FUNCTION OF BIOTIN IN BACTERIAL METABOLISM

by

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INTRODUCTION

Many microorganisms have been shown to require biotin for growth. The ability to synthesize biotin has been demonstrated for other organisms which are not dependent on an exogenous source of the vitamin. Thus biotin, like other members of the B vitamin complex, holds an important position in bacterial metabolism.

The members of this group of vitamins have been recognized as having great importance largely because the absence of one or more from the diet often causes severe changes in the physiology of the organism. The recognition of relationships between the vitamins and normal metabolism has outdistanced studies on elucidation of the manner in which each vitamin performs its duties. Until the specific chemical function is determined, however, knowledge of the vitamin is incomplete.

It was early recognized that vitamins must act as catalysts since only minute amounts were needed to produce effects on the physiology of a suitable organism. Many of the B vitamins have been shown to catalyze definite biological reactions. The first actual proof that a B vitamin was an integral part of an enzymatic reaction came in 1935 when a component of the "yellow enzyme" was identified as riboflavin. Other vitamins have been identified as parts

of enzyme systems and the coenzymes derived from thiamin, nicotinic acid, and pyridoxine, as well as riboflavin, have been isolated and their chemical structures determined. Identification of other coenzymes involving pantothenic acid and inositol is not as complete but similar relationships are indicated.

Biotin has been found associated with several molecules more complex than biotin itself. It seems probable that one or more of these complexes may be coenzymes for one or several reactions. On the other hand, it is possible that biotin as such may function in certain reactions.

Biotin has been reported to be involved in reactions involving the transfer of carbon dioxide. The purpose of this investigation was to study such a relationship, the primary consideration being the relationship of biotin to the fixation of carbon dioxide into oxalacetic acid.

HISTORICAL

Discovery of Biotin

The recognition of biotin as a chemical compound and specifically as a vitamin represented the convergence of three independent lines of investigation. In each case, a search was being made for a biologically active compound responsible for a certain effect. The responsible factor was proved each time to be biotin; the various investigators were merely dealing with different manifestations of the same chemical entity.

Wildiers recognized in 1901 that yeast required the presence of a certain factor in the growth medium and proposed the name "bios" for this essential. Bios was later found to consist of several fractions, one of which was further investigated by Kögl (1935) who isolated from egg yolk minute amounts of a crystalline substance possessing growth activity for yeast. Kögl and Tönnis (1936) presented a detailed account of an isolation procedure by which 1.1 milligrams of crystalline material were obtained from 250 kilograms of dried egg yolk and called the material biotin. This active principle was studied further by Kögl (1937-1938) who found that the crystals were the methyl ester of biotin. Kögl assigned to the compound the empirical formula

$C_{11}H_{18}O_3N_2S$.

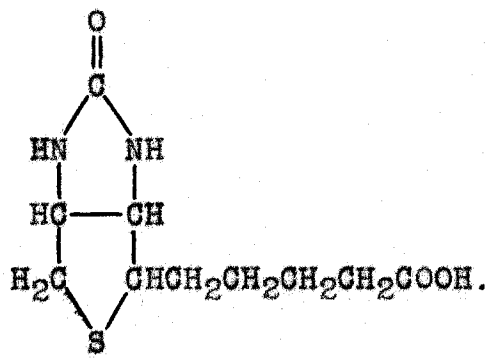
A second problem involved a growth factor for the symbiotic nitrogen fixing genus, Rhizobium, which Allison, et al, (1933) found present in various sources and named coenzyme R because of its respiration-promoting properties. On the basis of comparisons of crude concentrates West and Wilson (1939) suggested that coenzyme R and biotin were the same compound. Further evidence that the two were identical was found when Nilsson, et al, (1939) showed that a sample of Kögl's biotin possessed coenzyme R activity.

Investigation on a third branch of the biotin problem proceeded independently in other laboratories. Boas (1927) described the effects produced in rats fed on a diet including large amounts of dried egg white; loss of hair, dermatitis, skin hemorrhages, loss of weight, spasticity, and finally death resulted. It was soon recognized that many foods contained a factor which could cure or prevent these symptoms of "egg white injury". György in 1931 named this factor vitamin H because of the similarity of its action to that of vitamins known at the time. The possible identity of vitamin H with biotin and coenzyme R was first suggested by György, Melville, Burk, and du Vigneaud (1940) and more firmly established by vitamin H assays of a sample of Kögl's crystalline biotin conducted by du Vigneaud, et al, (1940). Final

confirmation was afforded by the demonstration by György, Rose, Hofmann, Melville, and du Vigneaud (1940) of the high vitamin H activity of pure biotin methyl ester isolated from liver concentrates (du Vigneaud, et al., 1941).

Chemical Properties of Biotin

The determination of the structure of biotin proceeded in the laboratories of du Vigneaud and of Kögl, and final elucidation was first presented by du Vigneaud (1942). The vitamin was found to be a monocarboxylic acid with two rings, the acid being valeric acid, and the rings derivatives of imidazole and thiophene. The assigned structure is



Harris, et al., (1943-1944) obtained the vitamin synthetically and found good agreement with values determined for chemical properties of the natural compound.

Biotin is a colorless, crystalline solid and crystallizes from water as long, thin needles. It is sparingly soluble in water, readily soluble in basic solutions due to

its carboxyl group, and insoluble in ordinary organic solvents such as chloroform, ether, or petroleum ether. The compound melts at 230-232°C. Biotin is readily dialyzable in the free form and easily absorbed on charcoal.

The vitamin is relatively stable. Brown and du Vigneaud (1941) found it to be inactivated by 20 per cent hydrochloric acid only after long periods of time and by 1 N potassium hydroxide after 17 hours at 120°C. It is destroyed by strong oxidizing agents such as peroxide or nitrous acid, but unaffected by acetylation, alkylation or carbonyl reagents. Recently, Axelrod and Hofmann (1950) demonstrated that hydrochloric acid has a more destructive action on biotin than was formerly believed. Sulfuric acid, however, exhibits no adverse effect on the vitamin. The destruction of biotin by rancid fats and fats with a high peroxide number was reported by Pavcek and Shull (1942) who found that the pure vitamin was almost completely inactivated in 12 hours. The inactivation appears to be an oxidation of the sulfur moiety to sulfoxide.

The chemistry of biotin has been adequately covered in reviews by Drumel and Hubert (1938), Hofmann (1943), and Melville (1944), and will therefore not be described in more detail.

Metabolic Functions of Biotin

Physiological action

Biotin has been found to be essential for various lower organisms, including bacteria, yeasts, molds, and protozoa. Many species which do not require an exogenous supply of biotin for growth have been shown to synthesize it in varying amounts. Many insects and higher animals, such as rats, chicks, turkeys, monkeys, rabbits, guinea pigs, dogs, and humans have been shown to require the vitamin for optimum growth.

The previously described symptoms of an avitaminosis which occur in rats on a diet which denies them biotin are manifested in closely related effects in other higher animals. Sydenstricker, et al, (1942) produced an experimental biotin deficiency in human volunteers by maintaining them on a diet in which about 30 per cent of the total calories were supplied as desiccated egg white.

Normally a biotin deficiency does not occur in animals because the synthesis of biotin by microorganisms in the digestive tract provides the animal with a sufficient supply. The deficiency can be produced experimentally by including in the diet bacteriostatic agents such as succinylsulfathiazole or sulfaguanidine (Black, et al, 1942;

Nielsen and Elvehjem, 1942) which are effective against intestinal microorganisms, thus inhibiting the synthesis of biotin. Raw egg white may be fed to the animal to produce the deficiency. There exists in egg white a protein which combines with biotin rendering the vitamin unavailable. This active constituent is avidin and was isolated and concentrated by Eakin, et al, (1940-1941) and by Woolley and Longsworth (1942). Hertz (1946) presented a thorough review of the avidin-biotin complex.

All of the possible optically active modifications corresponding to the structure of biotin have been synthesized. All are essentially inactive biologically except d-biotin which possesses the same activity as natural biotin. Kogl and ten Ham (1943) concluded that two isomeric forms exist in nature, α-biotin isolated from egg yolk and β-biotin isolated from liver. Many investigators, however, have disputed Kögl's conclusion. Krueger and Peterson (1948a), on the basis of growth experiments with several species of bacteria, molds, and yeasts, obtained evidence that the two forms are identical.

Williams, et al, (1950) reviewed the properties of many biologically active and inhibitory analogues of biotin. Oxybiotin, the oxygen analogue of biotin, and desthiobiotin, in which the sulfur atom is removed from the

tetrahydro-thiophene ring, appear to be able to replace biotin in the greatest number of cases. The effect of desthiobiotin appears to be that of a precursor. Dittmer, et al, (1944) suggested that desthiobiotin was converted to biotin in the case of Saccharomyces cerevisiae. The evidence included the disappearance of desthiobiotin from the culture medium and a corresponding increase in the concentration of biotin in the cells. Other analogues and derivatives reported to possess biotin activity in a few cases are biotin methyl ester, biotin sulfone, biotin sulfoxide, and the diamino acids obtained on hydrolysis of biotin, oxybiotin, and desthiobiotin.

Pimelic acid appears to be involved in the biosynthesis of biotin and can, therefore, like desthiobiotin, substitute for certain microorganisms. It was found by du Vigneaud, et al, (1942) that the diphtheria bacillus, for which pimelic acid was considered essential, could grow in the absence of this acid, provided biotin was added to the medium. Pimelic acid could not replace biotin for yeast, however. Eakin and Eakin (1942) showed that pimelic acid in the medium increased the biotin content of Aspergillus niger. Tatum (1945) noted an increase in desthiobiotin concentration after the addition of pimelic acid to cultures of Penicillium chrysogenum. This demonstration suggested that desthiobiotin is a normal intermediate in the synthesis of biotin from pimelic acid by this

mold and probably other organisms. The evidence indicates that pimelic acid serves as a precursor of biotin with the latter being synthesized by way of desthiobiotin. Other possible intermediates have not been determined. The ability of an organism to utilize these precursors of biotin would depend upon the presence of the correct enzymes to carry out the various steps involved in conversion of one compound to another.

Mode of action

Studies concerning the role of biotin in cellular metabolism have been particularly concerned with two relationships. One phase of work has dealt with the involvement of biotin in carbon dioxide fixation; a second line of investigation has been the connection between oleic acid and biotin. In addition, there have been a number of other reported functions for biotin, but little investigation has been undertaken concerning these possible roles.

Relation to carbon dioxide fixation. Burk, et al., (1941) studied the effect of biotin on various phases of yeast growth and metabolism. Yeast grown at low biotin levels possessed respiration and fermentation rates which were only one-twentieth of normal. Addition of biotin to these deficient cells caused fermentation rates to rise immediately, respiration to increase after one hour, and

growth increase could be noted after two hours. In order that these increases were to occur, a readily available source of nitrogen was necessary, ammonia being the best source. Winzler, et al., (1944) also showed that biotin uptake was decreased in the absence of glucose or phosphate. The authors suggested that biotin was concerned with the synthesis of nitrogenous material which was ultimately or cyclicly connected with the metabolism of glucose, or that biotin might function as a general coenzyme of carbon dioxide transfer (Burk and Winzler, 1943).

Pilgrim, et al., (1942) found that the rate of oxidation of pyruvic acid was decreased in liver slices from biotin-deficient rats. They considered this to be indirect evidence that biotin is a component of a system involved in pyruvic acid metabolism, or closely connected with its formation. Similar results were obtained by Summerson, et al., (1944) who found that the addition of biotin to biotin-deficient rat tissue respiring in the presence of lactic or pyruvic acid caused a rise in oxygen consumption and a striking change in the value of the respiratory quotient.

Koser, et al., (1942) found that aspartic acid partially substituted for the growth-stimulating effect of biotin in the nutrition of the yeast, Torula cremoris. The omission of biotin resulted in a pronounced delay of growth of the yeast in an ammonium phosphate-glucose-inorganic salts

medium. Under these conditions, aspartic acid, when supplied in place of biotin but in much larger amounts produced a distinct stimulation of growth amounting to approximately two-thirds that obtained when biotin was supplied. The authors speculated that biotin might be concerned with the formation of aspartic acid or that the amino acid was capable, to a limited extent, of performing a function of biotin.

Stokes, et al, (1947) definitely demonstrated that biotin is involved in the synthesis of aspartic acid by microorganisms. Biotin was found to substitute completely for aspartic acid in the growth of Lactobacillus arabinosus, Streptococcus faecalis, and related organisms. It was found, however, that biotin was required for metabolic functions other than those concerned with the synthesis of aspartic acid, though the latter need was much greater than that for the other function or functions. Resting cell suspensions of L. arabinosus formed aspartic acid by typical transamination reactions, but did not require biotin for these reactions. Oxalacetic acid was found to partially replace biotin in the growth of several organisms on biotin-deficient media, hinting that biotin might be involved in formation of oxalacetic acid.

Structural analogues of biotin were found to counteract the "biotin effect" on fermentation and growth of yeast but did not inhibit the stimulatory effect of aspartic acid.

Axelrod, Purvis, and Hofmann (1948) proposed that structural analogues exert their inhibitory effects on growth and fermentation by preventing the biosynthesis of an enzymatically active complex from biotin. One of the functions of this complex may be related to the synthesis of aspartic acid.

Lardy, et al, (1947) tested the possibility that the effect of biotin upon aspartic acid formation may be exerted on the fixation of carbon dioxide into oxalacetic acid which could then be converted to the amino acid by transamination. They found that bicarbonate elicits no response in a low biotin medium devoid of aspartic acid in the growth of L. arabinosus but that bicarbonate greatly stimulates growth in the presence of biotin.

Ochoa, et al, (1947) reported an enzyme in turkey liver which catalyzes the reversible conversion of malic acid to pyruvic acid and carbon dioxide and suggested that the enzyme also catalyzes the decarboxylation of oxalacetic acid to pyruvic acid and carbon dioxide. The authors further suggested the possibility that biotin might be involved in the synthesis of dicarboxylic acids by carbon dioxide fixation.

Blanchard, et al, (1950) partially purified from L. arabinosus an enzyme similar to that found in turkey liver. This enzyme decarboxylated malic acid rapidly, but was decreased in cells low in biotin. Reactivation of the system by biotin was slow. The authors suggested that biotin is not

involved directly in the reaction, but that the vitamin may be part of a system synthesizing the enzyme.

On the basis of values obtained with their inhibition analysis technique involving studies in competition for enzyme systems by biotin and various analogues, Shive and Rogers (1947) stated that biotin appeared to be involved in the biosynthesis of α-ketoglutaric acid from oxalsuccinic acid as well as in the carboxylation of pyruvic acid to form oxalacetic acid.

Lichstein and Umbreit (1947a) found that by holding cells of Escherichia coli at pH 4.0 in normal phosphate buffer for various lengths of time their ability to produce carbon dioxide from oxalacetic acid is decreased. This activity is restored upon the addition of biotin. The same stimulation by biotin was obtained with malic acid as the substrate. When large quantities of cyanide were present to bind oxalacetic acid, however, no biotin effect was noticed. This suggested that when oxalacetic acid was not decarboxylated, biotin was not needed. When small amounts of cyanide were added, permitting the decarboxylation of oxalacetic acid but largely inhibiting the decomposition of pyruvic acid, the effect of biotin could again be noted. On the basis of these results, the authors concluded that the site of the action of biotin was oxalacetic decarboxylase, with biotin functioning in the form of a coenzyme.

Potter and Elvehjem (1948), studying the relationship of biotin to aspartic acid and oleic acid, found that the two acids in combination could completely replace biotin in the nutrition of L. arabinosus, but when either acid was added separately only partial replacement took place. The biotin requirement for aspartic acid synthesis, however, was found to be at least ten times as great as that for the other functions of biotin. At pH 5.8 or below biotin could no longer cause aspartic acid synthesis, probably because of low carbon dioxide tension, although pH changes in this range did not affect the oleic acid-replacing activity of biotin. On the basis of the data presented, biotin appeared to catalyze the β-carboxylation of pyruvic acid. To test the hypothesis that biotin might function as a general carbon dioxide donor other biological syntheses involving the addition of one carbon atom were investigated. Biotin was not found to be involved in the conversion of anthranilic acid to indole or the formation of phenylalanine from carbon dioxide and β-phenylethylamine.

