LACTIC ACID FERMENTATION OF XYLOSE BY <u>ESCHERICHIA COLI</u>;

CARBON TRACER STUDIES ON THE C_2 + C_1

CONDENSATION REACTION

by

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TABLE OF CONTENTS

I. INTRODUCTION

The pentose molecule is an important constituent of biological systems. While there is little evidence to indicate that the five-carbon sugars normally occur in the unbound state, their presence as constituent parts of certain enzymes, nucleic acids and viruses is an established fact. The ubiquitous distribution of these latter substances in nature emphasizes the metabolic significance of pentoses.

The concept of the dynamic state of the living cell (Schoenheimer, 1946) suggests that the various cell constituents, including the pentose fractions, are undergoing continuous metabolic synthesis and decomposition. From this general point of view, it appears that any experimental evidence concerning the metabolic breakdown of the pentose molecule would be of value. Such information can be obtained through the use of a microbial metabolic system.

The value of the concept of comparative biochemistry and the use of microbial systems as tools in studies in the field of intermediary metabolism has been amply demonstrated (Kluyver, 1931; van Niel, 1940). It thus appears reasonable that investigations dealing with microbial enzymes functioning in the presence of a pentose as the sole substrate might contribute to the knowledge of pentose catabolism, and thus to the general field of intermediary metabolism. In addition to this broad consideration of the value of studies in microbial pentose metabolism, the structure of the pentose carbon chain suggests that experiments of this type could give information concerning the intermediary metabolism of a "C_2 fragment."

Metabolic studies during the past decade have conclusively demonstrated the importance and versatility of the C_2 fragment in enzymatic systems. For example, the role of the C_2 moiety in the phosphoroclastic

split of pyruvic acid (Lipmann, 1946), in both oxidation and synthesis of fatty acids (Breusch, 1948; Stadtman and Barker, 1949) and in the tricarboxylic acid cycle (Gurin, 1948) is well realized. More recently a C_2 intermediate has been shown to be directly involved in the formation of fumaric acid (Foster, et al, 1949) and citric acid (Foster and Carson, 1950). Furthermore, some of the early stages of photosynthetic CO_2 reduction may possibly involve a C_2 fragment (Calvin, Bassham and Benson, 1950).

The literature concerning the anaerobic breakdown of five-carbon sugars contains good evidence that one of the first reactions in the fermentation of pentoses is a carbon bond cleavage which results in the production of a C_3 and a C_2 fragment. (Fred, Peterson and Anderson, 1921; Dickens, 1938; Racker, 1948). Hence it would seem plausible that investigations dealing with a microbial system, capable of a fermentative breakdown of the pentose molecule, might give new information concerning the metabolic fate of the C_2 moiety.

II. SURVEY OF PENTOSE FERMENTATION BY MICROORGANISMS

The existent literature concerned with the fermentative action of microbial enzyme systems on carbohydrates is very extensive. In contradistinction with the voluminous number of researches devoted to the overall field of carbohydrate fermentation, the number of investigations which have dealt with the anaerobic breakdown of the pentose sugars is rather limited. For sake of clarity, the literature which deals with the anaerobic oxidation of five-carbon sugars may be loosely divided into three categories. First and most numerous are those publications concerned with the use of pentose sugars for taxonomic purposes. The second category is composed of studies devoted to an elucidation of the specific end products obtained from a given fermentation. The third category consists of those investigations directed toward an understanding of the intermediate stages and the detailed mechanisms of the anaerobic processes.

A. The use of pentose fermentations for taxonomic purposes.

The value of pentose fermentations as a tool for the differentiation of microorganisms is adequately illustrated in Bergey's Manual of Determinative Bacteriology (Breed et al, 1948). As is generally the case when carbohydrate fermentations are employed for purposes of classification, the major portion of the taxonomic studies involving pentose sugars require only acid production or acid and gas production as criteria indicative of fermentation. These criteria are of small value when one is interested in the enzymatic mechanisms involved. Koser and Saunders (1933), and Fulmer and Werkman (1930) have presented extensive qualitative data on pentose utilization by various species of bacteria and by baker's yeast while Camien, Dunn and Salle (1947) have investigated the quantitative production of acid from xylose and arabinose by several species of the genus Lactobacillus.

It is apparent that a number of different bacterial types, when examined under growing-culture conditions, possess the ability to utilize pentoses (Buchanan and Fulmer, 1930; Nicolle and Boyer, 1947; Porter, 1946; Stahly, 1936; Sternfeld and Saunders, 1937).

