

**THE FERMENTATION OF XYLOSE BY ESCHERICHIA COLI:
MECHANISMS OF SUCCINIC ACID BIOSYNTHESIS**

by

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LIST OF ABBREVIATED WORDS

- As 0.07 M sodium arsenite.
- C_n the biologically active intermediate of n carbon atoms whose exact chemical nature is unknown.
- Co A coenzyme A.
- c/s counts per second.
- DCAC dicarboxylic acid cycle (C₄ cycle) which requires the Thunberg or C₂ condensation as the initial step.
- BPN diphospho-pyridine-nucleotide (coenzyme I).
- ED ethylene diamine.
- K→S M/C . . the ratio of the activity in the methylene carbons of succinic acid obtained by the decarboxylation of alpha-ketoglutaric acid to the activity in the carboxyl carbons of this same succinic acid.
- MeB methylene blue.
- M/C the ratio of the activity in the methylene carbons of succinic acid to the activity in the carboxyl carbons of this same succinic acid.
- MFA sodium monofluoroacetic acid, 0.3 M, unless specified otherwise.
- M/M moles of end-product formed per mole of xylose utilized.
- OAA oxalacetic acid.
- S/K the ratio of the activity recovered in succinic acid to the activity recovered in alpha-ketoglutarate.
- TCAC tricarboxylic acid cycle (C₆ or Krebs cycle).
- TPN triphospho-pyridine-nucleotide (coenzyme II).

I. INTRODUCTION

The prominent position which the two carbon (C_2) fragment enjoys in studies of intermediary metabolism results from the fact that it represents a metabolic cross-read through which the pathways of anabolism and catabolism may, and oftentimes do, intersect.

The C_2 moiety is intimately associated with the synthesis of a variety of tissue constituents: glycogen, fatty acids, dicarboxylic amino acids, cholesterol, protoporphyrin and uric acid (Bloch, 1947). It has been implicated in the oxidation of fatty acids (Brady, Gurin, and Van Baalen, 1952), carbohydrates and proteins via the tricarboxylic acid cycle (TCAC) (Krebs, 1943).

The evidence which supports the participation of a C_2 fragment in the reversible phosphorelastic splitting of pyruvic acid ($C_2 + C_1 \rightleftharpoons C_3$) as adduced by Lipmann (1946) is complete as is also that of the reversible condensation of a C_2 fragment with oxalacetic acid (OAA) to form the initial tricarboxylic acid of the TCAC ($C_2 + C_4 \rightleftharpoons C_6$) (Stern, Shapiro, and Ochoa, 1950; Stern and Ochoa, 1951).

Furthermore several observations exist which are consistent with the possibility that acetate may:

(1) condense with itself to form succinate ($C_2 + C_2 \rightleftharpoons C_4$) according to the Thunberg (1920) hypothesis, and (2) with pyruvate to form aceto-pyruvate ($C_2 + C_3 \rightleftharpoons C_5$) (Krebs and Johnson, 1937).

Therefore, not only does the carbon skeleton of the C_2 fragment place it in a position of importance in metabolic studies but so also do its varied activities.

Evidence continues to accumulate that the concept of "comparative biochemistry" advanced by Kluver (1931) and supported vigorously by van Niel (1940), is fundamentally sound and that the contributions of investigations of intermediary metabolism wherein a microbial system has been used are found to have wide significance and validity. Therefore, an investigation of a microbial system in which the C_2 fragment plays an important role should yield valuable insight as to the metabolic fate of C_2 fragments in general.

Such a system is afforded by the fermentative dissimilation of xylose by Escherichia coli which gives rise to a C_2 fragment shown to be involved in the net synthesis of lactic acid by means of a $C_2 + C_1$ condensation and to be implicated in the biosynthesis of succinic acid. (Nutting and Carson, 1952).

The relationship between the C_2 fragment and the formation of succinate during the fermentation was

indicated by the finding that labelled acetate (acetate-2-C¹⁴) present as a tracer, i.e., in negligible amounts compared with the substrate xylose, gave rise to succinate labelled in the methylene carbons.

Since this system supports the net synthesis of lactate by C₂ + C₁ condensation, it was of considerable interest to determine whether the activity recovered in the succinate might have been introduced as a result of a net synthesis by C₂ + C₂ condensation.

Although at first glance, the isotopic labelling may indicate such a condensation, closer scrutiny reveals that there are at least two other possibilities for the origin of succinate, based on well-established mechanisms, which will produce similar results insofar as the isotope distribution is concerned.

Therefore, an attempt to elucidate the mechanism of the biosynthesis of this succinate must take into account the possible participation of at least three formative pathways: (1) the double fixation of carbon dioxide (C₁ + C₂ → C₃; C₁ + C₃ → C₄), (2) the TCAC and (3) the Thunberg (C₂) condensation.

II. SURVEY OF THE LITERATURE

The biosynthesis of succinate may occur by any of several metabolic pathways. Two of these pathways, the double fixation of carbon dioxide and the tricarboxylic acid cycle, are well documented and are believed to be present in E. coli. One other pathway, the Thunberg or C_2 condensation, has not been satisfactorily demonstrated although considerable favorable evidence has accumulated.

Together, these three metabolic pathways represent the major mechanisms responsible for the formation of succinate, and all fulfill the conditions observed by Nutting and Carson (1952 a,b) that the radioactivity present in the methylene carbon of acetate (acetate-2- C^{14}) is introduced into the methylene carbons of succinate.

A. The Double Fixation of Carbon Dioxide

The mechanism for the biosynthesis of succinic acid which most satisfactorily accounts for the observations of Nutting and Carson (1952 a,b) is one which involves the consecutive fixation of two CO_2 molecules, one on each "end" of the acetate molecule, to form OAA or malic acid. Presumably this latter C_4 acid would then be in equilibrium with succinate in accord with the Szent-Gyorgyi scheme.

This mechanism is presented in the series of reactions appearing in Figure 1.

In reaction (1) the C_2 fragment may be an activated form of acetate, the nature of which is uncertain. Lipmann (1946) has demonstrated that the reverse reaction, i.e., the oxidative decarboxylation of pyruvate involves orthophosphate and that the product of the reaction is an anhydride of acetate and phosphoric acid, and acyl phosphate. However attempts to demonstrate the synthesis of pyruvate in cell-free extracts of E. coli from acetyl phosphate and formate (Utter, Lipmann, and Werkman, 1945; Strecker, Weed, and Krampitz, 1950; Kaplan and Lipmann, 1948) have been unsuccessful even though small amounts of a keto acid have been demonstrated following the incubation of E. coli extracts with formate, adenosine triphosphate (ATP) and acetate. This failure may have been due to a requirement for coenzyme A (CoA) (Novelli, 1951).

Though formic acid appears to be the immediate C_1 fragment in this reaction, either formate or CO_2 may be considered in the case of E. coli, since this organism is able to form formate from CO_2 directly by means of either one of two enzymes, formic hydrogenlyase (Weeds, 1936) or formic dehydrogenase (Gale, 1939).

Once the C_3 compound, pyruvic acid, has been formed, there are two possibilities which may be considered

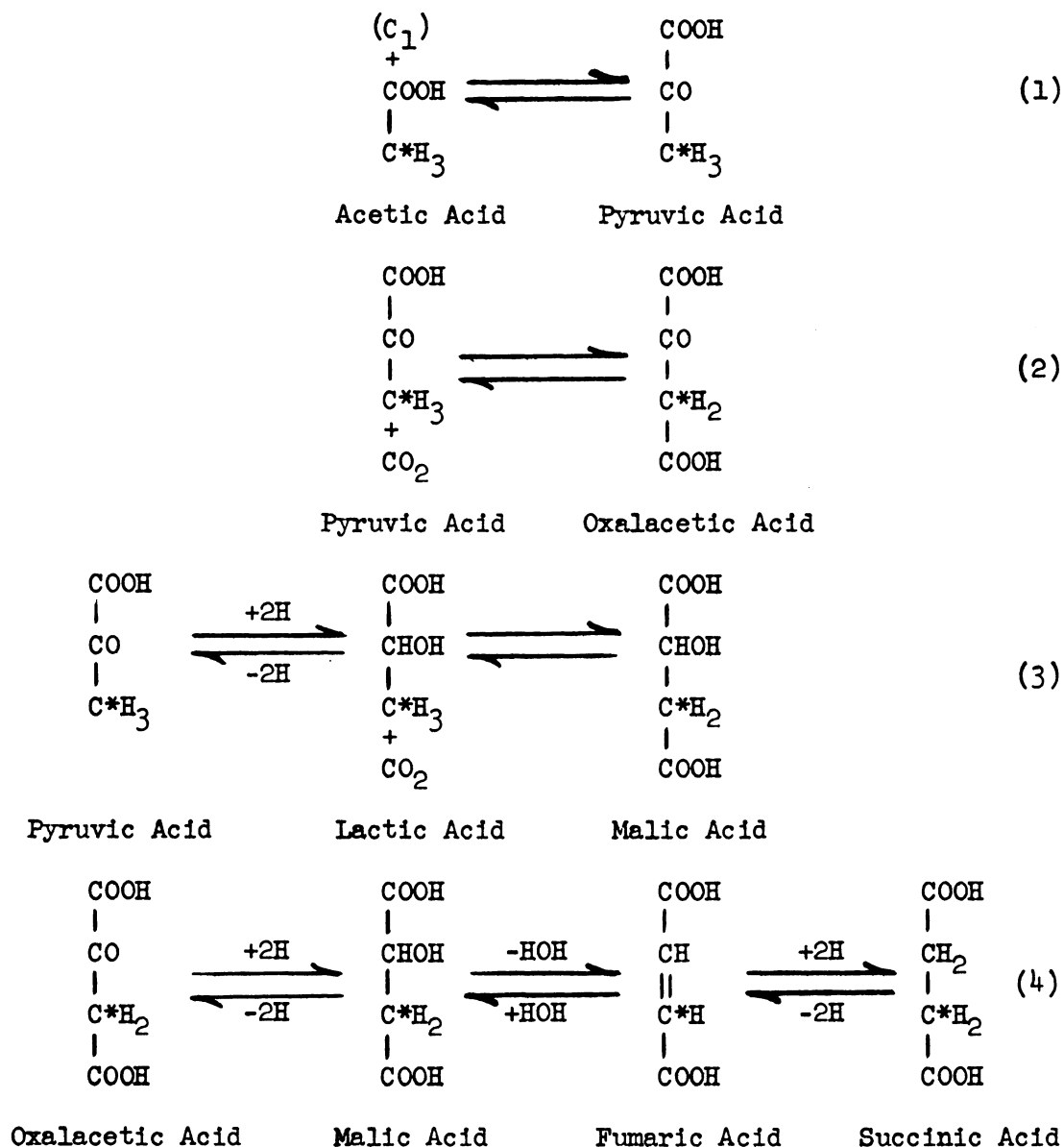


Figure 1. Succinic Acid Biosynthesis via the Double Fixation of Carbon Dioxide and the Dicarboxylic Acid Shuttle.

Note: Fumaric acid has been written in the cis (maleic acid) form for purposes of clarity and uniformity.

concerning the formation of the C_4 dicarboxylic acid from pyruvate and CO_2 . These are presented in reactions (2) and (3). The first of these (2) involves the direct beta carboxylation of pyruvate to form OAA. This reaction was first suspected by Wood and Workman (1936) in Propionibacterium pentosaceum. It has since shown to be catalysed by extracts of E. coli by Kaltenbach and Kalnitsky (1951). Although these latter authors conclude that the formation of OAA does not proceed via the mechanism presented in reaction (3), this mechanism cannot be overlooked as a possibility in view of the fact that the enzyme responsible for catalysing the fixation of CO_2 on lactate to form malate has been demonstrated in pigeon liver (Ochoa, Mehler, and Kornberg, 1947) and it has been found to be an adaptive enzyme in the bacterium, Lactobacillus arabinosus (Kerkes and Ochoa, 1948).

Once the initial C_4 dicarboxylic acid is formed, the pathway to succinate is clearly defined (reaction 4) and subject only to the equilibrium conditions which may prevail. The enzyme catalysing the reversible oxidation of malate to OAA was first clearly demonstrated by Green (1936) as it is found in animal tissues. It has been demonstrated and examined in cell-free extracts of E. coli by Gale and Stephenson (1939).

Fumarase, an enzyme discovered by Einbeck (1919) and shown by Quastel and Whetham (1924) to be present in E. coli, catalyzes the reversible dehydration of malate to fumarate.

The presence of succinic dehydrogenase in animal tissues was discovered by Batelli and Stern (1910) and, while there remains some question as to the mechanism of the interconversion of succinate and fumarate, this enzyme has been amply demonstrated in many types of cells and in E. coli in particular (Quastel and Whetham, 1924).

Thus, one may see that enzyme systems responsible for all of the reactions presented in Figure 1, with the possible exception of the malic enzyme of Ochoa (Ochoa, Mehler, and Kornberg, 1947), not only occur widely in all types of living cells but are actually present in E. coli.

If one now assumes that this is the pathway by which succinate is formed during the fermentation of xylose by E. coli in the presence of acetate-2-C¹⁴, let us examine the course of radioactive carbon. In Figure 1, the radioactive carbon is denoted by an asterisk. It is evident that the radioactivity is introduced into a methylene carbon of the succinate molecule wherein it randomizes, due to the symmetry of the molecule, between the two inner methylene carbons. At equilibrium one might expect then, that the succinate would be labelled entirely in the methylene

carbons and the acetate would have a lower methyl-carboxyl activity ratio (M/C ratio) than it had initially. This latter condition, of course, would depend entirely upon the equilibrium extant.

This mechanism is admirably suited to the anaerobic conditions under which the original observations appear to have been made (Nutting and Carson, 1952a,b). In conformity with these observations, this mechanism would not introduce activity into the carboxyl carbons of the succinate. Furthermore, it might be anticipated that this mechanism would be favored over either of the other two major pathways under the anaerobic conditions of the fermentation since both the TCAC and the C_2 condensation are essentially aerobic mechanisms.

B. The Tricarboxylic Acid Cycle (TCAC)

The TCAC does not fit the experimental conditions i.e., anaerobiosis, nearly as well as the first mechanism (double fixation of carbon dioxide). Nor does the TCAC explain the net synthesis of succinate during the fermentation as adequately as does the first mechanism, since for every molecule of succinate (C_4) synthesized one molecule of OAA (C_4) must be consumed. However, the TCAC does satisfy the condition that radioactivity introduced from acetate- $2C^{14}$ will be placed in the methylene carbons of

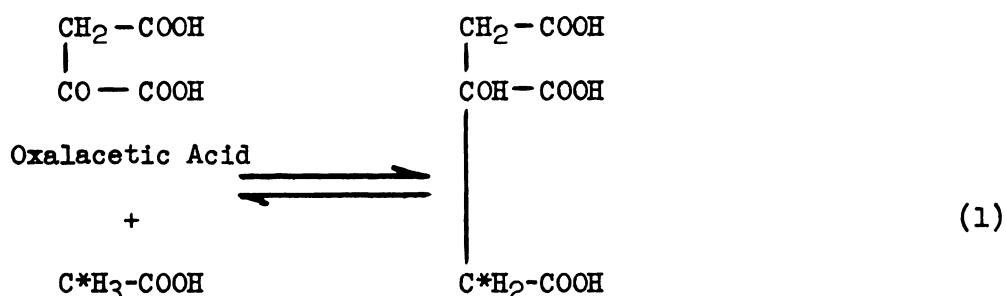
succinate, initially at least.

While the major credit for the development of the TCAC has been given to H. A. Krebs for his early researches on the subject (Krebs 1937, 1940), the intricate details of the step-by-step process have been elaborated through the combined efforts of many investigators.

In Figure 2, there is presented that portion of the TCAC which would be required to produce succinate.

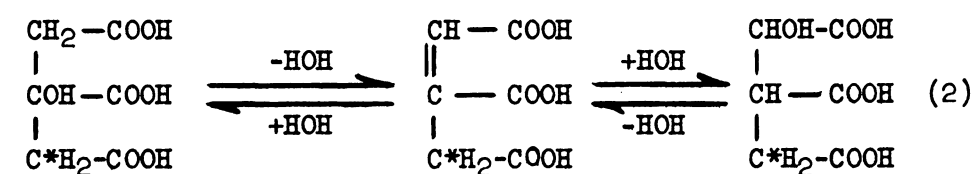
Reaction (1), the condensation of a C_2 fragment with OAA to yield citrate, has been fully demonstrated by Stern and Ochoa (1949). In the presence of an enzyme preparation from pigeon liver, supplemented with acetate, OAA, ATP, Co A, and magnesium ions, citrate was produced.

At first glance, it would appear that the citrate so formed would be symmetrical and that therefore isotopic carbon introduced in the C_2 fragment should be found to have randomized between the two acetyl radicals. Prior to 1948, this consideration appeared to rule out citrate as the product of the condensation since all the evidence then available indicated that the tricarboxylic acid intermediate involved was not symmetrical. However in that year, Ogston (1948) suggested that citrate might actually be unsymmetrical by virtue of the spatial arrangement of the three carboxyl groups and the manner in which citrate might be oriented in the enzyme-substrate complex.