In 1948 more data were added to the accumulating total concerning the mode of action of biotin when Olson, et al, (1948) studied the utilization of pyruvic acid and other members of the tricarboxylic acid cycle by heart muscle from normal and biotin-deficient ducks. The authors hypothesized that if biotin functions in the β-carboxylation of pyruvic

acid to oxalacetic acid, the utilization of the various substrates should be depressed in biotin-deficient tissue. Oxygen consumption was found to be lowered in the presence of heart muscle low in biotin when the tissue was metabolizing pyruvic or succinic acid, and a significant inhibition of evolution of carbon dioxide from succinic acid was observed under these conditions. It was suggested that all observed results might be connected with β-carboxylase activity.

Lardy, et al, (1949) added to the evidence that biotin functions as a catalyst in the Wood-Werkman reaction by carrying out a number of studies utilizing radioactive carbon as a tracer. Cells of L. arabinosus grown in the presence of optimum amounts of biotin were able to fix C^{14} from bicarbonate in the medium into cellular aspartic acid. When grown on a medium low in biotin the cells were unable to incorporate the tracer into the amino acid, a deficiency which could be overcome by adding biotin to the vitamin-deficient cells. MacLeod and Lardy (1949) conducted similar experiments with normal and biotin-deficient rats. The intraperitoneal injection of sodium bicarbonate containing radioactive carbon resulted in a larger C^{14} fixation in the adenine, guanine, arginine, aspartic acid, citric acid and bone carbonate of the control animal as compared with the biotin-deficient animal.

MacLeod, et al, (1949) showed that citrulline synthesis from ornithine by biotin-deficient rat liver homogenates was decreased to one-half the amount formed by the control. The addition of heated residue of normal rat liver homogenates increased the low rate of synthesis. Intraperitoneal injection of biotin into the animal brought the activity back to normal within 24 hours. Dietary deficiencies of riboflavin or vitamin B_6 did not affect the rate of citrulline synthesis.

Carbon dioxide is incorporated into arginine as the result of conversion of ornithine to citrulline in the Krebs-Henseleit urea cycle. It has been shown that carbamyl-L-glutamic acid rather than glutamic acid is the intermediate in citrulline synthesis from ornithine. Feldott and Lardy (1951) showed that the rate of citrulline synthesis in the presence of carbamyl glutamic acid is the same for either biotin-deficient or normal animals. It appears that biotin causes decreased conversion of glutamic acid to carbamyl glutamic acid; the influence of biotin is a step prior to where carbamyl glutamic acid functions in the conversion of ornithine to citrulline.

On the basis of these results, Lardy and his co-workers have suggested that biotin may be required as a coenzyme in several different enzymatic reactions involving the fixation of carbon dioxide, or that it is essential for the formation

of a single common precursor which could be incorporated into compounds such as arginine, aspartic acid, citric acid, and the purines.

Kaltenbach and Kalnitsky (1951) determined the optimum conditions for fixation of carbon dioxide into oxalacetic acid by extracts of Proteus morganii and E. coli. The authors have determined the optimum amount of biotin necessary for maximum formation of oxalacetic acid.

Broquist and Snell (1951) found that though many of the lactic acid organisms require biotin for growth in the absence of aspartic acid, such organisms as Lactobacillus fermenti and Clostridium butyricum require the same amount of biotin in the presence as in the absence of aspartic acid. In these organisms biotin apparently is not involved in aspartic acid synthesis since the amount of the amino acid formed is the same whether or not the vitamin is present. Small amounts of biotin are, however, necessary to satisfy other metabolic functions.

The possibility that biotin might be entering into biological carbon dioxide transferring mechanisms through an alternate opening and closing of the ureido ring system was first suggested by Burk and Winzler (1943). This hypothesis was tested by Melville, et al, (1949) by culturing L. arabinosus in the presence of radioactive biotin labeled with C^{14} in the ureido carbon atom. No detectable dilution

of the radioactivity resulted, indicating no replacement of the C^{14} of the radiobiotin. The authors concluded that the mechanism does not involve a transfer of the ureido carbonyl group of the biotin molecule.

Relation to oleic acid. An interesting relationship has developed concerning biotin and various unsaturated fatty acids, particularly oleic acid. While working on the development of a microbiological assay method for biotin in rice products Williams and Fieger (1945) noted stimulation of growth for Lactobacillus casei which they believed was due to lipoidal substances in the rice polish. Williams and Fieger (1946) found that oleic acid stimulated L. casei and that the organism could be maintained on an essentially biotin-free medium provided that oleic or elaidic acid was added to the medium. No synthesis of biotin from oleic acid could be demonstrated. Later studies (Williams and Fieger, 1947-1949) showed that biotin activity of rice polish lipids would not substitute for biotin in the nutrition of the chick and that oleic acid was slightly toxic to L. casei resulting in a lag period before stimulation ensued. The lag effect was completely overcome by the addition of bovine serum albumin to the medium.

Meanwhile Williams, et al, (1947) carried out further studies concerning oleic acid-biotin relationships in lactic acid bacteria. Oleic acid and several closely related

compounds, when supplied in non-toxic form as water-soluble esters, would partially replace biotin. The authors concluded that biotin catalyzes the synthesis of oleic acid.

Further evidence of a metabolic relationship between biotin and various fatty acids has accumulated. Axelrod, et al, (1947) isolated a vaccenic acid fraction from beef tallow which had biotin activity for several organisms. It was approximately one-fourth as active as oleic acid in the growth of L. arabinosus and had considerably higher activity for L. casei. This fraction was shown by Boer, et al, (1947) to have biotin activity for the rat. Hofmann and Axelrod (1947) obtained an ether soluble fraction with biotin activity from beef and human plasma. Oleic acid, on a weight basis, had approximately the same activity.

Trager (1947-1948) found that upon hydrolysis horse plasma yields a substance, extractable with ether, which, injected intramuscularly, reduces slightly dermatitis in chicks. The effect was not as great as that produced by a comparable biotin dose as determined by microbiological assay. Since oleic acid injections did not alleviate the symptoms of the disease, Trager concluded that the material was not a fatty acid or a related compound.

There remains, however, the possibility that the biotin activity of the substance could be explained in terms of

known fatty acids. This was found true for a fraction of human plasma studied by Axelrod, Mitz, and Hofmann (1950).

Trager (1948) concluded that biotin is essential for synthesis of oleic acid. Furthermore, the author proposed that biotin may function at more than one stage in the synthesis of fatty compounds. If, in vertebrates, fatty acids must be supplied in some already combined form in order to eliminate the need for biotin, the unidentified compound obtained from hydrolyzed horse plasma might fulfill such a requirement.

The oleic acid-biotin relationship is interesting in that it appears to be the other major role in which biotin is involved in addition to its function in carbon dioxide fixation and aspartic acid synthesis. The mechanism whereby biotin functions in fulfilling the oleic requirement is not well understood at present. The majority of investigators believe biotin functions in the synthesis of oleic acid. Williams and Williams (1949) suggested a surface-active nature for biotin, that oleic acid and related compounds function by means of adsorption on the surface of microorganisms, and that biotin substitutes for oleic acid in a similar manner. Axelrod, Mitz, and Hofmann (1948), however, dispute the surface activity theory, pointing out that many biotin analogues devoid of biotin activity should, structurally, be more surface active than biotin.

Recently Andrews and Williams (1951) have suggested a role for oleic acid in the synthesis of biotin. In contrast to the results obtained in earlier work, newer methods have revealed traces of biotin present when L. casei was grown without the vitamin but in the presence of oleic acid. Cells grown on oleic acid medium contained ten times as much biotin as uninoculated media.

Lichstein and Boyd (1951) studied the relationship of biotin and oleic acid in a manner considerably different from the usual growth methods. Utilizing manometric techniques the authors found that oleic acid is involved in the formic hydrogenlyase and formic dehydrogenase enzyme systems. The organism employed was a mutant strain of E. coli which required oleic acid or biotin for growth. When cells were grown in the presence of biotin the activity of the two enzymes was decreased and oleic acid stimulation of the systems was retarded. Biotin, however, exhibited some stimulatory effect upon formic hydrogenlyase activity of resting cells. The revelation of this unexpected property of oleic acid suggests an approach to the problem of the biotin-oleic acid relationship which may be useful in future studies.

Relation to amino acid deaminases. Lichstein and Umbreit (1947b) and Lichstein and Christman (1948) claimed that biotin functioned in the deamination of certain amino

acids. By the process of exposure to molar phosphate at pH 4.0, the ability of various bacterial cells to deaminate aspartic acid, serine, and threonine was markedly decreased. This activity could be restored upon the addition of biotin or adenylic acid. Lichstein (1949), Lichstein and Christman (1949), and Christman and Lichstein (1950), suggested the existence of a coenzyme form of biotin and by means of paper strip chromatography separated from yeast extract a substance which activates the deaminases of aspartic acid, serine, and threonine. Since the material was not adenylic acid or biotin as such, the authors concluded that the fraction contained the coenzyme for the reactions involving the deamination of the above amino acids. Upon hydrolysis of the fraction, biotin was made available to a strain of S. cerevisiae which does not respond to the unhydrolyzed material. Lichstein (1950) found that this so-called coenzyme form of biotin also stimulated oxalacetic and succinic decarboxylases.

The probability that biotin exists in a coenzyme form as reported by Lichstein is not surprising. By analogy with other coenzymes containing vitamins in their structure, biotin might well be expected to exist in such form. Biotin exists in strongly bound form and recently Bowden and Peterson (1949) confirmed and extended earlier work which showed that a type of bound biotin utilizable by L. casei had little growth activity for L. arabinosus until acid hydrolysis made

the biotin available to the latter organism. Hofmann, et al, (1950) characterized two biotin-containing fractions in beef liver, one a dialyzable fraction which possessed biotin activity not increased by acid hydrolysis, and the other a non-dialyzable material which exhibited activity only after acid hydrolysis. The latter was postulated to be a biotin-protein complex. The name biotoprotein was proposed for native biotin-containing proteins occurring in tissues.

The crystallization of a naturally occurring complex of biotin has been accomplished by Wright, Cresson, Skeggs, Wood, Peck, Wolf, and Folkers (1950) and named biocytin. The complex is available as a source of biotin to many organisms but is utilizable by others only after acid hydrolysis. Biocytin is heat stable, avidin combinable, and readily dialyzable. Lichstein, et al, (1950) found that biocytin differs from the coenzyme fraction reported to be active for the amino acid deaminases. Wright, Cresson, and Skeggs (1950), however, showed biocytin to be active in the deamination of aspartic acid. The authors believe that biocytin, or biotin, might be active in the formation of the coenzyme for the deamination of aspartic acid.

The relation of biotin to deaminase activity as claimed by Lichstein was challenged by Axelrod, Hofmann, Purvis, and Mayhall (1948) who were unable to reactivate cells previously

exposed to phosphate, pH 4.0, by the addition of biotin, although hot water extracts of bacteria cells or adenosine would reactivate the systems. Wright, et al, (1949) however, confirmed, with reservations, the claims of Lichstein. Cells could be reactivated if the reaction was run at pH 7.0 instead of pH 4.0. Though the mechanism of inactivation is not understood, the authors believe that biotin is in some way concerned with the deaminase systems of aspartic acid, serine, and threonine.

Other functions reported for biotin. Evidence indicating a multiplicity of functions for biotin has continued to accumulate. In addition to the relationships already considered, a number of possible other functions have been reported.

Gavin and McHenry (1941) reported that the feeding of a crude fraction from beef liver caused fatty livers in rats which could be prevented by including lipocaic, extracts of wheat germ, or inositol in the diet. Biotin was found to be the responsible fraction. Cholesterol, particularly, accumulated in the liver. The effect was rapid, being noticeable in 24 hours and maximum in five days. Okey (1946) found a similar relationship in that rats with incipient biotin-deficiency stored no liver cholesterol. While this relationship could merely be another manifestation of the role of biotin in carbon dioxide fixation, the effect has

not been further established.

Another effect which seems likely to be also due to the connection between biotin and carbon dioxide fixation is the influence of biotin on the rate of oxidation of glycerol by S. faecalis, (van Demark, 1950). The effect of biotin in this case is unexplained; in all probability it is an indirect effect.

Ajl, et al., (1950) found that the addition of biotin to dialyzed cell-free preparations of E. coli resulted in increased oxidation of succinic acid. The possibility that the effect may be due to oxidation of oxalacetic acid arising from succinic acid is ruled out on the basis that fumaric acid, which would arise from succinic acid, is not attacked appreciably and that carbon dioxide is not formed from succinic acid. Axelrod, et al., (1942) found no lowering of succinoxidase activity in liver tissue from biotin-deficient rats.

Delwiche (1950) demonstrated that biotin is concerned with the decarboxylation of succinic acid by Propionibacterium pentosaceum. Biotin-deficient cells showed decreased ability to decarboxylate this acid. The addition of biotin restored this activity. Lichstein (1950) also found a connection between biotin and succinic decarboxylase.

METHODS

Preparation of Cells and Juices

Micrococcus lysodeikticus was employed in all but a limited number of experiments. The organism was obtained originally from the laboratories of Dr. Marjory Stephenson of Cambridge University.

The cells were grown in Roux bottles on a solid medium described by Krampitz and Werkman (1941). Incubation time was 72 hours at 37°C. The cells were harvested by washing from the agar surface with distilled water, centrifuging, and re-washing with distilled water. Yields of cells averaged between 1.5 and two grams per Roux bottle. The cells were lyophilized and stored in the refrigerator. Acetone-treated cells, prepared according to Krampitz and Werkman (1941) were used in a few experiments.

Most experiments involved the use of an enzyme preparation obtained by lysing the cells to free the active material. The lysed cells were prepared as follows: lyophilized cells were suspended in distilled water and a solution containing 0.4 milligram of crystalline lysozyme per milliliter of normal saline was added. The concentration of lysozyme was one milligram to 100 milligrams of cells. The material was allowed to react for approximately

ten minutes at room temperature. The cells were well lysed after this length of time, lysis being indicated by changes in color, odor, and consistency. The lysed suspension was then added to the reaction flask, the diluting effect of other constituents tending to inhibit further action by the lysozyme.

In the preparation of lysed cells for use in fixation experiments employing relatively large amounts of cellular material, slightly greater concentrations (1.25 milligrams per 100 milligrams of cells) of lysozyme were used. Best results were obtained by first adding unlysed cells to the flask, adding a solution of lysozyme, and allowing lysis to proceed in the flask before adding other constituents. Large amounts of lysed cells are difficult to transfer because of the gelatinous character of the preparation; consequently, this procedure eliminates errors that might arise in pipetting large amounts of lysed cells.

Escherichia coli E-26 was employed as the source of enzyme in several experiments. The organism was grown on a medium described by Kalnitsky and Werkman (1943). Cell-free extracts were prepared according to Wiggert, et al. (1940).

Propionibacterium pentosaceum PE-14 has been used in a single set of experiments. Specific reference will be made to the growth of the organism and preparation of a cell-free

extract when the experiment is discussed.

Exchange Reactions Involving Heavy Carbon

Many of the experiments involve the fixation of carbon dioxide into oxalacetic acid. Since this acid is too labile to permit direct isolation of the compound after it has been formed, the reaction has been studied by utilizing an exchange reaction first described by Krampitz, et al, (1943). The method consists of incubating oxalacetic acid with heavy carbon sodium bicarbonate and allowing the reaction to proceed until approximately one-half of the oxalacetic acid remains. The reaction is then halted and any label incorporated into the compound is determined, giving a measure of the amount of carbon dioxide assimilated according to the reaction

$$CH_3COCOOH + C^{13}O_2 \rightleftharpoons HOOC^{13}CH_2COCOOH.$$

The reactions were carried out in 250 milliliter two-arm Erlenmeyer flasks which could be connected to Warburg manometers. Oxalacetic acid was neutralized with dilute sodium hydroxide at ice-bath temperature immediately before adding to the flask. Reaction flasks were gassed with nitrogen ten minutes, placed on the bath and equilibrated ten minutes and the oxalacetate added from the side-arm to the main chamber. The reaction proceeded for 20 minutes at which time approximately one-half of the oxalacetate had

been decarboxylated as measured manometrically by the evolution of carbon dioxide.

The reaction flasks were removed from the bath and 0.5 milliliter aliquots were immediately withdrawn for determination of residual oxalacetic acid. These aliquots were placed in small Warburg flasks to which all necessary reagents for aniline-citrate determination of oxalacetic acid had been added. Then four milliliters of ten per cent trichloracetic acid were added to each large reaction flask to stop the reaction and deproteinate the solution which was then centrifuged. Meanwhile, readings for residual oxalacetic acid were taken.

The supernatant from the deproteinated mixture was acidified to congo red with sulfuric acid and placed in an aeration chamber consisting of a pyrex tube equipped with a one-inch aeration stone. Carbon dioxide-free air was passed through the solution for 15 minutes to rid it of residual carbon dioxide. The released carbon dioxide was trapped in a small bead tower or a gas absorption boat containing ten milliliters of 2 N sodium hydroxide.