Inspection of the literature dealing with the fermentation of five-carbon sugars by yeasts brings out a divergence of opinion. This subject was reviewed by Abbott (1926) and it appears that the pentose sugars are not readily fermented by Saccharomyces cerevisiae in experiments of short duration (1 to 2 days), although there apparently is some anaerobic break-down in older cultures (15 to 20 days). According to Dickens (1938) pentoses are not fermented appreciably by either washed yeast cells or by Lebedew extracts but phosphorylated pentoses are fermented and, in the case of the d-ribose-5-phosphoric acid ester, the fermentation by yeast juice is about as rapid as that of glucose or hexose monophosphate. Lechner (1940) was able to demonstrate a breakdown of xylose by Torulopsis utilis and the yeast-like mold Monilia candida in the presence of inorganic nitrogen. Rose et al, (1939) deduced that the fermentation of pure pentoses by the true yeasts is doubtful but that members of the genus Torulopsis can decompose these sugars under anaerobic conditions. The findings of other investigators (Dickens, 1938; Lechner, 1940) are in support of this hypothesis. The investigations of F. F. Nord and his co-workers (1945) leave no doubt that the molds, particularly members of the genus Fusarium possess a quite vigor-ous enzymatic system for the fermentative breakdown of the pentoses. The ability of the filamentous fungi to ferment five-carbon sugars is compatible with the generally accepted fact of the metabolic versatility of these organisms.

B. The end products of pentose fermentation

Experiments designed for the elucidation of the end products resulting from the anaerobic breakdown of pentose sugars appear to have been initiated about 1912. Harden and Norris (1912) stated that Bacillus lactic aerogenes (Aerobacter aerogenes) and Bacillus cloacae are capable of producing acetymethylcarbinol and 2, 3 butylene glycol from arabinose. Arzberger, Peterson and Fred (1920) showed that Bacillus acetoethylicum ferments xylose in a peptone-phosphate medium with the production of acetic, formic, and lactic acids, ethanol, acetone, and carbon dioxide. These investigators also obtained the fermentation of pentose sugars by several other bacterial species. Bacillus vulgatus and Acetobacter xylinum fermented xylose with the production of acetone, ethanol, carbon dioxide and a small amount of organic acids. Fred, Peterson, and Davenport (1919) reported an interesting fermentation of xylose in which acetic acid was the main product obtained. Speakman (1923), working with Bacillus granulobacter pectinovorum in the presence of arabinose or xylose, obtained a fermentation yielding acetone and butyl alcohol in addition to acetic and butyric acids.

Studies carried out in the laboratory of C. H. Werkman (1929) showed that members of the genera Propionibacterium, Aerobacter and Escherichia, are capable of anaerobically dissimilating xylose. Propionibacterium pentosaceum yielded propionic acid, acetic acid and carbon dioxide. In the case of Aerobacter indologenes (Aerobacter cloacae) and Escherichia coli, the products of the fermentation of glucose and xylose were qualitatively the same.

As indicated previously, it appears that whole cells of Saccharomyces cerevisiae do not possess the enzymatic constitution necessary for

the fermentation of pentoses. However, Abbott (1926), using incubation periods of relatively long duration (15 to 20 days) and a pH somewhat higher than normally used for yeast studies (pH 5.5 to 7.0) obtained a positive reaction. The products were mainly a nonvolatile acid or acids, plus traces of carbon dioxide and ethanol. He also reported variable evidence for the production of a highly reducing, nonvolatile, nonacidic substance which he suggested might have been glyceric or glycollic aldehyde. From the literature pertaining to the products obtained from the anaerobic decomposition of pentose sugars by microorganisms it appears that the products are qualitatively the same as those obtained from the fermentation of glucose or other hexoses. Yeasts must be considered as exceptions to the rule as they apparently ferment free pentoses only slowly, if at all, although yeast juice will decompose the ribose-5-phosphate ester (Dickens, 1938).

Nord and co-workers (1945), employing _Fusarium_ species as the test organism and xylose as the substrate were able to show the accumulation of pyruvic acid although efforts to trap the expected two-carbon fragment were unsuccessful. Nevertheless, it was stated that glycolaldehyde would serve as a weak carbon source.

C. The intermediary metabolism of pentose fermentation

Metabolic studies dealing directly with the specific intermediary steps of pentose metabolism are not numerous. It is generally assumed, but not proven, that the initial cleavage of the pentose carbon chain is brought about by an aldolase type enzyme which yields a C_2 and a C_3 fragment per molecule of pentose split. Such a hypothesis appears reasonable in view of the aldolase cleavage occurring during the glycolytic breakdown of hexose sugars and indeed, this concept of a pentose aldolase is supported by several pieces of indirect evidence.