Acetic Acid

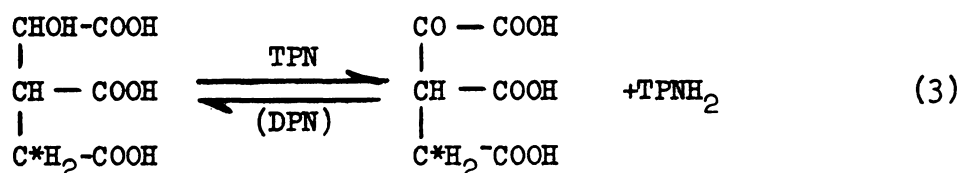
Citric Acid



Citric Acid

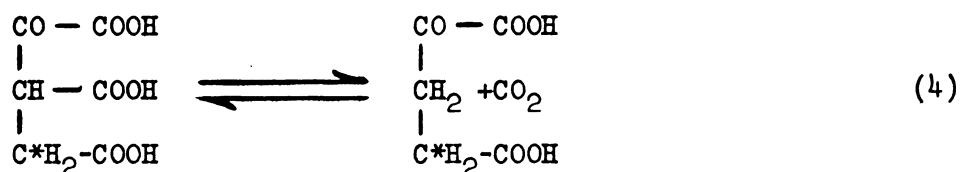
cis-Aconitic Acid

iso-Citric Acid

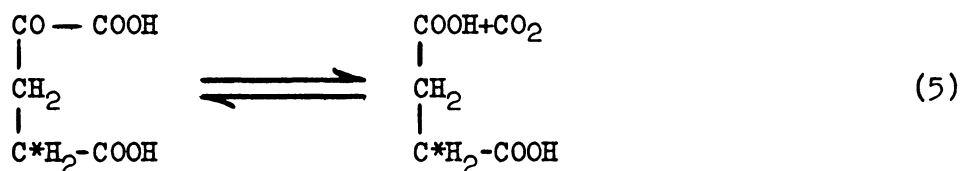


iso-Citric Acid

Oxalosuccinic Acid



Oxalosuccinic Acid alpha-Ketoglutaric Acid



alpha-Ketoglutaric Acid

Succinic Acid

Figure 2. Succinic Acid Biosynthesis via Steps of the Tricarboxylic Acid Cycle (TCAC).

The Ogsten concept was further confirmed for a microbial system by the work of Carson, Mesbach and Phares (1951). These latter workers found a wide divergence in the labelling of the primary carboxyls of citrate produced from sucrose by Aspergillus niger in the presence of sodium acetate-2-C¹⁴ and C¹³O₂.

Indeed, all of the available data indicate that both the synthesis and the degradation of citrate proceed on an asymmetric basis, so that entering C₄ dicarboxylic acid carboxyls become CO₂, and entering C₂ carboxyls become C₄ dicarboxylic acid carboxyls initially.

The initial condensation has also been demonstrated in E. coli by Novelli and Lipmann (1950) where according to Stern, Shapiro and Ochoa (1950), the overall reaction requires the participation of at least two enzymes. The first enzyme "activates" acetyl phosphate; the second enzyme catalyzes the condensation of this "active" acetyl phosphate with OAA to form citrate. This has been further elaborated (Stern and Ochoa, 1951) using soluble enzyme systems.

The next step in the cycle, reaction (2) involves the action of the enzyme, aconitase. The catalytic activity of this enzyme was initially described by Martius and Kneep (1937) and confirmed and named by Breusch (1937). Its presence in E. coli has been reported by Jacobsehn (1940). Aconitase catalyzes the interconversion of citrate and isocitrate through cis-aconitate.

Isocitrate is then dehydrogenated to oxalosuccinic acid as presented in reaction 3. This conversion was postulated by Martius (1937) who drew an analogy between this reaction and the biological dehydrogenation of other hydroxy acids to their corresponding keto acids.

The conversion of isocitrate to alpha-ketoglutaric acid has since been demonstrated in animal tissues (Martius, 1937; Krebs and Johnson, 1937 a; Adler, et.al., 1939; Ochoa, 1945, 1948), in higher plants (Ceithaml and Vennesland, 1949), and in the yeast (Kornberg and Pricer, 1951, Foulkes, 1951).

Reaction 4 is presumably catalysed by the enzyme, oxalosuccinic carboxylase, whose action has been demonstrated in animal tissues by Lynen and Scherer (1948) and by Ochoa and Weiss-Taberi (1945).

The first clear-cut demonstration of the continued conversion of a tricarboxylic acid to alpha-ketoglutarate in bacteria was obtained by Barban and Ajl (1952) in a species of Escherichia. Subsequently these same authors (Idem, 1953) have shown that the conversion is reversible and that oxalosuccinate is an intermediate.

The oxidative decarboxylation of alpha-ketoglutarate is catalysed by the enzyme alpha-ketoglutaric acid oxidase which has been obtained in soluble form from pig heart and has been examined by Sanadi and Littlefield (1951) and Sanadi, et. al. (1952) who believe the reaction to proceed through succinyl-coenzyme A. Besides catalytic amounts of CoA, DPN is required.

The presence of a similar, if not identical, enzyme system in Acetobacter vinelandii, strain O, has been reported by Lindstrom (1953). The co-factor requirements in the bacterial system are similar to those in the animal system and the differences appear to reflect, in part, dissimilarities in the degree of organisation; the bacterial preparation being soluble whereas the

the pig heart preparation requires solubilizing.

Thus, all of the reaction of the TCAC which are necessary to synthesize succinate have been shown to be catalysed biologically and although there is no direct evidence for the occurrence of isocitric dehydrogenase, oxalosuccinic carboxylase or alpha-ketoglutaric acid oxidase in E. coli, there is evidence for their occurrence in the same genus, and the concept of comparative biochemistry may allow us to assume a priori the probability that similar enzyme systems are present in E. coli.

If one examines the course of the labelled carbon of acetate-2-C¹⁴ through the series of reactions shown in Figure 2, one finds that the methyl group of the acetate should become randomized between the methylene carbons of the succinate.

This would agree with the finding of Nutting and Carson (1952 b) that the activity was present in the methylene carbons of succinate. However, since there was little or no activity present in the succinate carboxyl carbons, one must assume that if the succinate were formed by this mechanism, there was little or no recycling. For, if recycling had occurred, activity would become distributed evenly

between the two inner carbons of the OAA, one of which would become the carboxyl carbon of newly synthesized succinate, thus introducing activity into the carboxyl carbons.

The evidence, presented in the previous section, wherein all of the reactions between succinate and OAA are shown to be catalysed by E. coli would seem to eliminate the possibility of a break in the cycle at this point. However, the conditions under which any one enzyme may operate at its optimum rate may not be suitable for, and, indeed, may actually be detrimental to, the activity of another enzyme. Therefore, the possibility that the cycle may be present in part, sufficient to allow for the formation of the succinate but insufficient to permit recycling of the acid, cannot be overlooked.

Since it would appear that the OAA, which must condense with acetate to begin the TCAC, does not arise from preformed succinate, the question arises as to the origin of the OAA. That it does not arise by a double fixation of CO_2 on acetate as described in the previous section is supported by the same argument presented for the inability of succinate to serve as precursor for additional OAA: activity originally present in the methyl

group of the acetate would be found finally in the carboxyl group of the succinate formed. And this does not appear to be the case.

Thus, if we are to assume that the TCAC is the mechanism by which activity is incorporated into succinate when acetate-2-C¹⁴ is used as tracer, we must further assume either that the OAA which condenses with the acetate-2-C¹⁴ has been formed from carbon of the substrate, xylose, possibly by the fixation of CO₂ on a C₃ fragment (pyruvate), or doubtfully, that the OAA required represents pre-formed material already present within the cell.

It is also well to point out here that whereas all of the steps required for the formation of succinate via the TCAC are believed to be reversible, the formation of any intermediate followed by subsequent reversal of the cycle would involve the loss of the activity introduced during the condensation reaction (1).

C. The Thunberg Condensation and the Dicarboxylic Acid Cycle (DCAC).

The third major metabolic pathway which

may account for the observations of Nutting and Carson (1952 a,b) is the dehydrogenative coupling of two molecules of acetate through their methyl groups, sometimes referred to as the Thunberg (C₂) condensation. Although this mechanism would provide the simplest explanation for the results observed, the rigid proof of the occurrence of this reaction biologically remains to be satisfactorily demonstrated.

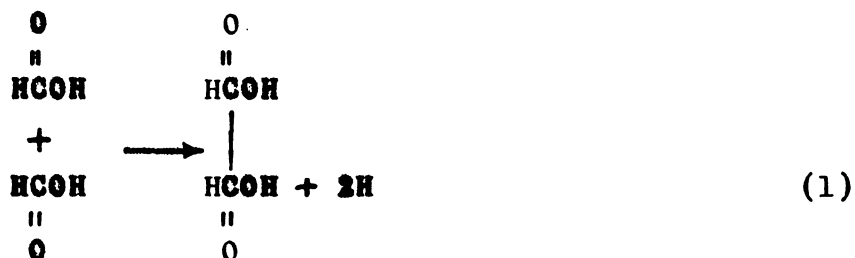
In 1920, Thunberg suggested that the oxidation of acetate was initiated by the dehydrogenative condensation of two molecules of acetate to form succinate, thus:



Thunberg based his hypothesis on the results which he observed when formate or acetate was incubated with muscle tissue in the presence of methylene blue (MeB).

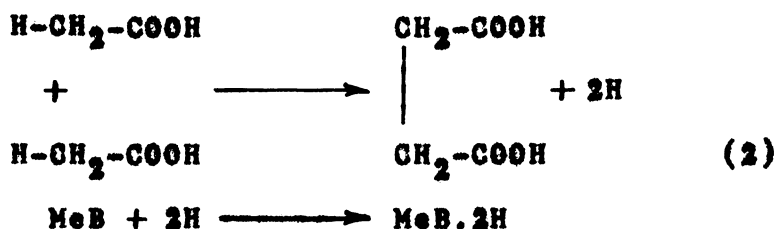
In the presence of formate and muscle tissue, MeB is reduced to its leuco form. Through the use of sodium formate, it was demonstrated that it was the C-H bond which was involved in the reduction and not the O-H bond.

Since two atoms of hydrogen are required for the reduction of MeB, they must have come from two molecules of formate. In that event, it was possible that the reaction involved an oxidative condensation of formate to oxalate, as in Equation 1:



This was, indeed, deemed to be the case when oxalate was subsequently isolated from the experimental medium.

Likewise, in the presence of acetate and the muscle enzymes, MeB became reduced. Thunberg reasoned that if the reaction were analogous to the case of formate, one should be able to isolate succinate from the experimental medium. This, however, was not accomplished. The failure to isolate succinate from the medium was explained as being due to the rapid metabolism of such an active metabolic intermediate. The reaction, however, was visualized as occurring as in Equation 2:



Since these original observations by Thunberg, many investigators have supported the occurrence of a C_2 condensation on equally tenuous evidence.

Indeed, the early researches of Thunberg (1920) and others, principally Wieland (1922) and Knoop (1923), led to the formulation of a cyclic mechanism for the oxidation of carbohydrate in which the key reaction is the condensation of the two-carbon compound, acetic acid, to form a four-carbon dicarboxylic acid, succinic acid. The scheme may be represented diagrammatically as in Figure 3.

As may be seen in the figure, for every two moles of acetate formed during carbohydrate breakdown, one mole is completely oxidized to carbon dioxide while the other is regenerated to maintain the cycle. All of the reactions required for this mechanism have been demonstrated and have adequate substantiating evidence except the all-important condensation step.

With the development of the TCAO (Krebs 1937, 1940), the participation and biological importance of

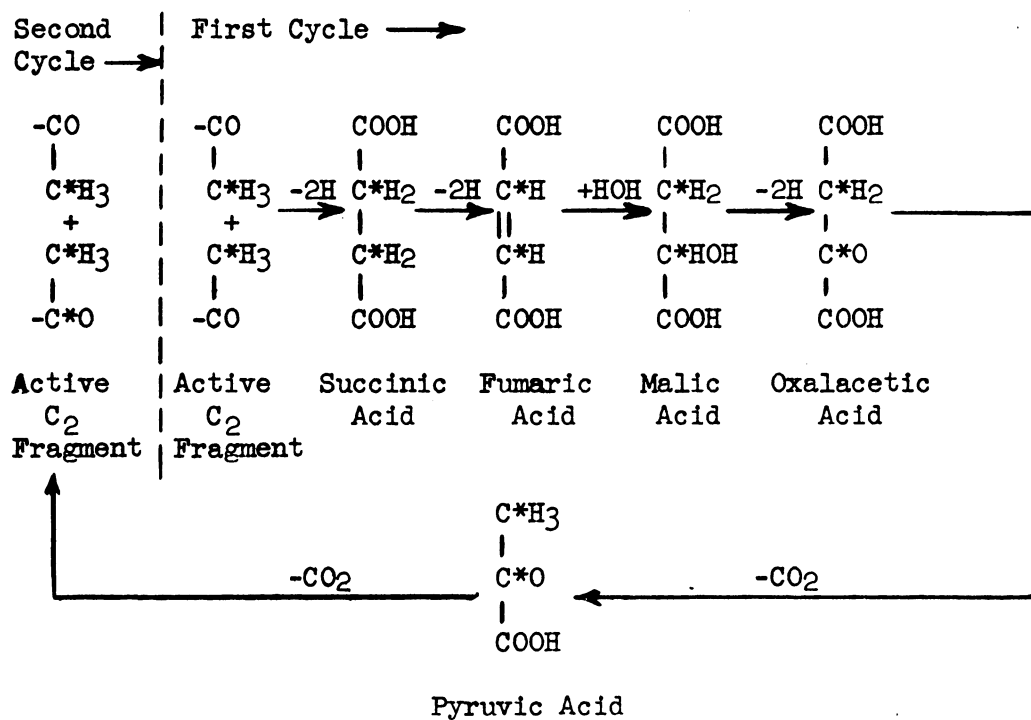


Figure 3. Succinic Acid Biosynthesis via the Thunberg (C₂) Condensation and the Dicarboxylic Acid Cycle (DCAC) from Foster and Carson (1950)

Note: Fumaric acid has been written in the cis (maleic acid) form for purposes of clarity and uniformity.

the C_2 condensation and the DCAC became overshadowed. the widespread availability of radioactive and stable isotopes which accompanied the recent progress of research in nuclear physics, however, permitted the re-investigation of many of the earlier mechanisms including the DCAC and the C_2 condensation.

Wieland and Sonderhoff (1932), working with yeast, found that acetate was transformed principally into succinate and citrate. The relative amounts of the products led them to conclude that the succinate was formed by means of a condensation of two acetate molecules in a manner similar to that proposed by Thunberg. They believed that citrate was formed by a side reaction not on the main pathway of acetate oxidation.

Later, Sonderhoff and Thomas (1937) repeated these experiments using trideuterio-acetate ($CD_3.COOH$) and again accumulated both succinate and citrate. However, in this case, the citrate isolated contained a higher percentage of deuterium than did the succinate, thus pointing to its participation in the pathway of acetate oxidation.

Lynen and Neciullah (1939) pointed out that the failure of yeast to dehydrogenate di- and tri-carboxylic acids was due to the impermeability of the

yeast cell wall to these acids. They froze yeast cells in liquid air and then demonstrated the rapid dehydrogenation of citric, alpha-ketoglutaric, succinic and malic acids.

Later, Lynen (1943) was able to inhibit acetate oxidation with malonate, and he concluded that acetate was oxidized via the TCAC.

That same year, Slade and Werkman (1943) reported the incorporation of isotope from labelled acetate ($C^{13}H_3COOH$) into the methylene carbons of succinate ($HOOC.C^{13}H_2.C^{13}H_2.COOH$) exclusively during the fermentation of glucose by Aerobacter indologenes. They considered this to be proof that a DCAC exists in these organisms.

In a similar type of experiment, Foster and Carson (1949, 1950) presented evidence for a condensation of labelled ethanol ($C^{14}H_3.CH_2OH$, $CH_3.C^{14}H_2OH$) yielding a net synthesis of fumaric acid in the presence of the mold, Rhizopus nigricans. They presented evidence to show that CO_2 fixation is negligible and that the amount of radioactive CO_2 incorporated is simply an expression of the operation of the equilibrium reaction:



The OAA being in equilibrium with fumarate through malate.

In a series of papers, Ajl and his co-workers (Ajl and Kamen, 1950; Ajl, 1950; Ajl and Wong 1951; Ajl and Kamen, 1951; Ajl, 1951) have reported evidence for the occurrence of the DCAC and the C_2 condensation in E. coli and A. aerogenes. They have based their conclusions principally upon adaptation studies wherein the increased activity of the organism upon DCAC substrates (acetate, pyruvate, and C_4 dicarboxylic acids) and absent or unmodified activity upon members of the TCAC inferred the importance and probable occurrence of a DCAC as a mechanism of respiration in these organisms.

Barron, Ardao, and Hearon (1950), using cells of Corynebacterium creatinovorans adapted to acetate, experienced results similar to those of Ajl et. al. (see preceding paragraph) and drew similar conclusions.

Recent "abstracts" by Swim and Krampitz (1951, 1952) which appeared during the period of the present research would indicate that under the conditions of their experiments the DCAC does not occur in E. coli. Their evidence, based on their

inability to find any doubly-labelled ethylene (prepared from the methylene carbons of succinate, according to the method of Weed, 1952) when acetate-2-C¹³ (53% G¹³) was used as a tracer, seems conclusive.