After this initial aeration, four milliliters of 0.2 N sodium bicarbonate containing no label were added and the aeration continued 15 minutes. The carbon dioxide was again trapped in alkali. This served as a rinse to flush out any remaining residual carbon dioxide.

The reaction mixture was then transferred to a 250 milliliter round bottom flask equipped with three ground glass joints. The flask was fitted with a reflux condenser and a small capillary through which a slight stream of carbon dioxide-free air was passed. The mixture was heated and kept at the boiling point for 30 minutes. This procedure released the carbon dioxide from the methylene carboxyl group of oxalacetic acid, and this was again trapped in alkali. Distilled water free of carbon dioxide was used in making up reagents used in the procedure, and air was passed through an alkali bead tower to avoid as much as possible dilution of the labeled compound with normal carbon. The bicarbonate rinse and sample itself were analyzed for isotope content, the rinse serving as a check so that isotope from the original bicarbonate was not carried over and measured with the sample. No label was ever found in any of the rinses.

The C^{13} content of the samples was measured on a Nier type mass spectrometer. Values in tables are given as atom per cent excess. This represents the difference between isotope content of the sample and a standard as represented by normal bicarbonate. Atom per cent = $\frac{C^{13}/C^{12}}{1 + C^{13}/C^{12}} \times 100.$

Analytical Methods

Succinic acid

The determination of succinic acid was carried out with a succinoxidase preparation made from beef heart according to Krebs (1937).

To prepare the reaction liquid for this determination, it was deproteinated by acidification to congo red with 10 N sulfuric acid, heating on the steam bath for 20 minutes, and centrifugation. The supernatant was continuously extracted with diethyl ether for 48 hours. The ether extract was evaporated to approximately five milliliters and made up to ten milliliters with distilled water. This solution was used for the determination of succinic acid.

For each determination two milliliters of sample were added to a Warburg cup along with 50 milligrams of the succinoxidase preparation in phosphate buffer, pH 7.4, and water to a total volume of 3.3 milliliters. The center well contained alkali for the absorption of carbon dioxide. Oxygen uptake was then determined with each millimole of succinic acid taking up two millimoles of oxygen.

Residual oxalacetic acid

The amount of oxalacetic acid remaining after an exchange reaction had been conducted was determined by the

aniline-citrate method. One milliliter of the solution containing oxalacetic acid, 0.3 milliliter of 50 per cent citric acid, and 0.5 milliliter of distilled water were placed in the main compartment of the Warburg flask. The side arm contained 0.4 milliliter of equal parts of freshly mixed aniline and 50 per cent citric acid. This was added after equilibration. Evolution of carbon dioxide was then measured to determine oxalacetic acid present.

Nitrogen

Cellular nitrogen was determined by the micro-Kjeldahl method of Pregl as modified by Johns (1941).

Microbiological Methods

Biotin assay

The method employed in the assay for biotin was that of Roberts and Snell (1946). Assays were carried out as outlined in their procedure with a standard curve being run each time with levels at 0.0, 0.0001, 0.0003, 0.0005, 0.0007, and 0.001 microgram of biotin. An uninoculated tube of medium served as a control. Materials to be assayed for biotin content were checked at a minimum of three levels at relative dilutions of one, ten, and 100, depending on the

biotin potency of the material.

Both the 72 hour titrimetric and the 16 hour turbidimetric methods were used and gave satisfactory results. The accuracy of the two methods is not greatly different, although the type of curve obtained is not the same. The titrimetric method is slightly more reproducible and is not affected by small differences in inoculum. Typical curves, in which 16 levels of biotin were used, are pictured in Figure 1. The lactic acid was titrated with 0.15 N sodium hydroxide with brom thymol blue as indicator. The turbidity of the cultures was read on a Klett-Summerson colorimeter using a 660 mμ filter.

The assay organism was Lactobacillus arabinosus 17-5. It was carried on a stock medium described by Krueger and Peterson (1948b).

Biotin was freed from combination by acid hydrolysis before carrying out an assay. For most cellular material this was accomplished by adding six milliliters of solution or suspension, containing approximately 0.2 gram dry weight of material, to six milliliters of 4 N sulfuric acid. In the case of purified enzyme much smaller amounts were used. The preparation was autoclaved two hours at 121°C and, after cooling, neutralized with an equivalent amount of barium hydroxide. The solution was then centrifuged to remove most of the barium salt and the cloudy solution resulting was

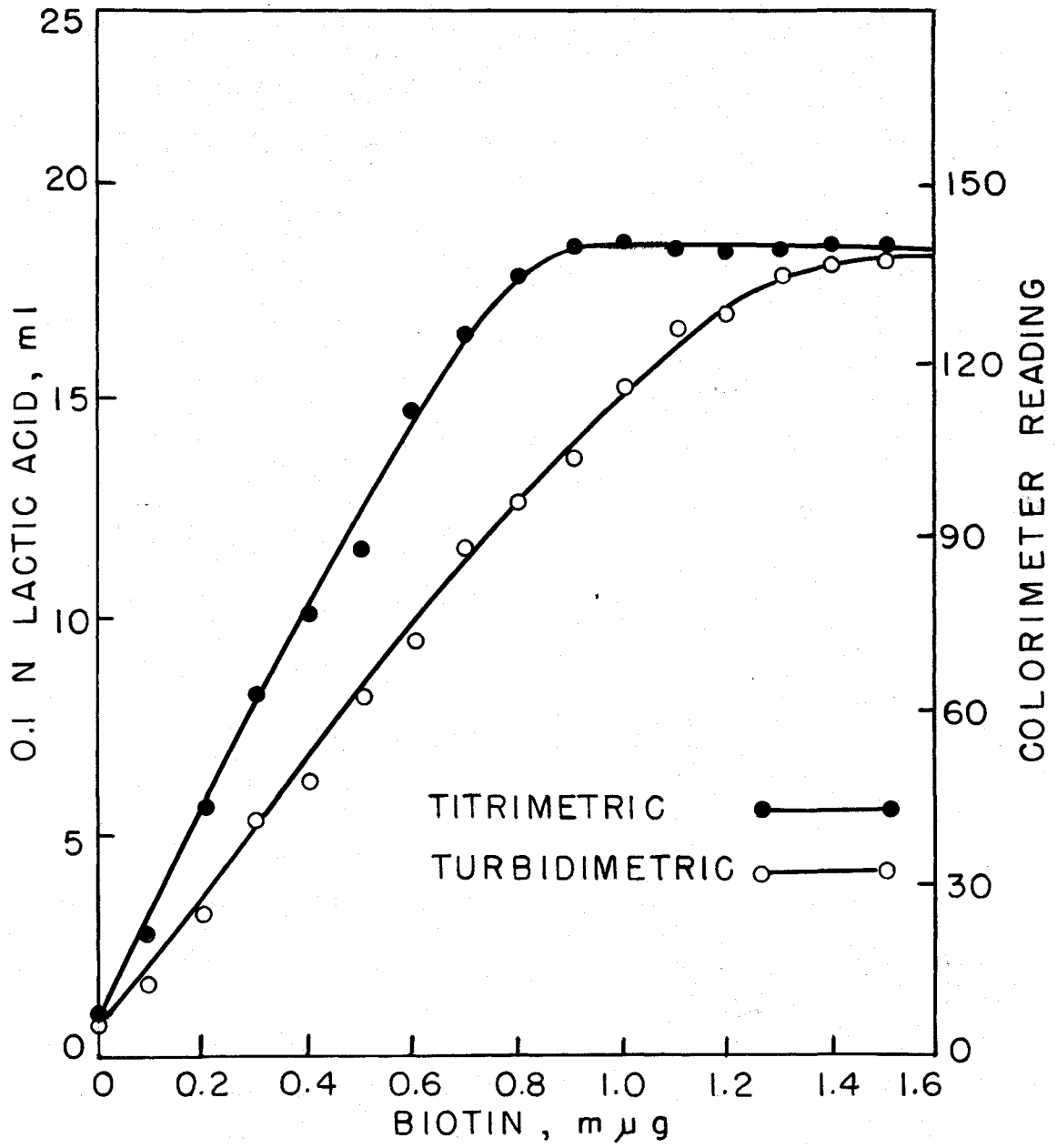


Figure 1. Standard Curves for Biotin.

filtered through No. 42 Whatman filter paper. The clear solution was then assayed for biotin.

Determination of aspartic acid

Aspartic acid was determined by the microbiological assay method of Steele, et al, (1949) using Leuconostoc mesenteroides P-60. After deproteinization by the sodium tungstate-sulfuric acid method outlined by Schurr, et al, (1950) assays were carried out in pyrex test tubes fitted with aluminum caps. After incubation at 37°C for 72 hours the lactic acid formed was titrated with 0.02 N sodium hydroxide using brom thymol blue as an indicator. Assays were run in duplicate.

Preparation of Materials

Concentration of avidin

Avidin was concentrated from egg white by slight modification of a procedure first developed by Eakin, et al, (1941).

The whites of ten dozen fresh eggs or an equal amount of frozen egg white served as starting material. This amounted to approximately three liters of egg white.

Two volumes of acetone were added and the mixture was stirred until the proteins had completely coagulated. The

material was filtered through cheese cloth and squeezed dry by hand. The acetone solution was refiltered through Filter-Cel for the recovery of additional protein. The filter pad and precipitate were added to the coagulum. The coagulum was broken up and suspended for one hour in one liter of distilled water. The coagulum was separated from the wash water as described for the acetone solution. Neither the wash water nor acetone solution contained biotin inactivating activity.

The washed coagulum was suspended for several hours in one liter of a two per cent solution of ammonium sulfate. The salt solution was then filtered as previously described. The extraction was repeated with an additional liter of ammonium sulfate solution. The filtrates were combined and contained the biotin inactivating activity.

Six liters of acetone were mixed with the filtrate from the salt extraction, giving a turbid solution as the protein precipitated. After 20 minutes, 20 grams of Filter-Cel were added and the suspension was filtered with suction. The solution was re-filtered through the same filter pad until clear. Usually the addition of a few drops of ten per cent acetic acid was necessary to coagulate the precipitated proteins in order that a clear filtrate would result.

The filter pad containing the precipitated proteins was suspended in 400 milliliters of a two per cent solution

of ammonium sulfate. Any sticky lumps of coagulated proteins were worked with a spatula until completely dissolved. The Filter-Cel was filtered off and discarded.

Fifty grams of ammonium sulfate were added to each 100 milliliters of solution and the solution stirred until the salt was dissolved. Small amounts of salt were added as rapidly as they would dissolve. With the increase in salt concentration the solution became opalescent, then various fractions of protein coagulated at the top of the solution. The coagulum which formed was colored, ranging from light pink to greenish-yellow, and was skimmed off with a spatula. When the solution was saturated with ammonium sulfate, the active fraction was almost completely precipitated. It did not coagulate, but remained suspended and was filtered off with the aid of Filter-Cel. The active protein and excess salt were dissolved in 20 milliliters of distilled water and freed from the Filter-Cel by filtration.

The active protein solution was freed from the salt by dialysis in sausage casing against running tap water for 24 hours. Four volumes of acetone were added to the dialyzed solution and the precipitated protein centrifuged off and dried in a vacuum. The active material was stored in a desiccator and retained activity for long periods of time.

Activities were determined as the amount needed to inactivate one part of biotin. Activities ranged from 500 to

1000, with the average approximately 800.

The activity tests were made by a slight modification of the biotin assay method of Roberts and Snell (1946). Known amounts of biotin were added to the assay tubes and the amounts of avidin varied. The point at which biotin was completely inactivated was determined by noting the lack of growth of the test organism, L. arabinosus. Table 1 shows a typical assay of this type.

Oxalacetic acid

Oxalacetic acid was prepared from sodium diethyloxalacetate by a modification of a method described by Krampitz and Werkman (1941). To 100 grams of the salt in 250 milliliters of distilled water an equivalent amount of concentrated sulfuric acid was added. The free ester was removed as an oily layer by means of a separatory funnel. The ester was extracted three times with ether, the total amount being 100 millliliters. The ether extracts were washed with 25 millliliters of distilled water and a small amount of anhydrous sodium sulfate was added to dry the ether extracts. After filtration, the solution was subjected to vacuum distillation and the ester, having a boiling point of 131-132°C, collected. The ester plus four volumes of concentrated hydrochloric acid, was vigorously shaken for two hours on a mechanical shaker. The solution was then placed

Table 1

Inactivation of Crystalline Biotin by Avidin

Biotin, µg	Avidin, µg	Growth Response *Colorimeter Reading	
Expt. 1			
0.001	0.0	**72.0	***70.0
0.001	0.1	64.0	60.0
0.001	0.5	42.0	33.0
0.001	1.0	9.0	7.0
0.001	10.0	4.0	3.0
0.001	50.0	5.5	2.0
0.001	100.0	5.0	2.0
0.001	200.0	4.0	3.0
Expt. 2			
0.001	0.0	73.0	76.0
0.001	0.1	65.0	64.0
0.001	0.25	55.0	59.0
0.001	0.50	43.0	41.0
0.001	0.75	8.0	7.0
0.001	1.0	7.0	6.0
0.001	10.0	5.0	4.0
0.001	50.0	6.0	6.0

*Klett-Summerson readings at 660 mµ.

**No incubation of avidin and biotin before inoculation.

***Incubation of avidin and biotin for one hour at 0°C before inoculation.

in a deep freeze for at least a day and the precipitated oxalacetic acid was filtered off at a low temperature using a sintered glass filter. Further purification of the acid was obtained by dissolving in a minimum amount of warm acetone and recrystallizing by cooling and the addition of chloroform.

Purity of the preparation, as determined by the aniline-citrate method, was about 90 per cent.

Sodium pyruvate

Crystalline sodium pyruvate was prepared from commercial pyruvic acid by the method of Robertson (1942). Purity was determined by oxidation with ceric sulfate according to Krebs and Johnson (1937).

Alumina C_γ

Alumina C_γ was prepared as described by Bertho and Grassmann (1938).

EXPERIMENTAL

The Biotin Requirement of *Micrococcus lysodeikticus*

At the time of this investigation the biotin requirement of *Micrococcus lysodeikticus* had not been clearly elucidated. Feiner, *et al*, (1946) found that biotin did not stimulate growth of the organism when the vitamin was added to a basal medium of casein hydrolyzate, cystine, tryptophan, adenine and inorganic salts. Allen (1951), however, conducted a series of studies on the nutrition of *M. lysodeikticus* and noted definite response of the organism to the addition of biotin.

The evidence presented here shows biotin to be markedly stimulatory to the growth of *M. lysodeikticus* on a synthetic medium. The organism, however, produces some growth even when the vitamin is excluded from the medium.

The method consisted of growing the organism in pyrex test tubes and measuring the response of the organism to biotin and compounds which have been reported to replace biotin in bacterial nutrition. The tubes were covered with aluminum caps to facilitate handling. Each tube contained a total of five milliliters of medium. The medium was varied by making additions to four milliliters of basal medium.

The basal medium was autoclaved at 15 pounds for ten minutes and stored under toluene in the refrigerator. Aliquots were withdrawn aseptically when needed.

The basal medium was comprised of the following:

	mg		mg
DL-alanine	100	NH_4Cl	500
L-arginine HCl	100	$MgSO_4 \cdot 7H_2O$	100
L-cysteine HCl	25	$FeSO_4 \cdot 7H_2O$	5
glycine	50	$MnSO_4 \cdot 4H_2O$	10
L-glutamic acid	100	NaCl	5
L-histidine HCl	50	K_2HPO_4	300
DL-isoleucine	100	KH_2PO_4	300
L-leucine	100		
L-lysine HCl	100		µg
DL-methionine	50		
DL-phenylalanine	50	p-amino benzoic acid	50
L-proline	100	calcium panto thenate	250
DL-serine	25	nicotinic acid	250
DL-tryptophan	25	pyridoxine HCl	500
L-tyrosine	100	riboflavin	250
DL-valine	100	thiamine HCl	250
adenine sulfate	5	folic acid	1
guanine HCl	5	distilled water to 400 ml	
uracil	5	pH adjusted to 7.0	
glucose	1500		

Inoculation of the assay medium was made as follows: a 24-hour culture of M. lysodeiktieus on a nutrient agar slant was washed into ten milliliters sterile normal saline, centrifuged, and again washed with saline. After centrifuging, the cells were resuspended in 10 milliliters saline and diluted one to ten. A single drop of this suspension added from a standardized pipette was used to inoculate each tube.