Investigations (Fred, Peterson and Anderson, 1921) conducted at the University of Wisconsin produced convincing evidence for the production of a C_2 and a C_3 piece from pentose. These investigators succeeded in isolating from silage a lactic acid organism (Lactobacillus pentosus) which was capable of fermenting arabinose or xylose to acetic and lactic acids. The two end products were equivalent to about 90 per cent of the pentose fermented and 98 per cent of the products isolated. The ratio of the acetic and lactic acid was approximately 1:1.

Recently Lampen and co-workers (1950) have extended and substantiated the original work of Fred et al by observing the action of the same organism on d-(+) - xylose-1-C^{14}. The reaction appears to be:

$$
\begin{array}{l}
HC^{14}O \\
|\ \\
HCOH \\
|\ \\
HOCH \\
|\ \\
HCOH \\
|\ \\
HCOH \\
H
\end{array}
\quad \xrightarrow{\text{L. pentosus}} \quad
\begin{array}{l}
C^{14}H_3 \\
|\ \\
COOH \\
+ \\
COOH \\
|\ \\
CHOH \\
|\ \\
CH_3
\end{array}
\quad
\begin{array}{l}
\text{acetic-a-}C^{14} \\
\text{acid} \\
\\
\text{lactic} \\
\text{acid}
\end{array}
$$

d-(+)-xylose-1-C^{14}
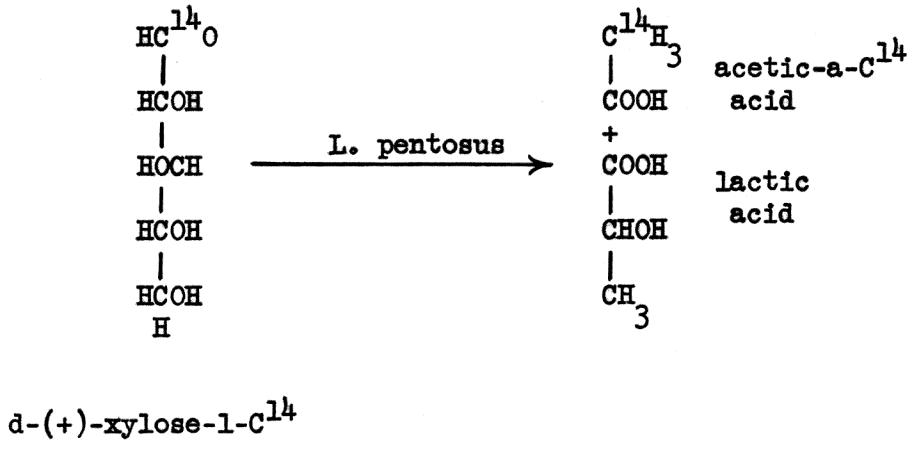
Dickens (1938) has investigated the metabolic decomposition of d-ribose-5-phosphate by a yeast juice, and has found that the fermentation of this compound is about as rapid as the fermentation of glucose. The products of the fermentation were carbon dioxide, ethanol and inorganic phosphate in a molar ratio of 1:1:1 per mole of d-ribose-5-phosphate decomposed. In addition, he obtained an unknown HIO_4-reducing compound and suggested that this may have been glycolaldehyde (C_2) though it was not further identified.

Nord and co-workers (1945) were successful in trapping pyruvic acid (C_3) from the nutrient medium of a Fusarium pentose fermentation, though they were not able to isolate glycolaldehyde which these investigators assumed would be formed by a C_3-C_2 split.

The bacterial degradation of ribose-phosphate has been investigated by Krampitz and Werkman (1947). Micrococcus pyrogenes var. aureus metabolized ribose phosphate from nucleic acid and nucleotides according to the equation:

$$C_5H_9O_5PO_3H_2 + 3O_2 \longrightarrow 3CO_2 + CH_3COOH + H_3PO_4 + 2H_2O$$
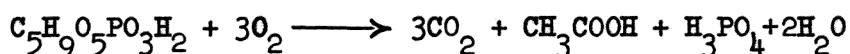
d-ribose-5-phosphate   acetic
                                        acid

Krampitz and Werkman also postulated the occurrence of an aldolase split and further reasoned that the resulting triose (C_3) is completely oxidized to CO_2 and H_2O while the suspected C_2 appears as acetic acid. Bacterial production of a C_3 piece from ribose-5-phosphate has been reported by Racker (1938). In this case the C_3 moiety was identified as triosephosphate and the same "bacterial extract" also catalyzed the oxidation of reduced diphosphopyridine nucleotide in the presence of glycolaldehyde, indicating that the C_2 moiety is capable of being further metabolized.

