although not so extreme as those observed with oleic acid. This pH sensitivity with NOPALCOL 6-O (and consequent disparity in acidity and turbidity) is especially interesting considering the widespread practice of adding such nonionic oleate detergents to various microbiological assay media. An examination of the data from which the
ratios of Table IV were calculated shows that the biotin activity of
Nopalea 6-0 decreases with increasing pH, maximum values being ob-
tained at pH 5.0.

6. Variation in the concentration of glucose.

In certain experiments utilizing hexose phosphates, both
alone and in combination with glucose, it was noted that a reduction
in the amount of glucose per tube (in the presence of biotin and not
oleic acid) had a marked effect on the turbidity-titration ratios.
Accordingly, this point was investigated further. Various concen-
trations of glucose ranging from 25 to 175 mg. per tube were used in
place of the 200 mg. usually supplied in the basal medium. Oleic
acid was not used in these tests, but 1,000 micromicrograms of biotin
were added per tube. Growth was compared with standards containing
200 mg. of glucose per tube. The results are given in Table V. When
compared with the standards, the low glucose tubes showed a remarkable
difference. The increase in the ratio from the usual value of 1.0
to approximately 2.0 occurred because turbidity comparable with that
in the standard tubes was achieved with less glucose than is ordinarily
supplied. These findings suggest that the similar effects observed
with oleic acid might be explained in several ways: (a) oleic acid
may act as an inhibitor at some point in the glycolytic cycle so that
only a portion of the glucose can be converted to lactic acid; (b)
oleic acid may offer a shunt mechanism for the production of energy
for cell growth; and (c) it is possible that the actual amount of
fermentation necessary to furnish energy for cell growth is less than
that involving the 20 grams of glucose per liter provided in the medium. Oleic acid thus may exert an effect of steric hindrance because of its attraction for the lipo-protein surface of the bacterial cell and prevent access of the excess glucose which is necessary to make the products of fermentation equivalent, in terms of apparent biotin content, to those of cell production. The latter hypothesis seems the

### Table V

**EFFECT OF GLUCOSE CONCENTRATION ON TURBIDIMETRIC-TITRIMETRIC RATIOS**

**FROM TUBES CONTAINING 1,000 MICROMICROGRAMS OF BIOTIN AND NO OLEIC ACID**

<table>
<thead>
<tr>
<th>Glucose Per Tube</th>
<th>0.1 N Acid Per Aliquot (Titrimetric)</th>
<th>Apparent Per Cent Biotin Transmission (Turbidimetric)</th>
<th>Apparent Biotin</th>
<th>Turbidimetric-Titrmetric Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>ml.</td>
<td>μg.</td>
<td>μg.</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>2.77</td>
<td>0</td>
<td>71.5</td>
<td>190</td>
</tr>
<tr>
<td>50</td>
<td>3.95</td>
<td>120</td>
<td>62.1</td>
<td>370</td>
</tr>
<tr>
<td>75</td>
<td>5.22</td>
<td>295</td>
<td>54.0</td>
<td>573</td>
</tr>
<tr>
<td>100</td>
<td>6.57</td>
<td>520</td>
<td>47.3</td>
<td>820</td>
</tr>
<tr>
<td>125</td>
<td>7.62</td>
<td>790</td>
<td>46.1</td>
<td>900</td>
</tr>
<tr>
<td>150</td>
<td>8.31</td>
<td>970</td>
<td>44.1</td>
<td>1,080</td>
</tr>
<tr>
<td>175</td>
<td>8.09</td>
<td>860</td>
<td>44.5</td>
<td>1,020</td>
</tr>
<tr>
<td>200</td>
<td>8.34</td>
<td>1,000</td>
<td>44.7</td>
<td>1,000</td>
</tr>
</tbody>
</table>
most probable considering the present concepts of surface adsorption and the data involving pH effects, differing oleic acid concentrations, and the contrast in the action of Nopalcol 6-0. The fate of the glucose that is not converted into lactic acid is unknown except that it can be accounted for as a reducing substance. This fact was ascertained in two ways: (a) by Shaffer-Hartmann determination of glucose (48); and (b) by use of Dreywood's anthrone reagent (42).