The tubes were incubated at 37°C for 72 hours. They were then thoroughly shaken to suspend the sedimented cells

and turbidity determined on the Klett-Summerson photoelectric colorimeter. Incubation without shaking results in only slight growth. Therefore, the tubes were shaken during incubation by means of a reciprocal shaker operating at approximately 125 three-inch strokes per minute.

Solutions of crystalline d-biotin in distilled water were used in the assays. Aspartic acid was neutralized before adding to the medium. Sodium oleate solutions were made up as follows: 100 milligrams of oleic acid were dissolved in four milliliters 95 per cent ethyl alcohol, titrated with 0.9 N NaOH and made to five milliliters with alcohol. The solution was diluted 1-100 with distilled water for use as a stock solution. Growth experiments indicated alcohol in the concentrations used had no effect upon M. lysodeikticus.

The results of typical assays are shown in Tables 2 and 3. The results of growth without shaking are included for comparison.

Response to a particular level of added material varied considerably from test to test. This has been found to be typical by other investigators working with M. lysodeikticus. Various factors, perhaps physical in nature, which are not well understood, appear to be responsible. Nevertheless, the data clearly demonstrate the stimulatory nature of biotin, even though the organism can produce fair growth in

Table 2

Biotin Requirement of Micrococcus lysodeikticus

Biotin, µg	Additions to Basal Medium								
	None			Na Oleate, 0.1 mg		Na Aspartate, 1 mg		Na Oleate, 0.1 mg Na Aspartate, 1 mg	
	*Colorimeter Readings								
0.00	**8	20	49	154	178	28	47	225	323
0.0001	12	21	57	178	180	30	100	224	310
0.005	12	30	85	258	221	56	150	236	305
0.001	13	42	116	325	270	98	201	230	305
0.005	20	63	178	325	318	140	243	241	321
0.01	22	79	190	325	320	157	282	210	310
0.05	22	111	180	307	290	344	281	250	299
0.1	24	141	247	330	331	324	286	243	331
0.5	30	169	279	321	327	328	307	261	337
1.0	37	306	289	316	298	294	302	255	296

*Klett-Summerson readings at 660 mµ.

**Values for growth of organism without shaking.

Table 3

Effect of Aspartic Acid on the Biotin Requirement of Micrococcus lysodeiktious

Na Aspartate, mg	Additions to Basal Medium			
	None		Biotin 0.0001 µg	
	*Colorimeter Reading			
0.00	22	38	25	50
0.001	24	38	25	56
0.005	29	44	31	61
0.01	28	39	34	61
0.05	34	40	39	69
0.1	29	42	47	80
0.5	38	54	55	93
1.0	43	63	50	109
2.0	40	60	48	100
3.0	44	69	53	110

*Klett-Summerson readings at 660 mµ.

the absence of the vitamin.

The effect of aspartic acid and of oleic acid, both of which can partially replace biotin in the nutrition of such organisms as Lactobacillus casei and Lactobacillus arabinosus is not as definite as the effect of biotin. Aspartic acid replaces biotin to an extent but the effect is not as pronounced as in the case of many organisms.

Oleic acid exerts a more pronounced effect than in the case of the lactic acid bacteria. Good growth occurs when sodium oleate is added to the medium, even when biotin is omitted. Oleic acid and aspartic acid, when added together, replace biotin completely.

Biotin and Carbon Dioxide Fixation in Oxalacetic Acid

Effect upon succinic acid formation

The first attempt to relate biotin and fixation of carbon dioxide was made by studying the effect of the vitamin on the formation of succinic acid during the anaerobic dissimilation of pyruvic acid by cell-free extracts of Escherichia coli. The growth of the organism and the preparation of cell-free extracts have been described in the section on methods. The preparations were frozen by immersion in a mixture of dry ice and acetone and stored in the deep-freeze for short periods.

The activity of the cell-free extracts was determined manometrically by measuring the amount of carbon dioxide evolved anaerobically from 0.1 millimole pyruvic acid in 20 minutes by 0.8 milliliter of the preparation.

Data were obtained for three sets of conditions. First, a control was provided in which the reaction proceeded in normal fashion with no change in the biotin available to the enzyme. Secondly, avidin was added to bind the biotin already present, thus at least partially denying the vitamin to the enzyme. Thirdly, avidin was again added but, in addition, biotin was supplied to compensate for that tied up by avidin. This arrangement of conditions showed any effect on the activity of the enzyme to be due to the binding of biotin and not to a different type of inhibition by the avidin.

The cell-free extracts were assayed for biotin content in order that the theoretical amount of avidin needed to bind the vitamin could be determined.

Reactions were conducted in 125 milliliter Erlenmeyer flasks equipped with double side arms. Sodium pyruvate was placed in one side arm, solutions of biotin in the other. These compounds were added to the other constituents in the main compartment of the flask after the equilibration period. The reactions were carried out with shaking in a Warburg bath at 30.4°C. At the end of the particular incubation

time chosen, the samples were immediately deproteinated by the addition of one-sixth volume of ten per cent trichloroacetic acid and centrifuged. The amount of succinic acid in the supernatant was then determined.

The results of four experiments are presented in Table 4. The amount of succinic acid formed is a function of the amount of carbon dioxide fixed in oxalacetic acid according to the system pyruvic acid + carbon dioxide $\rightleftharpoons$ oxalacetic acid $\rightleftharpoons$ malic acid $\rightleftharpoons$ fumaric acid $\rightleftharpoons$ succinic acid. Thus, differences in the amount of succinic acid formed will indicate differences in the amount of carbon dioxide fixed. Although the total amounts of acid formed were not large, differences in total formed were definite and regular. The effects of avidin and biotin can readily be seen. In every case the addition of avidin inhibited succinic acid formation. The addition of biotin brought the value back to normal. Since the amount of succinic acid formed depends on the amount of biotin available, and the amount of succinic acid formed also depends on the amount of carbon dioxide fixed, the data indicate a role for biotin in the Wood-Werkman reaction.

Incubation of avidin with the cell-free extract for long periods prior to the experiment had no additional effect upon the reaction. No greater inhibition of the formation of succinic acid was noted when avidin was added

Table 4

Effect of Biotin on Formation of Succinic Acid

Expt.	Additions	Cell-Free Extract Added: Activity, µl CO_2 per 20 min.	Cell-Free Extract Added: Biotin Content, µg per ml	Cell-Free Extract Added: Amount, ml	Succinic Acid Formed, mM
1	None	169	0.0250	4.0	0.026
	Avidin, 0.04 mg				0.021
2	None	259	0.0175	8.0	0.085
	Avidin, 0.075 mg				0.058
	Avidin, 0.075 mg; biotin, 0.14 µg				0.079
	Avidin, 0.075 mg; biotin, 0.60 µg				0.085
3	None	224	0.0205	4.0	0.104
	Avidin, 0.05 mg				0.063
	Avidin, 0.05 mg; biotin, 10 µg				0.117
4	None	260	0.0210	8.0	0.098
	*Avidin, 0.1 mg				0.047
	*Avidin, 0.1 mg; biotin 0.2 µg				0.099

*Avidin incubated with cell-free extract 13 hours at 12°C before experiment.

Each flask contained 1.0 mM pyruvate, 5.0 ml buffer, pH 6.8 (equal parts 0.1 M phosphate and 0.1 M $NaHCO_3$ saturated with CO_2), plus additions, and distilled water to volume. Temperature = 30.4°C.

Expt. 1. Volume = 20 ml, time = 120 minutes, atmosphere = H_2.
Expt. 2. Volume = 25 ml, time = 240 minutes, atmosphere = H_2.
Expt. 3. Volume = 20 ml, time = 180 minutes, atmosphere = 10% CO_2, 90% H_2.
Expt. 4. Volume = 25 ml, time = 240 minutes, atmosphere = 10% CO_2, 90% H_2.

13 hours before the enzyme was added to the substrate than when the period of incubation covered only the time of equilibration. This indicates that biotin is firmly bound by avidin within a relatively short time.

Utilization of avidin as an inhibitor in the Wood-Werkman reaction

Since previous work indicated that biotin may function in the fixation of carbon dioxide in oxalacetic acid, experiments were undertaken to study the relationship of the vitamin to this specific reaction. The experimental approach was similar to that used in the work on the effect of biotin on succinic acid formation in that avidin was again employed as an inhibitor.

Tracer experiments were set up utilizing C^{13} in the exchange reaction involving carbon dioxide and oxalacetic acid. The enzyme preparation was a lysed suspension of M. lysodeiktieus. Avidin was added to inactivate biotin present in the lysed preparation. The effect upon carbon dioxide fixation of the binding of available biotin was then determined by measuring differences in the amount of C^{13} fixed in oxalacetic acid. In a few experiments, egg white, a good source of avidin, was added instead of the purified compound.

The amount of avidin or egg white necessary to inactivate the biotin present in the cell preparation under experimental conditions was first determined. This involved a modification of the assay method for biotin. Lysed cells were added to the basal medium to serve as the source of biotin. Various concentrations of avidin or egg white were then added to the series of assay tubes.

The tubes were inoculated with L. arabinosus and incubated as in the usual assay for biotin. The amount of growth was determined by measuring turbidity or titrating the acid formed. Controls were especially necessary in the turbidimetric assays where the addition of lysed cells as a source of biotin would account for some turbidity in the assay tubes. The amount of avidin or egg white necessary to inhibit growth was then calculated. Tables 5 and 6 represent typical inactivation assays.

The calculated amount of avidin or egg white was added to the reaction mixture. Exchange reactions were conducted as previously described. Additions of crystalline biotin and the results of such experiments are indicated in Table 7. Equivalent biotin refers to biotin added to compensate for that inactivated by avidin or egg white; excess biotin indicates that the vitamin has been supplied considerably in excess of the amount bound. Biotin was added from the sidearm of the reaction flask after equilibration and incubation

Table 5

Inactivation of Biotin by Egg White

Cells, mg	Egg White, mg	Growth Response	
		*Colorimeter Reading	0.1 N Lactic Acid, ml
1.0	0.00	27.0	15.39
1.0	0.02	22.0	14.48
1.0	0.10	16.0	11.41
1.0	0.20	9.0	8.51
1.0	0.40	6.0	4.69
1.0	0.60	6.0	4.40

* Klett-Summerson readings at 660 mμ.

Solution of lysed M. lysodeiktieus as source of biotin.

Table 6

Inactivation of Biotin by Avidin

Cells, mg	Avidin, µg	Growth Response *Colorimeter Reading
1.0	0.0	37.0
1.0	0.1	33.0
1.0	0.5	19.5
1.0	1.0	11.5
1.0	10.0	11.0
1.0	50.0	10.5
1.0	100.0	9.0
1.0	200.0	10.0

*Klett-Summerson readings at 660 mµ.

Solution of lysed M. lysodeikticus as source of biotin.

Table 7

Effect of Biotin on the Fixation of $C^{13}O_2$ in Oxalacetic Acid

Experiment	1		2		3	
	*C^{13} Fixed	Oxal-acetate Remaining, mM	*C^{13} Fixed	Oxal-acetate Remaining, mM	*C^{13} Fixed	Oxal-acetate Remaining, mM
Normal exchange	0.14	0.648	0.17	0.681	0.42	0.702
Egg white added, 45 mg	0.02	0.678			0.11	0.752
Egg white, 45 mg + equivalent biotin, 0.2 µg	0.12	0.651			0.22	0.755
Avidin added, 0.3 mg			0.03	0.717		
Avidin, 0.3 mg + equivalent biotin, 0.2 µg			0.15	0.714		
Avidin, 0.3 mg + excess biotin, 0.5 µg			0.16	0.696		

*Values are given as atom per cent excess.

Each flask contained 1.50 mM oxalacetate, 0.75 mM phosphate buffer, pH 6.8 1.59 mM $NaHC^{13}O_3$ (5.13 atom per cent excess C^{13}), 300 mg lysed or acetone-treated cells, and distilled water to 30 ml. Time = 20 minutes. Atmosphere = N_2. Temperature = 30.4°C.

of avidin and cellular material.

The results show a marked decrease in the amount of $C^{13}O_2$ incorporated in oxalacetic acid when either avidin or egg white is added. In the first experiment the standard representing the amount of C^{13} in normal carbon dioxide was found to be 1.10 atom per cent. In a normal exchange in which neither avidin nor biotin has been added, the carbon dioxide from the β-carboxyl of oxalacetic acid contained 1.24 atom per cent C^{13} or 0.14 atom per cent excess. This shows a significant amount of carbon dioxide exchange and indicates fixation into oxalacetic acid. This value corresponds to values obtained by Krampitz, et al, (1943) under similar conditions.

When egg white was added to bind the biotin, the value for C^{13} content fell to 1.12 atom per cent or only 0.02 atom per cent above the value for the control. This amount is not significant as either experimental error or the sensitivity of the mass spectrometer could account for this small difference. The addition of biotin to relieve this inhibition resulted in a return to the value shown in a normal exchange. The amount of C^{13} found was 1.22, or 0.12 atom per cent excess, a difference of only 0.02 atom per cent from that obtained when neither inhibitor nor biotin was added.

Similar results were obtained in experiment No. 2 when purified avidin was used instead of egg white. Thus in

experiments of this type the addition of an inhibitor to bind biotin, thus making it unavailable, results in a 90 to 100 per cent decrease in fixation of carbon dioxide, an inhibition which is overcome when biotin is also added. The addition of biotin considerably in excess of that needed to compensate for the amount bound by the inhibitor does not result in larger amounts of carbon dioxide fixed.

These experiments were conducted with synthetic oxalacetic acid. In experiment No. 3 acetone-treated cells acted upon so-called "physiological oxalacetate" (Krampitz, et al, 1943) arising from the oxidation of fumaric acid. The amount of $C^{13}O_2$ is always higher under such conditions. Apparently the oxalacetic acid that is produced from the oxidation of fumaric acid differs in some way from that synthesized in the laboratory, and the "physiological oxalacetate" greatly facilitates the exchange reaction. The results, however, are similar to those obtained with synthetic oxalacetic acid.

The decrease in fixation upon addition of an inhibitor was not as complete as in the experiments employing lysed cells. The restoration of activity upon the addition of biotin was approximately 50 per cent, again not as complete. In all probability permeability factors entered the picture in cases where acetone-treated cells were used. These problems are largely avoided when lysed preparations are used.

The amount of oxalacetic acid remaining when the experiments were stopped after 20 minutes has been determined by means of the aniline-citrate method. The results show that approximately one-half of the oxalacetic acid remained in each case.

Since avidin, a specific inhibitor of biotin, reduces carbon dioxide fixation by a cell-free preparation of M. lysodeikticus and biotin removes this inhibition, the evidence indicates that biotin functions in the Wood-Werkman reaction. This has been reported by Wessman and Werkman (1950).

Oxalacetic Decarboxylase

Purification of the enzyme

Since biotin was shown to be involved in the carboxylation of pyruvic acid to form oxalacetic acid an attempt was made to effect some purification of the enzyme involved and thereby further study the relationship of the vitamin to the reaction. The enzyme oxalacetic decarboxylase, first discovered by Krampitz and Werkman (1941), was believed to catalyze the carboxylation of pyruvic acid as well as the decarboxylation of oxalacetic acid.

Several workers have partially purified the enzyme. Plaut and Lardy (1949) reported on the enzyme obtained from

Azotobacter vinelandii. Vennesland, et al. (1949) concentrated the enzyme found in parsley root. Byerrum, et al. (1950) crystallized a similar enzyme from seeds of squash, Cucurbita pepo. Herbert (1950) reported a high degree of purification of the enzyme from M. lysodeikticus but did not reveal the details of the purification process.

Fractionation of the enzyme by a series of changes in pH, followed by adsorption of the active principle on cupric hydroxide and elution with phosphate, was first tried. Lysed cells of M. lysodeikticus suspended in water were used as the source of enzyme. Changes in pH were made by slowly adding 0.1 N hydrochloric acid. Although considerable enhancement of activity was obtained with this method, the cupric hydroxide gel was difficult to handle and did not prove to be a good adsorption agent. Furthermore, fractionation by means of precipitation of protein with changes in pH tends to irreversibly denature considerable quantities of protein. It was shown, however, that the greatest amount of active material precipitates between pH 4.7 and 5.3 (Table 8). Therefore, a precipitation of inactive protein with acid was incorporated into the final purification scheme.