However, it is well to point out that minor differences in conditions of temperature, pH, substrate, hydrogen balance, and oxygen tension have enormous influence upon which mechanism may operate to the greatest extent in a given organism. Evidence of this is presented by Nutting and Carson (1952 a) with regard to the effect of pH upon the fermentation of xylose by this same organism, E. coli. Similar extreme effects of pH have been noted during the fermentation of glucose by Bacillus subtilis (Neish, Blackwood, and Ledingham, 1945). The effect of varying the substrate within the relatively narrow limits imposed using glucose, xylose and pyruvate has been demonstrated by Stanier and Adams (1944) using species of Aeromonas and appears to be considerable.

These few references make it clear that what may be said with certainty under certain

specified conditions with regard to a principal metabolic mechanism employed by a given organism may have to be modified with minor changes in the environmental conditions.

An examination of the pathway by which activity may be incorporated into succinate from acetate-2-C¹⁴ via the DCAC requires only the consideration of a single reaction: the direct dehydrogenative coupling of the acetate itself. This process is aerobic in character and, therefore, suffers from the same deficiency as does the TCAC when one attempts to fit this mechanism to conditions described by Nutting and Carson (1952a,b). Further, since the succinate produced under those conditions had no activity in the carboxyl carbons, it would appear that if the C₂ condensation is responsible for the biosynthesis of the succinate, there is little, if any, cycling. For, as in the case of the TCAC, cycling of the "once-formed" succinate allows activity to become distributed into the carboxyl carbons of the succinate. Thus, it can be seen that continued cycling will decrease the ratio of activity present in the methylene carbons to that present in the carboxyl carbons.

Although there is justification for supporting the view that a DCAC exists, no one has, as yet, shown conclusively that the DCAC is operating. To illustrate, let us examine the above reports a little more critically.

Thunberg (1920) was unsuccessful in his attempt to isolate succinate from among the products of his experiment. Further, as we know today, the TCAC could accomplish the same results through well-established mechanisms.

In the contradictory experiments of Wieland and Senderhoff (1932) and Senderhoff and Thomas (1937), there is little to corroborate Thunberg, and the oxidation of the TCAC intermediates using permeable yeast cells, (Lynen and Neciullah, 1939) in effect, points to the participation of the TCAC in producing the results of the former investigators.

The more recent isotopic tracer experiments have failed to adequately demonstrate the occurrence of a C_2 condensation, possibly because more than one pathway may account for the observed labelling in the succinate produced. Part of this difficulty stems from the fact that during the years prior to 1948 the question of the symmetry of citric acid per-

mitted the disregard of the TCAC as a pathway when the labelling observed required an asymmetrical citric acid molecule. Further difficulty has been introduced when investigators have failed to take into consideration, questions of cell permeability, particularly as regards citrate and alpha-ketoglutarate, and questions of equilibration, such as (1) the equilibration of added carrier TCAC compounds with the corresponding metabolically produced intermediates and (2) the equilibration between enzymatically formed compounds bound to the enzyme and a pool of the supposed intermediate. That these questions of equilibration are indeed of considerable importance has been demonstrated by the work of Sas and Krampitz (1951) and by the investigation of Kaufman, Korke, and Del Campillo (1951).

Thus, the simplest and oldest mechanism for the introduction of radioactivity from acetate-2-C¹⁴ into the methylene carbons of succinate, the Thunberg condensation, remains the most debatable, and yet, perhaps, the most intriguing of the mechanisms proposed.

III. PLAN OF THE INVESTIGATION

In order to determine the mechanisms by which E. coli biosynthesizes succinic acid during xylose fermentation, the research was divided into the following phases

- A. Carbon Tracer Techniques
- B. Confirmatory Experiments
- C. Inhibition Experiments
- D. Definitive Experiments
- E. Corroborative Experiments

which are described in the following paragraphs.

A. Carbon Tracer Techniques

Special methods peculiar to tracer research had to be mastered before even the confirmatory experiments could be undertaken; e.g., isolation, analysis, purification, and degradation procedures as applied to labelled compounds and C^{14} counting methods.

B. Confirmatory Experiments

The results of Nutting and Carson (1952a,b) were examined. A fermentation of xylose by E. coli

in the presence of acetate-2-C¹⁴ was analyzed with particular emphasis on a determination of the relative specific activities of the methylene and carboxyl carbons of the succinic acid, expressed as a ratio and referred to as the methylene-carboxyl ratio or simply as M/C.

If there had been no appreciable difference between the M/C of the succinate biosynthesized during the fermentation and the M/C of the tracer acetate introduced, then there could have been little or no cycling via either the TCAC or a DCAC. In this event, phase C, the inhibition experiments, could have been omitted.

Since the M/C of the succinate was considerably below that of the tracer acetate and since the atmospheric CO₂ was relatively inactive when compared with the succinate carboxyls, it was evident that either one or both of the oxidative metabolic cycles must be in operation and, therefore, steps must be taken to inhibit this cycling.

It was also observed that the presence of oxygen, introduced at the end of the fermentation, increased the percentage of activity incorporated into succinic acid, thus inferring the possible

operation of an aerobic mechanism.

For these reasons it became necessary to employ phase C, the inhibition experiments.

C. Inhibition Experiments

Since the operation of one or both of the oxidative cycles would effectively mask the operation of any pathway for the net synthesis of succinate, it was necessary to interrupt this cycling through the use of a suitable inhibitor.

A "suitable" inhibitor would be one which acts upon a single enzymatic step in preferably one, or possibly in both of the oxidative cycles, thus preventing cycling and affording an opportunity to study the pathway of net synthesis alone.

Among these enzyme inhibitors which have been used for this purpose and have exhibited a reasonably narrow spectrum of activity, fluoroacetic acid and arsenite (sodium salt) appeared to be the most suitable.

Monofluoroacetic Acid (MFA) has exhibited a higher degree of specificity than has arsenite and was, therefore, the initial inhibitor employed. Chenoweth (1949) has published a review of the literature prior

to 1949. The mechanism of MFA inhibition of citrate metabolism has been clarified by the recent studies of R. A. Peters and his collaborators (Buffa, Peters, and Wakelin, 1951; Lotspeich, Peters, and Wilson, 1951; Peters, Wakelin, and Buffa, 1952), who have shown that MFA competes successfully with acetate in the initial condensation reaction of the TCAC and the resulting fluoro-tricarboxylic acid specifically inhibits aconitase and thus the TCAC itself.

However, Martius (1949) has obtained data which lead him to conclude that MFA also inhibits reactions involved in the formation of OAA from succinate. Thus, a DCAC may also be inhibited by MFA.

The remarkable similarity between MFA and acetate geometrically, pointed out by Kalnitsky and Barron (1947), was also borne in mind, since it was possible that MFA might engage in other reactions normally involving acetate alone. That this might be the case has been emphasized by the work of Busch and Potter (1952) who have found that MFA inhibits the oxygen uptake, pyruvate utilization, and acetoacetate formation in a liver-malonate system in which no TCAC appears to operate.

Arsenite (As) The pharmacology of arsenicals

including sodium arsenite, has been studied by Veegtlin and his collaborators (Veegtlin, Dyer, and Leonard, 1925; Rosenthal and Veegtlin, 1930; Veegtlin, Rosenthal, and Johnson, 1931). They conclude that the poisonous action of these compounds is the result of a chemical union with the sulfhydryl groups of protoplasm (enzymes).

Krebs (1932) observed that arsenite specifically inhibited alpha-ketoglutaric acid oxidation in pigeon breast muscle. The general inhibition of alpha-keto acid oxidation by arsenite has been established by studies on the oxidation of acetoacetate (Quastel and Wheatley, 1935; Jewett and Quastel, 1935) and pyruvate (Ochoa, 1941; Peters, Sinclair, and Thompson, 1946).

It is evident that arsenite will interrupt both cycles in the event that both are in operation: the TCAC at the alpha-ketoglutarate $\rightarrow$ succinate step, and the BCAC at the pyruvate $\rightarrow$ acetate + CO₂ step. Therefore, the successful inhibition of cycling by arsenite is not of itself definitive.

Further, since arsenite affects sulfhydryl-containing enzymes in general, a broader spectrum of activity may be inferred than is represented by the alpha-keto acid oxidase systems.

The successful inhibition of cycling with 0.07 M sodium arsenite permitted the research to proceed to phase D, the differentiation between the two pathways of net succinate biosynthesis: C_2 condensation and the double fixation of CO_2 .

D. Definitive Experiments

Two approaches were made in an attempt to distinguish between the operation of a C_2 condensation and a double fixation of CO_2 .

First, the fixation of $Cl^{14}O_2$ in succinic acid was studied in order to determine how active this process was and what effect arsenite had upon it.

Second, evenly labelled acetate (acetate-1,2- Cl^{14}) was used as a tracer in order to ascertain whether there was any demonstrable C_2 condensation present in the event that both mechanisms for net synthesis were in operation.

From these experiments, it was clear that, in the presence of arsenite, the net synthesis of succinate via the mechanism of CO_2 fixation was negligible, and that the chief problem which remained was the differentiation between the TCAC mechanism and the C_2 condensa-

tion. The solution to this problem was attempted in phase E, the corroborative experiments.

E. Corroborative Experiments

Fermentations were carried out in the presence of labelled acetate and inhibitors in order to determine whether the TCAC via alpha-ketoglutaric acid was responsible for the labelling observed in the succinic acid produced.

Other experiments involving the use of alpha-ketoglutaric acid-1,2-C¹⁴ were also performed for the same purpose.

IV METHODS AND MATERIALS

A. Cultivation of the Organism

The organism used in the investigation was Escherichia coli, K-12, obtained from the laboratory of C. E. Clifton. The organism was cultivated according to the procedure of Nutting and Carson (1952a) except where noted otherwise.

The xylose used as a substrate throughout was Coleman and Bell d-(+)-xylose (designated l-xylose by Fischer) and will be referred to as xylose. The carbohydrate was sterilized for use as a 40 per cent solution by autoclaving at 15 lbs pressure for 20 minutes. It was then added to the separately autoclaved mineral salts solution, aseptically.

B. Preparation of Cell Suspensions

Non-proliferating cell suspensions were prepared according to the procedure of Nutting and Carson (1952a) except that in later experiments a suitable buffer or buffer-inhibitor solution was used to wash the cells instead of tap water. This was found to be particularly important when it became desirable to remove as

much dissolved oxygen as possible. This was achieved by aerating the buffer and buffer-inhibitor solutions with the proper CO₂-inert gas mixture prior to use, thus establishing the desired pH and freeing the solutions of dissolved oxygen.

C. Manometric Methods

All fermentations were carried out in Warburg respirometers in a manner similar to that reported by Nutting and Carsen (1952a). A typical fermentation carried out in a 160 ml, two-side-arm Warburg vessel generally involved the use of 25.0 ml of non-proliferating cell suspension in the main compartment (0.5 gm cells, dry weight, in buffer or buffer-inhibitor solution), 1.0 ml of substrate (0.5 M xylose) plus 1 ml of "tracer" solution, if used, in one side arm and 2.0 ml of inorganic acid (10 N H₂SO₄) in the second side arm.

If the tracer was C¹⁴O₂, a 170 ml three-side-arm vessel was used. The cell suspension and substrate volumes remained the same; however, the tracer, as BaC¹⁴O₃, was added to one arm of the double side arm and slurried in 1 ml of distilled water. Inorganic acid, 3.0 ml of 30 per cent phosphoric acid, was placed in the other arm of the double side arm from which it

could be tipped into the $\text{BaC}^{14}\text{O}_3$ slurry after temperature equilibration.

The use of phosphoric acid differs from the procedure used by Nutting and Carson (1952b) who used H_2SO_4 to release CO_2 from $\text{Na}_2\text{C}^{13}\text{O}_3$. H_2SO_4 was found to be highly unsatisfactory for this purpose when BaCO_3 was used, releasing only 15 per cent of the available CO_2 in five minutes compared with an almost instantaneous release of all available CO_2 by H_3PO_4 .

Buffer solutions were prepared from NaHCO_3 ; the usual concentration employed was 0.26 M. The gas atmosphere employed was usually 20 per cent CO_2 in He in order to achieve the desired pH of 8.0 (Umbreit, et al., 1949). The rather high buffer concentration was necessary because the normal production of organic acid (1 mE) from 0.5 mM xylose was sufficient to reduce the buffer capacity appreciably. At this concentration of buffer, 1 mE of organic acid would lower the pH from 8.0 to 7.7 during the course of the fermentation.

Buffer-inhibitor solutions were prepared by slurrying the dry inhibitor, either sodium fluoroacetate or sodium arsenite, in distilled water, followed by pH adjustment (8.0), NaHCO_3 addition, and the addition of distilled water to volume.

In those cases where it was desired to add oxygen to the atmosphere following the completion of the fermentation, a vacuum was created in the vessel by withdrawing a portion of the gas atmosphere by means of an evacuated bulb. The pressure was then equalized by opening the stopcock to a flow of pure oxygen.

This procedure usually involved the removal of approximately one-third of the gas atmosphere and the lowering of the CO₂ content of the gas phase from its high of 36 per cent (pH 7.7) at the end of the fermentation, to approximately 25 per cent (pH 7.8). As noted, little change in the pH was experienced. The final concentration of oxygen was approximately 20 per cent and not 33 per cent as one might expect since the pressure in the vessel was returned to atmospheric and not to the slightly elevated pressure present in the vessel at the end of the fermentation.

D. Methods for the Addition of Labelled Compounds

Labelled organic acids were neutralized and added in 1.0 ml volumes to the side arm of the Warburg vessel which contained the substrate. The carbon added in this form never exceeded four per cent of the carbon

available in the substrate. Labelled acetate (acetate-1-C¹⁴ and acetate-2-C¹⁴) was obtained from the Organic Chemistry Section of the Chemistry Division of the Oak Ridge National Laboratory. Acetate-1,2-C¹⁴ was prepared by mixing equal aliquots of acetate-1-C¹⁴ and acetate-2-C¹⁴, the molecular species having approximately equal specific activities. The alpha-ketoglutaric acid-1,2-C¹⁴ used in this investigation was prepared by Mr. E. F. Phares of the Biology Division, Oak Ridge National Laboratory. The synthesis of this latter compound involved an ester condensation between ethyl succinate and methyl oxalate followed by successive acid hydrolysis. Since the oxalate was prepared by the fusion of HC¹⁴OOH, it was evenly labelled and therefore, the alpha-ketoglutaric acid-1,2-C¹⁴ was also evenly labelled.

Labelled CO₂ was prepared from BaC¹⁴O₃. A suitable amount of radioactive material was mixed with inactive carrier and placed in one arm of the double side arm of a large Warburg vessel (170 ml) where the mixture was slurried in 1 ml of distilled water. The total amount of BaCO₃ employed (246 mg) was determined by the volume of the gas space (140 ml) and the percentage of CO₂ desired (20 per cent).

At temperature equilibration, the gas atmosphere (20 percent CO_2 in He) which had been used to maintain the pH was displaced by pure He. A partial vacuum was applied and the CO_2 released from the BaCO_3 by the addition of excess of H_3PO_4 from the other arm of the double side arm. Temperature equilibration was re-established and the remaining vacuum in the vessel was filled by opening the stop cock to a flow of pure He.

The inorganic acid was always present in excess so that there would be sufficient free acid remaining following this reaction to release all CO_2 from the buffer at the end of the experiment.

For radioactivity measurements on the CO_2 , the atmosphere was withdrawn at the end of an experiment by means of a 500 ml evacuated bulb. The CO_2 was absorbed in 0.5 N alkali and an aliquot was precipitated as BaCO_3 with BaCl_2 and taken for radioactivity assay according to the procedure of Anthony and Long (1952).

E. Separation and Analysis of Fermentation Products

The fermentation of xylose by E. coli grown under aerobic conditions is not accompanied by the liberation of gas (Nutting and Carson, 1952a) and the products may be initially separated into a neutral volatile fraction (ethanol), a volatile acid fraction

(acetate and formate), and a non-volatile acid fraction (lactate and succinate). A quantitative determination of each of these five products accounts for more than 95 percent of the xylose utilized.

In general, the procedure of Nutting and Carson (1952a) has been followed for the analysis and separation of the fermentation products.

Ethanol was determined by the standard chromic acid oxidation method. The neutral volatile fraction from an alkaline distillation of the fermentation medium, which usually had a volume of 20 ml and contained all of the ethanol (0.2-0.3M) was placed in a closed vessel with 0.5 gm CrO_3 and 3.5 ml conc. H_2SO_4 and heated on a boiling water bath for 20 minutes. The mixture was then cooled and transferred to a steam still from which the acetic acid was steam distilled and titrated with 0.1N alkali. The titration was equivalent to the alcohol present in the neutral volatile fraction.

Xylose was determined by the orcinol colorimetric procedure of Brown (1946) as modified by J. X. Rhym of the Oak Ridge National Laboratory (Personal communication).

A stock solution of 20 gm recrystallised orcinol and 13.5 gm ferric ammonium sulfate was made up to 500 ml in distilled H_2O and stored in the refrigerator.