7. **Comparison of *L. casei* with *L. arabinosus***

The turbidimetric-titrimetric disparity observed with *L. casei* was checked with *L. arabinosus*, and the data are given in Table VI. From two separate experiments conducted some weeks apart it may be seen that the effect with *L. arabinosus* is much less marked, so much so that it is doubtful whether the disparity exists in this case.

To summarize, one may then conclude that of the many substances and variations in procedure tested, not one was completely effective in removing the disparity observed in turbidimetric assay values obtained with oleic acid. The inclusion of oleic acid in the form of a high molecular weight ester, Nopalcol 6-0, was effective at pH 5.0 to 6.0. In general, turbidity values were approximately twice titration values under all other conditions. Increasing the pH from 5.5 to 7.0 resulted in an enormous increase in the turbidity-titration ratio because of the increased toxicity of oleic acid at higher pH values, particularly with respect to glycolysis. The only clue obtained with regard to the mechanism whereby oleic acid selectively inhibits
acid production was found in the case of tubes containing biotin and decreased glucose, but no oleic acid. When compared with tubes containing the usual amount of glucose and equal amounts of biotin, turbidities gave much higher apparent biotin values than did acidities. On this basis several possible explanations were offered for the strange disparity, the most probable of which is a physico-chemical one.
B. Oleic Acid in the Biotin Metabolism of Saccharomyces Cerevisiae.

Since the discovery by Williams and Fieger (45) that oleic acid is capable of replacing biotin for certain microorganisms, a study of the interrelationships of biotin and oleic acid in the metabolism of such microorganisms has been continuously underway in various laboratories. Although the original work involved the use of Lactobacilli, an extension of this phenomenon to yeast was attempted.

Of some twenty-five strains of yeast which were tested, S. cerevisiae Java showed the most pronounced response when oleic acid was substituted for biotin as a growth factor in the basal sucrose medium, and consequently it was chosen for study.

1. The ability of oleic acid to substitute for biotin.

In an attempt to determine whether oleic acid could adequately substitute for biotin, this organism was grown on the basal sucrose medium in the presence and absence of aspartate, glutamate, and succinate. Assays were performed in the following manner. The four media, i.e.,

(a) basal medium,
(b) basal medium + aspartate (1 mg./5 ml. medium),
(c) basal medium + glutamate and succinate (1 mg. each/5 ml. medium),
and (d) basal medium + aspartate, glutamate, and succinate (1 mg. each/5 ml. medium) were compared with one another on three bases: (a) no growth factor, (b) 0.01 microgram of biotin per tube, and (c) 100 micrograms of oleic acid per tube. Five milliliters of previously inoculated sucrose medium were added to each sterile tube, giving a total volume of 7 ml. per tube. The inoculum was made from a 24-hour
broth culture which had been washed three times with distilled water and diluted 1/10,000. Two drops of this suspension were sufficient for 60 ml. of medium. Tubes were incubated for 24 to 72 hours and the growth response determined by reading turbidity against a distilled water blank in the Beckman spectrophotometer at wave length 6000 Å and slit width 0.3 mm. Data from a representative experiment are illustrated in Figure 4. The growth of S. cerevisiae Java was stimulated by oleic acid on a biotin-deficient medium only in the presence of aspartic acid. The organism grew in the absence of aspartic acid if biotin was present, although for short periods of incubation the growth response was much lower. The presence of glutamate and succinate increased this growth response slightly, as might be expected.

2. Biotin Synthesis by S. cerevisiae Java.

When incubated for longer than three days, S. cerevisiae Java eventually achieved reasonably good growth in a biotin-deficient medium. Figure 5 shows growth curves of the organism on such media in the presence and absence of egg white. Oleic acid produced a similar amount of growth in a somewhat shorter time. Hydrolyses of cells and media showed synthesis of biotin when assayed by S. fragilis and L. casei. The hydrolyses were conducted in the following manner. Two liters of medium were diluted with 800 ml. of distilled water containing 40 mg. of oleic acid and were concentrated to 100 ml. An aliquot of this concentrate was hydrolyzed in 2 N sulfuric acid at
121° for two hours, neutralized with barium hydroxide, diluted to a final volume and filtered. A corresponding two liters of medium were diluted with an equal amount of oleic acid suspension, inoculated with 1 ml. of the usual 1/10,000 dilution of S. cerevisiae Java, and incubated for 72 hours. The cells were removed by centrifugation and hydrolyzed in 2 N sulfuric acid. The supernatant was treated in the same manner as described above for the un inoculated medium.

