STUDIES ON THE METABOLISM OF THE COLORLESS ALGA PROTOTHECA ZOPFII

By EDWARD H. ANDERSON

(From the Hopkins Marine Station of Stanford University, Pacific Grove)

(Received for publication, September 26, 1944)

INTRODUCTION

The first studies dealing with the physiology of the colorless alga *Prototheca zopfii* were those of Barker (1, 2) who demonstrated that the metabolism of this organism was essentially of an oxidative nature. He found that while it was capable of carrying on an anaerobic metabolism in that it could ferment glucose quantitatively to lactic acid, it was totally unable to develop under strictly anaerobic conditions. In this respect its metabolism may be compared with that of mammalian muscle tissue.

Barker made a detailed study of the metabolism of this alga in regard to its nutritional requirements for growth and to its ability to utilize a great variety of simple carbon compounds as the sole substrate.

Of the carbohydrates tested, only the monosaccharides were utilized by *Prototheca.* All of the fatty acids, with the exception of formic and isovaleric acid, appeared adequate as carbon sources, as did many of the alcohols and ketones. None of the nitrogen-containing compounds (glycine, asparagine, glucosamine, ethylamine, or yeast autolysate) was found to serve as a carbon source. The most surprising result was the fact that not a single substituted or dicarboxylic acid tested by Barker would serve as a utilizable substrate.

The study of the carbon nutrition was approached experimentally in two ways. Culture experiments in which the substance under investigation constituted the main carbon source of the medium showed what compounds could serve for growth. The effect of the addition of various organic substrates upon the oxygen consumption by suspensions of non-growing cells was studied with the manometric technique of Warburg-Barcroft.

In all cases of the oxidation of utilizable compounds, Barker was able to express the relationship between the quantity of substrate, oxygen, and carbon dioxide participating in the reaction in terms of a balanced chemical equation having simple stoichiometric relations. This relationship showed conclusively that those compounds which are attacked by *Prototheca* are not oxidized completely to carbon dioxide and water, but that a considerable fraction, from 50 to 80 per cent, is converted into a primary assimilation product having an over-all composition corresponding to that of a carbohydrate which is stored in the cells, probably as glycogen. Barker's experiments showed that the process of assimilation of simple organic substrates by this alga proceeds

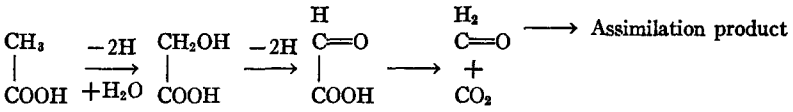
in two distinct stages. The first he found to consist of a very rapid oxidative conversion of the substrate into carbon dioxide and the stored material. The second stage comprises a slow decomposition of this primary assimilation product and its subsequent transformation into many different organic substances which make up the cell material.

This was the first convincing demonstration that, during the respiration of simple organic compounds by non-proliferating organisms, assimilation processes occur to an unexpectedly large extent. That a large oxidative assimilation is not restricted to the metabolism of a colorless alga was soon evidenced by the studies of Giesberger (3) on various *Spirillum* species and by Clifton (4, 5) on *Pseudomonas calcoacetica* and *Escherichia coli.*

Since the oxidation of such simple compounds as acetic acid by *Prototheca* resulted in so extensive a synthesis of carbohydrate-like materials, it suggested the possibility of an experimental approach to the problem of the mechanism of synthetic processes in general. The stoichiometric relationship seemed to indicate that the substrate would be partially oxidized, giving rise to intermediate products from which the synthesis to carbohydrate could proceed directly. In that case, a study of the behavior of the various organic compounds that could be postulated as being intermediate products in the synthesis of carbohydrate from acetic acid should reveal the general pathway of the metabolic reactions involved in the biochemical synthesis. This would appear to be a most fruitful approach, particularly in view of the fact that a vast number of studies have clearly demonstrated that the production of carbohydrate is one of the most important aspects of photosynthesis.

Prototheca is a member of the family Oocystaceae, order Chlorococcales, class Chlorophyceae (Chlorophyta), and represents an alga devoid of chlorophyll, and hence unable to produce organic cell materials from carbon dioxide as the sole carbon source. Nevertheless, in a primary assimilatory process such as is characteristic of the metabolism of *Prototheca*, the synthesis of carbohydrate from a simple organic compound shows a certain similarity to the photosynthetic reaction. Moreover, the great economy of carbon assimilation is not entirely restricted to photosynthesis, as is evidenced by the fact that Barker found *Prototheca* capable of assimilating such a large percentage of the carbon of a single substrate. One might expect the further conversions of the primary assimilatory product into numerous cell materials to proceed by much the same types of mechanism in both *Prototheca* and in the green plants, so that a detailed study of the metabolism of the former would ultimately aid in understanding that of the latter. Finally, heterotrophic organisms in general carry out synthetic reactions of various sorts. In view of the well established similarity of biochemical mechanisms in the most divergent types of organisms, a study of carbohydrate synthesis from acetate by *Prototheca* should be of decided value in contributing to a general clarification of such syntheses.

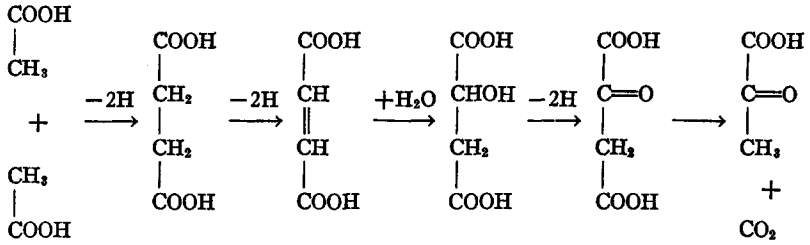
However promising the outlook, Barker's experiments appeared, at that time, to lead up a blind alley. This can best be appreciated by considering the oxidative metabolism of *Prototheca* in the presence of acetate. Two main pathways for the decomposition of this metabolite can be postulated. One would be through successive oxidations to glycolic and glyoxylic acids and its subsequent decarboxylation (Bernhauer (6)).



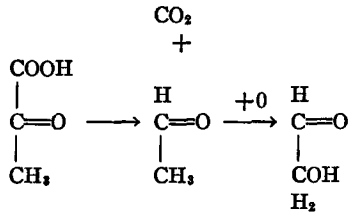
Synthesis of carbohydrate could then take place from the "formaldehyde," more or less in accordance with von Bayer's concept of carbohydrate formation in photosynthesis.

If *Prototheca* were to oxidize acetate in this manner it should follow that both glycolic acid and glyoxylic acid could be metabolized. Barker was forced to rule out this series of reactions, however, since *Prototheca* was incapable of oxidizing either intermediate.

The second manner in which acetate could be oxidized proceeds by way of succinic, fumaric, and oxalacetic acids, followed by decarboxylation to pyruvic acid (Thunberg (7)).



The further fate of pyruvic acid could be postulated to lead, through a second decarboxylation, to acetaldehyde from which, by oxidation to glycolaldehyde, carbohydrate might be formed by condensation:



This scheme too, would satisfy the experimental values for the relationship between acetate used, oxygen consumed, and carbon dioxide produced. But

this series proved no more tenable than did the first, for the intermediates here involved were likewise not metabolized when tested by Barker.

The identification of thiamin as the growth factor for *Prototheca* (8) made it possible to investigate its specific function in the oxidative metabolism of this organism. In the course of this work a number of new facts were discovered which gradually led to a skeptical attitude with respect to the general validity of some of Barker's findings. A reinvestigation of certain phases of his work was then undertaken which resulted in a much more satisfactory picture of the metabolism of the experimental organism. This, in turn, made it possible to carry out some preliminary experiments in connection with the assimilation problem proper.

Material and Methods

Organism.—The strain of *Prototheca zopfii* used in these investigations was No. 7322, one of several maintained in the pure culture collection of the Hopkins Marine Station, and is the same strain as that used in the studies on the growth factor requirements of this organism (8).

Medium and Methods of Culture.—Cultures of the organism were maintained on yeast agar containing 2 per cent dextrose.

"Normal" or vitamin-sufficient cells for use in making manometric measurements were grown on plates of the yeast dextrose agar medium incubated at 30°C. for 48 hours. The cells were washed once or twice by centrifuging and resuspended in sterile tap water at pH 7.0, or in M/15 phosphate buffers of the desired pH.

To obtain thiamin-deficient cells it is necessary to grow the organism in a medium in which thiamin is the limiting factor. Such cells were obtained by using a glycerol mineral medium (8) to which thiamin was added in sufficient amounts to allow good growth with limiting concentrations of the vitamin. The cells were grown in several rotating bottles, each containing 25 ml. volumes of glycerol mineral medium to which thiamin had been added. At the end of a 6 day period of incubation at 30°C. the cultures were pooled, centrifuged, washed once or twice, and resuspended in neutral sterile tap water, or in M/15 phosphate buffers, in a concentration suitable for use in making manometric measurements.

FUNCTION OF THIAMIN IN THE METABOLISM OF PROTOTHECA ZOPFII

Thiamin has been found to be essential for the normal development of *Prototheca* (8). Therefore the hypothesis that a growth factor or a vitamin represents the functional (active or prosthetic) group of an enzyme without which normal metabolism cannot proceed, leads to the assumption that the alga needs carboxylase and uses this enzyme in its metabolism but is unable to synthesize carboxylase unless supplied with thiamin or its component parts.

It was generally held, at the time the present work was undertaken, that the sole function of carboxylase was to catalyze the decarboxylation of α-keto acids. Furthermore, since the only known connection of thiamin with enzymes was its occurrence in carboxylase, it seemed logical to conclude that the de-

carboxylation of α-keto acids formed an integrant part of the metabolism of *Prototheca zopfii*.