The activity of the enzyme was calculated as microliters of carbon dioxide evolved from oxalacetic acid in 20 minutes. The tests were conducted in Warburg flasks

Table 8

Oxalacetic Decarboxylase Activity of Acid Precipitated Fractions

pH	Nitrogen in Sample, mg	CO_2 Evolved, µl per 20 min.	Activity, µl CO_2 per mg Nitrogen
6.5	0.018	4	222
6.0	0.117	25	214
5.3	0.182	112	615
5.0	0.202	141	698
4.7	0.209	390	1860
Supernatant at 4.7	0.072	30	417

Each flask contained 0.02 mM oxalacetate, 0.25 mM phosphate buffer, pH 6.8, 0.002 mM $MnSO_4$, 0.3 ml of 12 N H_2SO_4 in side arm, enzyme fraction as indicated and distilled water to 2.3 ml. Time = 20 minutes. Atmosphere = N_2. Temperature = 30.4°C.

under an atmosphere of nitrogen. In addition to 0.02 millimoles of oxalacetate in one side arm, each flask contained phosphate buffer at pH 6.8, manganese ions, enzyme, sulfuric acid in the other side arm, and distilled water. After equilibration, the oxalacetate was tipped into the main chamber, and carbon dioxide evolution measured for 20 minutes. At this time the sulfuric acid was added to the main reactants in order to stop the reaction and release any bound carbon dioxide.

Fractionation of the material containing the enzyme by means of precipitation with ammonium sulfate was carried out. Various amounts of ammonium sulfate were added to a solution containing the enzyme. The fractions which precipitated were collected and the oxalacetic decarboxylase activity of each determined. Table 9 shows the results obtained by this method. The fraction precipitating between 38 and 40.8 per cent saturation was the most active. This fraction was chosen for further purification.

After dialysis and treatment with acid to rid the preparation of as much inactive material as possible, further purification was effected by means of adsorption on alumina C_γ. Treatment of the preparation with a small amount of alumina C_γ, followed by treatment with a larger amount, resulted in great purification. The bulk of the activity was found in the second fraction. Third or fourth

Table 9

Oxalacetic Decarboxylase Activity of Ammonium Sulfate Precipitated Fractions

Per Cent Saturation with Ammonium Sulfate	Nitrogen in Sample, mg	CO_2 Evolved, µl per 20 min.	Activity, µl CO_2 per mg Nitrogen
16.5	0.069	13	188
26.5	0.062	20	323
33.4	0.084	77	917
36.4	0.099	99	1000
38.0	0.088	252	2864
40.8	0.050	287	5740
45.0	0.070	64	914
50.0	0.095	50	526
75.0	0.092	47	511
Cell, lysed, 10 mg	1.048	314	299

Each flask contained 0.02 mM oxalacetate, 0.25 mM phosphate buffer, pH 6.8, 0.002 mM $MnSO_4$, 0.3 ml of 12 N H_2SO_4 in side arm, enzyme fraction as indicated and distilled water to 2.3 ml. Time = 20 min. Atmosphere = N_2. Temperature = 30.4°C.

adsorptions contained little active enzyme. Elution with phosphate at a neutral pH recovered the activity from the alumina gel. A single elution was satisfactory; additional treatment did not recover further activity.

In final form the procedure for purification of the enzyme was as follows: ten grams of lyophilized cells of M. lysodeikticus were suspended in 200 milliliters of distilled water. Fifty milliliters of distilled water containing ten milligrams of lysozyme were added. The mixture was stirred mechanically for 60 minutes at 30°C at a rate just sufficient to keep the cells from settling out.

This operation was followed by stirring in an ice-bath at a temperature of 1-4°C for 90 minutes. All subsequent steps, including centrifugation, were carried out at this temperature. The material was then spun down in a refrigerated centrifuge operating at 2000 RPM for 60 minutes. Although the residue contained about 60 per cent of the decarboxylase activity, it was discarded because of difficulty in further separating the active principle from the particulate matter.

Solid ammonium sulfate was added slowly until the supernatant liquid obtained in the previous centrifugation was 75 per cent saturated and practically all the protein precipitated. This operation was carried out in an ice-bath with the solution being stirred constantly at a rate slow

enough to prevent frothing. The material was centrifuged at 2000 RPM for 60 minutes to separate the precipitated protein from the ammonium sulfate solution.

The residue from this centrifugation was dissolved in 75 milliliters of 0.1 M phosphate buffer, pH 6.8. Any debris which did not go into solution was centrifuged off at 10,000 RPM for 20 minutes and discarded. The previous centrifugations were somewhat difficult due to the large amount of material involved. It seemed highly desirable, therefore, to carry out this last centrifugation, since it involved smaller amounts of material and a higher speed, and thus, was more effective in freeing the solution of bits of cellular material which may have been carried along.

Solid ammonium sulfate was added to the supernatant liquid until 30 per cent saturation was reached. The solid reagent was used instead of a solution in order to keep the volume as small as possible. Beyond 30 per cent saturation, however, a saturated solution of ammonium sulfate was used in order to prevent the development of local areas of high saturation as the region was approached wherein the active enzyme would precipitate. When the degree of saturation reached 38 per cent the precipitated protein was centrifuged off at 10,000 RPM for 20 minutes. The supernatant solution was further fractionated with saturated ammonium sulfate until the degree of saturation reached 40.8 per cent. The

portion precipitating between 38 and 40.8 per cent was centrifuged off, again at 10,000 RPM for 20 minutes, and the supernatant material discarded.

The precipitated protein was dissolved in 20 milliliters of 0.1 M phosphate buffer, pH 6.8. The material was placed in sausage casing and dialyzed against four liters of distilled water. The water was changed three times during the dialysis. The operation was carried out in a refrigerator in order to keep the temperature from rising above 4°C. Total time of dialysis was nine hours, a period of sufficient duration so that any salts which might interfere in the alumina gel adsorption were removed. Dialysis also rids the preparation of interfering activities such as pyruvic decarboxylase.

The dialyzed fraction was adjusted to pH 5.1 by the addition of 0.1 N hydrochloric acid. The resulting precipitate was centrifuged off at 10,000 RPM for 20 minutes. The precipitate was discarded as it contained largely inactive protein. For each ten milliliters of supernatant liquid, 0.1 milliliter of alumina C_γ was added. The mixture was stirred slowly in an ice-bath for 30 minutes and then centrifuged. The operation was repeated on the resulting supernatant, except that one milliliter of alumina C_γ per ten milliliters of liquid was used. The step adsorbed the active fraction. Elution was carried out by stirring the

enzyme adsorbed on alumina C_δ with 20 milliliters of 0.1 M phosphate buffer, pH 6.8. The material was centrifuged and the supernatant retained. All centrifugation steps during the adsorption and elution procedures were carried out at 10,000 RPM for 20 minutes. The first alumina C_γ adsorption fraction and the supernatant liquid obtained after the second adsorption contained insignificant amounts of activity.

The active principle, dissolved in phosphate, was frozen and stored in the deep freeze. It retained practically full activity for at least a week. Total activity recovered during the procedure is only about ten per cent. At least a 100-fold increase in activity is obtained, however. The procedure also eliminates a number of other enzymes which might interfere in the determination of oxalacetic decarboxylase activity. Significant data involving the enzyme in various stages of purification are given in Table 10.

Characteristics of oxalacetic decarboxylase

A number of characteristics of the enzyme have been established in the past with preparations of less purity. Several of these characteristics were re-checked with the enzyme obtained in the procedure described. In most cases, comparable results were obtained and agree in most respects

Table 10

Enzymatic Activity of Various Fractions Obtained during the Purification of Oxalacetic Decarboxylase

Fraction	Total Nitrogen, mg	Nitrogen in Sample, mg	Carbon Dioxide Evolved, µl per 20 min.	Activity, µl CO_2 per mg Nitrogen	Per Cent Recovery
Lyophilized cells, 10 gm	1004.00	0.502	125	249	100
Lysing residue	891.46	0.217	37	171	60.98
Lysing supernatant	75.63	0.080	85	1062	32.13
75 per cent $(NH_4)_2SO_4$ saturation	71.08	0.105	116	1105	31.42
38 per cent $(NH_4)_2SO_4$ saturation	35.39	0.100	63	630	8.92
Supernatant from 38 per cent $(NH_4)_2SO_4$ saturation	31.56	0.091	144	1582	19.97
40.8 per cent $(NH_4)_2SO_4$ saturation	6.40	0.076	385	5066	12.98
Supernatant from 40.8 per cent $(NH_4)_2SO_4$ saturation	18.99	0.043	38	883	6.71
Precipitated at pH 5.1	1.84	0.067	60	896	0.66
First alumina C_γ adsorption	0.61	0.018	84	4667	1.13
Second alumina C_γ adsorption	0.99	0.016	409	25562	10.13
Supernatant from second alumina C_γ adsorption	2.26	0.041	38	927	0.81

For activity tests each flask contained 0.02 mM oxalacetate, 0.25 mM phosphate, pH 6.8, 0.002 mM $MnSO_4$, 0.3 ml of 12 N H_2SO_4 in side arm, enzyme fraction as indicated, and distilled water to 2.3 ml. Time = 20 minutes. Atmosphere = N_2. Temperature = 30.4°C.

with data secured with enzymes from other sources.

The effect of inorganic ions upon enzyme activity was studied. The enzyme is completely inactive in the absence of inorganic ions, preferably divalent ions. The activator of choice is Mn^{++}, although Mg^{++}also gives good results. The activity obtained in the presence of Co^{++} is approximately three-fourths that obtained with Mn^{++}. The effect of these ions upon enzyme activity is shown in Figure 2. These results are similar to results obtained by Krampitz and Werkman (1941) who first showed that Mg^{++} and Mn^{++} were activators for the enzyme found in M. lysodeiktieus. In the case of the oxalacetic decarboxylase of A. vinelandii, however, Plaut and Lardy (1949) could demonstrate only partial activation on the addition of Mg^{++}, although the enzyme was fully active in the presence of Mn^{++}, Co^{++}, or Zn^{++}. Speck (1949) studied in considerable detail the effect of various ions on the enzymatic decarboxylation of oxalacetic acid by a preparation obtained from parsley root. A number of divalent ions, particularly Mn^{++}, Mg^{++}, Ca^{++}, Ni^{++}, and Zn^{++} were effective, but trivalent ions failed to give significant activation.

Plaut and Lardy (1949) reported a 15-fold increase in activity of the enzyme from A. vinelandii when succinate-borate buffer was substituted for phosphate buffer. The authors believe that the enzyme is inhibited by phosphate,

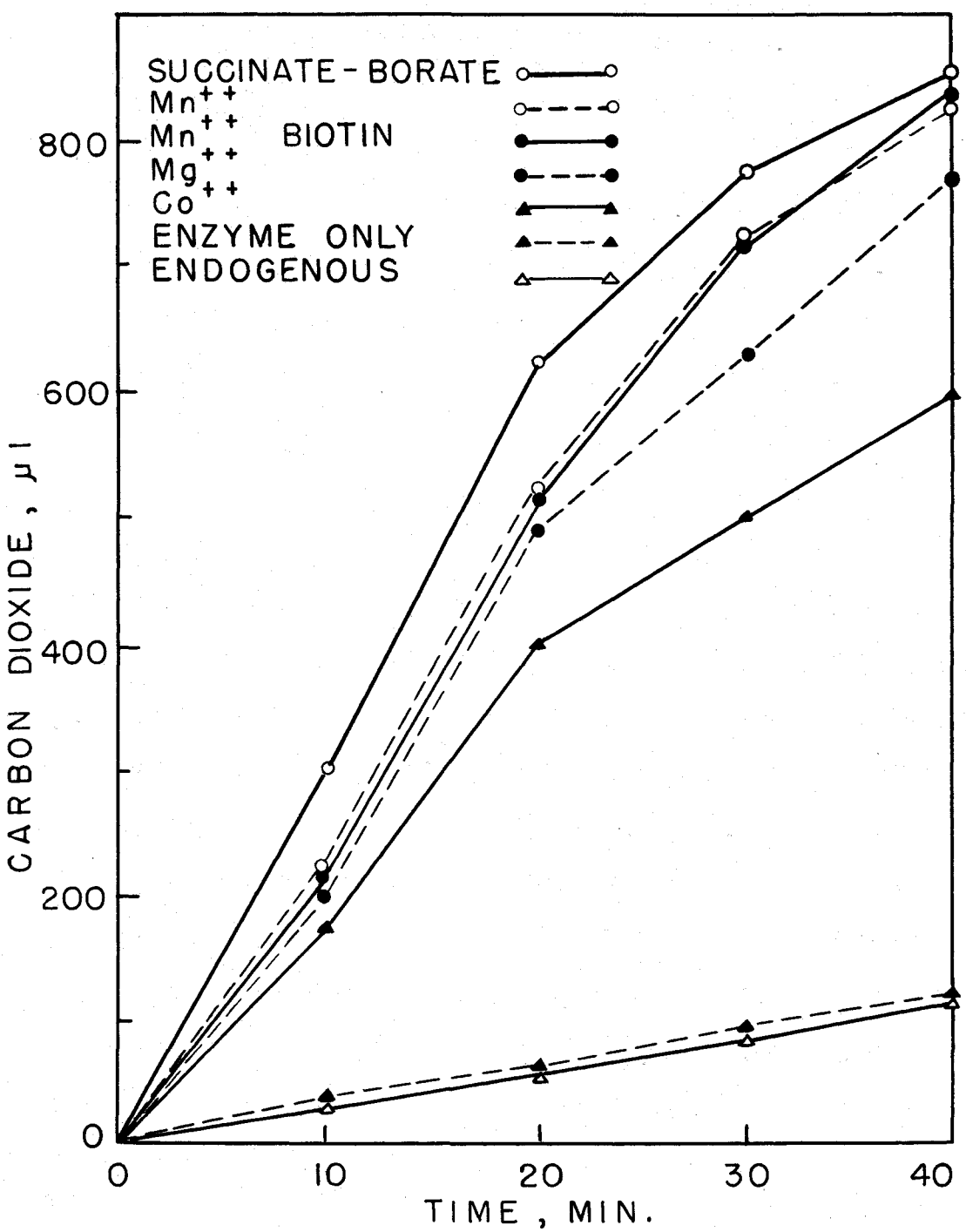


Figure 2. Effect of Inorganic Ions, Biotin, and Succinate-Borate Buffer on Oxalacetic Decarboxylase.

thus accounting for the difference in activity. These great differences in activity are not apparent with the enzyme of M. lysodeikticus. Slightly greater activity was obtained with succinate-borate buffer as shown in Figure 2. The activity obtained in phosphate buffer, however, is approximately 95 per cent that obtained when the reaction is carried out in succinate-borate buffer. On this basis, phosphate cannot be considered inhibitory to the system.

The data in Figure 2 were obtained by setting up Warburg flasks in replicate so that at the end of ten minute intervals acid could be added to a flask to release any carbon dioxide present in solution. Each flask contained 0.05 millimole of oxalacetate, 0.04 millimole of phosphate buffer of the pH indicated, 0.3 milliliter of 12 N sulfuric acid in the side arm, enzyme fraction containing 0.018 milligram of nitrogen, various additions and distilled water to 2.3 milliliters. The concentrations of the various additions were as follows: 0.002 millimole of manganous sulfate, 0.0005 millimole of cobaltous chloride, 0.002 millimole of magnesium sulfate, and 0.01 microgram of biotin. In the flasks involving succinate-borate, 0.04 millimole of the buffer was added.

Succinate-borate buffer was prepared by mixing 500 milliliters of 0.1 M sodium succinate and 500 milliliters of 0.1 M boric acid. The solution was adjusted to pH 6.8

by the addition of 0.1 N hydrochloric acid.

The enzyme is active over a relatively large range of pH. Maximum activity was found near neutrality but good activity was obtained between pH 5.3 and 8.0 as shown in Figure 3. Values were obtained by checking enzyme activity in the usual way, except that the pH of the buffer varied from flask to flask. Each flask contained 0.018 milligram of nitrogen in the enzyme fraction.

Many enzyme systems are affected by the age of the cells producing them. Oxalacetic decarboxylase is not influenced by this factor, however. In experiments conducted with lysed cells of M. lysodeiktieus rather than the pure enzyme, no differences in activity could be demonstrated in cells harvested at 12-hour intervals after incubation periods varying from 12 to 72 hours. Since the yield of cells is greatest after 60 to 72 hours incubation, it was feasible to use older cells when working with this enzyme.