For use, 5 ml of this solution was added to 95 ml of 30 percent HCl (1 part H₂O to 5 parts conc. HCl). A 3 ml sample containing less than 15 ug/ml was added to 9 ml of the orcinol reagent in a 20 ml test tube and placed in a boiling water bath for 20 minutes. The blue-green solutions were cooled, transferred to colorimeter tubes and the percent light (620 mμ) transmission determined using a Kromatrol photometer. All determinations of residual xylose were made on aliquots of the residue following the ether extraction of the non-volatile acid fraction. A standard curve was prepared for each determination using a solution whose xylose concentration was known.

Lactic acid analyses were made by the Barker and Summerson colorimetric method (Umbreit, et al., 1949) on aliquots of the fermentation medium.

Formic and acetic acid were determined by Duclaux distillation following steam distillations (McNair, 1947).

Succinic acid was determined manometrically using succinoxidase prepared from beef heart according to Umbreit, et al. (1949). Determinations were made on aliquots of the fermentation medium.

Although it was unnecessary to separate all of the individual products of the fermentation in order to determine their presence either qualitatively or quanti-

tatively, it was necessary to physically separate these products for purposes of degradation and radioactivity assay

This separation was accomplished by chromatographic techniques based on the investigation of Peterson and Johnson (1948) and the modifications reported by Phares, et. al. (1952).

F. Combustion Methods

All labelled compounds and fragments of compounds, except CO₂ itself, whose total radioactivity was of interest, were oxidized to CO₂ by the persulfate (wet) oxidation method of Osburn and Werkman (1932) as adapted for C¹⁴ measurements by Anthony and Long (1952).

G. Degradation Methods

The use of radioactive carbon for the delineation of the pathways of metabolism requires that one be able to locate and estimate the radioactivity in individual carbon atoms of the compounds concerned.

Therefore, it was necessary, at one time or another, to degrade all of the products of the fermentation, except formic acid, and, in addition alpha-ketoglutaric acid.

Acetate and ethanol (following its conversion to acetate as described above) were degraded by use of the Schmidt reaction according to the method of Phares (1951).

Lactate was degraded to acetate and CO_2 by acid permanganate oxidation in a closed system, in 100 per cent yield (Nutting, 1950). The acetate was further degraded as described above.

Succinate was degraded by two different methods during the course of these experiments. The method used initially involved the conversion of succinate to lactate which was then degraded as described above. The conversion of succinate to lactate was accomplished by means of the procedure of Foster and Carson (1950) which involves the conversion of succinate to malate using a succinoxidase preparation and the decarboxylation of malate to lactate and CO_2 by a resting cell suspension of Lactobacillus casei.

The second method for the degradation of succinate, more rapid and simpler than the first, was devised by E. F. Phares (Personal communication). Since this procedure has not yet been published, a description follows:

The method involves the adaptation of the

Schmidt reaction to the decarboxylation of a dicarboxylic acid, succinic acid. The apparatus required for the decarboxylation step is similar to that employed for the decarboxylation of acetic and propionic acids (Phares, 1951).

The recommended ratio of reactants is: 0.1 ml fuming H_2SO_4 and 20 mg NaN_3 per 0.1 mM succinate. A suitable sample based on the recovery of the methylene carbons as ethylene diamine would be 0.3 mM in order to assure a satisfactory sample of methylene carbon (0.2 mM) for subsequent radioactivity assay.

The sodium salt of succinic acid was dried under vacuum in the reaction flask and after cooling the flask to about 15° , the fuming H_2SO_4 was added carefully. (Note: The reaction is adversely affected by traces of water). The succinate was completely dissolved by warming and shaking in the stoppered flask. After recooling, the NaN_3 was added and the mixture was allowed to warm again with shaking until the azide was nearly dissolved.

At this point the vessel was connected to the acid permanganate and alkali traps and immersed briefly, in a 70° water bath. After the initial burst of gas, the flask was replaced on the water bath and the temperature raised to 100° slowly over a period of 20 minutes.

The system was then swept with CO₂-free air for 10 minutes at the elevated temperature. As noted in the paper by Phares (1951), two traps are required: a final trap containing 0.5 N alkali to trap CO₂, and a prior trap containing acid permanganate to trap oxides of sulfur as sulfate, since these would precipitate as BaSO₄ with the CO₂ when the latter was precipitated as BaCO₃ using BaCl₂.

This procedure has decarboxylated the succinate and converted the methylene carbons to ethylene diamine (ED). The yield of CO₂ is usually 60 to 70 percent of the theoretical and there is no contamination with methylene carbon based on results observed when methylene-labelled succinate is degraded.

In order to recover the resulting ED, the residue was diluted with one or two ml of distilled H₂O (to prevent layering of the alkali over the acid) and chilled in liquid N₂. A sufficient quantity of alkali (16N NaOH) was added to raise the pH to 10-11. The mixture was then frozen in a thin layer on the sides of the vessel with liquid N₂ and vacuum distilled. The distillate, ED, is collected in a liquid N₂ trap.

The precipitate of NaSO₄ should appear dry and white after ~ 45 minutes of pumping. The last traces of ED are removed by warming the vessel in hot tap water

and pumping for an additional 10 minutes.

The distillate is titrated with 0.1 N H_2SO_4 , using chlorphenol red as an indicator, and an appropriate aliquot taken for combustion. The yield of carbon was usually 30 percent of the theoretical (40 to 50 percent based on the carboxyl- CO_2 recovery), and there is no cross contamination with carboxyl carbon based on results observed when carboxyl-labelled succinate was degraded.

There is excellent agreement between the total specific activity of succinate, as determined by combustion, and the specific activities of the degradation products obtained by this method. This fact, coupled with the absence of cross contamination between methylene and carboxyl carbons, insures the validity of this method for the determination of methylene-carboxyl activity ratios (M/C) as required during this investigation.

It is to be noted that, while the first method for succinate degradation presented above degrades the molecule carbon by carbon, there is nothing to be gained in this investigation by such a differentiation since the succinate molecule is symmetrical and there is no difference between individual carboxyl carbons or individual methylene carbons found in the same molecule.

Alpha-ketoglutarate was decarboxylated to succinate and CO_2 with acid permanganate according to the method described by Calvin, et. al. (1949). The succinate was then degraded according to the method of Phares (Personal communication) presented above.

A summary of these degradation procedures appears in Figure 4.

H. Carbon Isotope Assay Methods

All counting was done on samples of BaCO_3 as nearly equal to infinite thickness as possible. The precipitate was recovered on porcelain bacteriological filters of about 1.8 cm^2 area. Mica end-window counters of 1.3 to 2.4 mg/cm^2 thickness were used with the sample centered within about 2 mm of the tube face. The counting efficiency of this arrangement was about 1.5 percent.

The background of the shielded counter with disk and inactive BaCO_3 in place was less than 0.4 c/s and no counts in this study were considered significant unless they were more than twice the background. All counts were run three or more times and each count was allowed to continue until it had accumulated at least 1000 events.

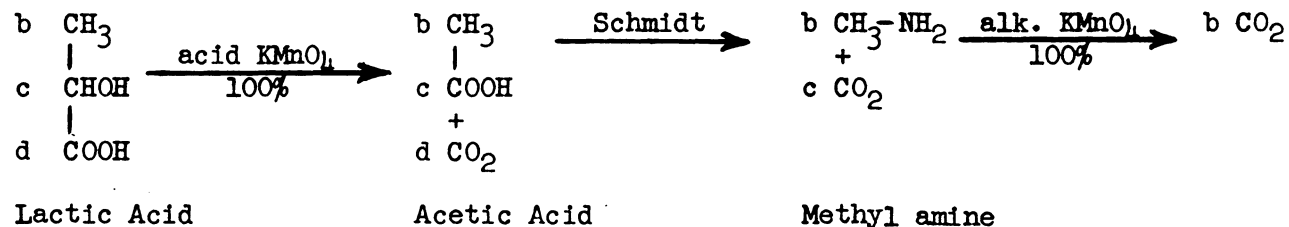
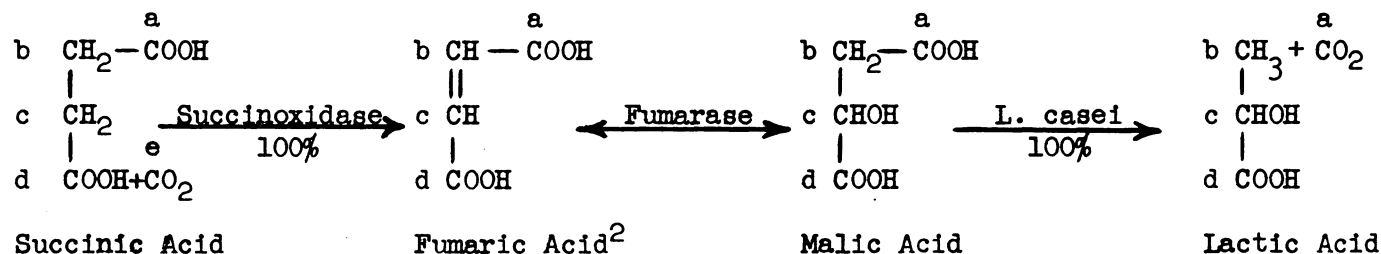
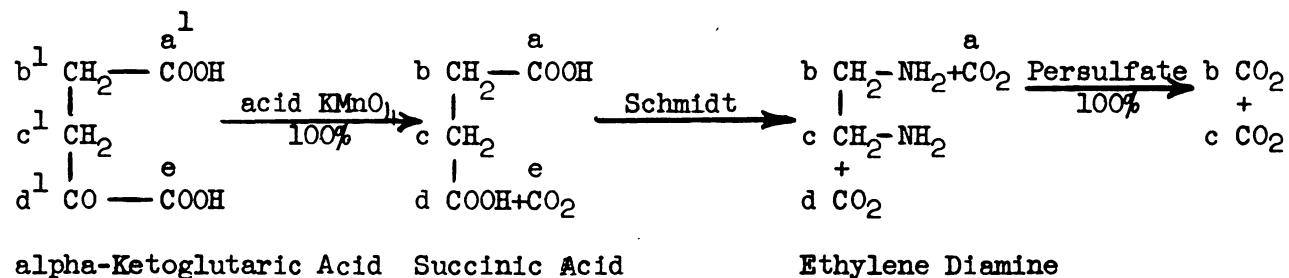


Figure 4. Summary of the Degradation Methods Employed in this Investigation

- Note: 1. Carbons a and d cannot be differentiated one from the other; the same is true of carbons b and c.
2. Fumaric acid has been written in the cis (maleic acid) form for purposes of clarity and uniformity.

Total activity refers to the total number of counts per second which would have been observed if the entire sample had been counted instead of the aliquot usually used. This figure does not change with dilution of the sample.

Specific activity refers to the number of counts per second observed divided by the weight of BaCO_3 being counted and is referred to as c/s/mg BaCO_3 . This figure will change with dilution of the sample.

Occasionally, with samples of low specific activity, the sample was converted back to CO_2 and introduced into a previously evacuated ionization chamber, which was connected to a vibrating reed electrometer and the amperes of ion current, converted to counts per second per mg of BaCO_3 were recorded (Anthony and Long, 1952).

When a quick, qualitative, or rough quantitative measure of the activity in a pure organic acid was desired, approximately 0.003 mE, as the sodium salt, was dried on a watch glass and its activity measured in a manner similar to that used for BaCO_3 on filter discs (Anthony and Long, 1952). This procedure was very satisfactory for estimating the relative activity of various fermentation products obtained from chromatographic columns.

V. DATA AND RESULTS

For purposes of brevity, the experiments discussed here will be accompanied by only those tentative conclusions which directed the course of the investigation. In the face of appearing repetitions, the most important conclusions reached will be summarized at the end of each phase of the investigation. The complete analysis of the research will be reserved for the discussion (Chapter VI).

A. Confirmatory Experiments

Experiment 1. The normal fermentation

The non-gaseous fermentation of xylose by E. coli in the presence of acetate-2-C¹⁴ was examined according to the procedure of Nutting and Carson (1952a, b) for comparison.

In the preparation of table 1, the figures presented are those obtained during the present investigation except for these figures in brackets which represent results reported by Nutting and Carson (1952a, b). The ratio M/C refers to the ratio of activity found in the methylene carbons (M) of succinate compared to the activity present in the carboxyl carbons (C). A low M/C value infers the operation of a cyclic mechanism as described in Chapter II, Survey of the Literature.

Table 1

Confirmatory Experiment 1. The normal fermentation

Buffer, NaHCO_3	0.16 M
Atmosphere	20% CO_2 in He
pH, Initial	7.8
Final	7.2
Average	7.5
Xylose Utilized	0.5 mM
Tracer	acetate-2- C^{14} (50,000 c/s)

End Product	Moles end product formed per mole of xylose fermented	Total Activity	Specific Activity c/s/mg BaCO_3
	M/M	c/s	
Succinic Acid	0.14 (0.13) ¹	234	4.23(1.92) ¹
Carboxyl Carbons	---	12	0.43(0.00)
Methylene Carbons	---	193	7.00(3.16)
M/C ²	---	16/1	16/1
Lactic Acid	0.28 (0.24)	127	1.53
Acetic Acid	0.71 (0.59)	43,500	310
Formic Acid	1.16 (1.17)	1	0.0
Ethanol	0.52 (0.61)	4,800	48
CO_2	---	1	0.0
Recovery of Carbon, % ³	100 (96)	---	---
Hydrogen, % ³	98 (96)	---	---
Activity, %	---	97	---

1. Figures in parentheses are those reported by Nutting and Carson (1952a,b).
2. See text, page 59.
3. See text, page 61.

The carbon and hydrogen balance has been calculated according to the method of Barker (1936). A recovery of 100 percent is equivalent to complete recovery of the substrate utilized.

Although comparisons will be made throughout this chapter between the results obtained by Nutting and Carson (1952a, b) and those observed during the present investigation, it is important for the reader to bear in mind that the interest of the former investigators was directed primarily toward an elucidation of the mechanism of lactate biosynthesis. The observations with regard to the production of succinate with which this investigation is chiefly concerned were chance observations only indirectly related to the problem studied by Nutting and Carson (1952a, b).

Results and Conclusions

(1) The similarity between the fermentation balance presented here and that reported by Nutting and Carson (1952a) indicates that the experimental procedure employed by these latter investigators has been repeated and confirmed.

(2) The presence of a significant amount of activity in the carboxyl carbons of succinate relative to that observed in the methylene carbons, combined with the

relatively low specific activity of the CO_2 of the atmosphere, infers the operation of a cyclic metabolic mechanism (TCAC or DCAC or both).

(3) The percent of the activity available in acetate-2- C^{14} which was fixed in succinate in the present experiment (0.47 percent) was less than half of that (1.1 percent) observed by Nutting and Carson (1952b) under similar conditions.

* * *

Since it was desirable to incorporate as much activity into succinate as possible for purposes of radioactivity assay, several experiments were performed in order to determine the effect of various factors such as pH, temperature, post-fermentation cycle time, and oxygen, on the incorporation of activity into succinate.

pH. It is reported by Nutting and Carson (1952a) that increasing the pH between 5.3 and 7.9 increases the production of succinate during the fermentation of xylose by E. coli. However, attempts to increase the incorporation of activity into succinate by increasing the initial pH from 7.8 to 8.1 were unsuccessful. In fact, a 70 percent decrease in the amount of activity recovered in succinate was observed over this range.

Temperature. Although the experiments of Nutting and Carson (1952a, b) were carried out at 30°, it was of interest to determine what effect increasing the fermentation temperature to 37° might have on the incorporation of activity into succinate, since the latter is the optimum temperature for the growth of E. coli, the test organism. The results observed, however, were unsatisfactory. Less than 70 percent of the activity incorporated at 30° was observed in succinate at 37°.

Experiment 2. The effect of post-fermentation "cycle time" and the presence of oxygen on the incorporation of activity into succinate from acetate-2-C¹⁴.

Post-Fermentation "Cycle Time". Since the results obtained in experiment 1 inferred that a cyclic metabolic mechanism was influencing the distribution of activity incorporated into succinate, it was of interest to determine what effect varying the length of time available for "cycling", called here "cycle time", might have on the incorporation of activity into succinate. The results observed regarding the incorporation of activity into succinate when the post-fermentation cycle time was increased from zero hours (at the time of 90 percent xylose utilisation) to two hours after 100 percent xylose utilization are presented in table 2.

Table 2

Confirmatory Experiment 2. The effect of post-fermentation cycle time and the presence of oxygen on the incorporation of activity in succinate from acetate-2-C¹⁴

Buffer, NaHCO ₃	0.16 M
Atmosphere	20% CO ₂ in He
pH, Initial	7.8
Xylose, Initial	0.05 mM
Tracer	acetate-2-C ¹⁴ (5,000 c/s)

Fermentation	1	2	3	4
Xylose Utilized, %	90	100	100	100
Cycle Time, hr	0	1	2	2 ¹
Succinic Acid, c/s	6.5	7.3	9.0	144.0
Activity Incorporated, %	0.13	0.14	0.18	2.9

1. Oxygen present during second hour of cycle time period.

Oxygen. Since the TCAC and the DCAC are both visualized as being aerobic mechanisms, the effect of the presence of oxygen during this post-fermentation cycle time was of interest. Accordingly, air was introduced during the second hour of a two-hour, post-fermentation, cycle-time period and the incorporation of activity into succinate was determined. The result also appears in table 2. The data appearing in table 2 will be referred to as experiment 2.