Prototheca is capable of using sugars as a substrate. From the accumulated data on the sugar metabolism of various organisms it was logical to assume pyruvic acid to be an intermediate substance in the decomposition of sugar. Therefore, carboxylase, and hence thiamin, could be expected to be needed in the metabolism of this alga. A serious difficulty was presented, however, by the fact that Barker had claimed *Prototheca* to be incapable of decomposing pyruvic acid. Consequently, Barker's experiments on the utilization of pyruvic acid by *Prototheca* were repeated.

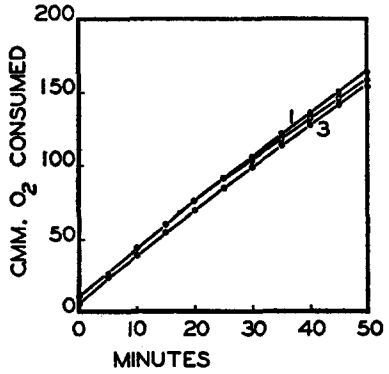


FIG. 1. Oxidation of pyruvate at pH 7.0 by non-deficient cells of *Prototheca*: (1) autorespiration; (2) 0.01 mM Na pyruvate; (3) 0.01 mM Na pyruvate plus 10 γ thiamin.

The oxygen consumption of 2.0 ml. volumes of normal cells in neutral tap water was measured by the manometric technique of Warburg-Barcroft, in vessels containing KOH in the center well. In the absence of an added substrate which the cells are capable of utilizing, the rate of oxygen consumption by such suspensions of non-proliferating cells is relatively low, and represents the rate of oxidation of cellular materials, such as reserve carbohydrates. The addition of a utilizable substrate greatly increases the rate of oxygen consumption (see also Barker (2)). Numerous experiments demonstrated that the addition of sodium pyruvate to such suspensions did not result in an increase in the rate of oxygen utilization. Even the addition of thiamin did not cause an acceleration of oxygen uptake. Fig. 1. represents the results obtained in one typical case.

The results were in complete agreement with the experiments reported by Barker. As a consequence of these unequivocal findings, Barker's conclusion that *Prototheca* was unable to decompose pyruvic acid was believed to be correct.

In view of the recognized function of carboxylase, it was difficult to understand why *Prototheca* needed thiamin as a growth factor and yet was unable

to decompose pyruvic acid. This strongly indicated that in the case of *Prototheca* thiamin might be used not for the synthesis of carboxylase, but for the synthesis of an enzyme concerned in the decomposition of organic compounds other than α-keto acids. The most characteristic metabolites for *Prototheca* are the unsubstituted fatty acids, especially acetic, as is attested by the abundant development of this organism in vinegar casks (Janke (9)). This natural occurrence of the alga indicates that acetic acid is a suitable enrichment substrate for *Prototheca*. Furthermore, the careful and extensive studies of Barker had shown that *Prototheca* was unable to utilize any but the fatty acids. Therefore, it seemed possible that thiamin might function in the decomposition of the fatty acids, and in particular in the oxidation of acetic acid. Consequently experiments were designed to determine whether thiamin functioned in the oxidation of fatty acids by *Prototheca*.

The method of approach was based on the technique developed by Lwoff (10) in his investigations on the rôle of blood in the nutrition of trypanosomes and later utilized by Hills (11) in his studies on the part played by thiamin, a growth factor for *Staphylococcus aureus*, in the metabolism of this organism.

Hills showed that staphylococci, grown with minimal amounts of thiamin, consumed oxygen in the presence of pyruvate as a substrate at a very low rate. The rate of oxygen consumption was immediately increased by the addition of minute amounts of thiamin. Cells grown with optimum amounts of thiamin were capable of a rapid decomposition of pyruvate and their oxygen consumption was not affected by the addition of the vitamin. These experiments made it clear that the oxidation of pyruvate by *Staphylococcus aureus* requires an enzyme which the organism can rapidly synthesize from thiamin. The fact that the anaerobic decomposition (decarboxylation) of pyruvic acid by "thiamin-deficient" cells was also greatly and immediately increased by the addition of thiamin to the cell suspension, showed that the main, if not only, function of the vitamin, in the metabolism of *Staphylococcus aureus*, is its conversion into carboxylase.

The above examples illustrate what could be expected from an application of Lwoff's "starvation" methodology to a study of the metabolism of *Prototheca*. Those substrates, for which a special thiamin-containing enzyme is required, would be decomposed slowly by cells that had been grown in media deficient in this vitamin. The addition of small amounts of thiamin to suspensions of such deficient cells should result in an immediate and rapid synthesis of the limiting enzyme system with a subsequent increase in the rate of utilization of the substrate.

Experiments with Thiamin-Deficient Cells

To determine if thiamin functioned in the oxidation of fatty acids by *Prototheca* the following experiment was carried out.

Thiamin-deficient cells were obtained by growing the organism in glycerol mineral medium to which 1×10^{-7} M thiamin had been added. Two ml. samples of the non-proliferating cells resuspended in neutral tap water were placed in the vessels of Warburg-Barcroft manometers. Each vessel contained KOH in the center well and a gas phase of air. Ten μg. of thiamin were added to the suspension in each of two vessels. Measurement of oxygen consumption in the absence of added substrate showed the cells without added thiamin to have a low rate of oxygen uptake. On the other hand, the cells supplied with thiamin showed a rate of oxygen utilization approximately twice that of the starved cells. This difference in autorespiration indicates that the addition of thiamin allows the cells to utilize some of their stored materials more rapidly, suggesting that the lack of the vitamin restricted the metabolism of these substances.

The addition of 0.01 mM of sodium acetate caused a rapid and nearly identical increase in oxygen consumption by the cells deficient in thiamin and by those to which thiamin had been added as well. In this case, therefore, thiamin had no effect whatever on the oxidation of acetate.

The addition of 0.01 mM of dextrose presented an entirely different picture. The thiamin-deficient cells consumed oxygen at a very low rate; the quantity utilized during the first hour was exactly the same as in the control. The extra oxygen consumption in the suspension containing sugar, which began at that time, increased slowly thereafter. The behavior of deficient cells was plainly different from that of normal cells in the presence of dextrose. Cells having access to thiamin showed an immediate increase in oxygen consumption in response to the addition of dextrose. The marked difference in rate of decomposition of dextrose by the deficient cells with and without added thiamin clearly indicated that thiamin is essential to *Prototheca* in its metabolism of dextrose. The results of a representative experiment showing the effect of thimain on the oxidation of acetate and of dextrose are presented in Fig. 2.

Apparently *Prototheca* could oxidize a representative of the fatty acids without benefit of thiamin, but it did need this substance, or its components, for growth and for the utilization of a sugar. The failure of thiamin to affect the oxidation of acetic acid indicated that there was no reason to ascribe to it a function in the metabolism of fatty acids. Its pronounced influence on sugar metabolism supported the possible relation of thiamin to the decomposition of pyruvic acid. It was almost certain that this α-keto acid would occur as an intermediate substance in the degradation of sugar by *Prototheca zopfii*. The strongest support for this contention was the extensive production of lactic acid from glucose under anaerobic conditions (Barker (1)). The entire body of evidence, amassed by Emden, Meyerhof, and others (12), made it reckless to suggest that the formation of this hydroxy acid would not proceed, in the case of *Prototheca*, by the well established mechanism of the reduction of pyruvic acid. In sharp contrast with these theoretical deductions was the experimental evidence, showing that pyruvate was not metabolized by *Prototheca*.

Experiments on the Effect of pH on the Decomposition of Pyruvic Acid

In all cases, determinations of the ability of *Prototheca zopfii* to utilize a given substrate had been carried out in neutral solutions. There is much evidence to show that cells are more freely permeable to undissociated molecules than to ions.

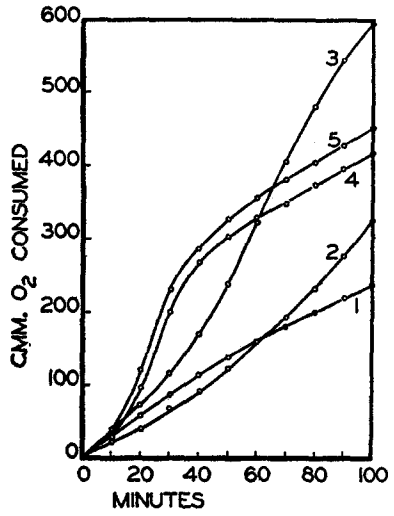


FIG. 2

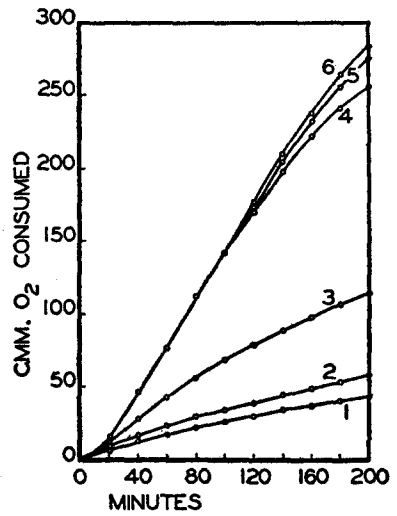


FIG. 3

FIG. 2. Effect of thiamin on oxidation of dextrose and acetate by vitamin-deficient cells of *Prototheca* at pH 7.0: (1) autorespiration; (2) 0.01 mM dextrose; (3) 0.01 mM dextrose plus 10 γ thiamin; (4) 0.01 mM Na acetate; (5) 0.01 mM Na acetate plus 10 γ thiamin.

FIG. 3. Effect of pH on pyruvic acid oxidation: (1) autorespiration at pH 4.0; (2) 0.01 mM pyruvic acid at pH 6.0 to 5.5; (3) at pH 5.5 to 4.5; (4) at pH 4.5 to 4.0; (5) at pH 4.0 to 3.5; (6) at pH 3.5 to 3.0.