Effect of biotin on decarboxylase activity

Since biotin functions in carbon dioxide fixation and oxalacetic decarboxylase supposedly catalyzes the fixation of carbon dioxide into oxalacetic acid as well as the decarboxylation of the acid, the relation of biotin to the purified enzyme was studied. Ochoa, et al, (1947) had shown

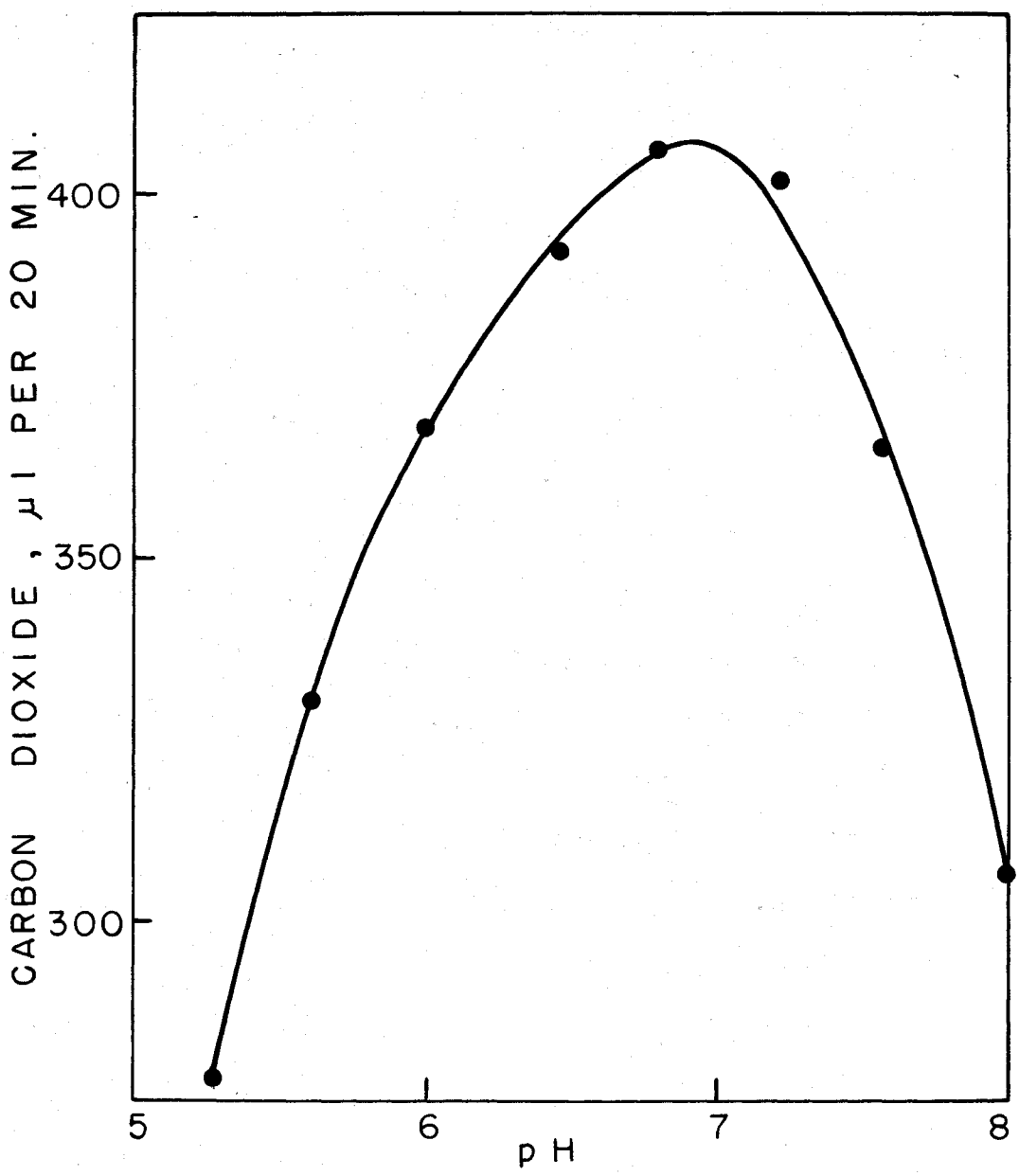


Figure 3. Effect of pH on Oxalacetic Decarboxylase.

that oxalacetic decarboxylase activity was diminished in the livers of biotin-deficient turkeys. Other workers also believed this enzyme to be dependent on biotin.

The addition of pure biotin to the enzyme in decarboxylation experiments failed to stimulate activity. Table 11 shows that no enhancement of oxalacetic acid decarboxylation resulted when concentrations of biotin varying from 0.0001 to 1.0 microgram per cup were added. In order to check the possibility that small amounts of residual biotin present in the enzyme preparation might be catalyzing the reaction, avidin was added to bind any of the vitamin present. No inhibition of activity resulted when purified avidin or a crude preparation of egg white was added. The results indicate that biotin is not involved in oxalacetic decarboxylase activity. Similar results have been shown by Plaut and Lardy and by Vennesland with enzymes from other sources.

As a further check on the possible relationship of biotin and oxalacetic decarboxylase, assays were carried out to determine the amount of biotin present at various stages in the purification process. As purification proceeds, the relative amount of biotin present should increase. As shown in Table 12, this was not found to be the case. The opposite seems to be true. The amount of biotin per unit of nitrogen as well as the amount on the basis of activity decreases. Most of the biotin originally present is lost

Table 11

Effect of Biotin on Decarboxylation of Oxalacetate by Purified Enzyme

	Carbon Dioxide Evolved, µl	
Enzyme only	367	254
Enzyme + .0001 µg biotin	376	247
Enzyme + .001 µg biotin	390	
Enzyme + .01 µg biotin	366	262
Enzyme + .1 µg biotin	384	
Enzyme + µg biotin	363	
Enzyme + 0.3 mg avidin	367	
Enzyme + 100 mg egg white		255
Enzyme + 100 mg boiled egg white		253

Each flask contained 0.02 mM oxalacetate, 0.25 mM phosphate buffer, pH 6.8, 0.002 mM $MnSO_4$, 0.3 ml of 12 N H_2SO_4 in side arm, enzyme as indicated and distilled water to 2.3 ml. Time = 20 minutes. Atmosphere = N_2. Temperature = 30.4°C.

Enzyme:
Expt. 1. 0.0327 mg N per flask.
Expt. 2. 0.0300 mg N per flask.

Table 12

Relationship of Biotin Content and Enzymatic Activity

Fraction	Biotin Content, mμg per mg Nitrogen	Activity, μl CO_2 per mg Nitrogen
Lyophilized cells	4.88	249
Lysing residue	3.87	171
Lysing supernatant	0.97	1062
75 per cent $(NH_4)_2SO_4$ saturation	0.92	1105
38 per cent $(NH_4)_2SO_4$ saturation	0.32	630
Supernatant from 38 per cent $(NH_4)_2SO_4$ saturation	0.29	1582
40.8 per cent $(NH_4)_2SO_4$ saturation	0.11	5066
Supernatant from 40.8 per cent $(NH_4)_2SO_4$ saturation	0.10	883
Precipitated at pH 5.1	0.08	896
First alumina C_δ adsorption	0.10	4667
Second alumina C_δ adsorption	0.02	25562
Supernatant from second alumina C_δ adsorption	0.12	927

during the purification process. The fact that biotin content does not increase along with enzyme activity is added evidence that the center of biotin activity is not oxalacetic decarboxylase.

Attempts to fix carbon dioxide in oxalacetic acid

The purified enzyme was checked to see if it had the ability to fix carbon dioxide. Exchange reactions involving heavy carbon were carried out as previously described. Table 13 shows the results of two separate experiments carried out with different enzyme preparations. Fixation did not occur in any case, even when biotin, adenosine triphosphate, or biotin plus adenosine triphosphate was added.

The results indicate two possibilities. First, that the conditions under which the experiments were run were not sufficient to permit fixation to occur; that is, that the addition of co-factors such as biotin or adenosine triphosphate was not adequate. Secondly, the enzyme oxalacetate decarboxylase may not be the enzyme, or, at least, not the only enzyme involved in carbon dioxide fixation. The possibility that a coupling enzyme system is necessary to carry out the fixation of carbon dioxide in oxalacetic acid must be considered.

Table 13

Fixation of $C^{13}O_2$ in Oxalacetic Acid by Purified Enzyme

Experiment	1		2	
	*C^{13} Fixed	Oxal-acetate Remaining mM	*C^{13} Fixed	Oxal-acetate Remaining mM
Enzyme only	0.04	0.728	0.02	0.663
Enzyme + 0.2 µg biotin	0.02	0.747	0.04	0.696
Enzyme + 0.02 mM ATP	0.01	0.718	0.03	0.714
Enzyme + 0.2 µg biotin + 0.02 mM ATP	0.04	0.690	-0.03	0.663

*Values are given as atom per cent excess.

Each flask contained 1.50 mM oxalacetate, 0.60 mM pyruvate, 0.75 mM phosphate buffer, pH 6.8, 1.20 mM $NaHC^{13}O_3$ (9.82 atom per cent excess C^{13}), .048 mM $MnSO_4$, and enzyme. Total volume = 30 ml. Time = 20 minutes. Atmosphere = N_2. Temperature = 30.4°C.

Enzyme:
Expt. 1. 0.159 mg N per flask. Activity = 21460.
Expt. 2. 0.200 mg N per flask. Activity = 25562.

Experiments with Cells Deficient in Biotin

Development of a medium deficient in biotin

Since it was shown that biotin stimulates the growth of M. lysodeiktieus, an attempt was made to develop a medium which contained a sub-optimum amount of biotin. Thus studies could be made comparing the reactions of cells low in biotin content with those of cells containing a normal amount of biotin.

A medium similar to that used in vitamin assays with L. arabinosus was investigated, with the addition of agar to provide a solid surface. Casein hydrolyzate served as the source of amino acids. Micrococcus lysodeiktieus failed, however, to produce more than a minimum amount of growth on this medium. Various other media were tried and best growth was obtained when peptone and yeast extract were included. Since these materials produced the best growth of the organism, it seemed logical that a good medium could be devised by including both peptone and yeast extract if the biotin in these materials could first be largely removed. Consequently, a method to eliminate most of the biotin by adsorbing it on charcoal was tried.

Five grams of peptone (or yeast extract) were dissolved in 100 milliliters of distilled water. The reaction of the solution was adjusted to pH 6.0 with glacial acetic acid.

Five grams of Norit-A were added and the mixture stirred mechanically for 30 minutes. The material was filtered with suction through Whatman No. 1 filter paper, the filtration being aided by the addition of Filter-Cel.

The pH of the filtrate was adjusted to 3.8 with glacial acetic acid. Two grams of Norit-A were added, the material stirred 30 minutes, and filtered as before. The residues from each filtration were washed with ten milliliters of distilled water and the washings added to the filtrate. The pH of the filtrate was adjusted to 6.8-7.0 with 10 N sodium hydroxide and diluted to 150 milliliters with distilled water so that each 30 milliliters of liquid contained approximately one gram of treated material. The solution containing peptone was easily filtered; the yeast extract, however, required four or five filtrations in order to obtain a clear filtrate.

Subsequent experiments showed that the treatment reduced drastically the amount of biotin present in either peptone or yeast extract. Other factors were also removed but could be added back so that a medium which would support good growth was obtained.

Table 14 shows the amount of biotin found in treated and untreated material as determined by means of assay with L. arabinosus. Figures obtained in the assay of unhydrolyzed material as well as those obtained after hydrolysis with

Table 14

Biotin Content of Various Constituents of the Medium

Constituent	Biotin per Gram of Dry Weight, µg	
	Unhydrolyzed	Hydrolyzed
Norit-treated yeast extract	0.007	0.034
Untreated yeast extract	0.290	0.952
Norit-treated peptone	0.004	0.037
Untreated peptone	0.150	0.440
Difco agar		0.002

sulfuric acid are included for comparison. Since agar was included in the medium on which M. lysodeikticus was grown, a sample of this material was also assayed for biotin. The value obtained is very low and eliminates the possibility of contamination of the medium by biotin present in the agar.

Experiments were carried out in which it was established that good growth of M. lysodeikticus could be obtained on a solid medium containing dextrose, tap water, agar, distilled water, charcoal-treated peptone and yeast extract, a solution of adenine, guanine and uracil, and vitamins, including biotin.

The method used in determining the over-all requirements for a medium producing optimum growth was as follows: the medium, totalling five milliliters, was slanted in pyrex test tubes and inoculated with a single drop from a standardized pipette. By tipping the tube so that the inoculum touched the entire surface of the agar, adequate inoculation was insured. The inoculum was prepared from a 24-hour culture of M. lysodeikticus grown on an agar slant. The cells were suspended in ten milliliters of distilled water, centrifuged, and resuspended in 15 milliliters of distilled water. A drop of this suspension was used for inoculation. The tubes were incubated at 37°C and checked visually at 24, 48, and 72 hours. The growth was washed from each slant after 72 hours and suspended in ten milliliters of distilled

water. The turbidity of the resulting suspension was read on the colorimeter.

Table 15 shows data obtained in this way. Poor growth was obtained when either the charcoal-treated peptone or yeast extract alone was added to the medium. The addition of both treated peptone and yeast extract or both plus biotin did not serve to enhance growth. The addition of the above constituents plus a solution of adenine, guanine, and uracil resulted in good growth. Slightly better growth was obtained with both treated yeast extract and peptone than with either separately. Treated yeast extract plus the other necessary additions, the purine-pyrimidine solution and biotin, resulted in considerably better growth than was obtained when treated peptone replaced the yeast extract. The vitamin solution did not have a great effect upon growth, although Allen (1951) has indicated that folic acid stimulates growth of M. lysodeikticus.

The addition of several other compounds to the medium did not produce greater growth. The following compounds were tried, the amounts listed being added per five milliliters of medium: ammonium chloride, 0.05 milligram, xanthine hydrochloride, 0.025 milligram, magnesium sulfate, 4 milligrams, sodium chloride, 0.2 milligram, ferrous sulfate, 0.2 milligram, and manganous sulfate, 0.8 milligram.

Table 15

Effect of Various Constituents upon the Growth of *Micrococcus lysodeikticus* on Solid Media

Additions per 10 ml								*Colorimeter Reading
Treated Peptone, 0.015 gm	Untreated Peptone, 0.015 gm	Treated Yeast Extract, 0.015 gm	Untreated Yeast Extract, 0.015 gm	Purine-pyrimidine Solution, 0.05 ml	Vitamin Solution, 0.01 ml	Biotin, 0.0001 µg	0.001 µg	0.01 µg	0.1 µg	1.0 µg	
X											15
	X										92
		X									12
			X								112
X		X									15
X				X							22
X				X	X						20
X				X	X					X	48
		X		X							18
		X		X	X						21
		X		X	X					X	94
X		X								X	17
X		X		X						X	15
X		X		X							23
X		X		X	X						18
X		X		X	X	X					49
X		X		X	X		X				67
X		X		X	X			X			83
X		X		X	X				X		97
X		X		X	X					X	99

*Klett-Summerson readings at 660 mµ.

"X" indicates inclusion in the medium.

Basal medium included 0.05 gm dextrose, 0.5 ml tap water, 0.075 gm agar, and distilled water to 10 ml.

Purine-pyrimidine solution contained 1 mg each of adenine, guanine, and uracil per ml.

Vitamin solution contained the following amounts per ml: PABA, 1 µg, Ca pantothenate, 5 µg, folic acid, 0.02 µg, nicotinic acid, 5.0 µg, pyridoxine HCl, 10 µg, riboflavin, 5 µg, and thiamine HCl, 5 µg.

From this information a medium was established which supported good growth of M. lysodeiktieus. The basal medium contained the following constituents: five grams of dextrose, 50 millliliters of tap water, 45 millliliters of treated yeast extract, 45 millliliters of treated peptone, five millliliters of a purine-pyrimidine solution, one millliliter of a vitamin solution, 15 grams of agar, and distilled water to one liter. The composition of the purine-pyrimidine and vitamin solutions are listed in Table 15.

Various amounts of biotin and other compounds were added to the basal medium and M. lysodeiktieus was grown on the medium in Roux bottles in the conventional manner. Cells were harvested after 72 hours incubation at 37°C. Yields of cells were markedly low when only 0.01 microgram of biotin was added to a liter of medium. The addition of 10 micrograms of biotin per liter resulted in good production of cells. Larger amounts of biotin did not increase the yield further.

Better results were gained, however, when aspartic and oleic acids were added to the medium. These two compounds together can substitute for biotin in the nutrition of M. lysodeiktieus. The yield of cells was in the range of that obtained when optimum amounts of biotin were added to the medium.

The cells grown on the various media were assayed for biotin content and considerable differences were noted. Cells grown on the medium to which no biotin was added contained very small amounts of biotin. Thus the medium to which oleic and aspartic acids were added provided considerably larger yields of cells but with lower biotin content than those grown on media having small amounts of biotin added. Table 16 records data concerning these experiments.

