TERMINAL RESPIRATORY PATTERNS IN MICROORGANISMS[1]

SAMUEL J. AJL

Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Missouri

In one form or another the general topic of terminal biological oxidation has been reviewed many times. In each case, however, animal tissues were emphasized and only brief reference was made to microorganisms. In this discussion the reverse will be attempted. Although a great number of the basic facts still remain obscure, enough progress has been made of late to warrant a comprehensive evaluation of the data thus far accumulated. A complete survey of the literature will not be attempted, but rather an effort will be made to evaluate critically certain reports and leave to the abstracting journals the compilation of references. It is likewise beyond the province of this discussion to evaluate the data that deal with the initial fermentative processes of substrate. This review will be concerned with the enzymatic reactions which affect the aerobic cleavage of organic molecules to carbon dioxide, the *main* end product of terminal respiration.

From what we know of the chemical constitution of most cells, it is to be expected that those interactions of cell constituents by which the organism obtains the energy necessary for continued existence should exhibit certain characteristics of continuity and recurrence, as does the cell itself. The utilization of foodstuffs by the cell frequently involves a cyclic chain of chemical transformations in which certain cell constituents, usually present in small and apparently constant amounts, facilitate the transformation of larger quantities of other metabolites in reactions releasing energy, or leading to the formation of the actual cell itself. In the past years such a cyclic series of reactions has been proposed for the mechanism of oxidation of carbohydrate in some tissues; more specifically, for the oxidation of pyruvic acid in voluntary musculature. This scheme, the so-called citric acid cycle, or the tricarboxylic acid cycle, was proposed by Krebs and Johnson, 1937 and Krebs, 1940. The tricarboxylic acid cycle is the only mechanism which has received widespread consideration as an explanation of the terminal chemical reactions involved in biological oxidation yielding CO_2 and water, and the view is expressed that data which speak against the occurrence of this cycle in some cells are dismissed as having little or no significance (see, for example, Ratner and Racker, 1950). The fact remains that although the cycle is well established in animal tissues, its occurrence in bacteria, for example, is still the subject of much investigation and some controversy.

The evidence and experimental proof for the Krebs cycle in animal tissues has been reviewed by Krebs (53), Wood (111), and Krebs (54), and need not be discussed here. Before describing in detail the experimental evidence for and

[1] This review stems, in part, from the author's contribution at the Symposium on Bacterial Respiration held at the Annual Meeting of the Society of American Bacteriologists, at Chicago, Ill., May, 1951.

against the participation of a tricarboxylic acid cycle in bacteria, molds and yeasts, several important theoretical contributions recently made in this field and presumably applicable to a number of different tissues will be discussed first.

GENERAL CONSIDERATIONS

A schematic presentation of the tricarboxylic acid cycle as originally formulated by Krebs, is shown in Fig. 1.

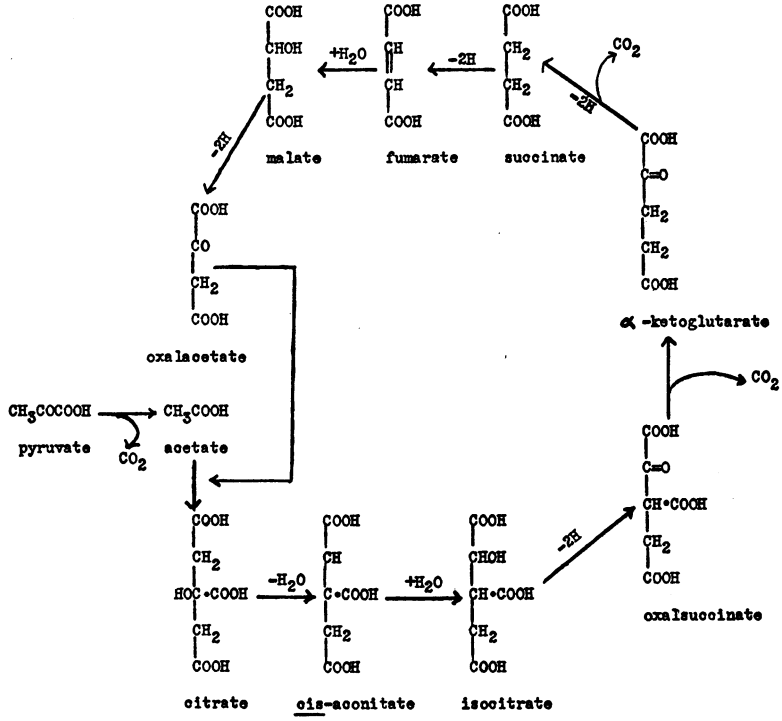


FIG. 1. The Tricarboxylic Acid Cycle

Wood *et al.* (112) showed that isotopic carbon, introduced as carbon dioxide together with pyruvate, led to the formation of α-ketoglutarate, which contained isotopic carbon only in the carboxyl group next to the keto group. On these grounds they excluded citrate as an intermediate. If citrate were an intermediate, they argued, passage through a symmetrical molecule (like citrate) would lead to the formation of α-ketoglutarate containing isotopic carbon in both carboxyls. The correctness of this reasoning was recently questioned on theoretical grounds by Ogston (77). He pointed out that this argument would be invalid if there was a "three-point contact" between substrate and enzyme. Under these conditions citric acid could be asymmetric in an enzyme-substrate complex, and could lead to an asymmetric distribution of isotope in the carboxyl groups of α-ketoglutaric acid.

The Ogston hypothesis is a logical extension of the "poly-affinity theory" of Bergmann and co-workers (17, 18), proposed some 15 years ago to explain the antipodal specificity of certain proteolytic enzymes. Wilcox *et al.* (110) believe that antipodal specificity of an enzymatic reaction demands the existence "of (1) three distinct and specific points of interaction between enzyme and substrate, and (2) some other condition such as steric hindrance, directed forces, or another point of interaction." The latter condition can be visualized as a restriction such that the catalytic complex can be formed on only one side of the plane that is defined by the three primary points of interaction.

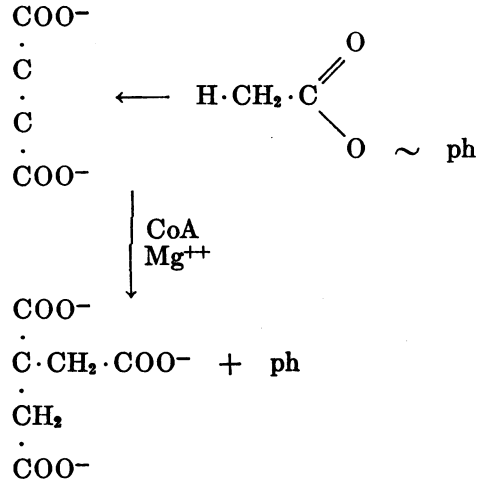
Experimental evidence in support of Ogston's theory was provided by Potter and Heidelberger (79), who isolated radioactive citric acid from a fortified rat liver homogenate into which $C^{14}O_2$ had been introduced. This labeled citric acid was now enzymatically converted by another rat liver homogenate, in the presence of arsenite, into α-ketoglutaric acid which contained all the activity in the α-carboxyl group. The asymmetric distribution of the isotope in the carboxyl groups presumably arose from an asymmetric configuration about the tertiary atom of the citric acid. Very recently Wilcox *et al.* (110) succeeded in chemically synthesizing asymmetric sodium citrate-1-C^{14} by the reaction of $NaC^{14}N$ with the levorotatory γ-chloro-β-carboxy-β-hydroxybutyric acid. The labeled citrate was carefully characterized and subjected to the enzymatic action of a fortified rat liver homogenate. The α-ketoglutarate which was isolated contained isotope only in the γ-carboxyl group. These data, coupled with those recently reported by Stern and Ochoa (95) and Novelli and Lipmann (72), showing the condensation of an acetyl derivative and oxalacetate to citrate, strongly suggest that this C_6 acid may actually be the first condensation product. In addition, the enzymatic formation of citric acid with C^{14}-labeled oxalacetate and acetate was recently reported by Lorber *et al.* (60).

The tricarboxylic acid cycle starts by the condensation of an "active" acetyl group with oxalacetate to form citrate. The active acetate can be formed by the oxidative decarboxylation of pyruvate, by the oxidation of fatty acids, by the breakdown of β-keto acids and from acetate under aerobiosis. Stern and Ochoa (95) described the synthesis of citrate anaerobically with soluble enzyme preparations from pigeon liver, in the presence of either acetate or β-keto acids, adenosine triphosphate and oxalacetate. In addition, this system required Mg^{++} or Mn^{++} and the pantothenic acid-containing coenzyme A recently discovered by Lipmann and coworkers (46, 70, 58). Work recently done further suggests that the active acetate may be an acetyl derivative of coenzyme A. Stadtman (92) isolated a transacetylation enzyme from extracts of *Clostridium kluyveri* which appears to catalyze the reversible transfer of the acetyl group of acetyl phosphate to coenzyme A. Chou *et al.* (28) showed the presence of transacetylase in other bacteria, e.g., *Escherichia coli* and *Clostridium acetobutylicum*, but not in animal tissues, with the possible exception of brain. Novelli and Lipmann (72) and Stern, *et al.* (97) have shown that citrate can be synthesized from acetyl phosphate and oxalacetate in the presence of bacterial transacetylase and of the condensing enzyme. (The latter appears to be widely distributed in nature since this enzyme

has been found in animal tissues, bacteria and yeast.) In addition, Mg^{++} or Mn^{++} and coenzyme A are the essential cofactors for the reaction. The condensing enzyme has recently been crystallized by Stern *et al.* (97). This enzyme is thought to catalyze the exchange of acetate from the acetyl-CoA complex to oxalacetate to form citrate plus coenzyme A. The following series of reactions (75) summarize these events.

1. Acetyl phosphate + CoA $\rightleftharpoons$ Acetyl-CoA + phosphate (Transacetylase).
2. Acetyl-CoA + oxalacetate $\rightleftharpoons$ citrate + CoA (Condensing enzyme).

The sum of these reactions, as pictured by Novelli and Lipmann (72), is,



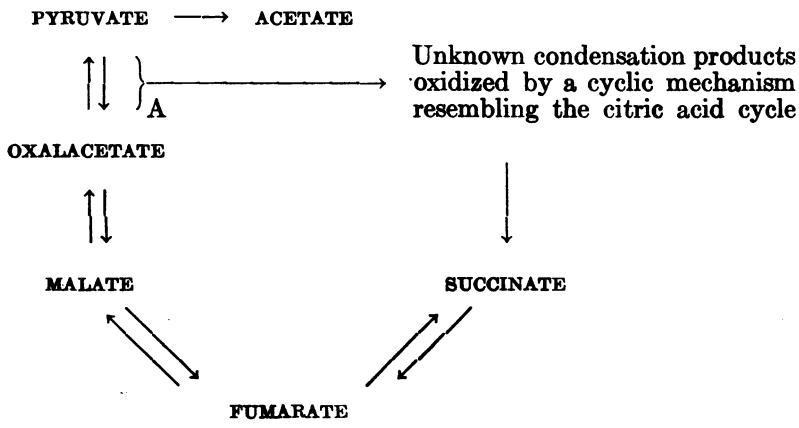
Using radioactive citrate, Stern *et al.* (97) demonstrated that reaction 2 is reversible to a limited extent.

Most of the experimental evidence supports the hypothesis that pyruvate enters into the Krebs cycle by being first oxidatively decarboxylated to an active acetyl derivative, which condenses with oxalacetate to form citrate, but if pyruvate and oxalacetate were to condense directly prior to the decarboxylation of pyruvate, a C_7 compound would have to be an intermediate. Several possible C_7 intermediates were postulated (53) and the most likely of these, oxalocitramalic acid, was actually synthesized by Martius (65) who found it completely inert in animal tissues. These findings, however, still leave unexplained several phenomena. The most important of these from the standpoint of *bacterial* metabolism are those reported by Umbreit and associates, who studied an inhibitory reaction of streptomycin on the respiration of *E. coli*. These results were interpreted on the basis of a specific inhibition of the pyruvate-oxalacetate condensation reaction on the tricarboxylic acid cycle.

Umbreit (101) first confirmed the work of Geiger (41) who showed that, following the oxidation of fumarate by a given strain of *E. coli*, there was a marked stimulation of serine oxidation. Now, if streptomycin was added during fumarate oxidation, the oxidation of serine was no longer stimulated. The same effect was obtained with threonine and with mixtures of fumarate and pyruvate. The

authors concluded that this antibiotic interfered with the formation of some intermediate which was required for the complete oxidation of serine, threonine or pyruvate. This substance presumably was oxalacetate. This work was continued by Oginsky *et al.* (76). It was found that freshly harvested *E. coli* possess an active oxalacetate decarboxylase which became much less active upon storage of the cells in distilled water in the cold. Such suspensions, however, continue to oxidize pyruvate to acetate and CO_2, and streptomycin has no effect on this oxidation. With both oxalacetate and pyruvate present as substrates, the mixture is oxidized rapidly and streptomycin strongly inhibits this oxidation. Oxalacetate as the sole substrate was slowly oxidized.