The work of Osterhout, Collander, and others has shown that in a neutral environment weakly dissociating acids and bases penetrate cells more rapidly than do strongly dissociating ones. Conditions that suppress the dissociation of a substance have a tendency to increase its penetration. There are indications in the literature that many organisms are unable to metabolize strong acids except in an acid environment. A striking example of this phenomenon is furnished by the photochemical nitrate reduction carried out by the green alga, *Chlorella*. Warburg and Negelein (13) found this reaction to take place only in solutions containing undissociated nitric acid. The anaerobic decomposition of pyruvic acid by yeast has also been shown to proceed most rapidly in an acid medium (14). Pyruvate is decomposed by cell-free yeast juice at a pH of about 6.0. Living yeast cells, however, fail to cause any appreciable decomposition of the keto acid until the acidity of the suspending medium is increased

to a value below pH 4.0 Such behavior may be explained as a result of the nature of the cellular membrane of certain cells which allows acids to penetrate only in the form of undissociated molecules.

Therefore experiments were conducted to test the possibility that pyruvic acid might be attacked when the suspension liquid was maintained at an acid reaction.

Vitamin non-deficient cells were obtained in the usual manner and resuspended in sterile tap water at a pH of 7.0. One-half ml. of the suspension, containing 12 mm.^{3} of cells, was measured into each of six Warburg vessels containing 1.5 ml. of a series of phosphate solutions at pH values ranging from pH 6.0 to 3.5. Each vessel contained NaOH in the center well and air as the gas phase. Autorespiration, determined over a period of 90 minutes, was quite similar throughout the range of hydrogen ion concentrations used. The lower pH values, however, did have a slight stimulatory effect on the rate of oxygen consumption, the cells at pH 3.5 using 43 mm.^{3} of oxygen during the initial 90 minute period, as against 35 mm.^{3} at pH 6.0.

The addition of 0.01 mM of pyruvic acid to the suspension of cells at pH 6.0 lowered the pH to 5.5 and caused a very slight initial increase of oxygen uptake which soon returned to a rate identical with that for the autorespiration (13 mm.^{3} per 50 minutes). At this stage the pH had risen to 5.7 as a result of the decomposition of a small amount of added pyruvic acid. At pH 5.5 the addition of the same amount of acid caused an increase in acidity to pH 4.5 with a subsequent initial rate of oxygen consumption of 38 mm.^{3} per 50 minutes which gradually fell off to a rate of 24 mm.^{3} Again at this point, the decomposition of a portion of the pyruvic acid had resulted in a decrease in acidity of the suspension; pH determinations showed it to be 5.2. The addition of the keto acid to the suspensions of organisms at pH 4.5, 4.0, and 3.5, now lowered to pH 4.0, 3.5, and 3.0 respectively, presented a distinctly different picture. In these three cases the rate of oxygen utilization immediately rose to 80 mm.^{3} per 50 minutes. This high rate was maintained until the pyruvic acid had been decomposed. Final pH determinations of these three suspensions showed that their acidity had returned to the initial values. The oxygen consumption for each suspension, from the time the pyruvic acid was added, is shown in Fig. 3.

Since the metabolism of pyruvic acid proceeded in nearly an identical manner at pH 4.0, 3.5, and 3.0, the highest value was chosen for use in all subsequent experiments in order to avoid any possible injury to the cells by an environment too strongly acid. Although Barker (1) reported an increase in hydrogen ion concentration to have an adverse effect on the rate of dextrose decomposition by *growing* cultures of *Prototheca*, further tests have shown that at pH 5.0, at least, the decomposition of dextrose by non-proliferating cells is certainly not inhibited but rather that the acid environment exerts a stimulatory effect.

This experiment was the first to demonstrate that *Prototheca* does possess enzyme systems capable of decomposing pyruvic acid. From the data recorded in Fig. 3, it is seen that a hydrogen ion concentration greater than that corresponding to pH 4.5 is necessary in order that pyruvic acid may be made available to the intracellular enzyme systems.

Effect of Thiamin on Pyruvate Decomposition

To determine whether thiamin is needed in the metabolism of *Prototheca* in the rôle ascribed to carboxylase, vitamin-deficient cells, with and without added thiamin, were tested for their ability to decompose pyruvic acid in an acid environment.

Deficient cells grown in glycerol mineral medium containing 1×10^{-8} M thiamin were centrifuged, washed twice in M/15 primary phosphate solution adjusted to pH 4.0 by the addition of H_2SO_4, and resuspended in the "buffer" in a concentration suitable for use in Warburg measurements. Two ml. of the suspension, containing 32 mm.^{3} of cells, were introduced in each of the vessels and the rate of oxygen consumption measured. The first vessel received no added substrate and, therefore, its rate of oxygen uptake is a measurement of the autorespiration of stored cellular materials. The cells in the second vessel received 0.01 mM of pyruvic acid. With the addition of the acid the rate of oxygen consumption was increased, indicating that the vitamin-deficient cells were able to decompose pyruvic acid. To the suspension of cells in the third vessel were added 10 μg. of thiamin. The addition of the vitamin to the deficient cells caused an immediate but slight increase in the rate of autorespiration. Addition of 0.01 mM of pyruvic acid to the cells now supplied with thiamin, caused a rate of oxygen uptake 2.5 times the maximum obtainable with the vitamin-deficient cells to which no thiamin was added. Suspensions of cells treated in a manner identical with that of deficient cells, except that they had been grown in a medium containing an optimum amount (1×10^{-6} M) of thiamin, were able to decompose pyruvic acid at a high rate without added vitamin. The vitamin-sufficient cells showed no increase in rate of oxygen consumption on the addition of the same amount of thiamin which caused a 2.5-fold increase in the rate with the deficient cells. The data obtained in one representative experiment are presented in Fig. 4.

Extensive investigations of the effect of thiamin on decomposition of pyruvic acid by suspensions of non-proliferating, vitamin-deficient cells have shown that the addition of the vitamin in general causes a 2.0 to 3.0-fold increase in the rate of oxygen consumption. The percentage of increase is dependent on the degree of vitamin deficiency and on the age of the "insufficient" cells.

Organisms grown in media containing 1×10^{-8} M vitamin B_1 tend to show a greater increase in rate of oxygen consumption on the addition of thiamin, while respiring pyruvic acid, than do cells grown in 3×10^{-8} M vitamin B_1. Likewise, cells grown for 96 hours in media containing 1×10^{-8} M vitamin B_1 tend to show a greater response than do cells grown in similar media for 72 hours. It is assumed that the older cells are more vitamin "starved" than are the younger cells, since the older cultures contain more cells per unit volume and, therefore, the available vitamin has been distributed to a larger number of organisms. Numerous tests have shown the addition of 10 μg. of thiamin to 2.0 ml. suspensions of vitamin-deficient cells to provide an arbitrary but entirely adequate amount of the vitamin to insure maximum rates of metabolism under all conditions of thiamin deficiency of the cells.

Evidence for the Occurrence of Pyruvic Acid As an Intermediate Product in the Metabolism of Prototheca

With the finding that *Prototheca zopfii* can utilize pyruvic acid the apparent discrepancies in the metabolism and growth requirements of the alga appeared to be solved. Thiamin was shown to immediately affect the metabolism of

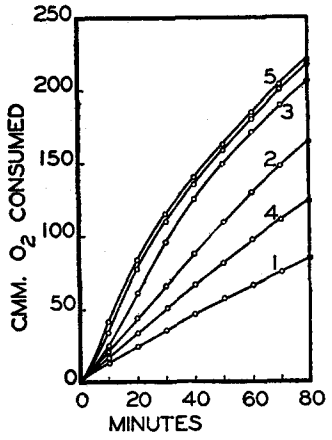


FIG. 4

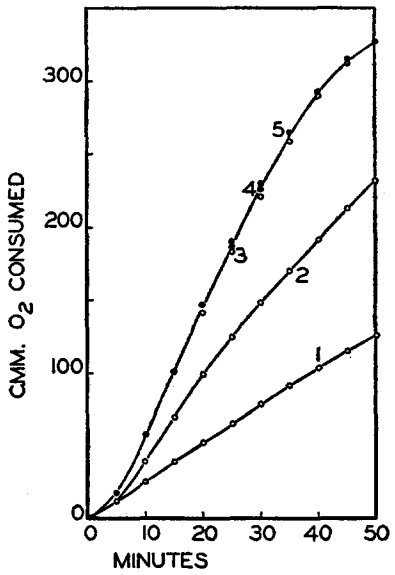


FIG. 5

FIG. 4. Effect of thiamin on pyruvic acid oxidation: (1) autorespiration of deficient cells; (2) deficient cells plus 0.01 mM pyruvic acid; (3) deficient cells plus 0.01 mM pyruvic acid plus 10 γ thiamin; (4) autorespiration of non-deficient cells; (5) non-deficient cells plus 0.01 mM pyruvic acid; (6) non-deficient cells plus 0.01 mM pyruvic acid plus 10 γ thiamin.

FIG. 5. Effect of pH on lactic acid decomposition: (1) autorespiration; (2) 0.01 mM lactic acid at pH 6.0; (3) at pH 5.0; (4) at pH 4.5; (5) at pH 4.0.

the alga in the presence of sugar and pyruvic acid, but not in the presence of acetate. Therefore, the conservative ideas of the function of the vitamin, or its components, as building blocks for the carboxylase, appear applicable in the case of *Prototheca.*

Lactic acid was tested and found to be attacked by non-proliferating cells of *Prototheca.* This hydroxy acid also was decomposed by the cells only in an acid environment.

Lactic acid ($K=0.031$) is not so strong an acid as is pyruvic ($K=0.56$), and consequently is not so highly dissociated in aqueous solutions. Therefore it would follow

that *Prototheca* suspensions might decompose lactic acid in an environment having a hydrogen ion concentration lower than that required to permit the decomposition of pyruvic acid. The data contained in Fig. 5 indicate that such might be the case. A hydrogen ion concentration corresponding to pH 5.0 will permit a maximum rate of oxygen consumption with lactic acid as a substrate while a pH below 4.5 is necessary for the maximum rate of oxidation of pyruvic acid.