Aliquots of the neutralized filtrates were taken for biotin assay by the methods mentioned above. A representative set of data is shown in Figure 6. It is obvious that the same general trend appears in each assay and that the amount of biotin in the cells cannot possibly have been concentrated from the medium. One must, therefore, conclude that biotin or its nutritional equivalent is synthesized to some extent by this organism.

The time interval of growth in a depleted medium was dependent to a great extent on the inoculum size. Since the medium was never found to be completely biotin-free, it was considered that growth might also have been dependent upon traces of biotin present. Cells grown in the presence of 2 ml. of egg white per liter of medium, however, showed appreciable synthesis of biotin.

Although Williams and Fieger had previously reported no detectable biotin in cells of L. casei grown on an oleic acid medium, the demonstrated biotin synthesis by yeast under similar conditions warranted repeating the original work using more sensitive techniques and extracts of greater concentration.
COMPARATIVE GROWTH STIMULATION OF *S. CEREVISIAE* JAVA BY BIOTIN AND BY OLEIC ACID ON BASAL MEDIA TO WHICH THE INDICATED ADDITIONS WERE MADE.
Figure 4

Comparative growth stimulation of *S. cerevisiae* Java by biotin and by oleic acid on basal media to which the indicated additions were made.
FIGURE 5

GROWTH OF SACCHAROMYCES CEREVISIAE JAVA ON BIOTIN-DEFICIENT MEDIA IN THE PRESENCE AND ABSENCE OF EGG WHITE
FIGURE 6
SYNTHESIS OF BIOTIN BY CELLS OF S. CEREVISIAE JAVA GROWN ON BIOTIN-DEFICIENT MEDIUM. ASSAYS PERFORMED BY L. CASEI AND S. FRAGILIS AS SHOWN
Washed cells, the corresponding supernatant medium, and uninoculated medium were each hydrolyzed in 2 N sulfuric acid for two hours at 121°C, neutralized with barium hydroxide, and filtered to remove barium sulfate. Aliquots of these concentrated filtrates were assayed with *Saccharomyces fragilis*, which does not respond to oleic acid, and with *L. casei*. Data representative of six such experiments are given in Table VII. Assay of the supernatant from the cells indicated approximately the same content as the uninoculated medium, whereas the cells contain considerably more biotin. Since this biotin cannot have been concentrated from the medium, it is assumed to have arisen from synthesis.

The higher values of biotin content for the supernatant and the uninoculated medium by *S. fragilis* assay as compared with the *L. casei* assay may be caused by (a) the difficulty in correcting for colored substances present in the extracts being analyzed turbidimetrically, or (b) the inhibitory effect of the larger quantity of concentrate necessary in the *L. casei* assays, or (c) simply the greater sensitivity of the *S. fragilis* assay.

The data obtained throughout the experiments with *S. cerevisiae* Java could not be reproduced upon either glucose, fructose, or acid-hydrolyzed sucrose media. Illustrative data are shown in Table VII. The yeast were grown upon media to which 1 ml. egg white per 100 ml. had been added. Monosaccharides were provided at a concentration of 10 grams per liter of medium. With the carbohydrates listed above as
substrates, no growth of *S. cerevisiae* could be obtained in the absence of biotin even on prolonged incubation.

In conclusion, the growth of *Saccharomyces cerevisiae* Java was stimulated by oleic acid on a biotin deficient medium in the presence of aspartate. Glutamate and succinate would not substitute for aspartate. When incubated for longer than three days, *S. cerevisiae* Java achieved fairly good growth in a sucrose medium lacking both oleic acid and biotin but containing aspartate. Analysis of hydrolyzed cells from such cultures showed synthesis of biotin. Small quantities of biotin, or its nutritional equivalent, have also been shown to be present in *L. casei* cells grown in the presence of oleate.