The concept of the site of streptomycin action on *E. coli*, as illustrated by Umbreit (102) is:



The hypothesis was that it acted upon reaction A, e.g., streptomycin inhibited the condensation between oxalacetate and pyruvate. In view of the fact that acetate oxidation was not inhibited by streptomycin, an oxidative decarboxylation of pyruvate could not have preceded the condensation reaction. If the experimental data warrant the conclusion these investigators have made, then here is a condensation reaction between a C_3 and C_4 compound, which has to be taken into consideration when the final conclusion is reached as to what the initial condensation product during pyruvate oxidation really is.

The system responsible for catalyzing the entire Krebs citric acid cycle has been obtained in cell-free form from liver, kidney, heart muscle and other tissues, and it has become possible to study separately each of the many reactions involved in the overall process (42, 73), but not with microorganisms. Generally speaking, the aerobic activity of cell-free extracts, obtained by grinding bacteria or yeast for example, is low and consequently makes this type of study extremely difficult. Not enough is known about the various cofactors involved in the aerobic breakdown of substrate by bacteria to enable one to reconstruct the complete system *in vitro*. Until such time then the observations with microorganisms are crude and much room for improvement remains. Neverthless, attempts to demonstrate the existence of a tricarboxylic acid cycle has been made in a great number

of different cells. Incorporation of isotopes into intermediates of the cycle, the accumulation of intermediates, the utilization by the metabolizing cell, as well as the actual demonstration of the enzymes participating in the cycle, have been used as criteria. It should be stressed that none of these approaches *alone* can be taken as proof for the existence of a tricarboxylic acid cycle, but together they may represent evidence either for or against such a cycle.

YEAST

Evidence is now available which suggests that acetate may be oxidized via a tricarboxylic acid cycle in yeast (106, 66, 40), although a Thunberg-Knoop condensation may function as an auxiliary reaction (106, 21).

Probably the first observation of the possible participation of citrate during acetate oxidation was the report by Wieland and Sonderhoff (109) on the accumulation of the C_6 tricarboxylic acid during the oxidation of barium acetate by brewers' yeast, in amounts approximating 10 per cent of the acetate utilized. The formation of succinate was also reported. These investigators, however, considered citrate formation as a side reaction on the acetate oxidation pathway of Thunberg and Knoop (100). Weinhouse and Millington (106) objected to this conclusion on the basis of their own experiments. First, the scheme as outlined below (fig. 2) calls for the dilution of acetate by "metabolic" non-isotopic acetate, arising from the two central oxalacetate carbons, during the oxidation of 1-C^{13}-acetate by yeast. This they found not to be true. Second, in an experiment they performed with isotopic magnesium acetate, 0.75 mM of citrate was formed, corresponding to 17 per cent of the acetate utilized. The C^{13} excess was 1.94, or 70 per cent of the acetate C^{13} excess. If acetate were oxidized via a Thunberg-Knoop condensation and citrate formation a side reaction, then the citrate should have had the same C^{13} content as the acetate, but it had only 70 per cent.

As indicated, Weinhouse and Millington (106) studied the oxidation of isotope-labeled acetic acid, $CH_3C^{13}OOH$, by yeast. From the reaction mixtures they isolated citric acid. The main product of yeast metabolism was CO_2, but some C^{13} was incorporated in lipids, and approximately 20 per cent of the acetate utilized was recovered as citrate. On the basis of 100 per cent C^{13} in the acetate carboxyl, there was found 80 per cent C^{13} in the primary carboxyls of citrate, 57 per cent in the tertiary carboxyl, and the remaining carbons contained no C^{13}. The C^{13} of the respiratory carbon dioxide was 55 per cent. These investigators have set up theoretical calculations based on different mechanisms of breakdown of an intermediary tricarboxylic acid, and have found good agreement between the observed C^{13} values and the calculated values, on the assumption that an asymmetrical molecule with six carbon atoms, rather than citrate itself, is on the direct path of acetate oxidation. Their results indicate that the tricarboxylic acid cycle may actually occur in yeast and that citrate is on a side reaction. More information, however, is required to prove conclusively the operation of a cyclic mechanism. If, for example, methyl-labeled acetate were used and equilibration between respiratory CO_2 and carboxyl groups of citrate obtained, the evidence for the operation of a cyclic mechanism during acetate

oxidation through a C_6 intermediate would have been very convincing. Further, we know little of the reactions between citrate and succinate. Krebs (53), for example, considered the possibility of the occurrence of the tricarboxylic acid cycle in yeast and concluded, on the basis of the apparent absence of the dehydrogenases for the various components of the cycle and the absence of fumarase and aconitase, that tricarboxylic acids are not involved in the oxidative processes of yeast. On the other hand, Weinhouse and Millington (106) found that yeast contains aconitase by extracting the enzyme with M/50 phosphate solution at pH 7.0 from cells previously frozen by immersion in liquid nitrogen. Further, Lynen and Neciullah (63) showed that the failure of yeast to oxidize intermediates of the Krebs cycle was simply a permeability problem. Using yeast, whose cell structure was destroyed by freezing in liquid air, they demonstrated a rapid dehydrogenation of citrate, α-ketoglutarate, succinate and malate. These results could be interpreted as favoring the occurrence of a Krebs cycle. However, it is believed that the mere presence of the enzymes for the breakdown of substrates which could be intermediates in the cycle, does not necessarily prove that this particular cycle exists. The actual metabolic relationships and interconversions between the intermediates would have to be thoroughly studied before any definite conclusions could be reached. It is, however, very likely that the data thus far accumulated strongly favor the occurrence of a tricarboxylic acid cycle in yeast, although Weinhouse and Millington (106) believe that their isotopic data could be interpreted in such a way that, concurrently with the operation of the Krebs cycle, a Thunberg-Knoop condensation for acetate oxidation co-exists. In fact, synthesis of a C_4 dicarboxylic acid is a necessity unless there is an endogenous source of C_4 acids, if citrate is formed via the tricarboxylic acid cycle.

Lynen (62) reached a similar conclusion with yeast, using 97.1 per cent deuterium-labeled acetate, CD_3COOH, and normal fumarate. In the presence of malonate and on the basis of 100 per cent deuterium in the acetate, the isolated succinate was found to contain 33.6 per cent deuterium, whereas if citrate was an intermediate the deuterium would have been expected to be 25 per cent as a maximum. The theoretical value, if aconitate is the intermediate, is 50 per cent deuterium. The fact that 33.6 per cent was observed is explained on the basis that part of the non-isotopic fumarate broke down to C_2 compounds, which in turn entered into the condensation reactions, thus giving rise to non-isotopic or "light" succinate. The quantitative data of the fumarate consumed and succinate formed are in agreement with such a hypothesis. The experiments with deuterium-labeled acetic acid of Sonderhoff and Thomas (86), on the other hand, are strongly indicative of a tricarboxylic acid cycle. These authors found that the oxidation of trideuterioacetate by yeast resulted in the accumulation of isotopic succinate and citrate with a higher deuterium content in the latter than in succinate; a result incompatible with the ideas that citrate is a side reaction in acetate oxidation.

In summing up the position regarding the mechanism of acetate oxidation in yeast, it must be said that it is still somewhat uncertain. From the various

reports presented, it would seem to follow that two oxidative systems take place in yeast, and the major one seems to be the Krebs cycle. Decisive conclusions on the most active oxidation system in yeast cannot be reached until the continued conversion of citrate to succinate through the accepted Krebs cycle intermediates, e.g., *cis*-aconitate, isocitrate, oxalosuccinate and α-ketoglutarate has been fully demonstrated. Only very recently (50, 40) have such reports begun to appear. Kornberg and Pricer (50), for example, observed the occurrence of two distinct and readily separable systems for the conversion of isocitric acid to α-ketoglutarate in yeast. One is triphosphopyridine nucleotide (TPN) specific, and is entirely comparable with that studied by Ochoa (74) in animal tissues. This enzyme system catalyzes the decarboxylation of oxalosuccinate, the reductive carboxylation of α-ketoglutarate, and the reduction of oxalosuccinate. The other enzyme system is diphosphopyridine nucleotide (DPN) specific, which fails to catalyze the reductive carboxylation of α-ketoglutarate, does not appear to have oxalosuccinate as an intermediate, and has an absolute and specific requirement for adenosine-5-phosphate. Foulkes (40) succeeded in obtaining a cell-free extract of bakers' yeast rapidly metabolizing citrate. Dialyzed extracts of this preparation yielded theoretical amounts of α-ketoglutarate when incubated with citric acid. The latter marks the first clear-cut demonstration of α-ketoglutarate formation by cell-free extracts of microorganisms (except protozoa) from a C_6-tricarboxylic acid.

Further, the question of citrate as an intermediate is just now becoming elucidated. The isotopic data of Weinhouse and Millington (106) were interpreted as definitely excluding citric acid on the direct pathway of acetate oxidation by yeast. Similar conclusions were reached by Lynen (61). He found the formation of citrate from acetate and oxalacetate by yeast to be promoted by the simultaneous oxidation of succinaldehyde, and believed that the function of this reaction is to convert either one of the components into a more reactive form. If the condensation takes place with an acetyl compound, $CH_3 \cdot COR$, a substituted asymmetrical citrate molecule may result which could be converted to *cis*-aconitate without elimination of the substituent, R. Lynen has pointed out that the data of Sonderhoff and Thomas (86) on the formation of labeled citrate and succinate in yeast also rule out a symmetrical citric acid. Succinate formed from trideuterioacetate by way of a symmetrical tricarboxylic acid could not have contained more than one atom of deuterium, but was actually found to contain two atoms. However, in view of the recent proposals of Ogston (77), and the demonstration of citrate synthesis *per se* the isotopic data may be explained on the basis of a "three point combination" of citrate and the enzyme surface, and make it necessary to *reconsider* the role of citrate in the cycle.

A most interesting report appeared by Lynen and Reichert (64), who claim to have isolated the so-called "active-acetate," from yeast. They allowed bakers' yeast to respire on acetic acid for some time. They then boiled this suspension briefly and subsequently extracted it with phenol and carried out several barium fractionations. The product, presumably free of transacetylase, was active in bringing about the acetylation of sulfanilamide by pigeon liver extracts. They

identified the "active acetate" as an "acetyl-CoA" compound and were able to identify the active component as CoA acetylated through the sulfur atom. They believe that the active grouping of CoA is the SH group and that the function of CoA is that of a co-transacetylase.

The mediation of acetate in glucose metabolism has also been demonstrated. Weinhouse *et al.* (107) adequately showed that acetate is an intermediate in the aerobic breakdown of glucose by resting cell suspensions of yeast. Evidence for the participation of acetate in carbohydrate metabolism of yeast was brought forward by Novelli and Lipmann (71) in connection with their studies of coenzyme A. They found that CoA-deficient yeast oxidized acetate at a lower rate than normal yeast, and that ethanol oxidation by CoA-deficient yeast resulted in the accumulation of acetate. Using glucose as substrate, they found that CoA-deficient yeast accumulated acetate.

MOLDS

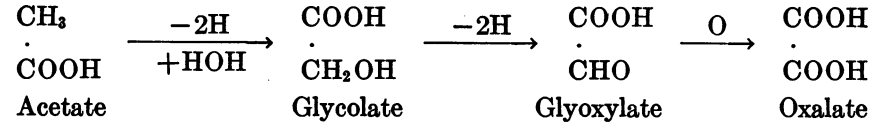
The terminal patterns of acetate oxidation in mold metabolism may involve any one or a combination of the following four pathways: (a) Breakdown of the C_2 acid to CO_2 and water via the aerobic tricarboxylic respiratory cycle; (b) oxidation of the C_2 fragment via its methyl group to oxalic acid; (c) oxidative condensation of two C_2 fragments to succinate, and (d) [which may be considered as the initial part of (a)] condensation of C_2 with C_4 from previous condensations to yield C_6, i.e., citric acid. For a detailed account of the evidence for and against these mechanisms and for what we know in general about mold structure, metabolism, and synthesis, the reader is referred to a recently published book by Foster (34).

A number of observations suggest that some steps of the tricarboxylic acid cycle, and in some organisms perhaps the whole cycle, may occur. Evidence for the occurrence of a dicarboxylic acid cycle is now accumulating (36, 35). Other reports (85, 69) indicate that molds of the wood destroying group oxidize acetate via glycolic and glyoxylic acids.