The rate of oxygen utilization of thiamin-deficient cells metabolizing lactic acid could also be increased by the addition of thiamin although the stimulatory effect of the vitamin was not so great as in the case of pyruvic acid. While the addition of thiamin caused a 2.0 to 3.0-fold increase in the rate of pyruvate decomposition, the rate for lactic acid decomposition was increased about 1.4 times. This difference finds a ready explanation in that the decomposition of lactic acid, with pyruvic acid as an intermediate, requires an oxygen uptake for the conversion of the hydroxy to the keto acid. This reaction should be unaffected by vitamin B_1 (carboxylase). The oxygen utilized in this conversion, however, would be included in the rate of oxygen consumption measured. Therefore, the portion of the oxygen consumption which could be expected to be directly influenced by carboxylase would be much less in the decomposition of lactic acid than in the decomposition of pyruvic acid.

The assumption that thiamin functions in the rôle of carboxylase in the normal metabolism of *Prototheca zopfii* is further substantiated by observations on cultures of the alga growing in glycerol media. Pyruvic acid was found to accumulate in the culture medium containing insufficient amounts of the vitamin, whereas not a trace of this substance could be detected in cultures that had grown in the presence of an optimum supply of the growth factor. This is in line with the observations of Platt and Lu (15) and others that pyruvic acid accumulates in tissues and body fluids of animals deprived of vitamin B_1 and also with the finding of Peters (16, 17) that avitaminotic brain tissues of pigeons oxidize pyruvic acid at a subnormal rate.

The experimental evidence that pyruvic acid accumulates in glycerol cultures growing in the presence of suboptimal concentrations of thiamin clearly indicates the formation of the keto acid as an intermediate product in the oxidation of glycerol and demonstrates the difficulty of vitamin B_1-deficient cultures in disposing of pyruvic acid.

Effect of Thiamin on the Metabolism of Acetate by Prototheca

Up to this point, studies on the metabolism of vitamin-deficient cells of *Prototheca zopfii* have shown the oxidation of glucose, pyruvic acid, lactic acid, and glycerol to be markedly increased by the addition of small amounts of vitamin B_1. Since pyruvic acid may be assumed to occur as an intermediate product in the oxidation of all these compounds, and has been demonstrated to accumulate during the "oxidation" of glycerol by thiamin-starved cells,

the results so far presented do not offer any indication that thiamin participates in the decomposition of substances other than α-keto acids. This is in complete harmony with the hitherto accepted function of carboxylase.

However, later experiments on the oxidation of acetate by non-proliferating, vitamin-deficient cells grown in the presence of 1×10^{-8} M thiamin showed the rate of oxidation of this substrate to be materially accelerated by the addition of thiamin. The results of five experiments, presented in Table I, appear contradictory to previous findings (see Fig. 2). The cells tested in the earlier experiments had been grown in media containing 1×10^{-7} M thiamin and, therefore, cannot be considered to have been so deficient as the cells which were produced in the presence of 1×10^{-8} M thiamin. It would appear, therefore, that a vitamin deficiency is manifest *first* in the metabolism of

TABLE I

Effect of Thiamin on the Oxidation of Acetate by Suspensions of Thiamin-Deficient, (1×10^{-8} M), Cells of Prototheca zopfii

Age of culture	O_2 utilization		Increase
	With thiamin	Without thiamin	
hrs.	*mm.³ per 50 min.*	*mm.³ per 50 min.*	*per cent*
72	117	85	138
72	137	95	144
90	137	87	157
96	100	65	154
96	75	47	160

pyruvic acid, and only later with acetate. Also it should be stated that the deficiency is more pronounced in the metabolism of pyruvic acid; a two- to three-fold stimulation was found with pyruvic acid as a substrate as against an average 1.5-fold increase with acetate.

The somewhat similar stimulatory effect of thiamin on vitamin-deficient cells oxidizing lactic acid was interpreted on the basis that pyruvic acid occurs as an intermediate product in the decomposition of the hydroxy acid and that, therefore, thiamin functions in the oxidation of the keto acid. Is it probable or possible that a similar explanation may be found to interpret the results obtained with acetate?

The mechanism of the oxidation of acetic acid is still unknown. However it is not improbable that some α-keto acid might be involved as an intermediate product in the decomposition of this simple fatty acid. From a consideration of the schemes for oxidation of acetate proposed in the introduction, either pyruvic acid or glyoxylic acid can be postulated to appear as intermediate products. In that event the effect of thiamin on acetate oxidation would be

similar to its function in the oxidation of lactate, glycerol, or dextrose. That is, thiamin would act in a secondary rôle.

The observation that thiamin can effect the oxidation of acetic acid by *Prototheca* is important in view of the demonstration by Quastel and Webley (18, 19) that vitamin B_1 appears to be essential for the oxidation of acetic acid by an unknown species of bacterium.[1] This organism, when grown on media containing suboptimal amounts of thiamin, responded strongly to additions of vitamin B_1. This stimulation was found to be particularly pronounced if the vitamin was added in the presence of magnesium and potassium ions (Mg^{++} and K^{+}).

On the basis of their results, Quastel and Webley have given an involved explanation of this combined vitamin and metal ion effect without, apparently, envisaging the possibility that the response might simply be due to thiamin functioning in the decomposition of intermediate products in the nature of α-keto acids. At first sight their data might seem to effectively rule out the function of thiamin in relation to α-keto acids. The stimulation of the rate of oxygen uptake for acetate appears to be much more pronounced than for pyruvate. While the rate of oxygen consumption in acetate oxidation is raised from 20.5 mm.^{3} of oxygen per hour per milligram of dried bacteria (Q_{O_2} 20.5), to 63.1 by the addition of K, Mg, and thiamin, the oxygen uptake with pyruvate is increased only from Q_{O_2} 19.9 to 37.1.

This difference in response, of course, is not a very solid argument against the participation of thiamin in the oxidation of pyruvate because the two substrates are *not* in the same "state of oxidation." As an example let us compare the effects obtained in the respiration of lactate and pyruvate. The maximum Q_{O_2} obtained on the addition of thiamin and metal ions was 37.1 for pyruvate oxidation, while for lactate the Q_{O_2} was 72. It is most significant that the addition of metal ions alone can increase the lactate Q_{O_2} from 35 to 53 while similar additions have no effect on the pyruvate Q_{O_2} which remains at about 20. On the other hand, the addition of thiamin alone raises the lactate Q_{O_2} from 35 to 52. The maximum increase in Q_{O_2} due to the addition of vitamin B_1 is therefore 17 for both lactate and pyruvate. Since Quastel and Webley have snown pyruvate to accumulate in suspensions of deficient cells fed lactate in the absence of thiamin, it may be asserted that the identical increase in Q_{O_2}, due solely to vitamin B_1 results from decomposition of pyruvate in both the lactate and pyruvate oxidation. A similar situation can be shown to exist in the case of succinate and fumarate.

In order to obtain vitamin-deficient cells Quastel and Webley grew their organisms on a deficient medium composed of Difco peptone, agar, and NaCl made up in distilled water and autoclaved for 1 hour at a pH of 9.0 to reduce the vitamin content. The

[1] Quastel and Webley claim the organism they used to be a propionic acid bacterium, *Bacterium acidi propionici*. This is most improbable, however, because it grows rapidly, aerobically, and is capable of growth in the absence of sugar or lactate as a substrate. Krebs and Eggleston (20) using the same strain, reported the organism incapable of producing propionic acid.

medium was then filtered through cotton and autoclaved again. As a result of this method of preparation it is obvious that the medium must have been deficient in most metals as well as in vitamin B_1. Bacterial cells, grown in such an environment, consequently would themselves be deficient in various metals known to play an important rôle in the activity of a number of enzyme systems. That this was so is shown by the fact that if the deficient bacteria were incubated aerobically in the presence of Mg and K for a short time and afterwards throughly washed, the oxidative powers of suspensions of such cells were increased by the addition of vitamin B_1, but not by the further addition of metal ions.

The picture of the combined vitamin and ion effect can best be presented by assuming that lactate, succinate, and fumarate must first be transformed into an oxidation product by reactions requiring the presence of metal ions rather than of thiamin. Following this, the further fate of the intermediate products would involve reactions in which thiamin (carboxylase) participates. The first phase in the decomposition of lactate, succinate, and fumarate would then be slow if the cells had been grown in a medium with an inadequate supply of minerals, but the rate of this oxidation should be increased by the addition of the necessary cations alone. Only when the rate of formation of intermediate substances exceeds the capacity of the carboxylase system present would the addition of thiamin have a stimulatory effect.

If, on the other hand, the rate of production of intermediate substances were high, due to the presence of sufficient enzymatic capacity for the initial reaction, the primary oxidation products would tend to accumulate in the presence of a limiting carboxylase supply. A case in point is the well known fact that pyruvic acid accumulates during the metabolism of sugar or lactate by thiamin-deficient organisms and tissues. The addition of thimain would then cause an increase in metabolic rate, due exclusively to an effect on the second phase. This scheme may be illustrated by the following diagram.

Phase 1. Limited by cation supply:

SUBSTRATE	Enzymes requiring special cations →	INTERMEDIATE PRODUCTS
(lactic acid, succinic acid, fumaric acid)		(pyruvic acid, oxaloacetic acid)

Phase 2. Limited by thiamin supply:

INTERMEDIATE PRODUCTS	Carboxylase →	FURTHER DECOMPOSITION PRODUCTS

In those cases where the rate of the first phase, although not maximal due to a cation deficiency, is nevertheless greater than the capacity of the enzymes operative in the second phase, the addition of either cations or of thiamin alone would cause an increase in the rate of oxygen consumption. However, the increase in rate of oxygen consumption due to the addition of thiamin alone can only be due to an increased

capacity for carrying out the second phase. Since, in the case of lactic acid decomposition, the rate of the second phase of the reaction can be determined directly by a study of the metabolism of pyruvic acid, the idea here developed can be tested experimentally. Fortunately, the publication of Quastel and Webley contains all the necessary data for such a comparison. These data have been assembled in Table II.