The cells used in the following experiments were grown on the basal medium to which aspartic and oleic acids, but no biotin, were added. The acids were neutralized before addition to the medium.

Oxalacetic decarboxylase activity of cells deficient in biotin

Cells of low biotin content, in which the amount of biotin present is only about five per cent that of normal cells, were used to check the possibility that biotin functions in the synthesis of oxalacetic decarboxylase. If biotin does function in this manner, cells grown on media low in biotin should contain reduced amounts of enzyme.

The decarboxylase activity of biotin-deficient cells was measured and compared with that of cells containing normal amounts of biotin. Table 17 incorporates data obtained in experiments of this type. In two experiments

Table 16

Yields and Biotin Content of Cells

Additions to Basal Medium, per Liter	Yield, Lyophilized Cells per Roux Bottle, mg	Biotin Content, µg
Biotin, 0.01 µg	25	0.160
Biotin, 10 µg	209	0.435
Biotin, 100 µg	195	0.410
Oleic acid, 40 mg, aspartic acid, 200 mg	162	0.020
Normal cells (grown on glucose nutrient agar)		0.460

Table 17

Decarboxylation of Oxalacetic Acid by Low Biotin and High Biotin Cells of *Micrococcus lysodeiktieus*

	Carbon Dioxide Evolved, µl	
	Expt. 1	Expt. 2
High biotin cells	247	211
High biotin cells + 0.001 µg biotin	243	209
High biotin cells + 2.5 µg avidin	237	225
Low biotin cells	244	220
Low biotin cells + 0.001 µg biotin	251	225
Low biotin cells + 0.1 µg avidin	244	203

Each flask contained 0.02 mM oxalacetate, 0.25 mM phosphate buffer, pH 6.8, 0.002 mM $MnSO_4$, 0.3 ml 12 N H_2SO_4 in side arm, 10 mg lysed cells, and distilled water to 2.3 ml. Time = 20 min. (Expt. 1), 15 min. (Expt. 2); Atmosphere = N_2; Temperature = 30.4°C.

Biotin content of cells:
High biotin cells - 0.435 mµg per mg cells.
Low biotin cells - 0.020 mµg per mg cells.

shown it will be noted that there is no difference in the ability of cells to decarboxylate oxalacetic acid even though extreme differences in the amount of biotin present in the cells exist. The addition of biotin had no effect upon either type of cell. Furthermore, the addition of avidin to bind biotin present in the cells does not inhibit decarboxylase activity. These experiments strongly indicate that biotin is not concerned with the synthesis of oxalacetic decarboxylase.

Before the development of a medium deficient in biotin for the growth of M. lysodeiktious, an attempt was made to utilize Propionibacterium pentosaceum for the purpose of studying biotin and oxalacetic decarboxylation. Delwiche (1950) described a medium low in biotin on which this organism could be grown. The cells were grown on this medium, in which casein hydrolyzate is the source of amino acids. To this medium the listed additions were made and the yields of wet cells shown were obtained.

250 ml basal medium + 12.5 µg biotin	2.6065 gm
250 ml basal medium + 1.0 µg biotin	1.0806 gm
250 ml basal medium + 1.0 µg biotin and 10 mg sodium oleate	1.9543 gm

These cells were tested for oxalacetic decarboxylase activity and no differences in the ability to decarboxylate oxalacetic acid was found. A gain in activity was obtained by preparing a sonic juice from the cells, but again the low

biotin and high biotin cells exhibited no differences in decarboxylase activity.

The results are shown in Table 18 and are included because they add weight to the evidence that biotin is not related to oxalacetic decarboxylase.

Fixation of carbon dioxide by cells deficient in biotin

The work with biotin-deficient cells added to the evidence established with the purified enzyme that biotin and oxalacetic decarboxylase are not connected. There appeared to be differences in other types of activity in the two types of cells, however, such as differences in the rate of oxidation of glucose. The Wood-Werkman reaction was checked, therefore, to determine if there were differences in the ability of the cells to fix carbon dioxide into oxalacetic acid.

Table 19 shows the results of these experiments. The inability of biotin-deficient cells to fix carbon dioxide is clearly indicated. When varying amounts of biotin are added, however, fixation proceeds, depending up to a certain point on the amount of vitamin present. The fact that biotin caused a rapid response in a short time experiment, plus the fact that the amount of fixation seems to correlate with the amount of biotin added, indicates that biotin functions in a rather direct manner in the reaction.

Table 18

Decarboxylation of Oxalacetic Acid by Low Biotin and High Biotin Cells of P. pentosaceum

	Carbon Dioxide Evolved, µl
High biotin cells	88
Low biotin cells	87
Cells grown with oleic acid	85
Sonic juice - high biotin cells	153
Sonic juice - low biotin cells	146

Each flask contained 0.02 mM oxalacetic acid, 0.25 mM phosphate buffer, pH 6.8, 0.002 mM $MnSO_4$, 0.3 ml 12 N H_2SO_4 in side arm, 0.5 ml of 10 per cent suspension of cells or 0.8 ml sonic juice and distilled water to 2.3 ml. Time = 60 min.; Atmosphere = N_2; Temperature = 30.4°C.

The juice was prepared by treatment in a Raytheon sonic vibrator using one-half as much ground glass as wet cells in twice the weight of distilled water. Time of treatment was 30 min. Centrifugation - 20 min. at 10,000 RPM.

Table 19

Fixation of $C^{13}O_2$ in Oxalacetic Acid by Low Biotin and High Biotin Cells of Micrococcus lysodeikticus

	*C^{13} Fixed
Expt. 1	
High biotin cells	0.14
Low biotin cells	0.01
Low biotin cells + 0.2 µg biotin	0.18
Expt. 2	
Low biotin cells	0.01
Low biotin cells + 0.0001 µg biotin	0.06
Low biotin cells + 0.001 µg biotin	0.08
Low biotin cells + 0.01 µg biotin	0.11
Low biotin cells + 0.1 µg biotin	0.19
Low biotin cells + 0.2 µg biotin	0.19

*Values are given as atom per cent excess.

Expt. 1. Each flask contained 1.20 mM oxalacetate, 0.45 mM pyruvate, 0.60 mM phosphate buffer, pH 6.8, 0.60 mM $NaHC^{13}O_3$ (C^{13} = 9.82 per cent), 220 mg of lysed cells and distilled water to 23 ml. Atmosphere = 10 per cent CO_2 in N_2; Time = 20 min.; Temperature = 30.4°C.

Expt. 2. Each flask contained 1.50 mM oxalacetate, 0.60 mM pyruvate, 0.75 mM phosphate buffer, pH 6.8, 1.20 mM $NaHC^{13}O_3$ (C^{13} = 9.82 per cent), 300 mg of lysed cells and distilled water to 30 ml.

Effect of biotin on the formation of aspartic acid

Since it was definitely shown that biotin is involved in fixation of carbon dioxide, further information was desired concerning the possiblity that it functions as a coenzyme. It has been shown in a number of cases that biotin and the synthesis of aspartic acid are closely related, the site of action, of course, being the formation of oxalacetic acid which is then converted to the amino acid. Growth experiments with M. lysodeikticus indicated such a relationship. Resting cell experiments were devised, therefore, in which pyruvic acid and sodium bicarbonate were incubated with high and low biotin cells and the aspartic acid formed measured.

The experiments were carried out in small Warburg flasks containing a total volume of three milliliters of reactants. The main compartment of the flask contained 0.05 millimole of phosphate buffer, pH 6.8, 0.1 millimole of sodium bicarbonate, 20 milligrams of lysed cells and various amounts of biotin or other additions. The side arm contained 0.1 millimole of sodium pyruvate which was tipped in after equilibration of the system. The atmosphere was ten per cent carbon dioxide in nitrogen and the temperature 30.4°C. At the end of 60 minutes the reaction mixture was

immediately deproteinated and assayed for aspartic acid content with Leuconostoc mesenteroides.

The results of experiments of this type are shown in Figure 4. The production of the amino acid by cells grown in the presence of an optimum concentration of biotin is compared to that produced by cells grown in the presence of only traces of biotin when various additions are made to the latter. Curve No. 1 represents cells grown with sufficient biotin to which various amounts of the vitamin were added. The other curves concern cells grown on the low biotin medium. Curve No. 3 represents low biotin cells plus various amounts of biotin, curve No. 5 low biotin cells plus various amounts of oleic acid, curve No. 2 low biotin cells plus 0.002 microgram of biotin and various amounts of oleic acid per flask, and curve No. 4, low biotin cells plus 0.1 milligram of oleic acid and various amounts of biotin per flask.

Cells containing low amounts of biotin synthesized only minute amounts of aspartic acid. The addition of biotin served to raise the amount of aspartic acid formed, the amount formed being proportional to the biotin added up to a certain point. Under the conditions employed, the optimum amount of biotin is slightly over 0.002 microgram per flask. Larger amounts failed to increase further the yield of aspartic acid.

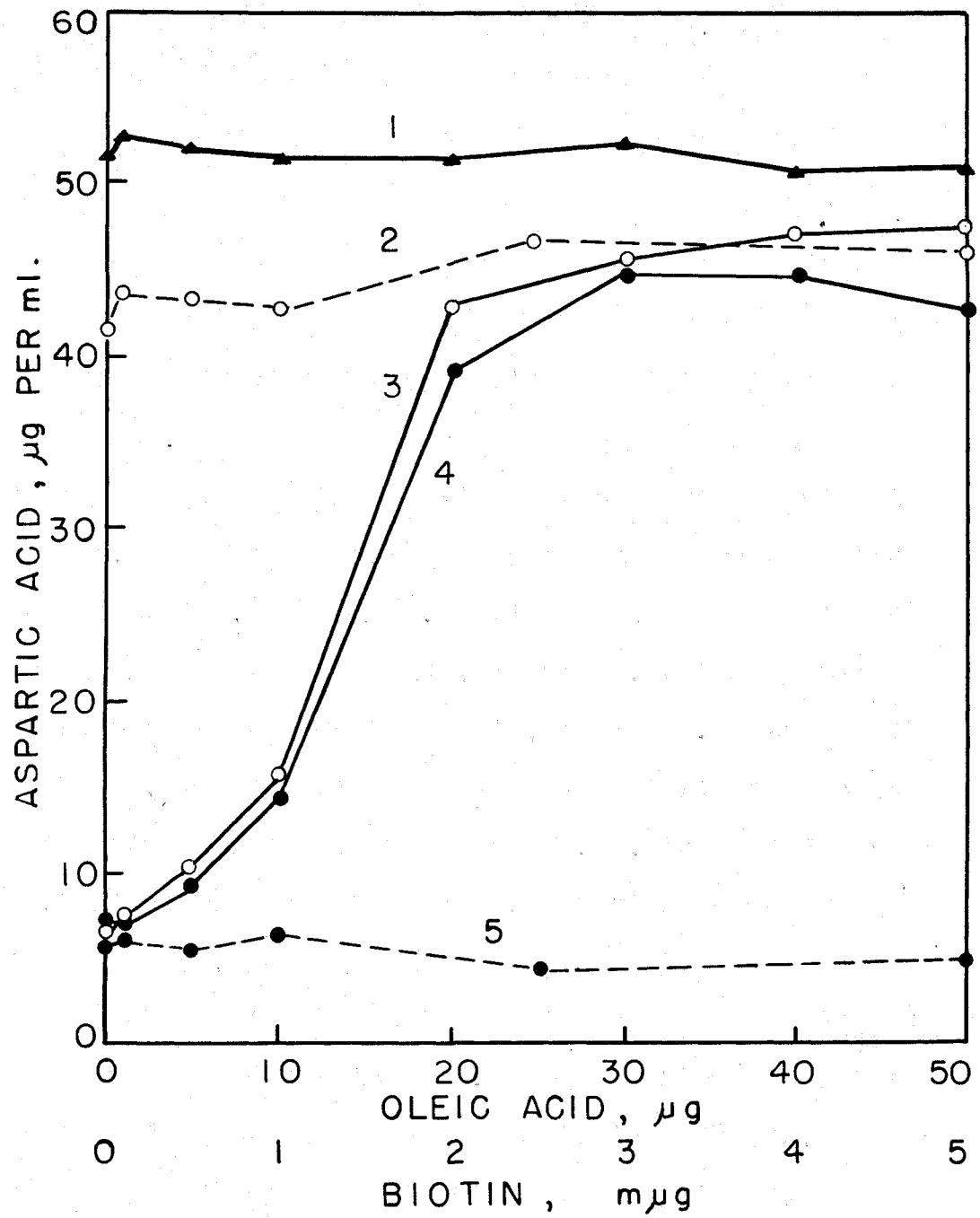


Figure 4. Effect of Biotin and Oleic Acid on the Formation of Aspartic Acid.

Cells of high biotin content produced considerably higher amounts of aspartic acid than the low biotin cells. The addition of biotin to these cells failed to increase the formation of aspartic acid. Apparently, these cells contain adequate amounts of the vitamin and further additions have no effect on the enzyme system. The maximum amount of aspartic acid formed by biotin-deficient cells upon the addition of biotin approached the amount produced by cells grown on media containing adequate amounts of the vitamin.

The results also show that oleic acid does not stimulate the formation of aspartic acid. Neither stimulation nor inhibition results when combinations of oleic acid and biotin are added to the system. Although a relationship between biotin and oleic acid has been shown to exist, the site of their interrelationship is not the system involving aspartic acid formation.

Figure 5 represents an experiment in which the aspartic acid formed has been measured as a function of time. Replicate Warburg flasks were set up in series and the amount of aspartic acid formed in cumulative ten-minute intervals measured by stopping the reaction in a flask at the end of such periods. Low biotin cells were used. Curve No. 3, representing cells without biotin, shows that only small amounts of aspartic acid were formed. It levels off after

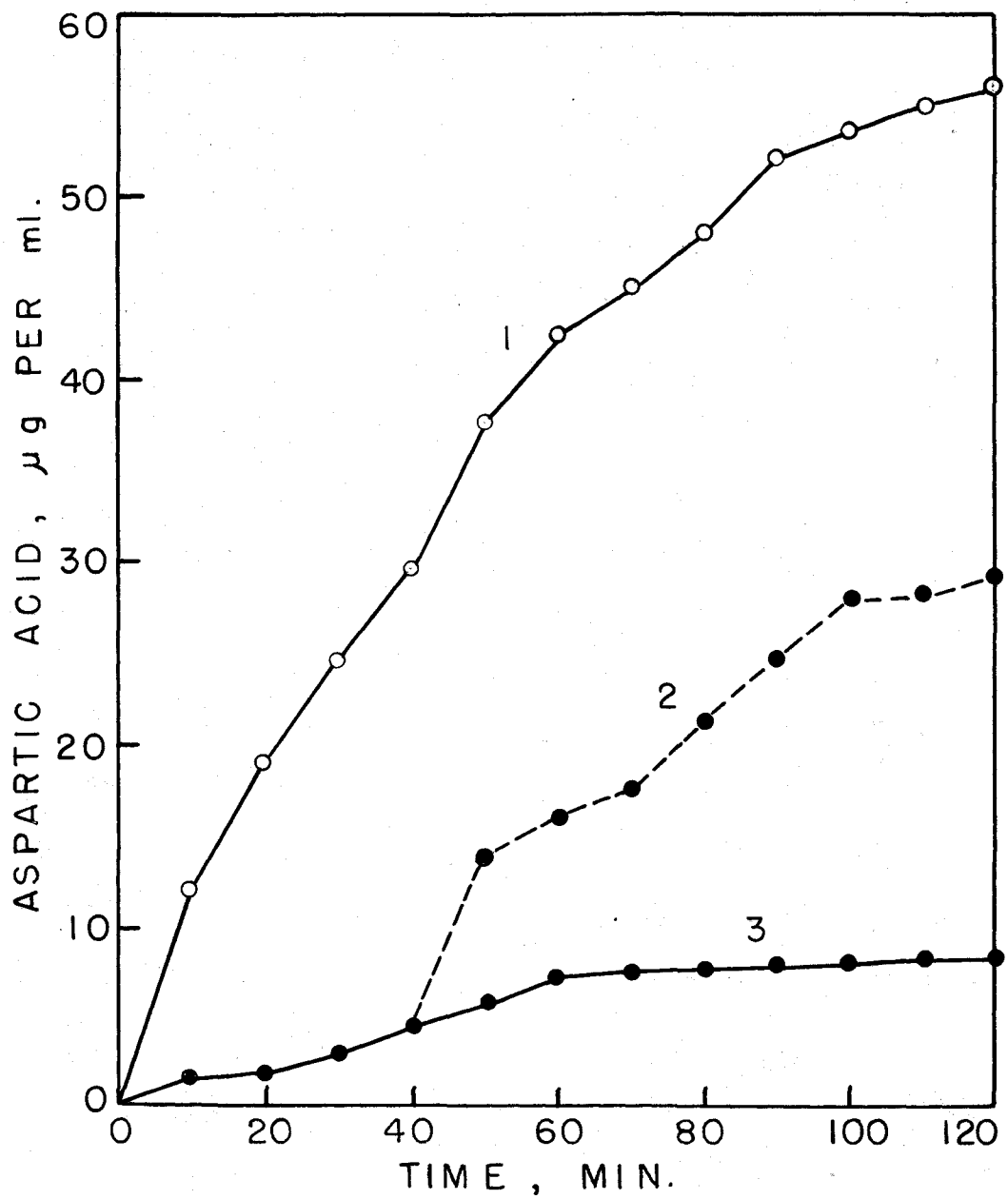


Figure 5. Effect of Biotin on the Rate of Formation of Aspartic Acid.

approximately an hour and fails to rise appreciably after that. Probably the small amounts of aspartic acid formed are dependent upon very limited amounts of residual biotin present in the cells.