Of the possible mechanisms described above, the evidence for the occurrence of a tricarboxylic acid cycle in the metabolism of mold mycelium is least convincing. Molds may, under certain conditions, accumulate citric and aconitic acids in their growth medium, when carbohydrate is the only organic substrate, and recently Chughtai *et al.* (29) reported the formation of acetate and pyruvate in young cultures of *Aspergillus niger* grown on glucose. Thus molds certainly form intermediates of the Krebs cycle. However, the mechanism of interconversion of these acids is not known. The oxidative decarboxylation of α-ketoglutaric acid, for example, a key reaction in the conventional Krebs cycle, has not yet been shown to occur. Likewise, the decarboxylation of oxalosuccinate and its possible role in mold metabolism has not yet been demonstrated. The condensation reaction to citrate has not yet been fully elucidated, although the experiments of Foster and coworkers (38, 39, 34) suggest that the mechanism of citric acid formation in Aspergillus, for example, is similar to that in pigeon liver, including the utilization of CO_2. Recently, however, Foster *et al.* (37)

demonstrated that isotopic citrate can arise by a C_2 and C_4 condensation, the C_4 compound in turn arising from two C_2 fragments. That citric acid degradation follows the classical Krebs cycle series of events is still mostly speculation.

The evidence that some molds can oxidize acetate via its methyl or tail end is much better. Quoting only one or two recent articles, one finds the report of Nord and Vitucci (69) who, working with Merulii and *Fomes annosus*, demonstrated the formation of oxalic acid from acetate and glycolate. The following sequence was suggested:



Using the decoloration of resazurin as an indication of a dehydrogenation reaction, and showing the formation of oxalate from glycolate, they postulated the intermediate formation of glyoxylic acid. Smith (85) furnished good experimental evidence for the formation of this acid in appreciable quantities on the pathway from glucose to oxalic acid by *Merulius lacrymans*. Although a great number of other molds can produce oxalic acid from acetate, Foster (34) does not believe that this mechanism provides for much of the oxidation of acetic acid.

A more prominent mechanism of oxidation of the acetyl derivative in molds appears to be via the Thunberg-Knoop condensation. The evidence for this mechanism in molds has been, to a large extent, the excellent work of Foster, Carson and their collaborators. Although data were available for quite some time indicating that a condensation of acetate to a C_4 dicarboxylic acid can occur in some molds, this question received rather exhaustive treatment in the past two years, employing isotopic labeling techniques.

Foster and Davis (39) demonstrated that fumaric acid formation from glucose by the mold *Rhizopus nigricans* involves at least two mechanisms, a $C_3 + C_1$ condensation, occurring primarily under anaerobic conditions, and a $C_2 + C_2$ condensation occurring aerobically. Subsequent to these observations, two papers appeared dealing chiefly with the precise mechanism of the $2C_2$ condensation. In the first of these (36) evidence was presented that fumaric acid synthesis from ethanol in the same strain of *Rhizopus* takes place by a direct condensation of $2C_2$ moieties, and that a tricarboxylic acid cycle is not involved in its synthesis. This conclusion was based upon the following findings: Starting with methyl-labeled C^{14}-ethanol, the specific activity of the formed fumaric acid methylene groups was equal to that of the ethanol methyl groups. However, one mole of fumarate formed from 2-C^{14}-ethanol contained more than the total radioactivity of the two moles of substrate; the carboxyl groups of fumarate contained the excess. This is explained by the fact that some of the alcohol was oxidized to CO_2 giving rise to labeled carbonate, and since the reaction was carried out in a closed system, the radioactive CO_2 might have entered the fumarate by the reversible decarboxylation of oxalacetate and reduction of the

latter via malate to fumarate. Starting with 1-C^{14}-ethanol, the approximate reverse was noted, e.g., a mole of fumarate contained less radioactivity than two moles of substrate; there was no activity, however, in the methylene carbons, and the carboxyls of the C_4 acid had all the activity. The foregoing was interpreted to mean that the fumarate produced at any instant from 1-C^{14}-ethanol could contain radioactivity equal to the carbinol of the alcohol. However, some of the alcohol is oxidized to CO_2 yielding two moles of the gas, only one of which is active. Unlabeled CO_2 also arises from endogenous mold metabolism. All these factors make the specific activity of the CO_2 in the closed vessel rather low. Consequently, during reversible decarboxylation, the carboxyls became diluted.

These data are strongly suggestive that a $2C_2$ condensation operates in fumarate synthesis. Acetate *per se* appears not to be the intermediate because of low conversion rates of acetate to fumarate. Succinate is probably the precursor of fumarate, but this has been shown only indirectly. Although mold mycelium is inert toward succinate, desiccated mycelium actively metabolizes the acid when tested manometrically for O_2-uptake. Oxygen uptake data indicated that fumarate is the end product of succinate oxidation by the dried preparations.

Two reasons are given for the elimination of the operation of a Krebs cycle in fumarate formation, namely, (a) theoretical molar yields of fumarate from C_2 fractions via a C_6 cycle are 67 per cent. Yields equaling or exceeding these have been obtained. A 67 per cent yield would actually never be obtained if a Krebs cycle were operating because of the obvious degradation outlets for the intermediates and the assimilation processes of the alcohol itself. (b) For each molecule of C_4 formed, the tricarboxylic acid cycle would require a C_4 molecule to begin with. This would have to come from ethanol, which is the only C_2 substrate available. Thus, a functioning Krebs cycle in this system would still require a C_4 synthesis from C_2 by some efficient method. The fixation of CO_2 may conceivably represent such a method. However, since the authors show that CO_2 fixation is not a major mechanism of fumarate synthesis, the $2C_2$ condensation as the origin of fumarate becomes even more apparent.

These studies are extended in their second paper (35). This publication deals chiefly with the incorporation of CO_2 into carboxyls of preformed C_4 dicarboxylic acids, which were synthesized by their $2C_2$ condensation reaction. According to these experiments demonstration in normal tissues of the Wood-Werkman formation of C_4 dicarboxylic acids, by locating the labeled CO_2 in the carboxyls of these acids, would not constitute unequivocal proof of net synthesis involving carbon dioxide. These findings prompted them to conduct Krebs-type malonate inhibition experiments with $C^{14}H_3 \cdot CO \cdot COOH$ and $C^{13}O_2$ (27). Malate, succinate, lactate and α-ketoglutarate were isolated and the specific activities of each carbon atom determined. They state that their results indicate that the $2C_2$ condensation reaction cannot be excluded from having taken part in the synthesis of the C_4 dicarboxylic acids. Further, they relate a rather unusual finding, e.g., in the malonate experiments the controls formed as much succinate

as the experimental containing substrate. Evaluation of these data will have to await the detailed report of their experimental findings.

In summing up the position regarding the terminal respiratory patterns of molds, it can be said that *the evidence favors the existence of a dicarboxylic acid cycle for the oxidation of the C_2 fragment.* The condensation of $2C_2$ fragments, with the subsequent formation of a C_4 dicarboxylic acid has been adequately demonstrated. The interconversion, however, of the dicarboxylic acids needs further elucidation, although the formation of fumarate from succinate and the decarboxylation of oxalacetate to pyruvate have been shown to occur.

BACTERIA

Introduction. In bacteria, the presence of a terminal respiratory cycle for the aerobic removal of carbon dioxide has always been a matter of some doubt. Even now little that is absolutely definite can be said undoubtedly, because the methods applicable to animal tissues do not always work with bacteria. The criteria upon which Krebs established the citric acid cycle in pigeon muscle cannot be readily applied here. For example, only few bacteria attack the tricarboxylic acids. However, this does not necessarily mean that the enzymes are not present. When the same bacteria are ground and the cell walls destroyed, individual enzymes attacking these acids are often found. By working with a cell-free preparation, the problem of permeability may be solved, but a more serious obstacle is then encountered. Cell-free extracts of bacteria generally do not exhibit appreciable aerobic activity. While the enzymes for some substrates (originally not attacked by the whole cell) are found, other enzymes originally present are destroyed. Not enough is known about conditions and cofactors operating in the terminal respiratory mechanism of bacteria to enable one to supplement these extracts in such a way as to make them active aerobically. The well-known malonate inhibition experiments of Krebs are inapplicable to bacteria, at least so far as the coli-aerogenes group is concerned. Occasionally a report appears (56) on the inhibition of some type of reaction occurring in the Krebs cycle by malonic acid in whole bacterial cells. This cannot always be assumed to be so as it has been our experience *never* to be able to inhibit succinic acid oxidation with malonic acid with whole *E. coli.* Malonic acid will inhibit the oxidation of succinic acid when a cell-free extract constitutes the enzymic preparation.

Irrespective of the disadvantages encountered with whole cells and cell-free preparations, the data obtained with each system have often been interpreted for or against the participation of a Krebs cycle in bacteria. For example, using whole cells, and based upon observations which will be discussed later in detail, Karlsson and Barker (49) came to the conclusion that the tricarboxylic acid cycle is not operative in *Azotobacter agilis.* Soon after, Stern and Ochoa (96) found that by disrupting the cell wall, the enzyme which condenses acetate and oxalacetate is present, and such a finding prompts one to conclude that a Krebs cycle does exist. A similar conflicting situation exists in *E. coli.*

Several tentative statements can be made in connection with the terminal

respiratory mechanisms in bacteria. (a) The available data suggest that a *cyclic* mechanism for pyruvate or acetate oxidation exists in some bacteria. (b) The tricarboxylic acid cycle does not occur in other bacteria, although some of the enzymes normally oxidizing Krebs cycle intermediates can be extracted from these organisms. (c) Some bacteria appear to possess both a tri- and a di-carboxylic acid cycle for the oxidation of pyruvate or acetate. It will be our purpose in the subsequent discussion to illustrate these statements.

Azotobacter agilis. That the tricarboxylic acid cycle apparently does not operate as an important oxidative pathway in all forms of life is demonstrated by two independent lines of evidence presented by Karlsson and Barker (49), one obtained by the study of adaptive enzymes, and the other by the isotope dilution technique. With regard to adaptive enzymes, it is assumed that if a compound occurs as an intermediate in metabolism, the cells must contain the requisite enzymes. Thus it will form adaptive or constitutive enzymes for all the intermediates (48, 93). Karlsson and Barker (49) found that when *A. agilis* was grown on acetate, it did not contain the enzymes necessary for the oxidation of compounds occurring in the tricarboxylic acid cycle, e.g., α-ketoglutarate, succinate, fumarate, etc. The bacteria fermented these substrates only after a considerable delay, indicating adaptation. However, when these cells were grown on succinate, they possessed the enzymes for succinate, acetate, fumarate, malate and pyruvate, but not for α-ketoglutarate. Cells adapted to α-ketoglutarate were adapted to all of the compounds just indicated. Postulations of permeability phenomena being involved, or modified compounds being the true intermediates, appear to be excluded in our opinion by the finding that adaptation to α-ketoglutarate results in simultaneous adaptation to all lower members of the cycle, as evidenced by immediate oxygen uptake. These results may also answer the arguments of Ratner and Racker (80), who maintain that in this case too permeability is the only factor which is responsible for the inability of *A. agilis* to utilize dicarboxylic acids when grown on acetate. The absence of the tricarboxylic cycle is further substantiated by the use of isotopes. The isotope dilution methods involved addition of C^{14}-labeled succinate and normal acetate to succinate-adapted cells. Although acetate was metabolized, apparently no intermediary succinate formed since the labeled succinate remained undiluted. The question here, however, may be whether complete equilibration took place between the extracellular labeled and intracellular unlabeled succinate. These findings were taken to indicate that succinate and the other C_4 dicarboxylic acids and α-ketoglutarate are not intermediates in the oxidation of acetate or pyruvate, and that the metabolism of acetate does not proceed via the Krebs cycle in this organism. What the mechanism of acetate oxidation may be is of interest since all oxidation products, such as glycolic acid, oxalic acid and formic acid, were found not to be attacked. It will take more research to correlate these findings with those of Stern and Ochoa (95), who demonstrated the existence of the condensing enzyme in these cells; and with those of Stone and Wilson (98), who have concluded from extensive investigation with *cell-free* preparations of *Azotobacter vinelandii* (in which both

manometric and isotopic techniques were used) that the tricarboxylic acid cycle operates. For the time being the available published evidence points against the occurrence of a tricarboxylic acid cycle in the metabolism of the strain of *A. agilis* used by Karlsson and Barker, but final judgment should perhaps be suspended until extensive experiments are carried out with cell-free preparations from their strain of *Azotobacter*.