Results and Conclusions

(1) Additional anaerobic, post-fermentation, cycle time has little effect upon the incorporation of activity from acetate-2-C¹⁴ into succinate.

(2) The presence of oxygen (20 percent) during the second hour of a two-hour, post-fermentation, cycle-time period produced a fifteen-fold increase in the activity incorporated into succinate from acetate-2-C¹⁴.

* * *

The data obtained in experiment 2 confirmed the oxidative nature of the mechanism incorporating activity from acetate-2-C¹⁴ into succinate and made it desirable to investigate the aerobic dissimilation of xylose in relation to the incorporation of activity into succinate from

acetate-2-C¹⁴.

Preliminary experiments revealed that the ability of E. coli to utilize xylose aerobically was an adaptive process. The more aerobic were the conditions employed for the growth of these organisms, the more readily these organisms were able to dissimilate xylose as determined by oxygen uptake and acid production (CO₂ evolution).

Cells, grown under conditions similar to those employed for fermentation studies, were able to dissimilate xylose, aerobically, at a rate equal to the fermentation rate, thus indicating that they were fully adapted. However, the activity recovered in succinate, under these conditions, was much less than that incorporated in experiment 1, and, therefore, this line of investigation was abandoned.

* * *

The results obtained thus far, strongly suggested that the process which incorporated activity into succinate from acetate-2-C¹⁴ might be an example of the "oxidative fermentation" of Bernhauer (1943) who uses the term to describe these fermentations which, in the presence of free oxygen, result in relatively more oxidized products, but which are not transformed into complete oxidation processes. Bernhauer, therefore, distinguishes between the

"oxidative fermentation" and the purely "anaerobic fermentation" and we shall adopt his terminology. A normal fermentation shall be referred to as an "anaerobic fermentation" and a fermentation to which oxygen has been introduced following the utilisation of xylose, as an "oxidative fermentation".

Recapitulation - Confirmatory Phase

At this stage of the investigation, it was evident that (1) the incorporation of activity from acetate-3- C^{14} in succinate is facilitated by the post-fermentation presence of oxygen and that (2) a cyclic mechanism is involved either partially or entirely in this process.

* * *

In order to determine the extent of the operation of the cyclic metabolic mechanism and, also, possibly, to uncover the operation of a mechanism for the net synthesis of succinate from acetate, it was necessary to interrupt this cycle. The most likely cycle involved was the TCAC and, therefore, monofluoroacetic acid (MFA) was the inhibitor of choice since this inhibitor has been shown to be specific for the TCAC and to be effective in low concentrations:

0.003M MFA inhibited pyruvate oxidation 52 percent by resting cells of E. coli (Kalnitsky and Barron, 1947).

B. Inhibition Experiments

Experiment 3. The selection of an inhibiting concentration of fluoreacetic acid.

While it was desirable to demonstrate inhibition of the fermentation by MFA in some manner, it was undesirable to have a pronounced effect on succinate production. Therefore, the effect of various concentrations of MFA was determined by observing the effect on both the rate of the fermentation and the production of succinate.

A preliminary experiment revealed that MFA inhibited the rate of the fermentation at concentrations between 0.1M and 0.5M, with little or no effect on the production of succinate. At 0.1M there was no inhibition observed whereas at 0.5M the rate of the fermentation was inhibited 85 percent. The percent inhibition reported is based upon the ratio of the volume of CO₂ evolved in the inhibited fermentation to the volume of CO₂ evolved in the uninhibited fermentation at the time that the latter fermentation was completed. The CO₂ evolution referred to here and elsewhere is, of course, the result of acid production freeing CO₂ since this is a non-gaseous fer-

mentation. The succinate produced is the actual production determined following the almost complete utilisation of substrate xylose and is reported in moles per mole of xylose utilised (M/M).

In order to select a suitable concentration of MFA for use, the effect of a series of concentrations between 0.1M and 0.5M was examined at 0.1M increments. These results appear in table 3 and will be referred to as experiment 3.

Results and Conclusions

(1) A concentration of 0.3M MFA was chosen for routine use because, although the rate of the fermentation was inhibited appreciably (42 percent), there was only a slight effect on the final amount of xylose utilised and on succinate production in moles per mole of xylose utilised (M/M).

* * *

The question arose as to whether 0.3M MFA might influence succinoxidase activity and, therefore, succinate determinations. A controlled experiment revealed, however, that this concentration of MFA has no inhibitory effect upon succinoxidase preparations as used for succinate analysis.

Table 3

Inhibition Experiment 3. The selection of an inhibiting
concentration of monofluoroacetic acid

Buffer, NaHCO_3 0.16 M
Atmosphere 20% CO_2 in Ne
pH, Initial 7.8
Xylose, Initial 1.0 mM

Monofluoroacetic Acid, M	0	0.1	0.2	0.3	0.4	0.5
Inhibition, % ¹	0	18	27	42	55	73
Xylose Utilized, mM	0.89	0.91	0.89	0.80	0.72	0.54
Succinic Acid, M/M	0.06	0.06	0.06	0.07	0.07	0.10

1. See text, page 68.

* * *

Experiment 4. The effect of 0.3M MFA on succinate production.

A slight tendency toward an increased production of succinate in the presence of increasing concentrations of MFA was observed in several experiments.

In order to establish whether this was a consequence of the presence of MFA or the extent of xylose utilisation or both, three normal fermentations and three MFA-inhibited fermentations were permitted to proceed to various stages of xylose utilisation and the production of succinate per mole of xylose fermented (M/M) was determined. In addition, a comparison was made between the production of succinate and the production of all organic acids relative to xylose utilisation. This latter relationship has been graphed in Figure 5 and all of the data are presented in table 4.

Results and Conclusions

(1) The presence of 0.3M MFA has little effect on the production of succinate per mole of xylose fermented under these conditions.

(2) Succinate represents a higher proportion (11.2 percent) of the organic acids produced early in the

Table 4

Inhibition Experiment 4. The effect of 0.3 M MFA on Succinate production

Buffer, NaHCO₃ 0.16 M
 Atmosphere 20% CO₂ in He
 pH, Initial 7.8
 Xylose, Initial 0.5 mM

Monofluoroacetic Acid, M	0	0.3	0	0.3	0	0.3
Xylose Utilized, mM	0.25	0.32	0.31	0.34	0.38	0.395
Succinic Acid Produced M/M	0.056	0.063	0.064	0.061	0.054	0.061
mM	0.028	0.041	0.032	0.041	0.041	0.047
Organic Acid Produced, mM	0.25	0.44	0.41	0.47	0.57	0.58
Succinic Acid(mM)	0.112	0.093	0.095	0.087	0.072	0.081
Organic Acid(mM)						

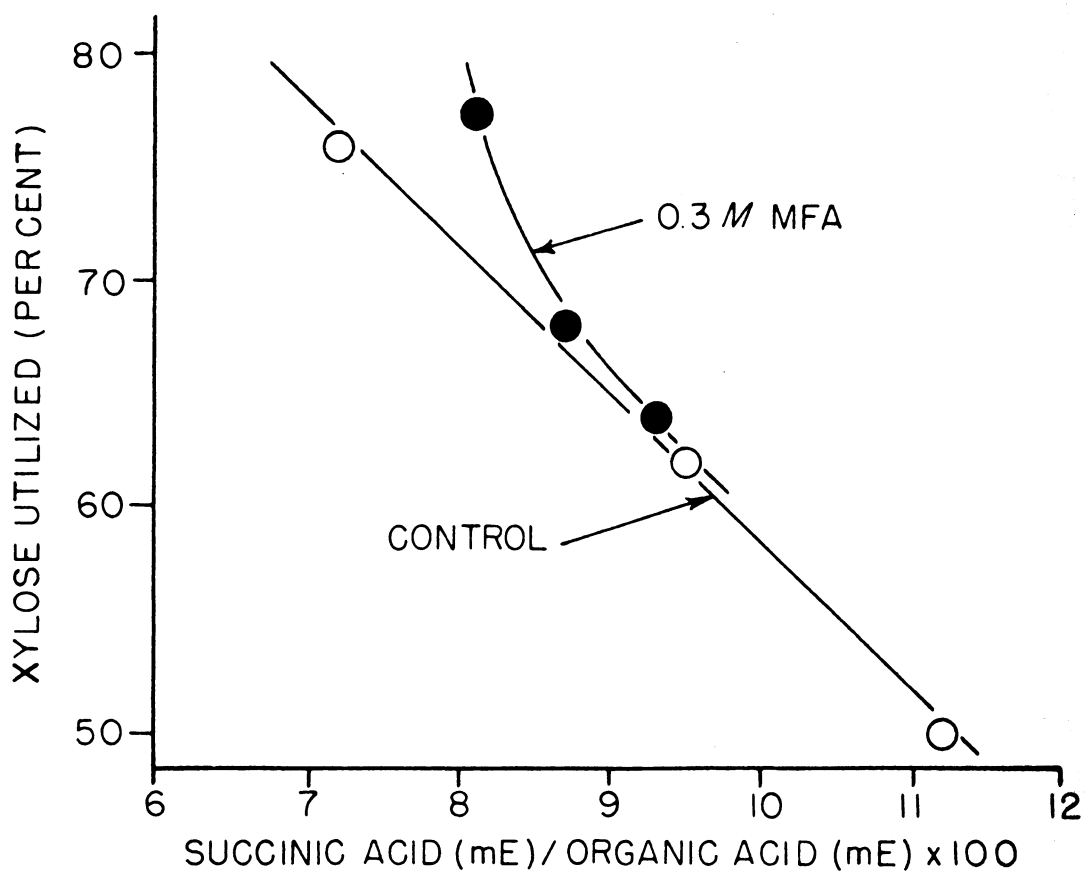


FIGURE 5 THE EFFECT OF 0.3 *M* MONOFLUOROACETIC ACID ON THE DECLINE IN THE PROPORTION OF SUCCINIC ACID PRESENT DURING XYLOSE UTILIZATION

fermentation (50 percent xylose utilization) than it does (7.2 percent) later in the fermentation (76 percent xylose utilization).

(3) From Figure 3, it is evident that while MFA does not influence the production of succinate, it does decrease the rate at which the proportion of succinate to all organic acids falls during xylose utilization. This observation infers an influence by MFA upon the pathway of succinate metabolism which is in accord with the reported influence of MFA on the TCAC.

* * *

The results obtained in experiment 4 indicated that 0.3 M MFA would inhibit the rate of fermentation significantly without affecting the production of succinate. It now became of interest to learn whether MFA would have any effect upon the fermentation balance, in general, and upon the incorporation of activity into succinate, in particular.

Experiments 5 and 6. The effect of MFA on the fermentation balance and the incorporation of activity into succinate.

In experiment 5, table 5, the effect of 0.01M MFA was determined in the event that the TCAC, while

present and susceptible to this concentration of inhibitor, might not be involved in the net synthesis of succinate but only serve to incorporate activity into succinate formed by another mechanism. In this case, inhibition of the cycle by MFA would be evident only in an altered M/C ratio of the succinate recovered. The rate of the fermentation and succinate production, used as measures of MFA inhibition heretofore, would not be valid.

Since, as is shown in table 5, 0.01M MFA did not exhibit any inhibitory effect on the fermentation, experiment 6, table 6, was carried out using 0.3M MFA.

Results and Conclusions

(1) Monofluoroacetic acid in concentrations of 0.01M and 0.3M has little or no effect on the fermentation balance, in general.

(2) Neither 0.01M nor 0.3M MFA affected the M/C ratio in succinate as observed in the normal fermentation although 0.3M MFA did decrease the total activity incorporated into succinate 85 percent. This indicates that MFA inhibits the incorporation of activity into succinate from acetate, probably by a process of competitive inhibition, but that the cyclic mechanism involved is independent of MFA inhibition (DCAC).

Table 3

Inhibition Experiment 3. The effect of 0.01 M MFA on the fermentation balance and the incorporation of activity in succinate

Buffer, NaHCO_3 0.16 M
 Atmosphere 20% CO_2 in He
 pH, Initial 7.8
 Xylose Utilised 0.5 mM
 Tracer acetate-3- C^{14} (50,000 c/s)

Monofluoroacetic Acid, M		0	0.01
Product			
Succinic Acid, M/M		0.14	0.13
	c/s	246	235
	M/C	16.3	16.3
Lactic Acid, M/M		0.28	0.26
	c/s	127	115
Formic Acid, M/M		1.16	1.16
	c/s	1	1
Acetic Acid, M/M		0.72	0.70
	c/s	43,500	39,000
Ethanol, M/M		0.52	0.54
	c/s	4,860	6,200
CO_2 (Atmosphere), c/s		8	6
Recovery of Carbon, %		101	99
Hydrogen, %		98	97

Table 6

Inhibition Experiment 6. The effect of 0.3 M MFA on the fermentation balance and the incorporation of activity in succinate

Buffer, NaHCO_3 0.16 M
 Atmosphere 20% CO_2 in N_2
 pH, Initial 7.8
 Xylose Utilized 0.5 mM
 Tracer acetate-2- C^{14} (21,000 c/s)

Monofluoroacetic Acid, M		0	0.3
Product			
Succinic Acid, M/M		0.10	0.12
	c/s	54	9
	M/C	12.4	12.3
Lactic Acid, M/M		0.42	0.48
	c/s	28	13
Formic Acid, M/M		1.09	1.18
	c/s	1	1
Acetic Acid, M/M		0.61	0.63
	c/s	12,500	13,900
Ethanol, M/M		0.53	0.38
	c/s	1,600	2,130
CO_2 (Atmosphere), c/s		12	6
Recovery of Carbon, %		101	102
Hydrogen, %		99	97

(3) Since the decreased incorporation of activity into succinate in the presence of 0.3M MFA was not accompanied by a similar decrease in production, it is probable that a monofluoroacetate-insensitive mechanism, not involving acetate, is responsible for the synthesis of the major portion of the succinate produced. A possible mechanism would be one involving the fixation of CO_2 on a C_3 fragment (pyruvate) from xylose to form a C_4 dicarboxylic acid which subsequently becomes reduced to succinate.

* * *

Since the MFA experiments thus far performed have involved only anaerobic fermentations, it was of interest to determine what effect MFA might have on the M/C ratio of succinate during an oxidative fermentation.

In addition, in view of the possibility that the cyclic mechanism was, indeed, insensitive to MFA, it was decided to investigate the effect of arsenite (sodium salt) under similar conditions. While this inhibitor would be expected to interrupt the TCAC at the alpha-ketoglutarate $\longrightarrow$ succinate step, it would also be expected to interrupt a DCAC at the pyruvate $\longrightarrow$ acetate plus CO_2 (C_1) reaction. The choice of the concentration of arsenite to be used was based on experience

in the use of this inhibitor with resting bacterial cells (S. F. Carson, Personal communication) and was 0.07M.

The procedure for the preparation of the non-proliferating cell suspension for use in this and future experiments was changed slightly from that used heretofore and reported by Nutting and Carson (1952a). Instead of tap water, buffer or buffer-inhibitor solution, already equilibrated with the CO₂-inert gas mixture and at the proper pH and temperature, was used to wash the cells as well as for the final cell suspension. In this way, the cells were kept under more anaerobic conditions prior to use and during the fermentation than had been the case previously.

Experiment 7. The effect of MFA and arsenite on the anaerobic and oxidative xylose fermentation.

The data observed when xylose is fermented in the presence of MFA or arsenite under either anaerobic or oxidative conditions are compared with data observed under similar conditions in an uninhibited fermentation and are presented in table 7.

Results and Conclusions

(1) In the arsenite-inhibited fermentations, xylose was converted entirely to lactate. The unexpected

Table 7

Inhibition Experiment 7. The effect of MFA and arsenite on the anaerobic and oxidative xylose fermentation

Buffer, NaHCO ₃	0.16 M
Atmosphere	
Anaerobic	20% CO ₂ in He
Oxidative	20% CO ₂ , 20% O ₂ , 60% He
pH, Initial	7.8
Xylose Utilised	0.5 mM
Tracer	acetate-2-C ¹⁴ (21,000 c/s)

Inhibitor		None		MFA		Arsenite	
Fermentation Type		-O ₂	+O ₂	-O ₂	+O ₂	-O ₂	+O ₂
Product							
Succinic Acid,	M/M ----	0.08	0.032	0.040	0.025	0.029	
	c/s ----	146	2	35	64	130	
	M/C ----	13/1	-----	14/1	31/1	38/1	
Lactic Acid,	M/M 0.45	0.58	0.79	0.61	2.10	1.63	
	c/s 20	25	11	12	14	19	
Formic Acid,	M/M 0.94	1.01	1.18	1.33	trace	trace	
Acetic Acid,	M/M 0.52	0.65	0.69	0.78	trace	trace	
Ethanol,	M/M 0.42	0.32	0.46	0.34	trace	trace	
Recovery of							
Carbon, %		84	100	119	111	127	101
Hydrogen, %		78	96	117	104	128	101

drastic effect of arsenite was heralded by the CO₂ evolution observed during the fermentation. Although 0.07M arsenite does not inhibit the fermentation rate, the total CO₂ evolution observed when 0.5mM of xylose is fermented approximates 0.8 mE instead of the 1 mE or more observed during the course of an uninhibited fermentation.