Curve No. 1, showing aspartic acid formed by low-biotin cells to which 0.002 microgram of biotin per flask has been added, rises much more rapidly and is still rising slowly at the end of 120 minutes. Approximately seven times as much aspartic acid was formed as when no biotin was added to the reaction.

When biotin was added to biotin-deficient cells after the experiment had run 40 minutes, aspartic acid formation was immediately enhanced (curve No. 2). A curve similar to that obtained when biotin is added at the beginning of the experiment results. The total amount of aspartic acid formed is, of course, considerably less. This is partially due to some enzyme inactivation as well as to a shorter time interval involved.

The shape of the curve resulting when biotin is added to the system seems to be an important indication as to the mode of action of biotin. If biotin functions in an indirect manner it would be expected that the curve would exhibit a definite and fairly long lag phase. Instead, the curve rises rapidly with no noticeable lag phase in a manner indicative of a coenzyme type of activity.

DISCUSSION

Action of Biotin in the Fixation of Carbon Dioxide in Oxalacetic Acid

Evidence has been presented which indicates that biotin functions as a catalyst in the Wood-Werkman reaction. Although other investigations have pointed to this as a site of action for biotin, the evidence here presented is more conclusive. First, this metabolic site has been narrowed to a more specific reaction; secondly, the use of avidin to bind biotin so that reactions could be observed under conditions wherein the vitamin is not available demonstrates a more specific attack.

The possibility that the effect of avidin on the reaction is due to its combination with a compound other than biotin merits consideration. Although avidin has great specific binding power for biotin, it will also unite with a number of analogues of the vitamin, although its affinity for these compounds is much less and combination, therefore, is of a much lesser degree. Wright and Skeggs (1947) and Wright, et al, (1947) studied the combinability of a large number of compounds having some similarity to biotin. Only a limited number exhibited measurable combination with avidin, emphasizing the relative specificity of the reaction.

Of the possible analogues of biotin for which avidin has some affinity, only two can be considered to be of any importance. Oxybiotin and desthiobiotin have been shown to partially substitute for biotin in a few cases, the latter as a precursor in the synthesis of the vitamin (Tatum, 1945). There is no evidence that oxybiotin occurs naturally; experimentally, it has always been supplied as a synthetic compound. Desthiobiotin has been reported to occur naturally, but the evidence for this is very questionable. Thus, although limited combination with avidin of certain structurally related compounds can occur, the possibility under our experimental conditions is remote. Therefore, the avidin-biotin relationship must be considered to be specific.

The inhibition of carbon dioxide fixation resulting when avidin is present is overcome upon the addition of biotin to the system. This reinforces the evidence that biotin is the compound responsible for the effect. The experiments are set up on the premise that avidin binds the biotin already present in the cells making it unavailable and that added biotin compensates for that bound by the protein. The question has been raised that in the case where both avidin and biotin are supplied, the added vitamin may merely be combining with the added avidin, leaving the original biotin present in the cell free to

carry out its function or functions. This seems unlikely. In the first place, it would not explain the action in the case where no biotin is added. Secondly, the reaction between biotin and avidin appears to be a rapid one. Recent studies by Launer and Fraenkel-Conrat (1951) on the kinetics of the avidin-biotin relationship support this contention. Utilizing radioactive biotin, the authors have shown that the reaction is practically complete with only limited dissociation occurring. In this investigation a sufficiently long period was allowed for the avidin to react with the cellular biotin. The use of lysed cells rather than whole cells eliminates errors which may arise due to permeability factors and the resulting binding of only a proportion of the biotin present in the cells.

Biotin exists in the bacterial cell in both combined and free form as shown by microbiological assay of hydrolyzed and unhydrolyzed material. The possibility exists that in order to function in the fixation of carbon dioxide biotin must be present in some combined form. Even if this were true, however, it would not change the validity of the experiments. Avidin is capable of reacting with certain combined forms of biotin. Wright, Cresson, Skeggs, Wood, Peck, Wolf, and Folkers (1950) have shown that biocytin, a naturally occurring complex of biotin, combines readily with avidin. Chang and Peterson (1951) have shown that

avidin reacts with several forms of soluble biotin complexes. It has further been shown that certain biotin complexes available to Lactobacillus casei are utilizable by Lactobacillus arabinosus only upon further hydrolysis. Yet the addition of avidin results in inhibition of growth for either of the organisms, illustrating that even the combined forms of biotin are bound by avidin, if they are present.

Blanchard, et al, (1950) have indicated that in L. arabinosus biotin functions in an indirect manner in relation to the "malic enzyme". This is the enzyme which reversibly decarboxylates malic acid to form pyruvic acid and carbon dioxide. In boiled extracts of normal liver or purified liver enzyme, reactivation of the enzyme is not brought about by the addition of biotin, suggesting that biotin does not function directly. If L. arabinosus is grown on a medium supplemented with malic acid, the cells catalyze the above reaction vigorously. This activity is decreased in biotin-deficient cells. The direct addition of biotin does not reactivate the system. A slow reactivation of malic acid dissimilation occurs in four hours when biotin is added together with a small amount of growth medium containing glucose. On the basis of such experiments, the authors concluded that biotin is not incorporated into a prosthetic group required by the malic acid dissimulation system. Rather, they believe that biotin functions at a

more basic level and that it may be a component of the prosthetic group of an enzyme system which synthesizes other prosthetic groups or apoenzymes, or that it is involved in some other way in the synthesis of these groups. An indirect mode of action of a similar type has recently been proposed by Christman and Williams (1952) concerning the relationship of biotin and aspartic acid deaminase.

The possibility that biotin may function in a comparable indirect manner in the Wood-Werkman reaction is largely ruled out on the basis of experiments with biotin-deficient cells and cells grown in the presence of normal amounts of biotin. The possibility that biotin may be involved in the synthesis of oxalacetic decarboxylase is eliminated since the two types of cells show no differences in ability to decarboxylate the acid. The cells grown with only minute amounts of biotin exhibit full ability to decarboxylate oxalacetic acid although the two types of cells do show definite differences, such as the total amount of cellular growth. This evidence indicates biotin does not function in the synthesis of the enzyme. The possibility that small amounts of biotin present may be sufficient to account for enzyme synthesized seems extremely doubtful in view of other differences apparent in the cells.

The cells exhibit great differences in their ability to catalyze the fixation reaction. Biotin-deficient cells

are unable to fix carbon dioxide in oxalacetic acid or to form aspartic acid. The addition of biotin, even in a short time experiment, results in activation of this ability. This is characteristic of a direct type of action, to be expected if biotin functions as a coenzyme.

The preparations employed are not fixing carbon dioxide by reductive carboxylation of pyruvic acid to form malic acid as suggested by Ochoa for certain systems. The preparations showed no "malic enzyme" activity. The presence of this enzyme in M. lysodeiktieus has been shown in young cells, and only in small quantity. It is at a maximum at about 12 hours and completely absent after 24 hours. McManus (1951) has also shown that the malic acid-triphosphopyridine nucleotide system does not normally operate in M. lysodeiktieus. Employing lysed suspensions and cell-free extracts of the organism, the author carried out a number of fixation experiments utilizing isotopic carbon. No evidence was found which indicated that any net reaction other than the oxalacetic carboxylase reaction occurred.

It has been shown that cells grown on a medium deficient in biotin fail to synthesize aspartic acid when incubated with pyruvic acid and carbon dioxide. This deficiency is overcome by the addition of biotin. The results of time experiments, wherein the amount of aspartic acid formed is plotted against time, showed a curve which

rose immediately upon the addition of biotin. This is additional evidence for a coenzyme form of action. If the vitamin functioned in an indirect manner, such as it would be doing if it was involved in catalyzing the synthesis of a coenzyme, then a lag period of considerable duration could be expected before aspartic acid would be formed. Such a lag period, for example, has been found by Blanchard, et al, (1950) to be required for synthesis of the "malic enzyme".

The fact that no lag period exists also suggests that biotin functions as such rather than in a combined form. It would be likely that even for the formation of a combined form of the vitamin a time interval of some length would be required. This, however, need not necessarily be true as rapid synthesis of coenzymes has been demonstrated in certain cases. Hughes and Williamson (1949) showed that nicotinic acid-deficient cells of L. arabinosus form cozymase rapidly when nicotinic acid is added to the system; Novelli and Lipmann (1947) noted that an immediate synthesis of coenzyme A resulted when pantothenic acid was added to Proteus morganii deficient in this vitamin. This hypothesis has not been tested concerning biotin and possible combined forms of the vitamin.

Oxalacetic Decarboxylase and the Fixation Reaction

The apparent lack of a direct relationship between biotin and purified oxalacetic decarboxylase from M. lysodeikticus appears to be in contrast to characteristics formerly attributed to this enzyme. Earlier work indicated an activation by biotin of this system in other species. Ochoa, et al, (1947) claimed a liver enzyme decarboxylating oxalacetic acid to be decreased in biotin-deficient turkeys. Lichstein and Umbreit (1947a) reported that the reduced ability of phosphate inactivated Escherichia coli to decarboxylate oxalacetic acid could be restored upon the addition of biotin. Yet all evidence in this work demonstrates no connection between the vitamin and this enzyme. It was shown that biotin does not stimulate the decarboxylation of oxalacetic acid, that it does not affect synthesis of the enzyme, and that no correlation exists between biotin content and enzyme purity. This lack of a definite relationship is similar to results found for oxalacetic decarboxylase present in other sources. Thus, Vennesland, et al, (1949) with parsley root, Plaut and Lardy (1949) with Azotobacter vinelandii, and Byerrum, et al, (1950) with a crystalline globulin preparation from seeds of Cucurbita pepo could demonstrate no activation by biotin.

It has been conclusively shown that biotin functions in the β-carboxylation of pyruvic acid to form oxalacetic acid. It has been assumed that the enzyme involved in this reaction also causes decarboxylation or the reverse of fixation. This was first indicated by Krampitz and Werkman (1941) and Krampitz, et al, (1943) for the system found in M. lysodeikticus. Evans, et al, (1943) believed the same to be true for a crude preparation obtained from pigeon liver. Subsequent work concerning the system has been done on the assumption that the same enzyme catalyzes fixation of carbon dioxide as well as oxalacetic acid decarboxylation. Yet, the purified enzyme from M. lysodeikticus failed to fix carbon dioxide into oxalacetic acid.

The inability of the purified enzyme to carry out the Wood-Werkman reaction, either alone, with biotin, with adenosinetriphosphate, or both, is surprising. Several explanations for this lack of action may be considered. First, the treatment employed in purification of the enzyme may have removed essential factors which were not added back. In view of the fact that only biotin and adenosinetriphosphate (plus manganese ions which were added in all cases) previously have been implicated in the fixation of carbon dioxide, this seems unlikely, although still a possibility.

McManus (1951) was not able to demonstrate that adenosinetriphosphate influences fixation in M. lysodeikticus. It has, nevertheless, been added to the system since it is necessary for the functioning of the enzyme found in other species. Utter and Wood (1946) demonstrated its action in catalysis of fixation by pigeon liver extracts. Vennesland, et al., (1947) partially purified the enzyme from pigeon liver and confirmed that adenosinetriphosphate stimulated carbon dioxide fixation and, in addition, inhibited the decarboxylation of oxalacetic acid. In the case, however, of a purified enzyme from parsley root, Vennesland, et al., (1949) did not find that adenosinetriphosphate stimulated fixation.

Likewise, the possibility exists that the fixation reaction may be more complex than the direct carboxylation of pyruvic acid to form oxalacetic acid. Wood, et al., (1942) suggested that a phosphorylated form of pyruvic acid, rather than the acid itself, may be the active metabolic compound involved. If this were true, or if some other intermediate were the precursor of oxalacetic acid, it appears possible that purified decarboxylase might not be able to catalyze the reaction in more than one direction because of the lack of a complete enzyme system.

Suppose the reaction is represented as follows:

$$\text{pyruvic acid} \overset{(a)}{\rightleftharpoons} \text{intermediate "X"} \overset{(b)}{\rightleftharpoons} \text{oxalacetic acid.}$$

In the purification the enzyme catalyzing (b) has been retained, but the enzyme responsible for (a) has been lost. Since this is true, no intermediate "X" is formed; therefore, no compound is available for carboxylation. The system, therefore, operates only to the left. The breakdown of compound "X" to form pyruvic acid must be considered to occur spontaneously. This does not seem unlikely since, even without an enzyme present, oxalacetic acid decomposes rapidly to form pyruvic acid.

The failure to fix carbon dioxide might also be attributed to the removal during purification of certain coupling enzymes which were present in the crude extracts of pigeon liver and M. lysodeiktieus. Possibly such a coupling system as a transaminase is missing. The coupling reaction may be necessary to supply the energy for the endergonic fixation of carbon dioxide with pyruvic acid.

Results obtained with enzymes from other sources provide evidence on both sides of the problem. Using radioactive bicarbonate in the exchange reaction with oxalacetic acid, Plaut and Lardy (1949) were unable to show any exchange with the purified enzyme from A. vinelandii. No fixation was observed when additions of adenosinetriphosphate, phosphate, pyruvic acid, or fumaric acid, either alone or in combination, were made. Vennesland, et al, (1949), however, obtained the opposite result with the parsley root enzyme.

Carrying out reactions in the same way, the authors were able to demonstrate fixation of carbon dioxide into oxalacetic acid. However, several facts indicate that the parsley root system was not very pure, and thus, the likelihood of a complete system being present is greater. No additions to the system were necessary in order that fixation could be carried out. The preparation also contained such activities as malic dehydrogenase, oxalsuccinic carboxylase and isocitric dehydrogenase.

The weight of evidence indicates that our oxalacetic decarboxylase is either not the enzyme mediating fixation of carbon dioxide or is only part of an enzyme system involved. The latter possibility is more likely.

SUMMARY AND CONCLUSIONS

1. Micrococcus lysodeikticus is stimulated by the addition of biotin to the medium. The organism will produce only scanty growth on a medium containing no biotin. The inclusion of aspartic and oleic acids in the medium eliminates the need for biotin.

2. Avidin prevents the fixation of carbon dioxide into oxalacetic acid by lysed cells of M. lysodeikticus by binding biotin present in the cellular material. The addition of biotin to compensate for that inactivated by the avidin results in a reactivation of the system. Micrococcus lysodeikticus, grown on a medium deficient in biotin, will not fix carbon dioxide or synthesize aspartic acid, deficiencies which are overcome by the addition of biotin. These experiments demonstrate the involvement of biotin in the Wood-Werkman reaction.

3. The lack of a lag period in the synthesis of aspartic acid when biotin is added to deficient cells demonstrates that biotin functions in a direct manner characteristic of a coenzyme.

4. Oxalacetic decarboxylase has been obtained from M. lysodeikticus in partially purified form. The enzyme rapidly decarboxylates oxalacetic acid with only manganese ions needed for its activation.

5. Biotin is not involved in the synthesis of oxalacetic decarboxylase. The vitamin has no direct connection with this enzyme, neither stimulating nor inhibiting the decarboxylation of oxalacetic acid.

6. Purified oxalacetic decarboxylase will not fix carbon dioxide into oxalacetic acid even in the presence of biotin, adenosinetriphosphate, or a combination of the two. The evidence indicates that oxalacetic decarboxylase is only part of a system carrying out the fixation of carbon dioxide.

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