Escherichia coli. With *E. coli* we are fairly certain that a cycle exists, but the question here is what is the precise nature of the cyclic mechanism involved. Recent experiments in our laboratory (2, 7, 8) support the view that a cyclic mechanism is involved in acetate oxidation by *E. coli*, but this mechanism appears to be an abridged Krebs cycle or a *dicarboxylic acid cycle* initiated by a

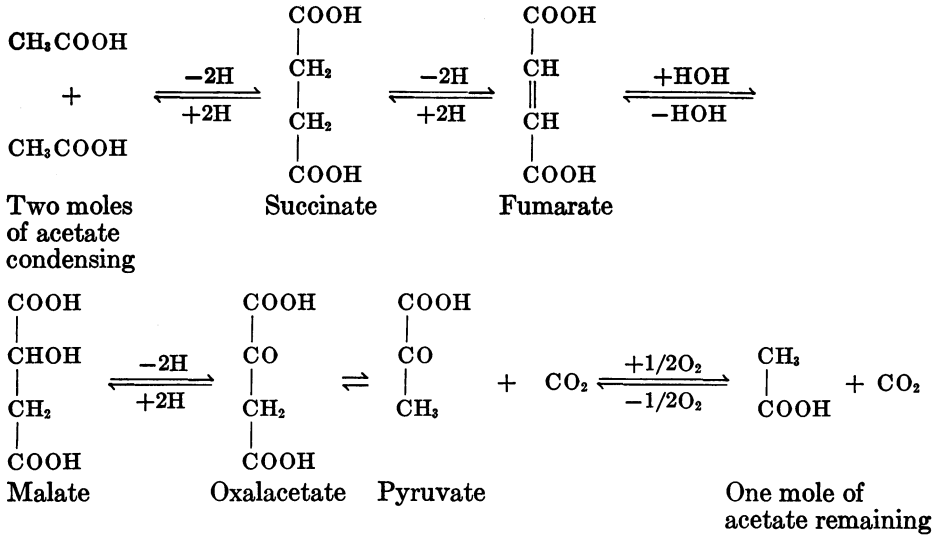


FIG. 2. A Dicarboxylic Acid Cycle for Acetate Oxidation

Thunberg-Knoop condensation. (See Fig. 2.) That such a condensation reaction may be operating in bacteria was suggested by the experiments of Slade and Werkman (84), who studied this process with C^{13}-containing acetate in *Aerobacter indologenes*. It was rather difficult to say with much certainty that this condensation really did occur because the presence of glucose in the medium made the interpretation of the isotopic data rather difficult. Subsequent to these experiments, Birkeland and Randles (19), in a short note, reported that *E. coli* grown under aerobic conditions in the presence of acetate yields cell suspensions in which the rate of reduction of methylene blue by C_4 dicarboxylic acids is greatly increased, compared to cells grown in the absence of acetate.

By applying the principle of "simultaneous adaptation", it has been observed (2) that *E. coli*, adapted to oxidize acetate rapidly, also oxidizes succinate, fumarate, malate, oxalacetate and pyruvate more rapidly than unadapted cells. However, the rate of oxidation of α-ketoglutarate or *cis*-aconitate

is not increased. These observations were interpreted as presumptive evidence for a Thunberg-Knoop condensation mechanism for acetate oxidation. Thus, two molecules of acetate are assumed to undergo oxidative coupling between adjacent methyl groups to form succinate, which is then degraded stepwise in the conventional Szent-Györgi scheme to yield one molecule of acetate, two molecules of CO_2 and two molecules of water. (See Fig. 2.)

Experiments of a more direct nature have subsequently been performed exploiting isotopic labeling techniques (7, 8). The method employed was similar to that used by Karlsson and Barker (49) and is based on the following considerations: The normal steady state concentration of intermediates involved in the metabolic reaction chain is so small that, if isotopically labeled acetate is oxidized, equilibration of isotopic material occurs at too low a level to be demonstrable, even if chemical procedures are adequate to isolate and purify the miniscule amounts of intermediate. However, if a large amount of any intermediate is added in unlabeled form then, providing the organism is permeable to this compound and complete mixing is obtained with the intracellular material, the total concentration of isotopic material should reach a much higher level determined by simple isotope dilution. At the same time isolation and purification is facilitated because chemically significant amounts of intermediate are supplied.

Thus, suppose acetate is oxidized by condensation of two molecules of acetate to succinate, with consequent oxidation back to CO_2, H_2O, and acetate, via fumarate, malate, oxalacetate and pyruvate, then partial oxidation of radioactive acetate in the presence of succinate will result in accumulation of C^{14} in the methyl groups of succinate. In a like manner, addition of fumarate will trap C^{14} in fumarate, etc. It may be noted that recycling of C^{14}-methyl labeled acetate results eventually in practically uniform distribution of C^{14} in all the carbon atoms of the intermediate compounds. The fact that *E. coli* attacks not only the C_4 dicarboxylic acids but to some extent α-ketoglutaric acid makes it possible to establish whether the reaction chain also involves this acid, and thus to furnish data on the possible participation of the conventional tricarboxylic acid cycle in acetate oxidation by this organism. Experiments were performed in which C^{14}-methyl labeled acetate was oxidized simultaneously with α-ketoglutarate, C_4 dicarboxylic acids, and pyruvate, singly or in combination. From chemical analysis of the residual acids, CO_2, and cell material, as well as manometric data on O_2-uptake, it was possible to show that when an appreciable fraction of acetate, as well as all other substrates, was metabolized, there was incorporation and distribution of labeled carbon into C_4 dicarboxylic acids by pyruvate in the amount to be expected on the basis of a Thunberg-Knoop condensation cycle, whereas there was no comparable incorporation into α-ketoglutarate. These results appear tentatively, at least, to exclude a conventional tricarboxylic acid cycle as a major pathway for acetate oxidation in *E. coli*.

A typical experiment in which 2-C^{14}-acetate was incubated with either inactive pyruvate, α-ketoglutarate or succinate is shown in table 1. It was con-

cluded that under conditions in which all three substrates were metabolized by the bacteria, acetate carbon was recovered only in succinate and pyruvate and not in α-ketoglutarate. Similar experiments were performed independently by Swim and Krampitz (99). Their experiments were done anaerobically with ferricyanide as the oxidant.

The experiment just cited shows that a mechanism exists during acetate oxidation for equilibration of acetate carbon with succinate and pyruvate, but not α-ketoglutarate. To establish quantitatively the nature of the condensation

TABLE 1

Distribution of Radioactivity in Products of Dissimilation of 2-C^{14} Acetate by Escherichia coli

EXP. NO.	PRODUCTS	CONCENTRATION		C^{14} CONTENT		AVG. SPECIFIC ACTIVITY OF ACETATE
		Initial	Final	Initial	Final	
		μM	*μM*	*cts/min*	*cts/min*	*cts/min/μM*
1	Acetate	1063	839	480,000	261,000	$\frac{\frac{480,000}{1063} + \frac{261,000}{839}}{2} = 380$
	Pyruvate	1000	630	0	13,900	
	Carbonate			0	2,400	
	Cells			0	2,420	
2	Acetate	1063	850	480,000	412,000	$\frac{\frac{480,000}{1063} + \frac{412,000}{850}}{2} = 930$
	α-Ketoglutarate	1000	973	0	<250	
	Carbonate			0	6,000	
	Cells			0	4,000	
3	Acetate	1063		480,000		
	Succinate	1000	860	0	12,000	
	Carbonate			0	1,500	
	Cells			0	2,500	

Total volume of reactants 11 ml containing 2 ml of a 10 per cent suspension of freshly harvested (acetate-grown) *E. coli;* 2 ml phosphate buffer 0.2 M, pH 7.0; substrates as indicated, and NaOH in center well. Aerobic. Temp. 30 C. Time of incubation, 6 hours.

reactions and the cyclic mechanisms involved, experiments were performed in which three substrates, one of which was labeled acetate, were oxidized simultaneously. Typical results are shown in table 2. These experiments were designed to supply definitive data on the question of participation of succinate on the one hand, and α-ketoglutarate on the other, in any cyclic mechanisms concerned with acetate oxidation. Consequently, the analysis of these results will be given in detail here, since they are important and not published elsewhere.

First, it should be remarked that the C^{14} content of the succinate was determined exclusively by enzymic means, to render as certain as possible the identification of the radioactive material. In calculating the consequences of the data given in table 2, it was noted that the average specific activity of acetate was

812 cts/min/μM, the initial and final specific activities being 1024 and 604 cts/min/μM, respectively. First, the dilution of acetate to be expected was examined. 14,500 cts/min were found in the succinate, of which 8,620 came from the central carbons and 5,840 from the carboxyl carbons. Thus, it appeared that sufficient cycling had occurred to fix appreciable methyl carbon of acetate

TABLE 2

Accumulation of Radioactive Succinate and Inactive α-Ketoglutarate During the Oxidation of 2-C^{14} Acetate by Escherichia coli

	PRODUCT	AMOUNT	C^{14} CONTENT	SPECIFIC ACTIVITY	MANOMETRIC DATA OXYGEN UPTAKE; μl	
		μM	*cts/min*	*cts/min/μM*	Expt.	Endogenous
Initial Conditions	Acetate	112	114,000 ± 2,500 (methyl carbon)	1020 ± 20	3649	1327
	Succinate	125	0	0	3649 −1327 = 2322 μl	
	α-Ketoglutarate	125	0	0		
	Carbonate		0	0	Calculated from substrates disappeared 3650 μl	
Final Conditions	Acetate	135	80,000 ± 2,500	604 ± 18		
	Succinate	97	14,500 ± 700			
	Methylene carbons		8,620 ± 400	44.4 ± 2.0		
	Carboxyl carbons		5,840 ± 300	30.1 ± 1.5		
	α-Ketoglutarate	95	120	<0.5		
	Cells (40 mg dry weight)		2,180 ± 100			
	Carbonate		1,830	16 (Avg. specific activity)		

The complete system consisted of 1 ml of 0.2 M phosphate buffer of pH 7.0; 1 ml of a 10 per cent suspension of freshly harvested (acetate-grown) *E. coli;* substrates as indicated; and NaOH in the center well. Total volume 10 ml Temp. 30 C. Time of incubation 4 hours. Recovery data: Residual acetate 80,000; succinate 14,500; cells, 2,180; carbonate 1,830. Total 98,410. Initial activity 114,000. Recovery, 86 per cent.

in succinyl carboxyl. (The magnitude of the C^{14} content was much too high to be accounted for as due to secondary fixation via carbonate.) Thus, 14,500/812 or 18 μM methyl carbon were incorporated into succinate, corresponding to formation of 9 μM succinate. In addition 30 μM succinate were formed because 30 μM of α-ketoglutarate were lost. Thus, 30 μM succinate extra must have been degraded to acetate, CO_2 and water, in addition to the 9 μM used up compensating for the acetate condensation and the actual 28 μM succinate

observed to be lost. The total oxygen uptake expected on this basis was 124.5 μM, and the total succinate oxidized was 67 μM.

Originally, acetate was present to the extent of 112 μM. Sixty-seven μM should have been formed from the succinate degradation. Therefore, the final amount of acetate should have been 179 μM. From this there was subtracted 18 μM used up in the condensation to succinate. In addition, the C^{14} content of the cells indicated 2,180/812, or approximately 3 μM assimilated. Subtracting 21 μM, there resulted 158 μM. Actually 135 μM were found. The difference, 23 μM, was the amount oxidized to CO_2 and water. This added 46 μM more of O_2 required, giving a total of 170.5 μM or 3,810 μl. This may be compared with the observed value of 3,650 μl which was not corrected for endogenous uptake.

Thus, it appeared that endogenous metabolism was completely repressed. If so, then the expected isotopic dilution of the acetate should have been that resulting only from the processes assumed in calculating oxygen uptake. This value could be calculated by procedures already described in the literature (15) involving simple dilution equilibria, but for the immediate purpose, it was sufficiently precise to calculate a maximal dilution by assuming that 44 μM labeled acetate were removed and replaced by the 67 μM of unlabeled acetate derived from succinate degradation. On this basis the final specific activity could not be less than $\sim$ (1440) 58/135 or 575 cts/min/μM. This agreed well with the observed value so that the dilution data supported the manometric data in indicating complete suppression of endogenous oxygen uptake.

Examination of the carbonate data revealed that the average specific activity of the CO_2 evolved was 16 cts/min/μM. The average specific activity of the succinyl carboxyl was 15 cts/min/μM (0 at the start, 30 cts/min/μM at the end). Thus, the isotope data were in accord with the notion that succinyl carboxyl was in equilibrium with evolved CO_2, as was to be expected on the basis of a cyclic mechanism operating by way of a condensation reaction.

The close correlation between moles oxidized (23 μM) and moles condensed (18 μM) argued strongly that practically all acetate must have been oxidized by way of the condensation to succinate or some compound in equilibrium with succinate.[2] At the same time, it was noted that $\sim$ 0.5 μM of acetate carbon entered α-ketoglutarate, so that it could be concluded that there was no appreciable participation of this keto-acid in acetate oxidation by *E. coli* after adaptation of the organisms to acetate.