Only two lactate determinations have been made following the initial observation of this phenomenon (both of which indicated at least the theoretical production of lactate from xylose), however, the recoveries of lactate from five anaerobic, arsenite-inhibited fermentations by column chromatography have corroborated these determinations.

It is also interesting that during an oxidative fermentation, a portion of the lactate formed during the anaerobic period becomes oxidized to pyruvate and the recovery of lactate plus pyruvate approaches the theoretical yield based on the xylose fermented.

In several instances, the column recoveries have exceeded the theoretical yield of C₃ compound based upon the xylose fermented and this possibility is also suggested by the lactate determinations presented in table 7. No explanation for this anomaly is apparent.

(2) The C₂ fragment arising from the fermentation of xylose as proposed by Nutting and Carson (1952a, b)

was not in equilibrium with the tracer acetate of the medium in view of the slight incorporation of activity into lactate in the presence of arsenite.

(3) Despite significantly reduced succinate production (75 percent) in the oxidative arsenite-inhibited fermentation, compared with the oxidative, uninhibited fermentation, there was little or no reduction in the total activity incorporated, possibly a result of the relatively higher specific activity of the tracer in view of the fact that in the presence of arsenite no dilution of the tracer results from the production of acetate during the fermentation.

(4) The M/C ratio again was unchanged by the presence of 0.3M MFA in the oxidative fermentation (where the only comparison was possible) although 0.07M arsenite increased the ratio significantly, both anaerobically and oxidatively.

(5) The absence of succinate production and the absence of the activity incorporation associated with it in the anaerobic uninhibited fermentation emphasizes the importance of oxygen for the production of succinate as well as for the process of activity incorporation from acetate established earlier. This is supported also by the results observed in the presence of each inhibitor.

(6) The presence of succinate in the anaerobic

inhibited fermentations is interpreted as being the result of the inhibition of the further metabolism of succinate once it is formed.

(7) A reduced succinate production in the presence of MFA and arsenite in the oxidative fermentation compared with that in the oxidative uninhibited control was noted. This is probably a result of both the more stringent anaerobic conditions present during the fermentation period proper and the restriction imposed on succinate production by the presence of the inhibitor itself during the post-fermentation oxidative period. It is very likely that MFA may inhibit succinate production by a process of competitive inhibition, whereas arsenite may inhibit indirectly by decreasing the concentration of available C_2 fragments.

Recapitulation - Inhibition Phase

At this point in the research, the following conclusions were reached:

(1) Oxygen, introduced at the end of the fermentation increases both the production of succinate and the incorporation of activity from acetate-2- C^{14} into succinate.

(2) Monofluoroacetic acid, an inhibitor of the TCAC, does not affect the fermentation balance signifi-

cantly even at a concentration of 0.3M, and has no effect upon the M/C ratio observed in succinate either anaerobically or oxidatively, although it does decrease the incorporation of activity from acetate-2-C¹⁴ into succinate 75 to 85 percent, possibly by competitive inhibition. It may or may not appear to decrease the production of succinate since this is apparently dependent primarily on the extent to which the "anaerobic" fermentation has been made anaerobic. These results lead to the conclusion that the TCAC is not important as a mechanism for the incorporation of acetate carbon into succinate.

(3) Arsenite, while decreasing the production of succinate presumably by decreasing the concentration of available C₂ fragments, inhibits the cyclic mechanism involved in the incorporation of activity from acetate-2-C¹⁴ into succinate without decreasing the incorporation of this activity. In fact, consideration of the figures for succinate production and activity incorporation coupled with an estimate of the average specific activity of the tracer acetate present may be interpreted as indicating that arsenite has no effect on the mechanism which synthesizes succinate and involves labelled acetate. The results observed in the use of arsenite, in addition to the failure of MFA to inhibit cycling, lead to the conclusion that the cycle which is concerned in the redistribution of

activity in succinate is the DCAC. The drastic effect of arsenite upon the xylose fermentation itself, i.e., the complete conversion of xylose to lactate, a C₃ compound, supports the view that the arsenite is obstructing the pyruvate $\longrightarrow$ acetate plus CO₂(C₁) reaction and thus interrupting the DCAC.

* * *

If the DCAC were present, conceivably, it could function on acetate alone as substrate. However, oxidative fermentations of substrate amounts (1 mM) of acetate-2-C¹⁴ in the presence and absence of arsenite failed to incorporate significant amounts of activity into succinate.

* * *

Returning to the oxidative fermentation of xylose and to the investigation of the most plausible pathways for the net synthesis of succinate, it is apparent that none of the evidence thus far obtained has reflected upon the participation of a mechanism of succinate biosynthesis involving the double fixation of CO₂ by acetate (C₂ + C₁ $\longrightarrow$ C₃; C₃ + C₁ $\longrightarrow$ C₄). The oxidative nature of the mechanism however, might be

viewed as evidence that a reductive process must play a relatively insignificant role if, indeed, any role at all.

Accordingly, having interrupted the cyclic metabolic mechanism through the use of arsenite, it was now possible to determine the participation and perhaps the relative importance of such a mechanism as the double fixation of CO_2 .

C. Definitive Experiments

Experiment 8. The fixation of CO_2 in succinate.

In order to demonstrate the participation of CO_2 fixation, or, possibly, CO_2 exchange, fermentations were carried out under various conditions in the presence of C^{14}O_2 . The results observed appear in table 8.

Results and Conclusions

(1) The introduction of CO_2 into succinate appears to be independent of the presence of oxygen. This suggests the possibility that the mechanism incorporating CO_2 into succinate is not a method of net synthesis but merely an expression of the equilibrium:

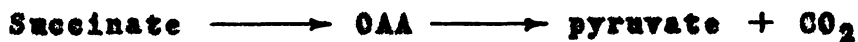


Table 8

Definitive Experiment 8. The fixation of CO₂ in succinate

Buffer, NaHCO ₃	0.16 M
Atmosphere	
Anaerobic	20% CO ₂ in He
Oxidative	20% CO ₂ , 20% O ₂ , 60% He
pH, Initial	7.8
Xylose Utilized	0.5 mM
Tracer	C ¹⁴ O ₂

Fermentation Type	Anaerobic		Oxidative	
Inhibitor	None		None	Arsenite
Tracer, c/s	105,000	52,500	52,500	
Product				
Succinic Acid, M/M c/s M/C	0.12 630 (0.6%) 1/170	-- 298 (0.6%) --	-- 40 (0.07%) --	
Lactic Acid, M/M c/s	0.44 60	-- 6	-- 158	
Formic Acid, M/M c/s	1.03 325	-- 90	-- 10	
Acetic Acid, M/M c/s	0.73 2	-- 1	-- 1	
Ethanol, M/M c/s	0.55 --	-- --	-- --	
Recovery of				
Carbon, %	107	--	--	
Hydrogen, %	107	--	--	

(2) The incorporation of CO_2 into succinate in the presence of arsenite is reduced to a degree similar to the reduction in succinate production. This may reflect a shift to the right in the equilibrium presented above which seems plausible in view of the accumulation of C_3 compounds (lactate and/or pyruvate) already observed in the presence of arsenite.

* * *

Experiment 9. The use of labelled acetate to determine the relative importance of CO_2 fixation for the biosynthesis of succinate.

While the use of labelled CO_2 has inferred the participation of CO_2 fixation or exchange in the biosynthesis of succinate, little information has been obtained regarding the importance of such a mechanism relative to the C_2 condensation (assuming that the DCAC is present and that the TCAC is absent or not significant).

It was thought that some indication of the relative importance of each of these mechanisms of net synthesis might be obtained by carrying out arsenite-inhibited oxidative fermentations in the presence of three types of labelled acetate: acetate-1- C^{14} , acetate-2- C^{14} , and acetate-1,2- C^{14} . In addition, an

arsenite-inhibited anaerobic fermentation was performed in the presence of acetate-1,2-C¹⁴. The data obtained appears in table 9.

The increased production of lactate observed in uninhibited fermentations in more recent experiments is believed to be in part the result of the more anaerobic conditions imposed during these fermentations. However, in order to maintain a firmer control over the pH fluctuation which may also be at fault, a more concentrated buffer solution (0.26M) was used which provided an initial pH of 8.0 and which, under ordinary conditions, permitted a drop of only 0.3 of a pH unit to 7.7.

Results and Conclusions

(1) The M/C ratios found in succinate produced in the presence of each of the three types of labelled acetate infer that little or no succinate could have been formed by the double fixation of CO₂ on acetate under these conditions either anaerobically or oxidatively.

(2) Only two pathways for the incorporation of acetate into succinate can satisfactorily account for the distribution of activity observed in this experiment:

(1) the Thunberg (C₂) condensation and (2) the TCAC, assuming that the OAA required arises by a mechanism not involving acetate.

Table 9

Definitive Experiment 9. The use of labelled acetate to determine the relative importance of CO₂ fixation for the biosynthesis of succinate

Buffer, NaHCO ₃	0.26 M
Atmosphere	
Anaerobic	20% CO ₂ in H ₂
Oxidative	20% CO ₂ , 20% O ₂ , 60% He
pH, Initial	8.0
Xylose Utilized	0.5 mM
Inhibitor	0.07 M sodium arsenite
Tracer	50,000 c/s

Fermentation Type		Oxidative		Anaerobic
Tracer, acetate-		1-C ¹⁴	2-C ¹⁴	1,2-C ¹⁴
Product				
Succinic Acid, c/s	1080	1320	1220	585
M/G	1/386	151/1	1/1.25	1/1.12
Alpha-keto-				
glutaric Acid, c/s	99	122	322	69
K→S M/C	---	127/1 ¹	---	---
S/K	4.4 ¹	6.4 ¹	2.2 ¹	5.1 ¹
alpha-COOH, c/s	---	---	11	---
Lactic Acid, c/s	50	59	56	23
Formic Acid, c/s	72	56	105	23
Acetic Acid, c/s	---	---	---	---
Ethanol, c/s	---	---	---	---
Pyruvic Acid, c/s	---	36	90	36
CO ₂ (Atmosphere), c/s	182	---	100	22

1. See text, page 93.

(3) The presence of activity in alpha-ketoglutaric acid recovered from the chromatographic column following the addition of the unlabelled alpha-ketoglutaric acid to the fermentation products inferred the possible participation of this key TCAC intermediate in the formation of succinate.

Recapitulation - Definitive Phase

The chief results of this phase of the investigation were:

(1) The mechanism of biosynthesis of succinate involving the double fixation of CO_2 was found to be unimportant as a mechanism for the incorporation of acetate into succinate in the presence of arsenite.

(2) Only two mechanisms fulfill the requirements regarding the distribution of activity into succinate when labelled acetate is used as a tracer: (1) the Thunberg (C_2) condensation and (2) the TCAC, if one assumes that the OAA required is inactive.

(3) Although the effect of arsenite on the fermentation, and the failure of MFA to inhibit the cyclic mechanism, both infer that the TCAC is relatively unimportant, the observation of significant amounts of radioactivity in alpha-ketoglutaric acid required further investigation of the TCAC as a possible mechanism for the

incorporation of acetate activity into succinate.

* * *

Since the distribution of activity in succinate produced from labelled acetate via the TCAC is indistinguishable by degradation methods from that produced via acetate condensation, relatively indirect measures are required to demonstrate the product-precursor relationship which exists between succinate and alpha-ketoglutarate.

D. Corroborative Experiments

Experiment 10. The effect of arsenite on the succinate-alpha-ketoglutarate activity ratio (S/K ratio).

Some evidence of the order of formation of succinate and alpha-ketoglutarate was gained by examining the effect of arsenite on the ratio of the activity incorporated into succinate to the activity incorporated into alpha-ketoglutarate (S/K ratio).

In addition, samples of succinate and alpha-ketoglutarate from each of the arsenite inhibited fermentations were degraded in order to compare the M/C ratio of succinate with that of the succinate derived from the decarboxylation of alpha-ketoglutarate ($K \longrightarrow S$ M/C).

Unfortunately, considerable non-enzymatic

decomposition of alpha-ketoglutarate occurs during analyses which is credited in part to the presence of traces of manganese. However, according to Kalnitsky (1953) only 10 to 20 percent of the alpha-ketoglutarate decomposed in this way appears as succinate. This was observed in later experiments using labelled alpha-ketoglutarate. However, in table 10, two figures are given for the activity recovered in succinate and alpha-ketoglutarate under each of the conditions examined. The first figure represents the actual activity recovered in the compounds corrected for chromatographic column losses. The second figure in brackets represents the activity present in alpha-ketoglutarate at the end of the fermentation and the activity in succinate if one assumes that all of the alpha-ketoglutarate decomposed after the fermentation has been converted to succinate. Obviously, the actual activity in succinate lies somewhere between the two values and therefore a weighted average has been used to calculate the succinate-alpha-ketoglutarate activity ratios (S/K ratio). This weighted average assumes that 20 percent (the maximum figure reported by Kalnitsky (1953)) of the decomposed alpha-ketoglutarate has been recovered as succinate. Lactate and CO_2 activities are recorded for comparison.

Table 10

Corroborative Experiment 10. The effect of arsenite on the succinate-alpha-ketoglutarate activity ratio

Buffer, NaHCO₃ 0.26 M
 Atmosphere
 Anaerobic 20% CO₂ in He
 Oxidative 20% CO₂, 20% O₂, 60% He
 pH, Initial 8.0
 Xylose Utilized 0.5 mM

Fermentation Type	Anaerobic		Oxidative	
Tracer (c/s)	acetate-1,2-C ¹⁴ (50,000)		acetate-2-C ¹⁴ (50,000)	
Inhibitor	None	Arsenite	None	Arsenite
Product				
Succinic Acid, c/s	14 (12)	182 (171)	226 (228)	472 (304)
M/C	--	1/1.05	--	131/1
alpha-Keto-glutaric Acid, c/s	14 (16)	30 (41)	12 (60)	56 (224)
K→S M/C	--	1/1.08		93/1
S/K	13.6/16 = 0.85	180/41 = 4.4	266/60 = 4.4	436/224 = 2.0
alpha-COOH, c/s	--	2.8	--	7.7
Lactic Acid, c/s	48	40	52	17
CO ₂ (Atmosphere), c/s	10	7	85	40

Results and Conclusions

(1) The slight incorporation of activity into succinate and alpha-ketoglutarate in the anaerobic, uninhibited fermentation agrees with the results observed in experiment 7.

(2) The determination of the M/C ratio of succinate, and of succinate obtained by the decarboxylation of alpha-ketoglutarate, in the oxidative, arsenite-inhibited fermentations reveals the difference between these acids and agrees with similar results obtained in experiment 9.

(3) The high M/C ratio in the succinate obtained from alpha-ketoglutarate infers the participation of relatively large amounts of inactive C₄ acid for the condensation reaction initiating the TCAC.

(4) The S/K ratio oxidatively reveals that arsenite is inhibiting the decarboxylation of alpha-ketoglutarate to succinate.

(5) Despite the inhibition of the decarboxylation of alpha-ketoglutarate, two to four times more activity is found in succinate than in alpha-ketoglutarate in the arsenite-inhibited fermentations.

* * *

Although the S/K ratio observed in experiment 10 was not reversed in the presence of arsenite as it might have been if the TCAC were responsible for all of the activity incorporated into succinate, it was decided to further demonstrate the relative unimportance of the TCAC by comparing the S/K ratios observed in the presence of arsenite with those obtained in the presence of both arsenite and monofluoroacetate.

Experiment 11. The effect of arsenite-monofluoroacetate on the succinate- α -ketoglutarate activity ratio (S/K ratio).

In table 11 are presented the results observed when both anaerobic and oxidative fermentations are performed in the presence of arsenite and arsenite-monofluoroacetate.

Results and Conclusions

(1) The comparison between the results of the anaerobic and the oxidative fermentations as regards the S/K ratio indicates strongly that two pathways are responsible for the introduction of activity into succinate: one, presumed to be the C_2 condensation, which functions under both anaerobic and oxidative conditions, and the second, presumed to be the TCAC

Table 11

Corroborative Experiment 11. The effect of arsenite-monoethylfluoroacetate on the succinate- α -ketoglutarate activity ratio

Buffer, NaHCO_3 0.26 M
 Atmosphere
 Anaerobic 20% CO_2 in He
 Oxidative 20% CO_2 , 20% O_2 , 60% He
 pH, Initial 8.0
 Xylose Utilized 0.5 mM
 Tracer acetate-2- C^{14} (50,000 c/s)

Fermentation Type	Anaerobic		Oxidative	
	Arsenite	Arsenite + MFA	Arsenite	Arsenite + MFA
Inhibitor				
Product				
Succinic Acid, c/s	370 (366)	10.3 (10.25)	201 (190)	31 (22)
M/C	-----	-----	-----	-----
α -Keto-				
glutaric Acid, c/s	13 (17)	0.24 (0.29)	47 (58)	21 (30)
K $\rightarrow$ S M/C	-----	-----	-----	-----
S/K	370/17 = 22	10.29/0.29 = 35	199/58 = 3.4	29/30 = 0.97
α -COOH, c/s	-----	-----	-----	-----
Lactic Acid, c/s	8.0	3.5	-----	10
CO_2 (Atmosphere), c/s	-----	-----	-----	-----

which functions only under oxidative conditions.