**C. A Linkage for Biotin in Carbohydrate Metabolism.**

During studies of the ability of oleic acid to substitute for biotin in the growth of *S. cerevisiae* Java, it was noted that data obtained on the basal sucrose media could not be duplicated if glucose, fructose, or hydrolyzed sucrose were substituted for sucrose as sources of carbohydrate. This appeared to indicate that biotin was essential for some phase of the monosaccharide glycolytic mechanism which is not involved in the utilization of sucrose. Data of similar implications have been published by Carlson and Whiteside-Carlson (35) from their studies with *Leuconostoc* species.
<table>
<thead>
<tr>
<th>9.0</th>
<th>7.7 x 10^-3</th>
<th>Micrograms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>7.7 x 10^-4</td>
<td>Micrograms</td>
</tr>
<tr>
<td>0.0</td>
<td>4.7 x 10^-4</td>
<td>Supplement</td>
</tr>
<tr>
<td>2.7</td>
<td>1.6 x 10^-3</td>
<td>Cells</td>
</tr>
</tbody>
</table>

(2 liters)

For **7. Case B** with a position-dependent medium:

**Bleomycin content of cells, supplement, and unincoculated medium**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Turbidimetric assay</th>
<th>Turbidimetric assay</th>
<th>Percent Tansmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>96.6</td>
<td>Fructose</td>
<td></td>
</tr>
<tr>
<td>2.7</td>
<td>61.9</td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>69.1</td>
<td>Hydrated Sucrose</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>79.0</td>
<td>Sucrose</td>
<td></td>
</tr>
</tbody>
</table>

No additions to 7. Case B.

**Growth upon basal media containing 1 ml. no white par 100 ml.**

Utilization of various sources of carbon dioxide by E. coli.

**Table VIII**

<table>
<thead>
<tr>
<th>Per Cent Transmittance</th>
<th>Carbohydrate Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

27
As far as the Meyerhoff-Embden pathway is concerned, there are two points in the glycolytic scheme at which biotin might function in monosaccharide utilization without being completely necessary for the glycolysis of sucrose. These are in connection with the action of the enzymes hexokinase and phosphoehemisomerase. The latter of the two enzymes would be necessary for the passage of glucose-1-phosphate through the Meyerhoff-Embden scheme, but a partial utilization of sucrose through its fructose portion might occur in the absence of biotin, should biotin be required for phosphoehemisomerase activity.

The problem of the connection of biotin to carbohydrate metabolism has been approached in three ways: (1) by studies of the glucose utilization of biotin-deficient L. casei cells, (2) by studies of the variation in the glucose utilization of cells of S. cerevisiae 139 grown on high- and low-biotin media, and (3) by studies of the activity of hexokinase in the presence and absence of (a) biotin and (b) biotin / adenylic acid.

1. **Glucose Utilization by Biotin Deficient Cells of Lactobacillus Casei**

   One of the standard methods of resolving enzyme systems is by growth of the enzyme source on materials devoid of building substances for the coenzymes. Since it appears probable from unpublished data of Christman and Williams that the coenzyme of aspartic acid deaminase (herefore considered a "biotin coenzyme") is derived from glucose, and that therefore glucose might be an essential building material for the coenzyme with which biotin may be linked in glycolysis, cells of
_L. casei_ were grown in the presence of Nopalcol 6-O on a low glucose medium. The washed cells were then incubated in buffered glucose (a) with no additions; (b) with the addition of 1 microgram of biotin, and (c) with the addition of 1 microgram of biotin / 1 microgram of adenylic acid. Details of the procedure follow.

The glucose content of the medium was reduced to 5 g. per liter, one eighth of its usual value. Nopalcol 6-O, 40 mg. per liter of double strength medium, was substituted for biotin as a growth factor. Cells grown on two liters of this low glucose medium containing Nopalcol 6-O were harvested after about 40 hours of incubation and washed once with distilled water. The cells were then suspended in approximately 20 ml. of distilled water and added in 2 ml. aliquots to tubes containing the following materials at 35°:

- 5 ml. of phosphate buffer, pH 7.15
- 2 ml. of glucose (2 mg./ml.)
- 1 ml. of additions.