Runs with labeled acetate and unlabeled pyruvate, fumarate, malate and oxalacetate, singly or in combination, all yielded results similar to those ob-

[2] Based on these observations, a simple and rapid method for the synthesis of isotopically labeled succinate from labeled acetate has been developed (9). A typical synthesis yielded the following results: Beginning with $\sim$10 μM acetate containing $\sim 2 \times 10^6$ cts/min in the methyl carbon and 125 μM unlabeled succinate, 60 μM succinate were isolated containing a total of 730,000 cts/min, a yield relative to original labeled acetate of some 37 per cent. Approximately uniform distribution of labeled carbon was observed.

tained with succinate. However, in experiments with fumarate, malate, and oxalacetate, an unexpected complication arose. It was observed that a large endogenous reduction of these compounds to succinate occurred, even though a rapid uptake of oxygen was noted and conditions were such that vigorous oxidation could proceed (with rapid shaking in air). Thus, in one experiment beginning with 100 μM of labeled acetate and 125 μM each of unlabeled fumarate and α-ketoglutarate, there remained 44 μM of acetate, 103 μM of α-ketoglutarate, and less than 30 μM of fumarate. 104 μM of succinate appeared. Thus, it seemed that practically all the fumarate had been converted to succinate, possibly through the intervention of endogenous hydrogen donors. Insufficient hydrogen was available from the acetate utilized to account for an appreciable fraction of the succinate formed. However, in early observations on *E. coli*, it has been shown that fumarate can undergo a dismutation under anaerobic conditions, 7 molecules of fumarate forming 6 molecules of succinate and 4 molecules of carbonate (57). A similar reaction may have occurred in this experiment, owing perhaps to the density of cell suspensions used, which may have resulted in maintaining cells much of the time under essentially anaerobic conditions, despite vigorous shaking in air.

Attempts to minimize this effect by using freshly harvested young cells (18 hours old) failed. It was found possible to demonstrate unambiguously entrapment of acetate carbon in malate and fumarate only when either relatively enormous quantities (2 mM) of these acids were used or when the experiment was carried out with constant aeration with tank oxygen (4).

From the data thus far cited, it seems rather certain that a cyclic mechanism for acetate oxidation exists, although more quantitative data are necessary to demonstrate the involvement of the intermediates of the cyclic mechanism. It seemed to us that a logical approach to the problem would be by studying the variation due to time in specific C^{14} content of at least three suspected intermediates during the oxidation of labeled acetate.

Zilversmit *et al.* (113), using differential equations, demonstrated that at least two criteria must be satisfied if a relationship between precursor and product is to be established. These include (a) the specific activity of a precursor of a given compound must be higher than that of the compound itself during the early interval after exposure of a labeled substance, and (b) if the specific activity of the precursor is maintained constant, the specific activity of the compound eventually becomes equal to that of the precursor.

Recent data, as yet unpublished, obtained in our laboratory indicate that both of the criteria are satisfactorily met, at least so far as succinate and fumarate are concerned, when these were metabolized in the presence of labeled acetic acid. So far as we can ascertain, no such data have yet been presented in connection with similar problems either in animals or microorganisms.

A conceivable mechanism of acetate oxidation could be via its methyl group, e.g., via glycolic and glyoxylic acids. Both isotopic and non-isotopic experiments were conducted and from the data obtained (6) it appears that they have no function as intermediates in acetate oxidation in *E. coli*.

The salient fact, which emerges from these studies, is that a cyclic mechanism is certainly involved in acetate oxidation and that this cycle is probably a *dicarboxylic acid cycle*, and not the conventional tricarboxylic acid cycle of Krebs. Siegel (83) presented some of the thermodynamic aspects of the proposed dicarboxylic acid cycle for acetate oxidation in *E. coli*. He comes to the general conclusion that our proposed scheme has a "thoroughly sound basis in thermodynamic formulations." If a citric acid cycle of the conventional type is assumed, then it is necessary to suppose that intracellular equilibration with extracellular α-ketoglutarate is lower by at least three orders of magnitude than that for succinate or pyruvate. Alternatively, one may postulate that some C_5 compound other than α-ketoglutarate is the actual intermediate. It is also possible that, when α-ketoglutarate enters the cell and attaches itself to the enzyme responsible for its breakdown, it never leaves that enzyme surface. Therefore, it never exchanges with radioactive α-ketoglutarate that would presumably be formed during the oxidation of radioactive acetate. A simpler explanation is that the primary condensation product is either succinate or a C_4 compound in equilibrium with it. However, before anything more definite can be said, the findings of other investigators, using the same organism (although often not the same strain), have to be explained. For example, as was stated previously, Novelli and Lipmann (72) found that extracts of *E. coli* formed citric acid. This datum may be interpreted as evidence for the occurrence of a Krebs cycle in this organism. That this does not necessarily follow may be explained. If a Krebs cycle were operating, then the citrate would have to be broken down via *cis*-aconitic acid, isocitrate, oxalosuccinate and α-ketoglutarate, to succinate, fumarate, etc. Now, aconitase and isocitric dehydrogenase have not yet been shown to be present in *E. coli*; consequently, even if citrate were formed, it may be broken down by the reverse of reactions 1 and 2 (cf., General Considerations). This type of citrate breakdown has been postulated for Aerobacter some ten years ago (25). In addition, unpublished experiments in our laboratory reveal that similar cell-free extracts of *E. coli* condense two molecules of acetate to a C_4-dicarboxylic acid, and that this is a diphosphopyridine nucleotide requiring reaction. Consequently, citrate formation is probably a side reaction occurring in *E. coli*, whose precise function in metabolism we as yet do not know. Alternatively, two cycles may co-exist in *E. coli* respiration (as appears to be the case in yeast), the dicarboxylic acid cycle predominating.

Somewhat more difficult is to correlate our experiments with those of Umbreit, *et al.* and Aubel, *et al.* In connection with their streptomycin studies in *E. coli*, Umbreit and co-workers (102) came to the general conclusion that this antibiotic inhibits the condensation reaction between pyruvate and oxalacetate. *Acetate oxidation was not inhibited.* This implies that the initial reaction of pyruvate oxidation is a condensation to some C_7 intermediate, and not an oxidative decarboxylation to acetate and CO_2. The C_7 intermediate was not isolated, neither could any citrate be detected. These findings seem to imply an alternate and completely different mechanism of pyruvate oxidation by

E. coli. It may be noted that some data have been published (2) against the occurrence of a condensation reaction of pyruvate prior to an oxidative decarboxylation to acetate and CO_2 in *E. coli*. Recently, Aubel *et al.* (12), based only on inhibition experiments, came to the general conclusion that neither a Krebs cycle nor a Szent-Györgyi cycle operates in *E. coli*. These conclusions were reached on the basis of gross inhibition type experiments and it is doubtful whether they tell much about mechanism.

In conclusion it may be said that *the available data, to date, can be best reconciled with the existence of a Thunberg-Knoop condensation reaction during acetate oxidation in E. coli, which is followed by an oxidation via the Szent-Györgyi system to CO_2 and water. The co-existence of other cycles is not excluded.*[3]

Corynebacterium creatinovorans. A similar situation exists with *C. creatinovorans* (16). Using whole cells and cell-free extracts, Barron and associates came to the conclusion that this organism oxidizes acetate via the dicarboxylic acid cycle. Some of the findings were confirmed in our laboratory, particularly those with cell-free extracts. We found that extracts of this organism will reduce methylene blue in the presence of acetate. However, dialyzed extracts require Mn^{++}, phosphate and diphosphopyridine nucleotide to do the same thing.

Micrococcus lysodeikticus. The findings just reported for *E. coli* and *C. creatinovorans* cannot be generalized. Results obtained by applying the present isotopic methods to *M. lysodeikticus* (10), an organism which oxidizes acetate readily without adaptation, are diametrically opposed to the findings with *E. coli* and can be brought into harmony with operation of a tricarboxylic acid cycle involving α-ketoglutarate. Unlike *E. coli*, α-ketoglutarate trapped acetate carbon. Upon degradation it was found that the radioactivity was evenly distributed throughout the molecule. In addition, it was found that all of the C_4 acids, as well as pyruvic acid, trap activity when oxidized in the presence of labeled acetate. Equilibration of labeled acetate with unlabeled dicarboxylic acids or pyruvate was roughly 10-fold less than observed in *E. coli*, and occurred to about the same extent in all substrates added. CO_2 evolved was not in equilibrium with carboxyl carbon. (See table 3.)

Saz and Krampitz (81) independently reported similar results and, in addition, they demonstrated the actual synthesis of citric acid from oxalacetate and radioactive acetic acid by lysed preparations of *M. lysodeikticus*. Recently, the same investigators (82) described conditions under which complete isotopic equilibration between substrates and respiratory CO_2 took place. Extension of these studies to *cis*-aconitate and oxalosuccinate are desirable. It is also ap-

[3] Although we have recently found that cell-free extracts of *E. coli* do attack citrate and the other C_6 tricarboxylic acids, the breakdown of the citric acid does not appear to occur via *cis*-aconitate, oxalosuccinate, and α-ketoglutarate. Until such data are obtained, the *conventional* tricarboxylic acid cycle cannot be assumed to be operating in the terminal respiration of this organism. Further, in successful experiments with cell-free extracts (8), it was shown that α-ketoglutarate never trapped acetate carbon, whereas succinate did become active. This further emphasizes the fact that to date no evidence exists for the operation of the conventional citric acid cycle in *E. coli*.

parent from these divergencies in the behavior of the organisms studied and by comparison with similar studies in yeast and *A. agilis* that no generalizations regarding oxidation of acetate by microorganisms should be made on the basis of the study of any single microorganism.

Rigorous elaboration of the cyclic mechanism in acetate oxidation by *M. lysodeikticus* will require study of the labeled intermediates formed without

TABLE 3

Qualitative Comparison of C^{14} Distribution and Metabolism of E. coli *and* M. lysodeikticus *when Oxidizing 2-C^{14} Acetate*

FUNCTION	CONDITIONS	*E. coli*	*M. lysodeikticus*
Endogenous Activity	+ Acetate	Increased rate	Increased rate
	+ Acetate and other substrates	Lowered rate	Lowered rate
Isotopic Dilution of Acetate	+ Acetate and α-keto-glutarate	Small	Small
	+ Acetate and C_4 acids	Large	Small
C^{14} content of evolved CO_2	+ Acetate and α-keto-glutarate	Large	Large
	+ Acetate and C_4 acids	Small	Large
C^{14} content of carrier	+ Acetate and α-keto-glutarate	Small	Small
	+ Acetate and C_4 acids	Large	Small
Equilibration of extracellular carrier and intracellular intermediates	Acetate + α-keto-glutarate	Complete	Very incomplete
	Acetate + C_4 acids	Complete	Very incomplete
Endogenous production of succinate during aerobiosis	Acetate + carrier acids	Considerable	None

addition of carrier. Development of reliable microanalytical procedures for the small quantities of intermediates encountered will be necessary. The best possibility at present seems to be the use of paper chromatography in conjunction with isotopic techniques.

Aerobacter aerogenes. An interesting situation exists in *A. aerogenes* (11, 5). Mixtures of 2-C^{14}-acetate and nonlabeled Krebs cycle intermediates were incubated aerobically with either acetate- or citrate-grown *A. aerogenes*. With the citrate-grown cells, (a) all intermediates contained radioactivity, (b) there

was lack of complete equilibration with unlabeled intermediates, and (c) the CO_2 evolved was not in equilibrium with carboxyl carbon. With acetate-grown Aerobacter the results were diametrically opposed, e.g., (a) only C_4-dicarboxylic acids and pyruvate contained radioactivity, although all compounds were metabolized, as evidenced by oxygen uptake; (b) complete equilibration took place with unlabeled carriers, and (c) the CO_2 evolved was in equilibrium with carboxyl carbon.

These results indicate the operation of different mechanisms for acetate oxidation by *A. aerogenes*, depending on the carbon source used for growth. The mechanism for citrate-grown cells appears to involve a conventional citric acid cycle, whereas acetate-grown cells appear to incorporate acetate carbon more readily via a dicarboxylic acid cycle.[4]

A. aerogenes was specially chosen for this investigation since this organism *normally* oxidizes citric acid and can use this acid as a sole source of carbon for growth. The other organisms which we have previously studied do not. It was, therefore, thought to be of particular interest to learn whether the pathway of acetate oxidation proceeds via citrate and, at the same time, to determine the mechanism of citrate oxidation. Thus far there has been no successful demonstration of α-ketoglutarate formation as a result of citrate oxidation in bacteria. Experiments were therefore performed (11) by oxidizing radioactive citrate in the presence of inactive α-ketoglutaric acid, just as in the case of acetate oxidation where 2-C^{14}-acetate was incubated in the presence of some unlabeled intermediate.