* * *

One more approach to the problem of whether the TCAC or the C_2 condensation was the more important pathway of succinate biosynthesis, was to study the effect of arsenite on the metabolism of alpha-ketoglutarate.

Experiment 12. The effect of arsenite on the utilisation of alpha-ketoglutarate.

In order to study the metabolism of this TCAC intermediate, it was introduced to the fermentation as alpha-ketoglutarate-1,2- C^{14} in trace amounts. The utilisation during the fermentation was determined by activity measurements on the atmospheric CO_2 and the decomposition following the fermentation was calculated from the specific activity of the recovered acid.

The data obtained appear in table 12. The figures presented are actual activity recoveries. The figures in brackets are the equivalent in activity of alpha-ketoglutarate-1,2- C^{14} . Since the latter acid is decarboxylated in the formation of succinate, only approximately one-half of the activity present in the tracer could possibly be fixed in the fermentation acids. For an activity balance, therefore, the activity found

Table 12

Corroborative Experiment 12. The effect of arsenite on the utilization of
alpha-ketoglutarate

Buffer, NaHCO₃ 0.26 M
Atmosphere
Anaerobic 20% CO₂ in He
Oxidative 20% CO₂, 20% O₂, 60% He
pH, Initial 8.0
Xylose Utilized 0.5 mM
Tracer alpha-ketoglutaric acid-1,2-C¹⁴

Fermentation Type	Anaerobic			Oxidative	
Tracer, c/s	1300			3900	
Inhibitor	None ¹	None	Arsenite	None	Arsenite
Product					
Succinic Acid, c/s M/C	16 (32) -----	55 (110) ~~1474~~	68 (136) 1/118	197 (394) -----	139 (278) -----
alpha-Keto- glutaric Acid, c/s	1219	924	654	----- ²	----- ²
Lactic Acid, c/s -COOH c/s	----- -----	47 (94) -----	87 (174) 87	14 (28) -----	33 (66) 33
Acetic Acid, c/s	7 (14)	1 (2)	1	5 (10)	1
Pyruvic and Formic Acid, c/s	42 (84)	44 (88)	18 (36)	48 (96)	88 (176)
CO ₂ (Atmosphere), c/s	11	120	120	240	275

Table 12 (Continued)

Fermentation Type		Anaerobic			Oxidative	
Tracer, g/s		1300			3500	
Inhibitor		None ¹	None	Arsenite	None	Arsenite
alpha-Ketoglutaric Acid-1,2-C ¹⁴						
Fermentative Decomposition, %		2	18	18	12	14
Total Decomposition to Fermentation Acids, %		10	22	27	13	13
Activity Recovery, %		104	94	77	-- ²	-- ²

1. Non-enzymatic decomposition in the absence of cells.
2. The alpha-ketoglutaric acid was precipitated with 2,4-dinitro-phenylhydrazine to reduce post-fermentation, non-enzymatic decomposition to fermentation acids.

in the fermentation acids should be doubled to correspond to the tracer utilized.

It should be emphasized that the activity incorporated into the fermentation acids represents the sum total of that activity introduced by the decarboxylation during the fermentation (best determined by activity measurements on the atmospheric CO_2) and that activity introduced by the decomposition of alpha-ketoglutarate during the post-fermentation separation of the fermentation products.

In table 12, the non-enzymatic decarboxylation of alpha-ketoglutarate during the fermentation is referred to as "fermentative decomposition." It is reported as the percent of the tracer accounted for by the activity recovered in the CO_2 of the atmosphere.

The sum total of the activity introduced into the fermentation acids by this "fermentative decomposition" plus the activity introduced by the decomposition of alpha-ketoglutarate during the post-fermentation separation of the fermentation products is referred to as "total decomposition to fermentation acids". It is reported as the percent of the tracer accounted for by the activity recovered in the fermentation acids.

Results and Conclusions

- (1) Based on the activity recovered in the

CO₂, 14 to 18 percent of the tracer alpha-ketoglutarate was decarboxylated during the fermentation.

(2) Arsenite did not exhibit any significant effect upon the decarboxylation thus indicating the non-enzymatic nature of the reaction.

(3) All activity present in lactate recovered from the oxidative, arsenite-inhibited fermentations was found in the carboxyl carbon; indicating that no reversal of the TCAC had occurred.

(4) Oxygen did not stimulate the decarboxylation of alpha-ketoglutarate. A further indication that the reaction observed was non-enzymatic in nature.

Recapitulation - Corroborative Phase

A further investigation of the TCAC as a possible mechanism for the incorporation of acetate activity into succinate has made possible the following conclusions:

(1) Anaerobically, in the presence of arsenite, the ratio of activity present in succinate to that present in alpha-ketoglutarate (S/K ratio) is considerably higher than the same ratio in the oxidative fermentation without a corresponding increase in the total activity incorporated into the two compounds. However, in no case, has more activity appeared in alpha-ketoglutarate than is recovered

in succinate. This indicates that two mechanisms are in operation, one functioning both anaerobically and oxidatively, presumed to be the C₂ condensation, and the other functioning primarily oxidatively, though of lesser importance, presumed to be the TCAC.

(2) MFA appears to inhibit both mechanisms but with greater effect upon the TCAC under anaerobic conditions.

(3) The inability of arsenite to reverse the succinate-alpha-ketoglutarate activity ratio (S/K ratio) in addition to the variation observed between the M/C ratio of succinate and the M/C ratio of succinate obtained by the decarboxylation of alpha-ketoglutarate, lend further support to the hypothesis that two mechanisms which incorporate acetate activity in a similar manner are in operation oxidatively: the C₂ condensation and the TCAC.

(4) The data obtained through the use of alpha-ketoglutarate-1,2-C¹⁴, while indicating the nature of the decomposition of this compound observed earlier, furnishes no information concerning the participation or importance of the TCAC as a pathway for succinate biosynthesis since, under the conditions employed, little if any, of the tracer compound was metabolised.

VI. DISCUSSION

The chance observation by Nutting and Carson (1952a, b) that succinate, produced during the fermentation of xylose by E. coli, in the presence of acetate-2-C¹⁴, was labelled entirely in the methylene carbons suggested that a net synthesis of succinate might have been accomplished by a condensation of two acetate molecules, methyl group to methyl group.

Some support for this suggestion was available in the demonstration that, under similar conditions, a net synthesis of lactate occurred which also involved a C₂ fragment similar to acetate.

In addition to the particular significance which the demonstration of such a reaction as the C₂ condensation would have for intermediary metabolism, the value of the further elucidation of the role played by the C₂ intermediate in biochemistry made an investigation of the mechanism of the biosynthesis of succinate, under the conditions described, of especial interest.

A. Mechanism of Succinate Biosynthesis

Three metabolic pathways have been considered which were deemed to be the most likely ones by which acetate-2-C¹⁴ might become incorporated into succinate.

All three routes satisfy the requirement that all of the activity present in the succinate molecule be confined to the methine carbons. They are: (1) the double fixation of CO_2 on acetate, (2) the TCAC (carried only as far as succinate), and (3) the Thunberg or C_2 condensation.

1. The double fixation of CO_2

The data which have been obtained concerning the participation of this mechanism for the formation of succinate are far from complete, but a conclusion regarding the relative importance of such a mechanism may be reached, based upon the following observations:

a) Despite the ready incorporation of radioactivity present in CO_2 into lactate (oxidatively, in the presence of arsenite), little incorporation of acetate activity has been observed under similar conditions, although the substrate, xylose, is converted almost entirely to lactate. This observation would infer that the C_2 plus C_1 condensation reported by Nutting and Carson (1952 a,b) may not be the principle mechanism for the production of lactate in excess of one mole per mole of xylose fermented.

b) The M/C ratio found in succinate when it is synthesized in the presence of acetate-1,2- C^{14} (either anaerobically or oxidatively in the presence of arsenite), indicates that no dilution of the carboxyl carbon with

inactive carbon (CO_2) has occurred.

c) The M/C ratio, found in succinate when it is synthesized in the presence of acetate-1- C^{14} and arsenite, oxidatively, is so minute (1/386) as to almost completely exclude the participation of a process involving the double fixation of CO_2 on labelled acetate.

d) The effect of arsenite on the fermentation as a whole, would lead one to suspect that the C_3 plus $\text{C}_1 \longrightarrow \text{C}_4$ reaction was inhibited in view of the maximum production of C_3 compounds. The fact that both precursor and product of such a reaction are likely to be alpha-keto acids (pyruvate and OAA) also infers that the reaction will be susceptible to arsenite inhibition.

Since all of the observations regarding the double fixation of CO_2 on acetate presented here are based on experiments conducted in the presence of arsenite, one can only conclude that in the presence of arsenite, there is no indication that a mechanism occurs which involves the double fixation of CO_2 on acetate.

2. The tricarboxylic acid cycle

The distribution of activity in succinate following the use of labelled acetate implicated the TCAO. The participation of this cycle was thought to be established when labelled alpha-ketoglutarate, isolated by the addition of unlabelled carrier alpha-ketoglutarate to the fermenta-

tion medium, was found to contain significant amounts of activity (up to 50 per cent of that recovered in succinate when arsenite was present as an inhibitor).

However, several observations have been made which testify to the relative unimportance of this mechanism as a pathway for the incorporation of acetate activity into succinate:

a) The TCAC does not satisfy the requirement that the oxidative mechanism be responsible for the net synthesis of succinate as well as the incorporation of acetate activity. For every mole of succinate formed by the TCAC, one mole of an equivalent acid (OAA) must be utilized and thus no net synthesis is possible.

b) MFA, in concentrations up to 0.3M, does not influence whatever cyclic mechanism is in operation either anaerobically or oxidatively, as evidenced by the M/C ratio observed in succinate, despite a 42 percent inhibition of the rate of the fermentation, and a 75 to 85 percent inhibition in the total activity incorporated into succinate. The relatively high concentration of 0.1M MFA has little or no effect even upon the rate of the fermentation. The inhibition of the incorporation of acetate activity by 0.3M MFA (absent at 0.01M) can be explained satisfactorily by a contemplation of the similarity of structure and the recognition of the principle of competitive inhibition.

c) While 0.07M arsenite exhibited an almost complete inhibition of the cyclic mechanism (it would be expected to inhibit a DCAC as well as the TCAC), there was no inhibition of the mechanism incorporating activity into succinate from acetate-2-C¹⁴. On the average, succinate, produced during an arsenite-inhibited fermentation might be expected to have a specific activity four times greater than succinate produced in the uninhibited fermentation, if arsenite had absolutely no effect upon the mechanism which incorporates acetate activity. In experiment 7, succinate, produced in the oxidative, arsenite-inhibited fermentation, has a specific activity which is almost three times the specific activity of succinate produced in the uninhibited, oxidative fermentation.

d) If one decarboxylates alpha-ketoglutarate recovered from an oxidative, arsenite-inhibited fermentation carried out in the presence of acetate-2-C¹⁴, one finds that the succinate so obtained has a lower M/C ratio than does succinate isolated directly from the fermentation medium. This infers that succinate, arising during the fermentation, could not be formed entirely from alpha-ketoglutarate.

In view of the results presented above, two conclusions appear to be valid:

(1) The TCAC is present in E. coli and it participates in the incorporation of acetate activity into preformed succinate.

This conclusion is interesting in that Ajl and Kamen (1950, 1951) were unable to demonstrate the incorporation of acetate activity into alpha-ketoglutarate in intact cells of this organism and they concluded that the TCAC was not present.

(2) Although present, the TCAC does not appear to be the only mechanism for the incorporation of acetate activity into succinate.

3. The Thunberg or C_2 condensation

The evidence obtained in the use of labelled acetate in the presence of arsenite clearly demonstrated that, in the formation of succinate, the acetate molecule is used in its entirety: the methyl carbon becoming the methylene carbon of succinate and the carboxyl carbon becoming the carboxyl carbon of succinate with no interchange between the two carbons. Two possible pathways can account for the incorporation of the acetate molecule in this manner: the TCAC and the Thunberg or C_2 condensation. Since succinate, produced by one of these mechanisms cannot be distinguished by degradation methods from succinate produced by the other, relatively indirect

methods have been employed in an attempt to uncover the possible operation of the C_2 condensation in the presence of the TCAC.

While it is clear that no one observation by itself can satisfactorily demonstrate the participation of such a mechanism, it is hoped that the sum total of many observations may allow a proper conclusion.

Several observations which have already been considered with regard to the occurrence of the TCAC will be reiterated here since the two mechanisms obviously intersect at many points.

a) The C_2 condensation, in contradistinction to the TCAC, satisfies the requirement that the oxidative process increase both the yield of C_4 acid (succinate) and the incorporation of acetate activity.

b) In view of the failure of MFA to inhibit the cyclic mechanism involved in the incorporation of activity into succinate, the successful inhibition of this cyclic mechanism by arsenite can be ascribed to its effectiveness in inhibiting the decarboxylation of pyruvic acid, an integral reaction in the DCAC. That this may be the actual case, is emphasized by the effect of arsenite on the fermentation in general, i.e., to prevent any and all decomposition of C_3 fragments.

c) The failure of arsenite to decrease the acetate

activity incorporated into succinate (while partly a result of the increased relative specific activity of the tracer due to non-formation of unlabelled acetate from substrate), may also reflect the nature of the reaction. Whereas arsenite would not be expected to influence the formation of succinate via the C_2 condensation, its formation via the TCAC and alpha-ketoglutarate should be significantly affected, particularly in view of the effectiveness of arsenite on the cyclic mechanism as indicated by the M/C ratio observed in succinate following the use of acetate-2- C^{14} as a tracer.

d) Despite the presence of arsenite and its demonstrated effectiveness against the cyclic mechanism and alpha-ketoglutarate decarboxylation, the activity recovered in alpha-ketoglutarate never exceeded 50 per cent of that observed in succinate. Anaerobically, this figure dropped to 5 to 25 per cent, and in the presence of both arsenite and monofluoroacetate, anaerobically, only three per cent of the activity present in succinate was present in alpha-ketoglutarate.

The extent of the chemical or non-enzymatic degradation of labelled alpha-ketoglutarate (13 to 27 percent) infers that if succinate were formed by the TCAC: (1) the demonstrated inhibition of cycling, (2) the noted inhibition of alpha-ketoglutarate enzymatic

decarboxylation, and (3) the very low value of this non-enzymatic decomposition, should lead to the accumulation of significantly more activity in alpha-ketoglutarate even exceeding that which is observed in succinate. This however, has never been observed.

e) The post-fermentation addition of oxygen to an arsenite-inhibited fermentation, while increasing the incorporation of acetate activity into succinate significantly, seldom more than doubles the fixed activity whereas, in the case of alpha-ketoglutarate, the fixed activity increases more than five-fold. This supports the hypothesis that two mechanisms are responsible for the incorporation of activity into succinate: the one, functioning both anaerobically and oxidatively, the C_2 condensation, and the other, functioning principally oxidatively, the TCAC.

f) The non-gaseous nature of the fermentation as described by Nutting and Carson (1952a) and confirmed during the present investigation further emphasizes the suitability of the C_2 condensation as a mechanism of succinate synthesis in view of the fact that the TCAC would involve the release of two moles of CO_2 per mole of succinate formed.

The results obtained in the present investigation and summarized above lead to the following over-all conclusions:

1) In the presence of arsenite, the biosynthesis of succinate via a process involving the double fixation of CO_2 on acetate is not an important mechanism for the incorporation of acetate activity into succinate.

2) In the presence of arsenite, the TCAC is functioning, primarily during the oxidative, post-fermentation, cycle-time period but it is not the principle mechanism for the incorporation of acetate activity into succinate.

3) In the presence of arsenite, the principle mechanism for the incorporation of acetate activity into succinate is the Thunberg or C_2 condensation, and the cyclic mechanism principally concerned in the distribution of activity is the DCAC.

B. Other Mechanisms of Succinate Biosynthesis

It is to be emphasized that the conclusions reached above refer to the situation as it appears in the presence of 0.07M arsenite and are concerned only with these mechanisms which may introduce acetate activity into succinate.

However, it is important to note, that in the absence of arsenite considerably more succinate is formed although there appears to be no significant effect on the incorporation of acetate activity. While it is difficult

to compare the acetate-activity incorporated and the total succinate produced in arsenite-inhibited and uninhibited fermentations, it has been observed that, in the presence of 0.3M MFA, the incorporation of activity in succinate may be inhibited without inhibiting succinate production. These observations would lead one to conclude that other mechanisms, not involving acetate, may synthesize succinate. However, a possible explanation for this might be that the C_2 fragment obtained in the aldolase-like cleavage of xylose as proposed by Nutting and Carson (1952a,b) is not in rapid equilibrium with the tracer acetate in the medium and succinate may be formed directly with relatively little opportunity for the introduction of tracers. The fact that there is little equilibration between the C_2 fragment and tracer acetate even during the formation of the theoretical amount of lactate from xylose in the presence of arsenite lends some support to this possibility.

Doubtless other mechanisms for succinate synthesis not involving acetate or a C_2 fragment may be in operation. The most probable mechanism of this type would be the fixation of CO_2 on a C_3 fragment (pyruvate) from xylose followed by subsequent reduction via the C_4 acid shuttle to succinate. The fixation of CO_2 in the carboxyl carbons of succinate observed during the investigation may

be considered a priori evidence that such a reaction can occur.