It can be seen that the predictions agree with the actual measurements. When the rate of the first phase is not altered, the effect of the addition of thiamin is identical for the oxidation of both lactate and pyruvate. This is seen to be true at both a low and high rate of the first phase.

The same effects can be shown for succinic and fumaric acids, where oxaloacetic and pyruvic acids could be expected as intermediate products. The data for succinate and fumarate are recorded in Table III.

TABLE II

Quastel and Webley's Data for the Oxidation of Pyruvic (PA) and Lactic (LA) Acids, Arranged to Show the Possibility of Interpreting the Oxidation of Lactic Acid to Occur in Two Phases—the Rate of the First Governed by the Presence of Cations (C), the Second by Thiamin (T)

Addition to deficient cells	Reaction involving cations	Reaction involving thiamin	Q_{O_2}	Difference in Q_{O_2} due to thiamin
PA	None	Slow	20	17
PA + C + T	None	Fast	37	
LA	Slow	Slow	35	17
LA + T	Slow	Fast	52	
LA + C	Fast	Slow	53	19
LA + C + T	Fast	Fast	72	

In the oxidation of succinic and fumaric acids by deficient cells the increase in rate of oxygen consumption caused by the addition of thiamin is not so high when cations are also present; *i.e.*, when phase 1 occurs at a rapid rate. A simple explanation for this discrepancy is based upon the assumption that the carboxylase system may now have become limiting. Although adequate to cope with the supply of intermediate products furnished by phase 1 when this proceeds slowly, it may not be supplemented sufficiently by thiamin addition to cause a commensurate decarboxylation of these products when phase 1 proceeds at a maximum rate. Here the comparison with the decomposition of pyruvic acid, involving a single decarboxylation, is not entirely justified because in the breakdown of succinic and fumaric acids *via* oxaloacetic acid two decarboxylation reactions occur.

As another possibility it may be assumed that the over-all metabolic rate in the presence of both cations and thiamin is actually limited by the capacity of the final oxygen-activating systems. A decision between these alternative hypotheses would rest upon the evaluation of the significance of the Q_{O_2} increments. The higher value

with fumarate favors the latter explanation, but the difference in increment for succinate and fumarate in the presence of cations is too small to make this a convincing argument.

The stimulatory effect of thiamin and metal ions on the oxidation of acetate by Quastel and Webley's organism is different from that observed with lactate, pyruvate, succinate, and fumarate. In the case of the oxidation of this fatty acid, neither the cations nor thiamin alone permit the remarkable stimulation obtained when both are added together. This situation may be explained by assuming that a preliminary reaction, in which vitamin B_1 is involved, is required prior to the formation of a

TABLE III

Quastel and Webley's Data for the Oxidation of Pyruvic (PA), Succinic (SA), and Fumaric (FA) Acids, Arranged to Show the Possibility of Interpreting the Oxidation of Succinic and Fumaric Acid to Occur in Two Phases—the Rate of the First Governed by the Presence of Cations (C), the Second by Thiamin (T)

Addition to deficient cells	Reaction involving cations	Reaction involving thiamin	Q_{O_2}	Difference in Q_{O_2} due to thiamin
PA	None	Slow	20	17
PA + C + T	None	Fast	37	
SA	Slow	Slow	30	19
SA + T	Slow	Fast	49	
SA + C	Fast	Slow	58	9
SA + C + T	Fast	Fast	67	
FA	Slow	Slow	28	17
FA + T	Slow	Fast	45	
FA + C	Fast	Slow	53	12
FA + C + T	Fast	Fast	65	

compound which can undergo dehydrogenation, a reaction for which metal ions are needed. The reactions involved in the oxidation of acetic acid may be represented by a series of steps.

$$\text{Substrate} \xrightarrow{\text{Thiamin}} \text{Intermediate product 1} \xrightarrow{\text{Metals}}$$

$$\text{Intermediate product 2} \xrightarrow{\text{Thiamin}} \text{Further decomposition products}$$

It is improbable that such a preliminary step would involve a decarboxylation of acetic acid because biochemical decarboxylations have been observed exclusively with keto acids. Since carboxylase is likely to function in the carboxylation of acids as well as in their decarboxylation, the preliminary step involved in the oxidation of acetate by these bacteria might be the linking together of two acetate molecules to form acetoacetate. This reaction may be considered as equivalent to a carboxy-

lation and hence might require carboxylase (*i.e.* thiamin). The consumption of oxygen in the decomposition of acetate by this scheme, should, therefore, be preceded by a step in which thiamin functions. The oxidation proper would involve the cooperation of the cation-requiring systems. In such a manner the observed effect of thiamin and cations on acetate oxidation by Quastel and Webley's organism could be readily harmonized with the concept that here, too, thiamin functions only as a building block of carboxylase.

Theoretical Considerations on the Rôle of Thiamin in the Metabolism of Prototheca zopfii

The experiments demonstrating a thiamin effect on the oxidation of acetate by *Prototheca zopfii* cannot as yet be interpreted in so detailed a manner. So far as is known, this alga has never been cultured in mineral-deficient media and, therefore, it is impossible to interpret the available data on the oxidation of acetate in the light of specific stepwise reactions. Whether thiamin has an *initial* or a *secondary* effect on the oxidation of this simple fatty acid by *Prototheca* must be left out of consideration for the present. At any rate, the function of thiamin in the oxidation of acetate need not be considered to be other than as a building block of carboxylase.

Krebs and Eggleston (21) have presented the hypothesis that the principal function of carboxylase in animal tissue, and also in certain higher plants, molds, and bacteria, is not directly concerned with the oxidation of pyruvate but with the preparatory reaction. The reaction, for which only indirect evidence has been presented, is a carboxylation in which oxaloacetic acid is synthesized from pyruvic acid and carbon dioxide. The oxaloacetic acid so formed is then used in the 4-carbon-dicarboxylic acid cycle of hydrogen-transporting substances known as the "Szent-Györgyi cycle." It also forms the basis of the citric acid cycle postulated by Krebs as an integral part of oxidative metabolism. Smyth (22) has presented supporting evidence for such a mode of oxidation of pyruvic acid in the case of *Staphylococcus aureus*. Using vitamin-deficient cells, Smyth found that thiamin could be replaced by the addition of a 4-carbon-dicarboxylic acid. That is, the addition of either thiamin or a compound such as oxaloacetic acid increased the rate of oxidation of pyruvic acid by vitamin-deficient cells of the staphylococcus.

Although Krebs' hypothesis for the mechanism of pyruvic acid oxidation is most attractive, its general occurrence has not been well substantiated as yet. It is to be remembered that this scheme would hardly fit in with the convincingly established fact that the enzyme carboxylase itself causes the quantitative decarboxylation of pyruvic acid. If thiamin is postulated to act principally as an enzyme system for the synthesis of oxaloacetic acid, the importance of the decarboxylation mechanism recedes entirely into the background. It is true that the mechanism for the oxidative degradation of pyruvic acid, pro-

posed by Krebs in connection with the "citric acid cycle," would make a decarboxylation unnecessary. However, there are entirely too many facts, supporting the view that decarboxylations, too, are of primary importance in metabolism, to permit the discard of this mechanism.

In view of the significant position pyruvic acid holds as an intermediate product in the metabolic processes in general, experiments were conducted to determine whether the findings of Smyth on the substitution for thiamin of members of the 4-carbon-dicarboxylic acid are applicable in the case of *Prototheca*. The results of experiments in which succinic, fumaric, or malic acids were added in catalytic amounts to suspensions of vitamin-deficient cells oxidizing pyruvic acid do not support Krebs' postulate. In no case did the addition of these acids result in stimulation of the rate of oxygen utilization. The presence of thiamin seems essential to permit the oxidation of pyruvic acid by *Prototheca zopfii*. This observation tends to throw additional doubt on the general validity of the citric acid cycle as the only mechanism for the oxidative decomposition of pyruvic acid.

The results obtained with *Prototheca*, therefore, lead to the conclusion that the function of thiamin as a building block for carboxylase implies the regular occurrence of genuine decarboxylation reactions. Whether these must be considered as straight decarboxylations or as oxidative decarboxylations remains for further investigation. In the event that an oxidative decarboxylation is involved in the metabolism of *Prototheca*, the combined function of carboxylase and an additional hydrogen acceptor would, of course, be indicated (see, *e.g.*, Long and Peters (23) and Peters (24)). However, the main argument put forward here to stress the importance of thiamin (carboxylase) as a decarboxylating agent, remains unaltered. The experimental results definitely indicate that the need of thiamin by *Prototheca* is immediately concerned with the decomposition of α-keto acids.

STUDIES ON THE OXIDATION OF GLYCOLIC ACID BY PROTOTHECA ZOPFII

Barker's investigation of the mechanism involved in the synthesis of carbohydrate from simple fatty acids, such as acetic, by *Prototheca* resulted in a deadlock because any scheme that could be postulated for the oxidative degradation of acetate involved acids as intermediate products which the organism was incapable of utilizing as substrates. When the alga was found capable of oxidizing such acids, and conditions could be specified under which a study of their metabolism is possible, an experimental reexamination of conceivable mechanisms for the decomposition of simple fatty acids became feasible.

An investigation of the mechanism involved in the oxidative assimilation of a utilizable substrate must of necessity consider two important phases of the process—the decomposition of the substrate and the synthesis of cell material.

The theoretical basis upon which the subsequent experiments were conducted will be developed in the following section.