When citric acid, containing 84,700 cts/min, was oxidized in the presence of inactive α-ketoglutarate and acetate, both compounds became radioactive at the end of the incubation period. The fact that radioactive citrate, when incubated with inactive α-ketoglutaric acid, causes the formation of active α-ketoglutarate, lends support to the idea that the keto acid may lie on the pathway of citrate breakdown. In addition, succinate was found as a product of the reaction. Similarly, experiments were performed by incubating radioactive α-ketoglutarate in the presence of inactive succinate, radioactive succinate in the presence of inactive fumarate, and radioactive fumarate in the presence of inactive malate. In addition, normal acetate was added to all of these as well. The results we have obtained clearly indicate that, in each case, the inactive compounds become active. Further, as was the case with radioactive citrate, where, in addition to the carrier substance becoming active, succinate formed as a product of the reaction; so did citric acid form as an additional product when radioactive succinate was incubated with inactive carrier.

An interesting point in connection with these experiments is that acetate becomes active. The tricarboxylic acid cycle does not require the formation of active acetate. Therefore, in cells grown on citrate, both a di- and tricarboxylic acid cycle may co-exist. That a similar situation may exist in yeast has already been discussed.

[4] In a short note Dagley *et al.* (33) expressed the opinion that a dicarboxylic acid cycle operates in *A. aerogenes*. Their complete data are to appear in a future issue of the J. Gen. Microbiol.

From the above studies it is clear that the oxidation of acetate or pyruvate in bacteria is a function of the organism in question, as well as the substrate upon which the organism is grown. Any one of these possibilities may be involved:

(a) Operation in whole or in part of a conventional tricarboxylic acid cycle, as in the case of *M. lysodeikticus*, citrate-grown *A. aerogenes*, and *A. vinelandii*;[5]

(b) operation in whole or in part, of a Thunberg-Knoop cycle as in the case of *E. coli*, *C. creatinovorans* and acetate-grown *A. aerogenes*;

(c) operation of neither cycle, as tentatively appears to be the case with the strain of *A. agilis* used by Karlsson and Barker.

OTHERS

Protozoa. Of all the classes of microorganisms, the evidence for the existence of a tricarboxylic acid cycle is most convincing for the malarial parasites. Some general aspects of the metabolism of malarial parasites have been reviewed by Moulder (68). A detailed account of the mechanism of pyruvate oxidation in protozoa is given by Speck *et al.* (90).

The initial stages of carbohydrate breakdown in the trypanosomes and malarial parasites that have been studied follow essentially the same pattern as found in the tissues of many free-living organisms. Whether or not the glycolytic enzymes of these parasites are in every respect similar to those of vertebrate tissue remains to be studied.

The mechanism of aerobic respiration in malarial parasites is also similar to that of their vertebrate hosts. The lactic (89) and malic (90) dehydrogenases of *Plasmodium gallinaceum* require diphosphopyridine nucleotide for maximum activity, and flavin adenine dinucleotide is probably formed by *P. knowlesi* (13), suggesting the presence of both pyridinoproteins and flavoproteins in plasmodia. The oxygen uptake of several species of *Plasmodium* is almost completely inhibited by low concentrations of cyanide (67, 22), and the respiration of *P. knowlesi* is lessened in the presence of CO (67). These observations strongly suggest that malarial parasites utilize O_2 by means of a heavy metal respiratory enzyme.

In general all these studies support the tentative conclusion that oxygen transport in malarial parasites is catalyzed by iron-porphyrin proteins, flavoproteins, and pyridinenucleotides similar to those demonstrated in other forms of life.

The mechanism of pyruvate oxidation has been studied by Speck, *et al.* (90) in considerable detail. We have already indicated that in animal tissues the tricarboxylic acid cycle as a mechanism of pyruvate and acetate oxidation is rather well established. Krebs (53) has listed the fundamental observations which support the tricarboxylic acid cycle mechanism in pigeon breast muscle: (a) All the component acids of the cycle are metabolized by minced pigeon breast muscle; (b) these acids catalyze the oxidation of pyruvate when added

[5] Campbell and Stokes (26) report the presence of the enzymes necessary for the oxidation of members of the tricarboxylic acid cycle in cells of *Pseudomonas aeruginosa* and from manometric data conclude the operation of a tricarboxylic acid cycle in this organism.

to breast muscle preparations in catalytic amounts; (c) citrate and α-ketoglutarate are formed from pyruvate and dicarboxylic acids; (d) pyruvate oxidation is inhibited by malonate and in the presence of malonate, succinate is oxidatively formed from fumarate and pyruvate. When this approach was followed with free malarial parasites, similar results were obtained. In free parasites, the acids of the tricarboxylic acid cycle are oxidized at rates comparable to the rate of pyruvate oxidation. The oxidation of pyruvic acid is catalyzed by the dicarboxylic acids and is strongly inhibited by malonate, with the subsequent accumulation of succinate. The malonate inhibition could be reversed by the addition of fumaric acid. When pyruvate and oxalacetate were incubated with free or parasitized erythrocytes, a small amount of citrate was formed. It is, therefore, clear that all the criteria that have been set up for pyruvate oxidation by Krebs could be met by the malarial parasites.

The observations concerning the cofactors for pyruvate oxidation by free parasites are of special interest. The finding that Mn^{++} stimulates pyruvate oxidation in the free parasites are in agreement with previous work on the participation of this ion in enzyme systems metabolizing pyruvate, α-ketoglutarate, oxalacetate and oxalosuccinate. The acceleration of oxidation produced by adenosine triphosphate is strongly suggestive that pyruvate oxidation is linked with esterification of inorganic phosphate. The stimulative effects of thiamine provides evidence for the participation of thiamine in the metabolism of these parasites. The function of thiamine is concerned with the oxidation and decarboxylation of pyruvate and other keto acids in animal tissues. The function may be similar here.

The question of pyruvate oxidation to acetate has also been considered by Speck *et al.* (90). Acetic acid, in small amounts, was consistently formed from pyruvic acid by free parasites. Catalytic amounts of C_4 dicarboxylic acids decreased the formation of acetate. There are two apparent explanations for this observation. The first explanation is based on the view that the initial step in the oxidation of pyruvate is the formation of a 2-carbon compound. Once formed, the 2-carbon unit may condense with oxalacetate and be oxidized through the tricarboxylic acid cycle, or it may be converted to acetate. The authors believe, however, that it is the damage to the enzymatic mechanism of the tricarboxylic acid cycle in free parasites that is responsible for the formation of acetate from pyruvate by the preparations they used. Addition of small amounts of dicarboxylic acids, by partially restoring the tricarboxylic acid cycle mechanism, decreases the proportion of the 2-carbon particle converted to acetate. This explanation visualizes essentially a competition between two systems for the 2-carbon unit formed from pyruvate, with the oxidation through the tricarboxylic acid cycle predominating. The second explanation is based on the assumption that two independent mechanisms co-exist for pyruvate oxidation, e.g., one is the conversion to acetate, perhaps by a direct oxidative decarboxylation, and the other the tricarboxylic acid cycle. These two mechanisms might then compete for the intact pyruvate molecule in the manner indicated previously for the 2-carbon particle derived from pyruvate. The data presented do not make it possible to distinguish between the two schemes in the malarial

parasite. However, the authors express doubt whether the formation of acetate constitutes an important metabolic pathway under physiological conditions.

In summing up the position regarding the terminal respiratory pattern of protozoa, it can be said that *the evidence favors the operation of a tricarboxylic acid cycle for pyruvate oxidation.* Since acetate accumulates, it appears not to play a significant role in the metabolism of these cells.

Rickettsiae. Although far from being conclusive, recent work by Bovarnick and co-workers suggest that at least some of the steps of the tricarboxylic acid cycle occur in partially purified preparations of typhus rickettsiae. Studies yielding information concerning the growth and metabolism of obligate parasites should be of obvious theoretical and possibly practical importance.

About two years ago Bovarnick and Snyder (24) reported that partially purified rickettsiae are capable of oxidizing glutamate and that the rate of this reaction is proportional to the number of organisms present. Glucose is not metabolized. These findings were extended and in a more recent publication (Bovarnick and Miller, 23) the general conclusion was reached that glutamate is oxidized first via α-ketoglutarate, and ultimately to CO_2 and water through the citric acid cycle. The evidence for this conclusion, not too convincing and only indirect, is simply this. During the oxidation of glutamate, some aspartic acid forms which, they claim, together with the evidence for the presence of a glutamate-aspartate transaminase implicates oxalacetate as a metabolite in the respiration of the rickettsiae. The fact that α-ketoglutarate does not accumulate during the oxidation of glutamate and that both pyruvate and succinate are being oxidized by their partially purified preparations is all taken as evidence for the operation of a tricarboxylic acid cycle. On the basis of these indirect findings such a conclusion is premature since, for example, neither fumarate, malate, oxalacetate, α-ketoglutarate or citrate is not attacked. The authors suggest that this is due to permeability factors, but no evidence for this assumption is given. Further, as the authors themselves point out, there is no evidence whatsoever for the formation of citrate.

Phage and Virus. Even though the assumption concerning the possible operation of a tricarboxylic acid (TCA) cycle in the respiration of rickettsiae is premature, at least it indicates that progress is being made. With phages or viruses, however, we know as yet little concerning their respiratory mechanism, or whether they exhibit an independent metabolism in general. Recently, Ajl (3) confirmed earlier findings that partially purified phage exhibits no independent metabolic activity on any of the TCA cycle intermediates, as measured by oxygen uptake or methylene blue reduction. The same is true with viruses, although it is noteworthy that evidence has very recently been presented (1) which indicates that the functioning of the TCA cycle is essential for the propagation of influenza virus.

ASSIMILATORY MECHANISMS IN MICROORGANISMS

The mechanism of the respiratory production of CO_2, one of the major two end products of terminal respiration, has been discussed. Cellular respiration

is the fundamental mechanism by which potential energy of organic compounds is released and made available for work alone or with synthesis of cell material. The latter represents the *second major* end product of microbial oxidative metabolism (94). Organic foods are oxidized in a stepwise manner by a series of catalysts, the respiratory enzymes, and the potential energy is likewise released in a series of graded steps. The stepwise liberation of energy results in efficient utilization, for the transfer or coupling of the energy will be most efficient when the reactions are operating at or near their equilibrium points. Each partial reaction of formation and of degradation must have a velocity at least equal to the velocity of the overall reaction of respiration.

The main purpose of biological oxidation is to yield energy to the system. Much of the kinetic energy produced is in the form of heat, and this energy is useless for cellular work and is wasted, except as it serves to maintain body temperature in the mammals. However, the cell possesses contrivances for utilizing some of the liberated energy by channeling it through the so-called adenylic acid system, which serves as a mediator in energy transfer.

The central problem of cellular respiration—the mechanism of using the energy liberated in oxidation for the work of the cell, including synthesis—is a virtual mystery. In no single case has an adequate and complete explanation of the mechanism of the energetic coupling between the energy-liberating reaction and the cellular machinery been given. However, although we know little about the interaction of the various enzyme systems which bring about the synthesis of protoplasm, a great deal of progress is being made in understanding the synthetic mechanism involved in building up the individual components which ultimately make up cell material. In fact, a pronounced shift from catabolic to anabolic research has taken place in the last several years and fruitful results are already becoming apparent.

Oxidative Assimilation. It has long been known that oxidative assimilation occurred in growing cells, and that assimilation also takes place in nonproliferating cells was clearly shown by the work of Barker (14), Clifton and Logan (31), Pickett and Clifton (78) and others.

Clifton and associates (30, 31) have also been able to separate the "coupling" of oxidation and assimilation by poisoning the cells with 0.0025M NaN_3 or 0.0005M dinitrophenol. The poisoned cells oxidize the substrate completely to CO_2 and water. This widespread oxidative assimilation must be at the very basis of the chemistry of growth, and the fundamental problem of how the energy coupling occurs is now being elucidated.

Phosphorylation Due to Microbial Oxidation. It has been shown repeatedly that the oxidation of Krebs cycle intermediates in animal tissues is linked with the uptake of inorganic phosphate, resulting in the formation of energy-rich phosphate compounds. Relatively few such studies, however, have thus far been presented with microorganisms.

The early work of Lipmann, who showed the formation of an energy-rich compound, acetyl phosphate, as a result of the oxidative decarboxylation of pyruvate by *Lactobacillus delbrückii* has been reviewed previously (57). This

observation probably represents the first example of a coupled oxidation and phosphorylation in bacterial metabolism. The anaerobic formation of acyl phosphates in a number of bacteria has also been reviewed (75). Experiments have recently been conducted which deal with the formation of energy-rich compounds as a result of dehydrogenation reactions.