Several series of three tubes were prepared, each series to be removed from the temperature bath after definite time intervals. The tubes contained (a) no additions, (b) 1 microgram of biotin, and (c) 1 microgram of biotin / 1 microgram of adenylic acid. At the end of the incubation period each tube was heated in boiling water for one minute to inactivate the enzyme systems. Suspended cells were then centrifuged out and 5 ml. aliquots of supernatant removed for precipitation with zinc sulfate-barium hydroxide.
Figures 7 and 8 illustrate the rate of glucose utilization from two such washed cell incubations. At incubation periods of 10 to 15 minutes it becomes obvious that some stimulation of glucose utilization is produced by biotin alone, and that an even more pronounced stimulation occurs in the presence of a combination of biotin and adenylic acid.


Saccharomyces cerevisiae 139 was grown for 48 hours on two media differing only in biotin content. The high biotin medium contained 2 micrograms of biotin per liter. The low biotin medium contained 2 x 10^-2 micrograms per liter. Approximately 29 g. of cells were obtained from 10 liters of the former medium as compared with 9 g. from 10 liters of the latter. Cells were suspended in 3 ml. of water per gram of cells.

The following three incubation flasks were set up for cells from both high- and low-biotin media:

(a) 50 ml. of M/20 phosphate buffer, pH 7, containing no glucose.
(b) 50 ml. of M/20 phosphate buffer, pH 7, containing 20 mg. glucose.
(c) 50 ml. M/20 phosphate buffer, pH 7, containing 40 mg. glucose.
Nine milliliters of cells were added to each flask and 10 ml. aliquots were removed from each at intervals of 0, 5, 10, and 15 minutes. These samples were immediately centrifuged to remove the cells, and the supernatants were analyzed for glucose. The rates of glucose utilization are shown in Figure 9. During initial stages of glycolysis it is obvious that the rate of glucose utilization of the biotin-deficient cells is much slower than that of the cells which were grown on the high-biotin medium.

3. Effect of Biotin on Hexokinase.

Yeast hexokinase was prepared several times. Enzyme assays were performed in the manner described in Chapter II and analysis made for the disappearance of glucose. The enzyme obtained showed either such a low level of activity that stimulations could not be observed or an exceedingly high degree of activity with no evidence of stimulation in the presence of biotin or biotin with adenylic acid. The lack of a stimulative effect is not, however, conclusive evidence that biotin is not necessary for the function of hexokinase. It is entirely possible that the enzyme system as isolated is not sufficiently resolved.

To summarize, a study of the linkage of biotin to the glycolytic cycle has been made. That biotin has a stimulative effect upon at least one of the enzyme systems involved has been demonstrated by the use of two different microorganisms. Washed cells of L. casei which had been grown on a low glucose medium in the presence of Nopalcool 6-0 showed an increased utilization of glucose upon the addition of biotin or biotin with adenylic acid to the glycolyzing suspension. A
FIGURES 7 AND 8

THE RELATIVE STIMULATORY EFFECTS OF BIOTIN AND BIOTIN PLUS ADENYLIC ACID UPON GLUCOSE UTILIZATION BY L. CASEI CELLS GROWN ON LOW GLUCOSE BIOTIN-DEFICIENT MEDIA
FIGURE 9

RELATIVE RATES OF GLUCOSE UTILIZATION BY CELLS OF *S. CEREVISIAE* 139 GROWN ON HIGH- AND LOW-BIOTIN MEDIA
similar increased rate of fermentation was shown by cells of *S. cerevisiae* 139 grown on a high-biotin medium when compared under similar conditions with cells produced in the presence of a much lower concentration of biotin.

Because of the differences observed in the utilization by yeast of the di- and the monosaccharides in the absence of biotin, and because of a somewhat similar phenomenon noted in the metabolism of *Leuconostoc* species by Carlson and Whiteside-Carlson (35), it has been postulated that biotin must be linked to the action of one of two enzymes: either hexokinase or phosphohexoisomerase, most probably the former. The preparations of hexokinase tested were not stimulated by the addition of biotin or of biotin with adenylic acid. Since, however, the enzyme system may have remained unresolved, this was not regarded as a negation of the hypothesis.
CHAPTER IV

SUMMARY

Previously unexplored phases of the relationships of biotin and oleic acid in the metabolism of certain microorganisms have been studied along three different lines: (a) the anomaly between turbidimetric and titrimetric microbiological assays of oleic acid-containing media has been examined under various conditions; (b) the phenomenon of oleic acid stimulation of microorganisms in biotin-free systems was studied with yeast; and (c) the relationship of biotin to some phase of carbohydrate metabolism has been demonstrated for several microorganisms.