Furthermore, the presence of the inhibitor, arsenite, may have created an artificial situation, which, although satisfactory for the purposes of this study, does not reflect the true importance of the various biosynthetic pathways available for the formation of succinate. Thus, mechanisms which appear of little or no importance in the presence of arsenite, may assume broader roles in the uninhibited fermentation.

Suffice it to say, that while the mechanisms of succinate biosynthesis which involve the incorporation of acetate in the presence of arsenite are relatively clear, other mechanisms responsible for the production succinate in the absence of arsenite may not have been exposed or their importance truly represented.

C. Suggestions for Future Investigations

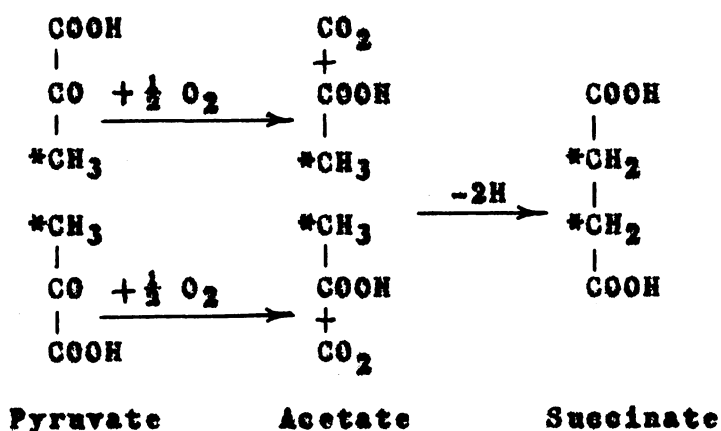
With regard to the further investigation of the mechanisms by which succinate is biosynthesized, several possible lines of investigation may be suggested:

a) If the DCAC plays a major role in the biosynthesis of succinate, continued cycling should result in a significant decrease in the M/C ratio of acetate as well as succinate when acetate-2-C¹⁴ is used as a tracer.

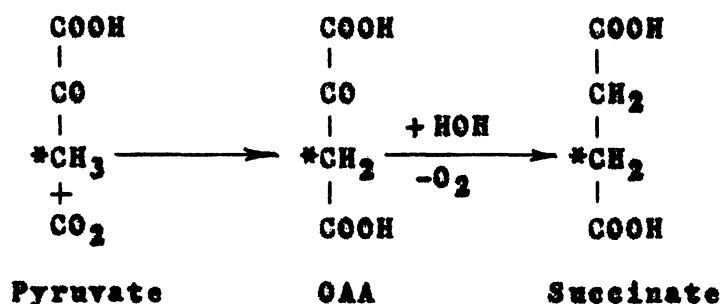
In this event, while a direct determination of the M/C ratio of the tracer may prove impracticable, it should be possible, by a suitable dilution procedure, to demonstrate the increased activity present in the carboxyl of acetate recovered from the fermentation compared with that initially present in the tracer.

It is to be noted that the TCAC will not produce a similar effect since no redistribution of the activity in acetate occurs as a result of the operation of this cycle.

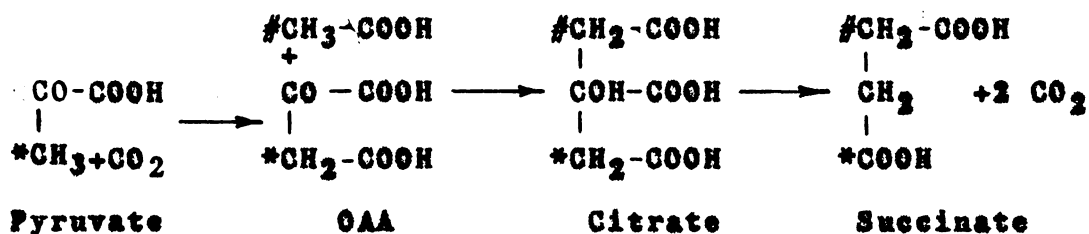
b) The relative importance of the DCAC compared with the TCAC might be further established if pyruvate-3-C¹⁴ were used as a tracer. In the event that succinate is formed either by a condensation of acetate obtained from pyruvate by decarboxylation as required in the DCAC:



or by CO₂ fixation on pyruvate followed by the reduction of the resulting C₄ acid to succinate:



the use of pyruvate-3-C¹⁴ would result in a predominantly methyl-labelled succinate. However, in the event that succinate is formed by the TCAC, the formation of labelled OAA (*) by the fixation of CO₂ on the pyruvate-3-C¹⁴, followed by its condensation with unlabelled acetate to form citrate, will result in the formation of entirely carboxyl-labelled succinate:



If any of the acetate required in the above condensation originates from the pyruvate-3-C¹⁴, it will be methyl-labelled (#) and it will introduce activity into the methylene carbons of succinate. However, it is unlikely that the activity incorporated from acetate in this manner would exceed the activity incorporated directly from pyruvate and, therefore, the succinate should remain predominantly carboxyl-labelled.

The relative amounts of activity in the methylene and carboxyl carbons of succinate, formed in the presence of tracer pyruvate-3-C¹⁴, should enable one to distinguish between the operation of the TCAC and the DCAC, if one or the other predominates.

c) A third suggestion, which might be considered in an effort to uncover the operation of the C₂ condensation in the presence of the TCAC, is the use of the procedure developed by Weed (1952).

Succinate may be decarboxylated such that the methylene carbons are obtained as acetate. If this succinate were produced in the presence of acetate-2-C¹³ and the methylene carbons, obtained as acetate, were converted to ethylene, it might prove possible to determine the relative abundance of a doubly-labelled ethylene by mass spectrometer analysis. This would be excellent evidence for the occurrence of a condensation of acetate-2-C¹³ with itself, methyl group to methyl group.

However, the detection of succinate (ethylene) actually containing two heavy carbons might be extremely difficult in view of the minute amount of activity incorporated during the usual fermentation. For less than one per cent of the succinate formed involved tracer acetate and only one-quarter of this could, on the average, be expected to involve the linking of two heavy carbons, using acetate-2-C¹³ containing 50 percent C¹³.

B. The Arsenite Inhibited Fermentation

The results observed during the fermentation of xylose by E. coli in the presence of arsenite deserve special mention.

Although the effect of arsenite on pyruvate oxidation has been amply demonstrated in animal tissues (Ochoa, 1941; Long, 1945; Peters, Sinclair and Thompson, 1946) comparatively little evidence has been presented concerning the activity of arsenite on pyruvate oxidation in microbiological systems. Wood and Werkman (1940) observed that 0.04M arsenite did not inhibit pyruvate oxidation by propionic acid bacteria. However, in the study of the pyruvate oxidation factor, alpha-lipoic acid, Gunsalus (1952) reported the striking inhibition of pyruvic acid oxidation in dried cells of Streptococcus faecalis by 20 uM arsenite. This inhibition was reversible by 1,2 dithiols (BAL) and a strong possibility exists that the inhibition of arsenite may simply involve a reaction with the coenzyme, alpha-lipoic acid, rather than with the monothiol groups of enzymes as has been previously suggested (Veegtlin, Rosenthal, and Johnson, 1931).

During the course of this investigation, the use of arsenite has reemphasized its effectiveness in the pyruvate oxidation system. Normally, the fermentation of carbohydrate, xylose included, by E. coli is of the

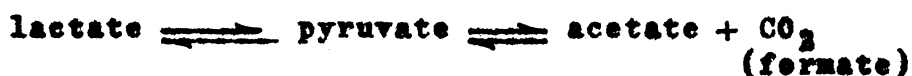
heterolactic type, i.e., while lactic acid may be a principle product of the fermentation, significant amounts of other products, such as acetic acid, ethanol, formic acid, succinic acid, etc., are also produced. However, in the presence of 0.07 M arsenite the fermentation of xylose by E. coli becomes a typical homolactic fermentation, i.e., lactic acid is the sole product of the fermentation.

While this result might have been expected following the use of this inhibitor, a similar effect has heretofore not been reported although several observations concerning the conversion of a heterolactic fermentation into a homolactic fermentation do exist for other organisms and other inhibitors.

The effect of arsenite is even more striking when one considers that the substrate, xylose, is a five-carbon sugar. A homolactic fermentation, resulting only in three-carbon fragments, thus requires the fixation of a C_1 fragment at some stage during the dissimilation of the substrate.

The evidence presented by Nutting and Carson (1932a,b) suggests that the C_1 fragment is fixed via a C_2 plus C_1 condensation. However, one or two observations made during the present investigation suggest the possibility that a C_5 plus C_1 condensation may be involved.

First, despite the theoretical yield of lactate observed in many instances, little equilibration between the hypothetical C₂ fragment and the tracer acetate occurred based on the amount of tracer activity incorporated into lactate. The presence of small amounts of activity in lactate may be ascribed to the presence of the equilibrium reaction.



Second, the present conception of the action of arsenite is that it inhibits the decarboxylation of preformed pyruvate by inactivating the dithiol group of alpha-lipoic acid, a coenzyme required for the oxidation. This is however, not to be construed as indicating that it favors or accelerates the formation of pyruvate from its oxidation products, for this has never been observed.

Third, the net uptake of CO₂ and the incorporation of C₁ (CO₂, HCOOH) activity into the carboxyl carbon of lactate observed by Nutting and Carson (1952 a,b) can be explained by a mechanism involving the fixation of CO₂ on the five carbon sugar to form a six carbon "sugar" if one assumes, contrary to the general case, that the fixed carbon becomes the carboxyl of lactate following the oxidation of the six-carbon "sugar" to two three-carbon fragments. A reaction similar to that postulated here involving the fixation of CO₂ by ribulose-5-phosphate to

form 6-phosphogluconate has recently been described by Herecker (1952).

The observation, in several instances, that more than the theoretical yield of C_3 fragment (lactate and pyruvate) was recovered requires further examination. However, if one accepts the possibility that CO_2 may be fixed on xylose to form a six-carbon "sugar", it is only one step further to suggest that a mechanism such as this may actually be a requirement for the subsequent oxidation of xylose. In this event, the production of lactate approaching two moles per mole of xylose fermented would not be unexpected.

Although the suggestion presented above concerning a mechanism involving the fixation of CO_2 on xylose may prove unwarranted, the use of the arsenite-inhibited fermentation of xylose by E. coli offers unusual opportunities for the study of mechanisms of lactate biosynthesis and CO_2 fixation.

VII. SUMMARY

Few metabolic intermediates have received the intensive study which has been accorded the C₂ fragment. As a result of this scientific preoccupation, however, the biological activity and importance of the C₂ fragment has been recognized in all phases of metabolism: anabolism, catabolism, and respiration.

The equilibrium observed between the C₂ fragment and acetic acid, isotopically-labelled, has enabled investigators to follow many of the varied activities of the former ubiquitous metabolic intermediate without actually disclosing its exact chemical nature.

In their study of lactic acid biosynthesis during the fermentation of xylose by *Escherichia coli* K-12, Nutting and Carson (1952) observed that succinic acid, produced in the presence of acetate--2-C¹⁴ was labelled almost exclusively in the methylene carbons. In addition, the C₂ fragment, necessarily produced in the aldolase-like cleavage of xylose supported by these latter authors, was labelled by equilibrium with the tracer and appeared to be involved in the net synthesis of lactic acid.

These results implied that the C₂ fragment might also be involved in the net synthesis of succinic

acid and that this reaction could be followed isotopically.

The present investigation was undertaken in an effort to determine the mechanisms responsible for the biosynthesis of succinic acid by *Escherichia coli* during the fermentation of xylose.

Three well-recognized metabolic pathways which could have accounted for the incorporation of acetate (C_2) activity into succinic acid were examined with the aid of tracer technique and enzyme inhibitors. They are:

- A. The double fixation of carbon dioxide on acetic acid
- B. The tricarboxylic acid cycle
- C. The Thunberg (C_2) condensation and the dicarboxylic acid cycle

* * *

A. The double fixation of carbon dioxide on acetic acid appeared to represent an unimportant mechanism for the synthesis of succinic acid based on evidence obtained from fermentations carried out in the presence of arsenite and labelled acetate or carbon dioxide. However, the fixation of $C^{14}O_2$ in the carboxyl carbons of succinic acid was observed and a synthetic mechanism involving carbon dioxide may play a significant role in the uninhibited fermentation.

B. The tricarboxylic acid cycle appears to be present in this organism and thus, to incorporate acetate activity into succinic acid, particularly under oxidative conditions. This conclusion is based primarily on the recovery of appropriately labelled alpha-ketoglutaric acid from fermentations carried out in the presence of labelled acetate.

C. The Thunberg (C_2) condensation and, thus, the dicarboxylic acid cycle, on the basis of evidence given here, is believed to be present in this organism and to be the major mechanism for the incorporation of acetate activity into succinic acid in the presence of arsenite. This conclusion is based primarily on the following observations:

1. The presence of 0.3 M monofluoroacetic acid, a specific inhibitor of the tricarboxylic acid cycle, fails to inhibit the cyclic mechanism in operation, despite a 75 to 85 percent inhibition of the mechanism which incorporates acetate activity.

2. The presence of 0.07 M arsenite inhibits the cyclic mechanism almost completely without inhibiting the mechanism which incorporates acetate activity.

3. Despite the almost complete inhibition of the cyclic mechanism by 0.07 M arsenite, the acetate activity incorporated into alpha-ketoglutaric acid, which

varies directly with the presence of oxygen, represents only 5 to 50 per cent of the activity incorporated into succinic acid.

* * *

In the course of this investigation it has further been observed that arsenite (0.07 M) exhibits a striking effect on the fermentation of xylose, presumably by inhibiting the decarboxylation of pyruvic acid (an integral reaction in the dicarboxylic acid cycle) which results in the production of theoretical amounts of lactic acid (1.66 moles per mole of xylose fermented). Since this result requires the fixation of carbon dioxide at some stage during the dissimilation of the substrate, the use of this system is advocated for future studies of lactate biosynthesis and carbon dioxide fixation.

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ABSTRACT

THE FERMENTATION OF XYLOSE BY ESCHERICHIA COLI: MECHANISMS OF SUCCINATE BIOSYNTHESIS

by

James Miller Adams

In their study of lactic acid biosynthesis during the fermentation of xylose by Escherichia coli K-12, Nutting and Carson (1952) observed that succinic acid, produced in the presence of acetate-2-C¹⁴ was labelled almost exclusively in the methylene carbons. In addition, the C₂ fragment, necessarily produced in the aldolase-like cleavage of xylose supported by these latter authors, was labelled by equilibrium with the tracer and appeared to be involved in the net synthesis of lactic acid.

These results implied that the C₂ fragment might also be involved in the net synthesis of succinic acid and that this reaction could be followed isotopically.

The present investigation was undertaken in an effort to determine the mechanisms responsible for the biosynthesis of succinic acid by Escherichia coli during the fermentation of xylose.

Three well-recognized metabolic pathways which could have accounted for the incorporation of acetate (C_2) activity into succinic acid were examined with the aid of tracer techniques and enzyme inhibitors. They are:

- A. The double fixation of carbon dioxide on acetic acid
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A. The double fixation of carbon dioxide on acetic acid appeared to represent an unimportant mechanism for the synthesis of succinic acid based on evidence obtained from fermentations carried out in the presence of arsenite and labelled acetate or carbon dioxide. However, the fixation of $C^{14}O_2$ in the carboxyl carbons of succinic acid was observed and a synthetic mechanism involving carbon dioxide may play a significant role in the uninhibited fermentation.

B. The tricarboxylic acid cycle appears to be present in this organism and thus, to incorporate acetate activity into succinic acid, particularly under oxidative conditions. This conclusion is based primarily on the recovery of appropriately labelled alpha-ketoglutaric acid

from fermentations carried out in the presence of labelled acetate.

C. The Thunberg (C_2) condensation and, thus, the dicarboxylic acid cycle, on the basis of evidence given here, is believed to be present in this organism and to be the major mechanism for the incorporation of acetate activity into succinic acid in the presence of arsenite. This conclusion is based primarily on the following observations:

1. The presence of 0.3 M monofluoroacetic acid, a specific inhibitor of the tricarboxylic acid cycle, fails to inhibit the cyclic mechanism in operation, despite a 75 to 85 per cent inhibition of the mechanism which incorporates acetate activity.

2. The presence of 0.07 M arsenite inhibits the cyclic mechanism almost completely without inhibiting the mechanism which incorporates acetate activity.

3. Despite the almost complete inhibition of the cyclic mechanism by 0.07 M arsenite, the acetate activity incorporated into alpha-ketoglutaric acid, which varies directly with the presence of oxygen, represents only 5 to 50 percent of the activity incorporated into succinic acid.

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In the course of this investigation it has further been observed that arsenite (0.07 M) exhibits a striking effect on the fermentation of xylose, presumably by inhibiting the decarboxylation of pyruvic acid (an integral reaction in the dicarboxylic acid cycle) which results in the production of theoretical amounts of lactic acid (1.66 moles per mole of xylose fermented). Since this result requires the fixation of carbon dioxide at some stage during the dissimilation of the substrate, the use of this system is advocated for future studies of lactate biosynthesis and carbon dioxide fixation.