It has been possible to demonstrate that succinic acid oxidation (43) is appreciably reduced in the absence of inorganic phosphate by cell-free extracts of *E. coli*. On the addition of inorganic phosphate, a 7-min. acid hydrolyzable compound formed, which has recently been identified as a compound having the same R_f as known adenosine triphosphate (ATP) (44). The reaction was carried out by enzyme preparations, which did not oxidize succinate beyond the fumarate:malate equilibrium, and, consequently, the formation of ATP appears to be the result of a single dehydrogenation reaction between succinate and the fumarate:malate equilibrium. Incorporation of P^{32} into carrier ATP was used as an index of phosphorylation. Data have also been obtained in our laboratory which show the requirement of inorganic phosphate for the oxidation of acetate to succinate. This was done by incubating radioactive acetate in the presence of carrier succinate, with and without phosphate. If the concentration of succinate is kept sufficiently high so that its concentration for all practical purposes remains constant, then the incorporation of 2-C^{14}-acetate carbon into it, with or without phosphate, becomes a measure of phosphate requirement for the condensation reaction. The results indicated that phosphate was essential for the dehydrogenation reaction. This dehydrogenation is of particular interest for it combines an energy-yielding (dehydrogenation) and energy-requiring (condensation) reaction. The intermediate formation of an energy-rich compound has not yet been detected.

A dissociation of respiratory hydrogen transfer and phosphorylation by the action by such compounds as dinitrophenol (59), gramicidin (45), and sodium azide (91) has been demonstrated with various tissue preparations. These results explain the earlier data of Clifton and coworkers on the inhibition of anabolic processes by these compounds. They further imply the participation of energy-rich phosphates in oxidative assimilations, although no evidence has been obtained that there is an appreciable accumulation of a primary assimilatory product rich in phosphate-bond energy, since no appreciable phosphate uptake is observed in the course of oxidative assimilation.

Utilization of high energy phosphate for synthetic reactions. It has become rather clear, mainly from the studies of Lipmann, that energy delivery from fermentative, as well as from oxidative, metabolism is geared through the generation of energy-rich phosphate compounds. However, until recently, there existed a vacuum on the receiving side. For a long time only relatively few instances of phosphate bond utilization were recognizable. During the last few years, however, the number of synthetic reactions driven by an influx of energy-rich phosphate bonds has increased considerably. Peptide formation (20), the synthesis of urea (32), the synthesis of α and β-keto acids (47, 87), and the synthesis of glutamine (88), to name only some of the important advances, appear now to belong to this group.

In microbial metabolism, too, several examples on the utilization of high energy phosphate for synthetic reactions may be cited.

A series of extremely interesting experiments on the utilization of oxidative energy has been reported some years ago by Vogler, Umbreit and collaborators with the autotroph *Thiobacillus thiooxidans* (103, 104, 105). The processes of energy release through oxidation (of sulfur in this case) and of energy utilization could be separated. The oxidative phase was found to be accompanied by phosphate fixation, and the reductive phase of carbon dioxide fixation by a release of phosphate.

The utilization of oxidative energy for synthetic reactions is now being studied in our laboratory (108). Using cell-free extracts of *E. coli*, it was found that the incorporation of 2-C^{14}-glycine into glutathione during vigorous succinate oxidation is from two to four times greater than in its absence. The formation of ATP due to the oxidation of succinate by similar cell-free extracts has also been shown (43). It is, therefore, believed that a system has been reconstructed *in vitro* which can utilize biological energy for the immediate synthesis of a peptide bond.

REFERENCES

1. ACKERMANN, W. W. 1951 Concerning the relation of the Krebs cycle to virus propagation. J. Biol. Chem., **189**, 421–428.
2. AJL, S. J. 1950 Acetic acid oxidation by *Escherichia coli* and *Aerobacter aerogenes*. J. Bact., **59**, 499–507.
3. AJL, S. J. 1950 Metabolic studies on T_2H *Escherichia coli* bacteriophage. J. Bact., **60**, 393–399.
4. AJL, S. J. 1951 Studies on the mechanism of acetate oxidation by bacteria. V. Evidence for the participation of fumarate, malate, and oxalacetate in the oxidation of acetic acid by *Escherichia coli*. J. Gen. Physiol., **34**, 785–794.
5. AJL, S. J. 1951 Studies on the mechanism of acetate oxidation by bacteria. VI. Comparative patterns of acetate oxidation by citrate-grown and acetate-grown *Aerobacter aerogenes*. J. Gen. Physiol., **35**, 119–127.
6. AJL, S. J. 1951 Studies on the mechanism of acetate oxidation by bacteria. VII. Time-function relating specific activity of precursor to product considerations. To be published.
7. AJL, S. J., AND KAMEN, M. D. 1950 Studies on the mechanism of acetate oxidation by bacteria. Fed. Proc., **9**, 143–144.
8. AJL, S. J., AND KAMEN, M. D. 1951 Studies on the mechanism of acetate oxidation by *Escherichia coli*. J. Biol. Chem., **189**, 845–857.
9. AJL, S. J., AND KAMEN, M. D. 1951 A simple and rapid biosynthesis of isotopically labeled succinic acid. J. Am. Chem. Soc., **75**, 2349–2350.
10. AJL, S. J., KAMEN, M. D., RANSON, S. L., AND WONG, D. T. O. 1951 Studies on the mechanism of acetate oxidation by *Micrococcus lysodeikticus*. J. Biol. Chem., **189**, 859–867.
11. AJL, S. J., AND WONG, D. T. O. 1951 Studies on the mechanism of acetate oxidation by bacteria. IV. Acetate oxidation by citrate-grown *Aerobacter aerogenes* studied with radioactive carbon. J. Bact., **61**, 379–387.
12. AUBEL, E., ROSENBERG, A. J., AND SZULMAJSTER, J. 1950 Contribution a l'étude de la fermentation et de la respiration de *Escherichia coli*. I. Action de quelques inhibiteurs sur la respiration et la fermentation de *Esch. coli*. Biochem. et Biophys. Acta, **5**, 228–254.

13. BALL, E. G., MCKEE, R. W., ANFINSEN, C. B., CRUZ, W. O., AND GEIMAN, Q. M. 1948 Studies on malarial parasites. IX. Chemical and metabolic changes during growth and multiplication in vivo and in vitro. J. Biol. Chem., **175**, 547–571.
14. BARKER, H. A. 1936 The oxidative metabolism of the colorless alga, *Prototheca zopfii*. J. Cell. Comp. Physiol., **8**, 231–250.
15. BARKER, H. A., AND KAMEN, M. D. 1945 Carbon dioxide utilization in the synthesis of acetic acid by *Clostridium thermoaceticum*. Proc. Natl. Acad. Sci., **31**, 219–225.
16. BARRON, E. S. G., ARDAO, M. I., AND HEARON, M. 1950 The mechanism of acetate oxidation by *Corynebacterium creatinovorans*. Arch. Biochem., **29**, 130–153.
17. BERGMANN, M., AND FRUTON, J. S. 1937 On proteolytic enzymes. XII. Regarding the specificity of aminopeptidase and carboxypeptidase. A new type of enzyme in the intestinal tract. J. Biol. Chem., **117**, 189–202.
18. BERGMANN, M., ZERVAS, L., FRUTON, J. S., SCHNEIDER, F., AND SCHLEICH, H. 1935 On proteolytic enzymes. V. On the specificity of dipeptidase. J. Biol. Chem., **109**, 325–346.
19. BIRKELAND, J. M., AND RANDLES, C. I. 1947 The effects of malic and malonic acids on methylene blue reduction by bacteria. J. Bact., **54**, 275.
20. BLOCH, K. 1949 The synthesis of glutathione in isolated liver. J. Biol. Chem., **179**, 1245–1254.
21. BOLCATO, V., SCEVOLA, M. E., AND BOSSO, M. 1951 Pyruvic acid intermediate product in acetate oxidation by yeast. Experientia, **7**, 69–71.
22. BOVARNICK, M. R., LINDSAY, A., AND HELLERMAN, L. 1946 Metabolism of the malarial parasite with reference particularly to the action of antimalarial agents. I. Preparation and properties of *Plasmodium lophurae* separated from the red cells of duck blood by means of saponin. J. Biol. Chem., **163**, 523–533.
23. BOVARNICK, M. R., AND MILLER, J. C. 1950 Oxidation and transamination of glutamate by typhus rickettsiae. J. Biol. Chem., **184**, 661–676.
24. BOVARNICK, M. R., AND SNYDER, J. C. 1949 Respiration of typhus rickettsiae. J. Exptl. Med., **89**, 561–585.
25. BREWER, C. R., AND WERKMAN, C. H. 1939 Oxidation of citric acid by coli-aerogenes bacteria. Proc. Soc. Exptl. Biol. Med., **41**, 185–188.
26. CAMPBELL, J. R., AND STOKES, F. N. 1951 Tricarboxylic acid cycle in *Pseudumonas aeruginosa*. J. Biol. Chem., **190**, 853–858.
27. CARSON, S. F., FOSTER, J. W., ANTHONY, D. S., AND PHARES, E. F. 1950 Genesis of C_4 dicarboxylic acids in the tricarboxylic acid cycle. Fed. Proc., **9**, 159.
28. CHOU, T. C., NOVELLI, G. D., STADTMAN, E. R., AND LIPMANN, F. 1950 Fractionation of coenzyme A-dependent acetyl transfer systems. Fed. Proc., **9**, 160.
29. CHUGHTAI, M. I. D., PEARCE, A. A., AND WALKER, T. K. 1950 The mechanism of formation or organic acids by mould fungi. IV. The formation of acetic and pyruvic acids in *Aspergillus niger* growing in glucose media. Biochem. J., **47**, 135–139.
30. CLIFTON, C. E. 1937 On the possibility of preventing assimilation in respiring cells. Enzymologia, **4**, 246–253.
31. CLIFTON, C. E., AND LOGAN, W. A. 1939 On the relation between assimilation and respiration in suspensions and in cultures of *Escherichia coli*. J. Bact., **37**, 523–540.
32. COHEN, P. P., AND HAYANO, M. 1948 The enzymatic steps in urea synthesis. J. Biol. Chem., **172**, 405–415.
33. DAGLEY, S., MORRISON, G. A., AND DAWES, E. A. 1951 The "Dicarboxylic acid cycle" in bacterial metabolism. Arch. Biochem. and Biophys., **32**, 231–232.
34. FOSTER, J. W. 1949 "Chemical Activities of Fungi." Academic Press, Inc., New York.