Theoretical Considerations

Although it has long been known that a definite relationship exists between the breakdown and assimilation of foodstuffs, oxidation and synthesis have been considered as more or less separate reactions in which the assimilation reactions, giving rise to products possessing greater free energy than that of the substrate, can acquire energy from the simultaneously occurring dissimilation process in which the free energy decreases. As long as the amount of energy liberated in the catabolic process is in excess of that required by the anabolic reactions, energetically coupled reactions of this type are quite possible on the basis of thermodynamic considerations.

A clearer understanding of the mechanism involved in biochemical processes concerned with the breakdown of foodstuffs has resulted in the possibility of also interpreting assimilatory processes as chemically intelligible step-reactions, not materially different from those operative in catabolism. The general principles of the mechanisms involved in catabolic processes may be briefly summarized. Such a biochemical process can be considered to consist of a chain of individual step-reactions, each step constituting a simple, chemically understandable type of reaction whose common property is the transference of hydrogen from one constituent to another. Each step is a thermodynamically exergonic reaction.[2] This attempt to interpret anabolic and catabolic processes as being closely intermingled chemical reactions does not deny the existence of energetic relations but aims at elucidating the chemical mechanisms for energy transfer which would obviate the necessity of considering the energetic coupling of two sets of chemical reactions which are not known to possess any material link.

The first broad attempts to formulate a chemical mechanism of energy transfer were those of Kluyver (26, 27) who opened the way for further advances. Although the proposed "mechanisms" may no longer be considered as tenable or probable, the fundamental principle remains a valuable working hypothesis. This embodies the idea that, in the course of the degradation of the substrate, intermediate products arise which can be exergonically converted into the products of assimilation. Thus the structure of the intermediate products is all important in the process of synthesis while the gradual degradation of the initial substrate down to the point of the formation of the essential product plays no *direct* rôle in the assimilation process itself, but is merely the preparation of the essential building block for synthesis. Giesberger (3) developed this idea in his concept of "chip-respiration" in which carbon dioxide and water formed during the oxidative process are regarded as "chips" or waste products of the main reaction. This concept of oxidative assimilation, therefore, places the main emphasis upon the structure of the raw material. Any compound which could be postulated to give rise to an intermediate product possessing the characteristics required to enable it to serve as an initial substrate for exergonic synthetic reactions, would serve the purpose of synthesis to the extent to which it

[2] The term "exergonic" is used in preference to "exothermic" in accordance with Coryell's proposal (25).

could contribute the necessary intermediates. Although little work has been done on a study of the mechanisms of assimilation there are indications in the literature which lend strong support to the above views.

Clifton and Logan (28), investigating the oxidative assimilation of various compounds by *Escherichia coli*, found that the oxidation of lactic acid proceeds in a manner similar to that of pyruvic acid. The oxidation of these two compounds can be represented by the following equations:

$$CH_3COCOOH + 1.5\ O_2 \rightarrow 2CO_2 + H_2O + (CH_2O)^*$$
$$CH_3CHOHCOOH + 2O_2 \rightarrow 2CO_2 + 2H_2O + (CH_2O)$$

*(CH_2O) is here used to represent a compound having the empirical formula of a carbohydrate.

It is a general observation that, in the course of its oxidation, lactate passes through the stage of pyruvate by the loss of two atoms of hydrogen. Therefore, it may be assumed that the same intermediate products would arise in the oxidation of each of these two acids. This is substantiated by the above equations which show that *Escherichia coli* converts one-third of the total carbon of both lactate and pyruvate into primary assimilation products, despite the fact that the concomitant oxygen consumption is materially greater with lactate than with pyruvate. This implies that simple energetic considerations fail to account satisfactorily for the situation because more energy becomes available in the oxidation of lactate than in the oxidation of pyruvate. Conversely, the formation of carbohydrate storage products from pyruvate would require more energy than from lactate. If, therefore, catabolic and anabolic processes represented merely two types of reactions, coupled energetically, then the extent of assimilation should be appreciably greater with lactate than with pyruvate. The very fact that equal portions of both substrates appear in the form of assimilation products thus strongly supports the contention that the nature of special intermediate products is far more important than energetic relationships.

Clifton and Logan also found that the same amount of assimilation occurs during the oxidation of succinic as during the oxidation of fumaric acid, although the free energy of succinate is greater than that of fumarate.

The results of Doudoroff's (29) studies on the oxidation of various substances by *Pseudomonas saccharophila* have shown that this organism carries out an oxidative assimilation in much the same manner as that described by Barker (2), Giesberger (3), and Clifton and Logan (28) for other microorganisms. The broader studies of Doudoroff showed that sugars, both hexoses and disaccharides, as well as lactic and pyruvic acids are respired with the complete oxidation of one-third of the substrate and the assimilation of two-thirds. The reaction for each substrate could be represented by a simple stoichiometric relationship, as shown by the following equations:

Pyruvic acid

$$C_3H_4O_3 + 0.5\ O_2 \rightarrow CO_2 + 2(CH_2O);$$

Lactic acid

$$C_3H_6O_3 + O_2 \rightarrow CO_2 + H_2O + 2(CH_2O);$$

Glucose

$$C_6H_{12}O_6 + 2O_2 \rightarrow 2CO_2 + 2H_2O + 4(CH_2O);$$

Sucrose

$$C_{12}H_{22}O_{11} + 4O_2 \rightarrow 4CO_2 + 3H_2O + 8(CH_2O).$$

From these equations it is apparent that the thermodynamic efficiency with which these substances are assimilated increases in the order carbohydrate, lactate, pyruvate, and that the actual extent of synthesis is directly dependent upon the number of carbon atoms contained in each. Here again, a comparison of the energy released in the oxidation of carbohydrates, pyruvate, and lactate indicates that synthesis is consequently dependent on a chemical mechanism concerned with the intermediate products of metabolism rather than on a purely energetic coupling between separate reactions of oxidation and of synthesis.

By the demonstration that pyruvic acid could be isolated as an intermediate product in the oxidation of glucose, Doudoroff was able to support the indications, presented in the equations for the oxidative assimilation of the sugars, that the metabolism of the mono- and disaccharides would proceed by way of the three-carbon compounds.

Lactate and Pyruvate Oxidation by Prototheca zopfii

The above examples clearly show that a comparison of the decomposition of structurally related compounds has, in the case of *Escherichia coli* and *Pseudomonas saccharophila*, supported the idea that a chemical mechanism is operative in processes of oxidative assimilation. Since previous experiments on the oxidative metabolism of *Prototheca zopfii* had not included such structurally related compounds, a comparative study was made of the oxidation of pyruvic and lactic acids by this colorless alga.

The oxidation of these two acids by *Prototheca* was found to correspond to that for *Escherichia coli* and to differ markedly from that for *Pseudomonas saccharophila*.

During the rapid oxidation of pyruvic acid by *Prototheca*, the ratio of carbon dioxide production to oxygen consumption was found to be between 1.20 and 1.37 with an average of 1.31 for four experiments. The theoretical value required by the equation is 1.33. The R.Q. decreased gradually to that found for autorespiration at the time when the acid was completely used up. Data from a number of experiments indicate that autorespiration is completely suppressed during the oxidation of pyruvic acid. The R.Q. for lactate oxidation was found to be approximately 1.0, which also agrees with the theoretical.

Again, the above experimental results fit in much better with a chemical than with a strictly energetic concept of the mechanism for an assimilatory process. The equations show that the conversion of lactate to pyruvate may well proceed without being accompanied by the formation of reserve materials. It is even possible to postulate further a rational pathway for the subsequent

decomposition of pyruvic acid which is entirely in harmony with the experimental results.

The experiments on the influence of thiamin on pyruvic acid decomposition by *Prototheca zopfii* make it logical to accept the occurrence of a decarboxylation mechanism in the oxidation of pyruvic acid. In view of the complete lack of alcohol production by *Prototheca* under anaerobic conditions, the most likely fate of pyruvic acid would appear to be an oxidative decarboxylation. This would result in the production of equimolar amounts of carbon dioxide and acetic acid and would involve the utilization of one-half mol of oxygen per mol of pyruvic acid:

$$CH_3COCOOH + 0.5\ O_2 \rightarrow CH_3COOH + CO_2.$$

The oxidation of acetic acid has been extensively investigated by Barker, and can be expressed by the equation:

$$CH_3COOH + O_2 \rightarrow (CH_2O) + CO_2 + H_2O$$

The sequence of stages in the oxidation of pyruvic acid could then be represented by a summation of these two equations. This yields a final equation identical with that experimentally determined.

Similarly the consecutive steps for the oxidation of lactic acid could be formulated as follows:

$$\begin{array}{ll} (1) & CH_3CHOHCOOH + 0.5\ O_2 \rightarrow CH_3COCOOH + H_2O \\ (2) & CH_3COCOOH \quad + 0.5\ O_2 \rightarrow CH_3COOH + CO_2 \\ (3) & CH_3COOH \quad\quad + O_2 \rightarrow (CH_2O) + CO_2 + H_2O \\ \hline & CH_3CHOHCOOH + 2O_2 \rightarrow (CH_2O) + 2CO_2 + 2H_2O \end{array}$$

Again, the final equation is in complete agreement with the one derived from experimental data. The first two steps are simple reactions for which much evidence had been accumulated in a number of instances and with a variety of organisms. These steps may be considered as elementary ones whose intimate mechanism can be investigated only by special enzyme studies. This is, however, not true for the third state, which not only leaves the question of intermediate products in the acetate oxidation unanswered, but which also "hides" the mechanism of the assimilation process proper. It is, therefore,apparent that the reasons which prompted the postulation of these series of reactions for the decomposition of pyruvic and lactic acids inevitably led to the desire to study the mechanism of acetate oxidation in more detail.