The turbidimetric-titrimetric disparity which exists in Lactobacillus casei assays of oleic acid-containing medium was examined under various conditions. Under most of the conditions tested the apparent biotin contents as measured by turbidities were approximately twice the values as measured by titration. The phenomenon showed some pH sensitivity, the turbidimetric-titrimetric ratios increasing as the pH was raised to 7.0 probably because of the increased toxicity of oleic acid with decreasing acidity. An increase of turbidities as compared with acidities was obtained upon lowering the glucose content of the basal medium in the presence of biotin, not oleic acid. Only
the substitution of a high molecular weight ester of oleic acid for
the latter compound in the assay medium completely eliminated the
anomaly. On the basis of these findings several explanations were offered
for the disparity, the most probable of which appears to be a physico-
chemical one.

The ability of oleic acid to stimulate growth of Saccharomyces
cerevisiae Java on a biotin-deficient sucrose medium in the presence of
aspartic acid was demonstrated. Synthesis of biotin by this organism
grown on a similar medium was shown to occur. Cells of L. casei grown on
a biotin-deficient medium were also found to contain low concentrations
of biotin or its nutritional equivalent. Reproduction of S. cerevisiae
Java occurring upon sucrose medium in the absence of biotin, did not
occur if glucose, hydrolyzed sucrose, or fructose were substituted for
sucrose as carbohydrate sources.

From the latter observation the third line of research was
developed: the relationship of biotin to carbohydrate metabolism.
Stimulation of glucose utilization was observed when biotin or biotin
with adenylic acid was added to cells of L. casei grown on a low-glucose
medium in the presence of Kopalcol 6-0. The rate of glucose utilization
by cells of S. cerevisiae 139 grown on media containing 2 micrograms of
biotin per liter was found to be considerably higher than that of cells
grown in the presence of $2 \times 10^{-2}$ micrograms of biotin per liter. A
linkage of biotin to hexokinase function was postulated, but could not
be demonstrated in the purified enzyme under the conditions used.
Several conclusions were drawn.

(1) The turbidimetric-titrmetric disparity observed in microbiological assays of oleic acid-containing media may be removed by the substitution of a high molecular weight ester of the oleic acid for the latter compound in assay media. The anomaly is probably due to an effect of steric hindrance by oleic acid adsorbed on the cell membrane.

(2) Oleic acid is capable of stimulating the growth of *S. cerevisiae* Java on a biotin-deficient sucrose medium. Synthesis of biotin or its nutritional equivalent occurs when the organism is grown for a prolonged period on a biotin-deficient sucrose medium. Cells of *L. casei* grown on a biotin-deficient medium also synthesize small quantities of biotin. Growth of *S. cerevisiae* Java on medium lacking in biotin cannot be produced if the monosaccharides listed above are substituted for sucrose as sources of carbohydrate.

(3) Biotin exhibits a stimulative effect upon glucose utilization by cells of *L. casei* and *S. cerevisiae* 139. A biotin linkage for hexokinase has been postulated.
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VITA

Emilie Anne Andrews was born at Hickory, North Carolina, on February 7, 1925. She was educated in the public schools of Morganton, North Carolina, and graduated from Morganton High School in 1942. The same year she enrolled in Southeastern Louisiana College and graduated from that institution in June of 1946 with the Bachelor of Science Degree in chemistry. In September, 1946, she entered Louisiana State University as the recipient of an honor scholarship awarded at her graduation from Southeastern Louisiana College. She was a graduate assistant in the Department of Chemistry from 1947-1949. In June of 1949 she received the Master of Science Degree with a biochemistry major. Since that time she has been employed as a research assistant in the Department of Agricultural Chemistry and Biochemistry of the Louisiana Agricultural Experiment Station, Louisiana State University, and has continued her studies toward the degree of Doctor of Philosophy in the field of biochemistry.
EXAMINATION AND THESIS REPORT

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Major Field: Biochemistry

Title of Thesis: Biotin and Oleic Acid in the Nutrition and Metabolism of Certain Microorganisms

Approved:

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Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

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Date of Examination:

November 19, 1951