35. FOSTER, J. W., AND CARSON, S. F. 1950 Metabolic exchange of carbon dioxide with carboxyls and oxidative synthesis of C_4 dicarboxylic acids. Proc. Natl. Acad. Sci., **36**, 219–229.
36. FOSTER, J. W., CARSON, S. F., ANTHONY, D. S., DAVIS, J. B., JEFFERSON, W. E., AND LONG, M. V. 1949 Aerobic formation of fumaric acid in the mold *Rhizopus nigricans:* Synthesis by direct C_2 condensation. Proc. Natl. Acad. Sci., **35**, 663–672.
37. FOSTER, J. W., CARSON, S. F., AND JEFFERSON, W. E. 1950 The $2C_2$ condensation reaction in citric acid formation by a strain of *Aspergillus niger*. Fed. Proc., **9**, 172.
38. FOSTER, J. W., CARSON, S. F., RUBEN, S., AND KAMEN, M. D. 1941 Radioactive carbon as an indicator of carbon dioxide utilization. VII. The assimilation of carbon dioxide by molds. Proc. Natl. Acad. Sci., **27**, 590–596.
39. FOSTER, J. W., AND DAVIS, J. B. 1948 Anaerobic formation of fumaric acid by the the mold *Rhizopus nigricans*. J. Bact., **56**, 329–338.
40. FOULKES, E. C. 1951 The occurrence of the tricarboxylic acid cycle in yeast. Biochem. J., **48**, 378–383.
41. GEIGER, W. B. 1947 Interference by streptomycin with a metabolic system of *Escherichia coli*. Arch. Biochem., **15**, 227–238.
42. GREEN, D. E., LOOMIS, W. F., AND AUERBACH, V. H. 1948 Studies on the cyclophorase system. I. The complete oxidation of pyruvic acid to carbon dioxide and water. J. Biol. Chem., **172**, 389–403.
43. HERSEY, D. F., AND AJL, S. J. 1951 Phosphorylation due to the oxidation of succinic acid by cell-free extracts of *Escherichia coli*. J. Gen. Physiol., **34**, 295–304.
44. HERSEY, D. F., AND AJL, S. J. 1951 Adenosine triphosphate formation due to the oxidation of succinic acid by bacteria. J. Biol. Chem., **191**, 113–121.
45. HOTCHKISS, R. D. 1944 Gramicidin, tyrocidine and tyrothricin. "Advances in Enzymology and Related Subjects," **4**, 153–199.
46. KAPLAN, N. O., AND LIPMANN, F. 1948 The assay and distribution of coenzyme A. J. Biol. Chem., **174**, 37–44.
47. KAPLAN, N. O., AND LIPMANN, F. 1948 The acetyl precursor in pyruvate synthesis in *Escherichia coli*. J. Biol. Chem., **176**, 459–460.
48. KARLSSON, J. L. 1947 Thesis. University of California.
49. KARLSSON, J. L., AND BARKER, H. A. 1948 Evidence against the occurrence of a tricarboxylic acid cycle in *Azotobacter agilis*. J. Biol. Chem., **175**, 913–921.
50. KORNBERG, A., AND PRICER, W. E., JR. 1951 Di- and triphosphopyridine nucleotide isocitric dehydrogenase in yeast. J. Biol. Chem., **189**, 123–136.
51. KREBS, H. A. 1937 The role of fumarate in the respiration of *Bacterium coli commune*. Biochem. J., **31**, 2095–2124.
52. KREBS, H. A. 1940 The citric acid cycle. Biochem. J., **34**, 460–463.
53. KREBS, H. A. 1943 The intermediary stages in the biological oxidation of carbohydrate. "Advances in Enzymology and Related Subjects," **3**, 191–252.
54. KREBS, H. A. 1948–49 The tricarboxylic acid cycle. The Harvey Lecture Series, **44**, 165–199.
55. KREBS, H. A., AND JOHNSON, W. A. 1937 The role of citric acid in intermediate metabolism in animal tissues. Enzymologia, **4**, 148–156.
56. LENTI, C. 1946 Oxidation of pyruvic acid by *Bacterium coli communis*. Boll. Soc. Ital. biol. Sper., **22**, 332–333. (Cited in Chem. Abstracts, **41**, 787).
57. LIPMANN, F. 1939 An analysis of the pyruvic acid oxidation system. Cold Spring Harbor Symp. of Quant. Biol., **7**, 248–459.
58. LIPMANN, F., KAPLAN, N. V., NOVELLI, G. D., TUTTLE, L. C., AND GUIRARD, B. M. 1950 Isolation of coenzyme A. J. Biol. Chem., **186**, 235–243.

59. LOOMIS, W. F., AND LIPMANN, F. 1947 Reversible inhibition of the coupling between phosphorylation and oxidation. J. Biol. Chem., **173**, 807–808.
60. LORBER, V., UTTER, M. F., RUDNEY, H., AND COOK, M. 1950 The enzymatic formation of citric acid studied with C^{14} labeled oxalacetate. J. Biol. Chem., **185**, 689–699.
61. LYNEN, F. 1942 Zum biologischen Abbau der Essigsäure. I. Über die "Induktionszeit" bei verarmter Hefe. Ann. duetsch. chem. Gesell. **552**, 270–306.
62. LYNEN, F. 1948 Zum biologischen Abbau der Essigsäure. III. Ein versuch mit Deutero-Essigsäure. Ibid. **558**, 47–53.
63. LYNEN, F., AND NECIULLAH, N. 1939 Zum Abbau von Bernsteinsäure Äpfelsäure und Citronensaure durch Hefe. Ibid. **541**, 203–213.
64. LYNEN, F., AND REICHERT, E. 1951 Zur chemischen Structur der "aktivierten Essigsäure." Angewandte Chemie, **63**, 47–48.
65. MARTIUS, C. 1943 Der Mechanismus der Citronensäurebildung im Tierkörper im Zusammenhang mit dem Abbau der Brenztraubensäure. Z. Physiol. Chem., **279**, 96–104.
66. MARTIUS, C., UND LYNEN, F. 1950 Probleme des Citronensäurecyklus. "Advances in Enzymology and Related Subjects," **10**, 167–222.
67. MCKEE, R. W., ORMSBEE, R. A., ANFINSEN, C. B., GEIMAN, Q. M., AND BALL, E. G. 1946 Studies on malarial parasites. VI. The chemistry and metabolism of normal and parasitized (*P. knowlesi*) monkey blood. J. Exptl. Med., **84**, 569–582.
68. MOULDER, J. W. 1948 The metabolism of malarial parasites. Ann. Rev. Microbiol., **2**, 101–120.
69. NORD, F. F., AND VITUCCI, J. C. 1948 Certain aspects of the microbiological degradation of cellulose. "Advances in Enzymology and Related Subjects," **8**, 253–298.
70. NOVELLI, G. D., KAPLAN, N. O., AND LIPMANN, F. 1949 The liberation of pantothenic acid from coenzyme A. J. Biol. Chem., **177**, 97–107.
71. NOVELLI, G. D., AND LIPMANN, F. 1947 The involvement of coenzyme A in acetate oxidation in yeast. J. Biol. Chem., **171**, 833–834.
72. NOVELLI, G. D., AND LIPMANN, F. 1950 The catalytic function of coenzyme A in citric acid synthesis. J. Biol. Chem., **182**, 213–228.
73. OCHOA, S. 1944 α-ketoglutaric dehydrogenase of animal tissues. J. Biol. Chem., **155**, 87–100.
74. OCHOA, S. 1948 Biosynthesis of tricarboxylic acids by carbon dioxide fixation. III. Enzymatic mechanisms. J. Biol. Chem., **174**, 133–157.
75. OCHOA, S. 1951 Biological mechanisms of carboxylation and dicarboxylation. Physiol. Rev., **31**, 56–106.
76. OGINSKY, E. L., SMITH, P. H., AND UMBREIT, W. W. 1949 The action of streptomycin. I. The nature of the reaction inhibited. J. Bact., **58**, 747–759.
77. OGSTON, A. G. 1948 Interpretation of experiments on metabolic processes, using isotopic tracer elements. Nature, **162**, 963.
78. PICKETT, M. J., AND CLIFTON, C. E. 1943 On the relation between the oxidation and assimilation of simple substrates by yeast. J. Cell. Comp. Physiol., **21**, 77–94.
79. POTTER, V. R., AND HEIDELBERGER, C. 1949 Biosynthesis of "Asymmetric" citric acid: A substantiation of the Ogston concept. Nature, **164**, 180–181.
80. RATNER, S., AND RACKER, E. 1950 Carbohydrate metabolism. Ann. Rev. Biochem., **19**, 187–214.
81. SAZ, H. J., AND KRAMPITZ, L. O. 1950 Acetate oxidation in *Micrococcus lysodeikticus*. Bact. Proc., 126.
82. SAZ, H. J., AND KRAMPITZ, L. O. 1951 Acetate oxidation by *Micrococcus lysodeikticus*. Fed. Proc., **10**, 243.
83. SIEGEL, B. V. 1950 Thermodynamic aspects of a proposed mechanism for acetate oxidation in bacteria. Proc. Natl. Acad. Sci., **36**, 515–517.

84. SLADE, H. D., AND WERKMAN, C. H. 1943 Assimilation of acetic and succinic acids containing heavy carbon by *Aerobacter indologenes*. Arch. Biochem., **2**, 97–111.
85. SMITH, V. M. 1949 On the mechanism of enzyme action. XXXVI. Dehydrogenation studies on *Merulius lacrymans* and *Marasmius chordalis* and the mechanism of oxalic acid formation. Arch. Biochen., **22**, 275–287.
86. SONDERHOFF, R., AND THOMAS, H. 1937 Die enzymatische Dehydrierung der Trideutero-essigsäure. Ann. deutsch. chem. Gesell. **530**, 195–213.
87. SOODAK, M., AND LIPMANN, F. 1948 Enzymatic condensation of acetate to acetoacetate in liver extracts. J. Biol. Chem., **175**, 999–1000.
88. SPECK, J. F. 1947 The enzymatic synthesis of glutamine. J. Biol. Chem., **168**, 403–404.
89. SPECK, J. F., AND EVANS, E. A., JR. 1945 The biochemistry of the malaria parasite. II. Glycolysis in cell-free preparations of the malaria parasite. J. Biol. Chem., **159**, 71–81.
90. SPECK, J. F., MOULDER, J. W., AND EVANS, E. A., JR. 1946 The biochemistry of the malaria parasite. V. Mechanism of pyruvate oxidation in the malaria parasite. J. Biol. Chem., **164**, 119–144.
91. SPIEGELMAN, S., KAMEN, M. D., AND SUSSMAN, M. 1948 Phosphate metabolism and the disocciation of anaerobic glycolysis from synthesis in the presence of sodium azide. Arch. Biochem., **18**, 409–436.
92. STADTMAN, E. R. 1950 Coenzyme A dependent transacetylation and transphosphorylation. Fed. Proc., **9**, 233.
93. STANIER, R. Y. 1947 Simultaneous adaptations: A new technique for the study of metabolic pathways. J. Bact., **54**, 339–348.
94. STANIER, R. Y. 1950 Problems of bacterial oxidative metabolism. Bact. Rev., **14**, 179–191.
95. STERN, J. R., AND OCHOA, S. 1949 Enzymatic synthesis of citric acid by condensation of acetate and oxalacetate. J. Biol. Chem., **179**, 491–492.
96. STERN, J. R., AND OCHOA, S. 1950 Enzymatic synthesis of citric acid. Fed. Proc., **9**, 234–235.
97. STERN, J. R., SHAPIRO, B., AND OCHOA, S. 1950 Synthesis and breakdown of citric acid with crystalline condensing enzyme. Nature, **166**, 403–404.
98. STONE, R. W., AND WILSON, P. W. 1951 Personal communication.
99. SWIM, H. E., AND KRAMPITZ, L. O. 1950 Evidence for the condensation of acetic acid to succinic acid in *Escherichia coli*. Bact. Proc., 125–126.
100. THUNBERG, T. 1920 Intermediary metabolism and the enzymes concerned therein. Skand. Arch. Physiol., **40**, 1–91. (Cited in Chem. Abstr., 1921, **15**, 876–877.)
101. UMBREIT, W. W. 1949 A site of action of streptomycin. J. Biol. Chem., **177**, 703–714.
102. UMBREIT, W. W. 1950 The metabolic action of streptomycin. New York Acad. Sci., **53**, 6–12.
103. VOGLER, K. G. 1942 Studies on the metabolism of autotrophic bacteria. II. The nature of the chemosynthetic reaction. J. Gen. Physiol., **26**, 103–117.
104. VOGLER, K. G., LEPAGE, G. A., AND UMBREIT, W. W. 1942 Studies on the metabolism of autotrophic bacteria. I. The respiration of *Thiobacillus thioöxidans* on sulfur. J. Gen. Physiol., **26**, 89–102.
105. VOGLER, K. G., AND UMBREIT, W. W. 1942 Studies on the metabolism of autotrophic bacteria. III. The nature of the energy-storage material active on the chemosynthetic process. J. Gen. Physiol., **26**, 157–167.
106. WEINHOUSE, S., AND MILLINGTON, R. H. 1947 Acetate metabolism in yeast, studied with isotopic carbon. J. Am. Chem. Soc., **69**, 3089–3093.
107. WEINHOUSE, S., MILLINGTON, R. H., AND LEWIS, K. F. 1948 Oxidation of glucose by yeast, studied with isotopic carbon. J. Am. Chem. Soc., **70**, 3680–3683.

108. WHEAT, R. W., AND AJL, S. J. 1951 Peptide bond synthesis in bacteria. Bact. Proc., 130.
109. WIELAND, H., UND SONDERHOFF, R. 1932 Uber den Mechanismus der oxydationsvorgänge. XXXII. Die enzymatische Oxydation von Essigsäure durch Hefe. Ann. deutsch. chem. Gesell., **499**, 213–228.
110. WILCOX, P. E., HEIDELBERGER, C., AND POTTER, V. R. 1950 Chemical preparation of asymmetrically labeled citric acid. J. Am. Chem. Soc., **72**, 5019–5024.
111. WOOD, H. G. 1946 The fixation of carbon dioxide and the interrelationships of the tricarboxylic acid cycle. Physiol. Rev., **26**, 198–246.
112. WOOD, H. G., WERKMAN, C. H., HEMINGWAY, A., AND NIER, A. O. 1941 Mechanism of fixation of carbon dioxide in the Krebs cycle. J. Biol. Chem., **139**, 483–484.
113. ZILVERSMIT, D. B., ENTENMAN, C., AND FISHLER, M. C. 1943 Calculation of "Turnover time" and "Turnover rate" from experiments involving the use of labeling agents. J. Gen. Physiol., **26**, 325–331.