Experiments on the Oxidation of Glycolic Acid by Prototheca zopfii

Of the two main pathways for the decomposition of acetic acid outlined in the introduction the one involving the successive oxidation to glycolic and glyoxylic acids appears to be the simpler. If the oxidative metabolism of

acetic acid were to proceed through glycolic acid as an intermediate, an investigation of the oxidation of glycolic acid could be expected to show that its decomposition occurs in a manner similar to that described for acetic acid, and an oxidative assimilation as a result of the decomposition of glycolic acid could be expected which would be even more spectacular than that found in the case of acetic acid. The formation of assimilation products from such simple compounds would involve only the methylene group, the —COOH group being lost. The former group is more oxidized in the case of glycolic acid than it is in acetic acid and therefore more nearly conforms to the empirical formula of the assimilated material. Therefore, the same proportion of assimilation might occur per mol of glycolic as per mol of acetic acid, while being accompanied by an oxygen consumption of only one-half the magnitude. This situation is similar to that discussed above in the comparison of the oxidation of lactic and pyruvic acids.

On the basis of simple stoichiometric relationships, the equation for glycolic acid might be represented as:

$$CH_2OHCOOH + 0.5\ O_2 \rightarrow CO_2 + H_2O + (CH_2O).$$

Since the energy obtainable from the oxidation of acetic acid is twice the amount obtainable from glycolic acid, a demonstration that the oxidative assimilation of glycolic acid could be represented by the above equation would effectively rule out the occurrence of coupled catabolic and anabolic reactions in a strictly energetic sense.

The addition of 0.01 mM of glycolic and 0.01 mM of glyoxylic acid to aliquot suspensions of non-proliferating cells of *Prototheca zopfii* resulted in an increase in the rate of oxygen consumption over that of control suspensions. The higher respiratory rates show *Prototheca* to be capable of utilizing the two acids postulated as occurring as intermediates in the Bernhauer scheme for the oxidation of acetic acid.

Quantitative Studies on the Oxidation of Glycolic Acid

Attempts to establish a balanced equation for the oxidation of glycolic acid by *Prototheca* resulted in the unexpected observation that the amount of oxygen consumed was far in excess of the amount of oxygen that would be required to bring about a *complete* combustion of this substrate.

The complete oxidation of 0.01 mM of glycolic acid, in agreement with the following equation:

$$C_2H_4O_3 + 1.5\ O_2 \rightarrow 2CO_2 + 2H_2O$$

requires the uptake of 336 mm.^{3} of oxygen.

The addition of 0.01 mM of glycolic acid to suspensions of cells having a moderately high rate of autorespiration, resulted in the utilization of 1.89 times

the amount of oxygen needed for complete combustion. If the oxygen consumed is uncorrected for autorespiration, this value is increased to 2.44 times. The addition of 0.01 mM of glycolic acid to suspensions of cells having a lower rate of autorespiration resulted in a value of 1.54 times corrected for autorespiration and 1.85 times if no correction is made.

The addition of different amounts of glycolic acid to several equal portions of cell suspensions showed conclusively that in all cases the oxygen consumption attained values greatly in excess of those required for complete oxidation of the added substrate. Data from nine different experiments, in which suspensions of cells having quite different rates of autorespiration were used, show that the amount of oxygen consumed during the oxidation of equal amounts of glycolic acid varied from 1.34 to 3.57 times the amount of oxygen that would be required for complete oxidation of this substrate. Each of these values is that obtained after correction for autorespiration of control suspensions.

Explanation of the Excess Oxygen Consumption in the Oxidation of Glycolic Acid

The unexpected oxygen consumption upon the addition of glycolic acid to suspensions of non-proliferating cells of *Prototheca* indicates that this acid must exert a catalytic effect on the metabolism of this organism.

To test this hypothesis, aliquot portions of heavy suspensions of washed cells were suspended in phosphate buffer solution at pH 4.0. Each portion was placed in shallow layers in rotating bottles and incubated at 30°C. for 12 hours. One portion was given no added substrate but allowed to carry on endogenous respiration only, resulting in the production of "starved" cells. During the incubation period, small amounts of glycolic acid were added to the second portion at intervals of sufficient duration to insure that meanwhile the previous addition had been completely oxidized.

The autorespiration of the normal "starved" and the glycolic acid-treated organisms was measured at the end of the incubation period. The rate of respiration of the organisms which had previously been oxidizing glycolic acid was found to be but 72.2 per cent of the rate of the "starved" cells. This observation indicates that the reserve cell material had been oxidized more rapidly in the presence of glycolic acid than in its absence, and that the residue remaining available for autorespiration was sharply reduced in the experimental organisms.

Addition of 0.001 mM of glycolic acid to 2.0 ml. suspensions of both types of cells resulted in a rapid uptake of oxygen. The cells previously treated with glycolic acid consumed a volume of oxygen, over that for autorespiration, equal to 1.75 times the amount needed for complete combustion of the acid added. In contrast, the normally starved cells utilized an amount of oxygen equal to 2.47 times that needed for complete oxidation. This may also be

taken as an indication that the oxidation of glycolic acid by non-growing cells of *Prototheca* causes the oxidation of cell material in addition.

In two experiments carried out with cells having a very high rate of autorespiration the addition of 0.001 mM of glycolic acid resulted in the uptake of an amount of oxygen, corrected for autorespiration, equal to 3.57 and 3.22 times the amount necessary for total combustion of the acid. This may be compared with an average of 1.92 for four experiments carried out with cells having a more "normal" rate of autorespiration.

That the oxidation of glycolic acid by *Prototheca* does affect the autorespiration is further evidenced by the results obtained in experiments in which two successive additions of 0.001 mM of glycolic acid were made to suspensions of cells having a high rate of autorespiration. The first addition resulted in the uptake of a quantity of oxygen equal to 3.16 times the amount needed for complete oxidation of the acid. An equal amount of the acid added to the suspension at the time the first portion was completely decomposed caused an uptake of oxygen equal to but 2.08 times that necessary for total oxidation. The addition of 0.002 mM of glycolic acid to the suspensions resulted in the uptake of the same amount of oxygen as when the substrate was added in two equal portions.

To determine whether glycolic acid has an effect on the ability of the cells to carry out an oxidative assimilation, and also to test if glycolic acid could cause an oxidation of newly assimilated cell materials, experiments were carried out in which the oxygen consumption of aliquot suspensions of cells treated in four different ways, was compared. To one sample 0.001 mM of glycolic acid alone was added; another, initially treated in the same way, was supplied with 0.01 mM of acetate after the glycolate had been consumed; both acids, in the above stated amounts, were added simultaneously to the third portion; and in the fourth suspension the glycolate was introduced following the decomposition of an initial supply of 0.01 mM of acetate. The data so obtained are presented in Fig. 6.

The addition of glycolic acid alone to the suspensions of cells used for these experiments caused an oxygen uptake of 1.34 times the amount required for the complete oxidation of the acid. The subsequent addition of acetic acid resulted in the consumption of 1 mol of oxygen per mol of acetate which is identical with the values obtained for the oxidative assimilation of this substrate by "normal" cells. The suspension to which both acids were simultaneously added, showed a total oxygen uptake of the same amount as the total consumed by the suspension to which glycolic acid was added prior to the addition of acetic acid. The results of these two experiments indicate that glycolic acid has no effect on the ability of *Prototheca* to assimilate a typical substrate. In the case in which glycolic acid was added after the suspension had completed its oxidative assimilation of acetic acid alone, the addition of

0.001 mM of glycolic acid resulted in the uptake of 1.64 times the amount of oxygen required for the complete oxidation of the glycolic acid. A comparison of this value with that obtained for the oxidation of glycolic acid by an aliquot portion of cells which had not oxidized acetic acid (1.34) indicates that glycolic acid caused the oxidation of some of the cell material assimilated during the oxidation of acetic acid and may be considered as additional evidence that the

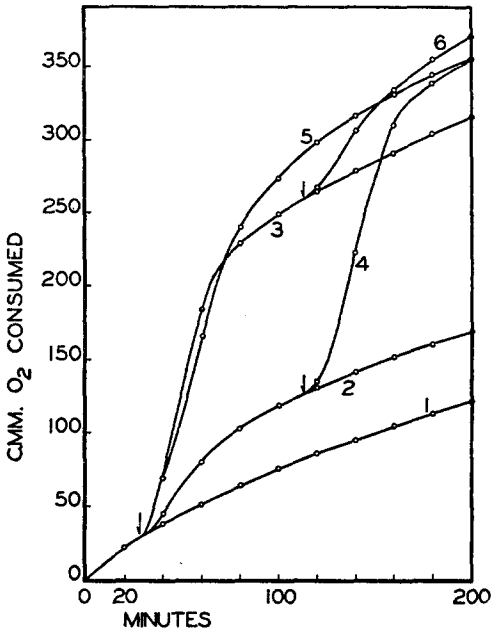


FIG. 6. Influence of glycolic acid on the oxidative assimilation of *Prototheca zopfii*: (1) autorespiration; (2) 0.001 mM glycolic acid; (3) 0.01 mM acetic acid; (4) 0.01 mM acetic acid added after decomposition of 0.001 mM glycolic acid; (5) glycolic acid and acetic acid added simultaneously; (6) 0.001 mM glycolic acid added after decomposition of 0.01 mM acetic acid. Arrows indicate time at which substrates were added.

oxidation of glycolic acid by *Prototheca* has an effect on the autorespiration of these cells.

Comparison of the Action of Glycolic Acid with That of Known Biochemical Catalysts

With the exception of glycolic acid, all the substrates so far tested with *Prototheca zopfii* are oxidized in such a manner that a simple stoichiometric relationship exists between the number of substrate molecules disappearing, and the number of molecules of oxygen consumed and of carbon dioxide pro-

duced. These relationships permit the formulation of simple, balanced equations, representing the over-all result of the metabolic activity. Also, in all cases, with the exception of glycolic acid oxidation, the quantity of oxygen consumed appears to be a definite fraction of that required for complete oxidation of the substrate.

The unexpectedly high values obtained for oxygen consumption during the oxidation of glycolic acid can be explained only on the basis that this substance functions as a "respiratory catalyst." Thus it becomes attractive to compare the action of glycolic acid with that of substances known to be biochemical catalysts.

Since the general effects of glycolic acid on the oxidative metabolism of *Prototheca zopfii* are so very similar to those which led to the proposal of the Szent-Györgyi (30–32) and Krebs cycles (33, 34) the question arises whether it is possible that glycolic acid also might participate in a similar catalytic cycle. The two previously postulated cycles depend largely upon the occurrence of related hydroxy and keto acids. Such relations are readily conceivable for the system glycolic-glyoxylic acids:

$$\begin{array}{lcl} \mathrm{H} & & \mathrm{H} \\ \mathrm{HCOH} & \overset{-2\mathrm{H}}{\rightleftharpoons} & \mathrm{C{=}O} \\ | & \overset{+2\mathrm{H}}{} & | \\ \mathrm{COOH} & & \mathrm{COOH} \end{array}$$

Therefore, the possibility of a glycolic acid-glyoxylic acid cycle, functioning as a simple type of hydrogen-transporting system, is far from remote.

Against this interpretation is, however, the fact that glyoxylic acid is respired in a "normal" manner by *Prototheca*. This substrate does not appear to possess any catalytic properties such as it would demonstrate if it were a participant in a cyclic reaction.

While this observation makes it, therefore, unlikely that the catalytic effect of glycolic acid on cellular respiration by *Prototheca* is due to its action as a factor of a simple hydrogen-transporting system, a more complicated manner of its participation in metabolism may be considered. The function of glycolic acid in a mechanism similar to that postulated for oxaloacetic acid in the Krebs cycle would imply that glycolic acid is condensed with some oxidizable substance resulting in the synthesis of a compound which is more easily oxidizable than the metabolite itself. The nature of this type of mechanism as applied to glycolic acid is not clear. It is theoretically possible that two mols of glycolic acid could couple to form malic acid in a manner similar to the postulated synthesis of succinic acid from two mols of acetic acid in the Thunberg scheme. However, the addition of malic acid to suspensions of non-proliferating cells of *Prototheca* does not produce a catalytic effect as would be expected if this substance were to arise from glycolic acid "condensation."

The observed action of glycolic acid does suggest an interplay of this substance with some oxidizable cell constituent. However, the nature of the latter is completely unknown and therefore further speculation at this time would seem futile.

It is impossible to evaluate the available quantitative data to determine the relation between glycolic acid used, oxygen consumed, and carbon dioxide produced because it is impossible to separate the gaseous exchange due to the oxidation of glycolate from that arising as a result of the induced oxidation of cell material. For a detailed discussion of the general difficulties encountered in evaluating the portion of metabolism to be ascribed to autorespiration, reference is made to the work of Barker (2), Doudoroff (29), and Thomas (35).

All that can be definitely concluded from the present studies on the oxidation of glycolic acid is that this substance acts in a catalytic capacity and, therefore, the mechanism of acetate oxidation does not go through glycolic acid as an intermediate.

The discovery that *Prototheca* can utilize substituted acids has made possible an investigation of the intermediate stages of acetate oxidation. However, the first approach based on Bernhauer's scheme for acetate degradation, yielded information which makes it necessary to discard this as a likely pathway. With the mechanisms proposed for acetate breakdown thus restricted, studies on other possible intermediates are necessary. Preliminary investigations of one of the condensation reactions of acetate, that of Thunberg, have yielded certain results which indicate this scheme also to be unlikely. By elimination, therefore, the most profitable mechanism for acetate degradation remaining for future investigation is that of a condensation reaction with the formation of acetoacetate and its subsequent oxidation.

SUMMARY

The metabolism of *Prototheca zopfii* was investigated in an attempt to establish the specific function of its growth factor, thiamin. A study of the oxidative decomposition of various substrates by this organism demonstrated that the addition of catalytic amounts of thiamin to vitamin-deficient cells causes a pronounced stimulation in the rate of oxygen utilization during the degradation of certain compounds.

The phosphoric ester of thiamin is known to be the prosthetic group of carboxylase. The fact that this enzyme is involved in the decomposition of pyruvic acid suggested that this α-keto acid might be an important intermediate product in the metabolism of *Prototheca*. Pyruvic acid, however, was not included in the list of organic substances which Barker had reported as utilized by this alga. Barker's observations were confirmed, but subsequent experiments led to serious doubts as to the validity of his interpretation. Further investigations resulted in the establishment of environmental condi-

tions which permit this alga to readily decompose pyruvic acid, as well as nearly all other organic acids tested. This can be accomplished by providing a millieu of sufficiently low pH to insure the presence of undissociated acid molecules.

The stimulatory effect on the rate of oxygen consumption, caused by the addition of minute amounts of thiamin to suspensions of vitamin-deficient cells of *Prototheca* respiring pyruvic acid, indicates that the presence of thiamin results in the synthesis of enzyme systems which are involved in the decomposition of pyruvic acid.

Experimental data on the oxidation of pyruvic acid and other organic compounds are discussed in the light of various hypotheses which have been advanced concerning the rôle of carboxylase in the decomposition of pyruvic acid. The conservative conclusion which can be drawn from the available information is that there appears to be no justification for a belief that thiamin and carboxylase are functional in biochemical reactions other than in decarboxylation and carboxylation processes.

The discovery of the ability of *Prototheca* to utilize substituted and dicarboxylic acids led to further studies on themechanism of oxidative assimilation. The results of these investigations are in agreement with those of Clifton and Logan, and of Doudoroff, and indicate the existence of a relatively simple chemical mechanism of assimilation rather than of a strictly energetic coupling of catabolic and anabolic reactions.

A consideration of possible mechanisms for the oxidative assimilation of pyruvic and lactic acids indicates acetic acid as the most likely starting point for the assimilatory process proper.

Experimental investigations of the mode of acetate breakdown began with studies on the oxidation of glycolic acid. This substance is shown to be an oxidation catalyst in the metabolism of *Prototheca zopfii*. The exact nature of the catalytic function has not yet been determined.

It is a great satisfaction to be able to thank Dr. C. B. van Niel for suggesting the present study and for the stimulating advice, the sagacious criticism, and the encouragement which he gave so unstintingly during the time it was my privilege to work under his inspiring direction. In addition, I am very much indebted to my associates and to various members of the staff of the Hopkins Marine Station for their numerous courtesies and willing assistance at all times.

LITERATURE CITED

1. Barker, H. A., *J. Cell. Comp. Physiol.*, 1935, **7**, 73.
2. Barker, H. A., *J. Cell. and Comp. Physiol.*, 1936, **8**, 231.
3. Giesberger, G., Beiträge zur Kenntnis der Gattung *Spirillum Ehgb.*, Dissertation Utrecht, 1936.
4. Clifton, C. E., *Enzymologia*, 1937, **4**, 246.

5. Clifton, C. E., *Nature*, 1937, **140**, 318.
6. Bernhauer, K., Die oxydativen Gärungen, Berlin, Springer, 1932.
7. Thunberg, T., *Skand. Arch. Physiol.*, 1920, **40**, 1.
8. Anderson, E. H., *J. Gen. Physiol.*, 1944–45, **28**, 287.
9. Janke, A., *Österr. Bot. Z.*, 1929, **78**, 97.
10. Lwoff, A., *Zentr. Bakt., 1- Abt., Orig.*, 1934, **130**, 498.
11. Hills, G. M., *Biochem. J.*, 1938, **32**, 383.
12. Meyerhof, O., *Ergebn. Physiol.*, 1937, **39**, 10.
13. Warburg, O., and Negelein, E., in Uber die katalytischen Wirkungen der lebendigen Substanz, Berlin, Springer, 1928, especially p. 368.
14. Harden, A., Alcoholic fermentation, London, Longmans, Green and Co., 4th edition, 1932, especially 95 .
15. Platt, B. S., and Lu, G. D., *Biochem. J.*, 1939, **33**, 1525.
16. Peters, R. A., *Biochem. J.*, 1936, **30**, 2206.
17. Peters, R. A., *Biochem. J.*, 1937, **31**, 2240.
18. Quastel, J. H., and Webley, D. M., *Biochem. J.*, 1941, **35**, 192.
19. Quastel, J. H., and Webley, D. M., *Biochem. J.*, 1942, **36**, 8.
20. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, 1941, **35**, 676.
21. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, 1940, **34**, 1383.
22. Smyth, D. H., *Biochem. J.*, 1940, **34**, 1598.
23. Long, C., and Peters, R. A., *Biochem. J.*, 1939, **33**, 759.
24. Peters, R. A., *Nature*, 1939, **146**, 387.
25. Coryell, C. D., *Science*, 1940, **92**, 380.
26. Kluyver, A. J., The chemical activities of micro-organisms, University of London Press, 1931.
27. Kluyver, A. J., *Arch. Mikrobiol.*, 1930, **1**, 181.
28. Clifton, C. E., and Logan, W. A., *J. Bact.*, 1939, **37**, 523.
29. Doudoroff, M., *Enzymologia*, 1940, **9**, 59.
30. Szent-Györgyi, A., *Z. physiol. Chem.*, 1935, **236**, 1.
31. Szent-Györgyi, A., *Z. physiol. Chem.*, 1936, **244**, 105.
32. Szent-Györgyi, A., in Perspectives in biochemistry, (J. Needham and D. E. Green, editors), Cambridge University Press, 1937, 165.
33. Krebs, H. A., and Johnson, W. A., *Enzymologia*, 1937, **4**, 148.
34. Krebs, H. A., in Advances in enzymology and related subjects of biochemistry, (F. F. Nord and C. H. Werkman, editors), New York, Interscience Publishers, Inc., 1943, **3**, 191.
35. Thomas, J. O., The anaerobic carbohydrate metabolism of *Tetrahymena geleii*, Dissertation, Stanford University, 1943.