Reynolds and Werkman (1937) made comparisons, with growing cultures, between the fermentations of glucose and xylose by both Aerobacter indologenes and Escherichia coli. In discussing the anaerobic breakdown of xylose by Escherichia coli these investigators made special note of the fact that approximately one mole of lactic acid is formed for each mole of xylose fermented. This was taken as good evidence that the initial cleavage of the pentose molecule produced a C_3 and a C_2 fragment; the C_3 fraction being converted into lactic acid and the C_2 fraction giving rise to end products other than lactic acid.

Barker (1944) has investigated the fermentation of xylose by Clostridium thermoaceticum as a part of the general phenomenon of carbon dioxide utilization. Similar to glucose, the bulk of the xylose was converted to acetic acid by this organism. It seems plausible, as suggested by Barker, that a C_3 fragment arises from the initial split of the pentose; this C_3 moiety then gives rise to C_2 + CO_2 and the CO_2 is ultimately reduced to acetate. Barker and Kamen (1945) later showed, by isotopic carbon tracer techniques that the reduction of CO_2 to acetic acid does indeed take place during fermentation of glucose.

The most recent papers dealing with the bacterial anaerobic decomposition of pentoses are those of Stanier and Adams (1944), which are concerned with the fermentations of Aerobacillus polymyxa and Aeromonas hydrophila using xylose as substrate. As Stanier and Adams have pointed out, it is difficult to interpret their data on the basis of a simple C_3-C_2 cleavage, though such a split followed by a resynthesis of the C_2 piece to a higher moiety would be in line with their observations.

In summarizing the present status of the general problems of pentose fermentation by microorganisms the following points may be reiterated:

1. Quite a large number of species of microorganisms are capable of inducing some type of anaerobic decomposition of the pentose sugars. 2. The data concerning the fermentation of pentoses by yeasts are conclusive though it appears that _Torulopsis_ species are able to metabolize these carbohydrates while _Saccharomyces_ species are not. 3. Fermentation analyses of the anaerobic dissimilation of five-carbon sugars show few qualitative differences from the results obtained with hexose sugars. The main differences between the fermentative breakdown of the two types of carbohydrates are quantitative in nature and often these differences are not large. 4. An initial carbon bond cleavage to a C_3 moiety and a C_2 moiety is indicated; (though the subsequent metabolic fate of the two fragments is largely a matter of comparison with what is known concerning hexose intermediary metabolism).

III. MATERIALS AND METHODS

A. Cultivation of the organism

The organism selected for these investigations was <u>Escherichia</u> <u>coli</u> K-12 obtained from the laboratory of C. E. Clifton. Preliminary observations indicated that this organism would (1) give an excellent yield of cells from a mineral salts medium with a single carbohydrate as a source of carbon and energy, and (2) yield a luxuriant crop of "resting cells" with pentoses as the carbon source (aerobic). A most important attribute of this strain is its ability to ferment pentoses; such is not always true for organisms which can grow on pentoses. The pentose used throughout these investigations was Pfanstiehl d-(+)-xylose (designated l-xylose by Fischer) and will be referred to as xylose.

The organism was always grown at 37° C. for 20-24 hours. Stock cultures were maintained on nutrient agar slants transferred at intervals of 3 to 4 months and stored at 9° C. All other cultures were grown in a liquid medium (Anderson, 1948) of the following composition: NH_4Cl 0.05%, NH_4NO_3 0.2%, Na_2HPO_4 (anhydrous) 0.6%, KH_2PO_4 0.3%, $MgSO_4 \cdot 7H_2O$ 0.02%, $CaCl_2$ 0.005 %, carbohydrate 0.5%, distilled water. The mineral salts portion of the liquid medium was sterilized by autoclaving at 20 lbs for 20 minutes, whereas the carbohydrate portion was sterilized by filtration as a 40 per cent solution and added asceptically to the mineral salts solution.

Liquid cultures, to be harvested for the preparation of the non-proliferating suspensions, were inoculated with a 10 per cent inoculum of liquid medium which had previously been seeded from a stock agar slant.

These liquid cultures were continuously aerated by means of a sintered glass sparger immersed in the liquid.

B. Preparation of nonproliferating cell suspensions.

The cells were removed from the growth medium by centrifugation

in either a refrigerated International centrifuge or in a refrigerated Sharples centrifuge. Volumes exceeding 2 liters only were manipulated through the Sharples apparatus. The cells were washed twice in tap water and the final suspension made up in the desired buffer.

C. Manometric experiments.

With the exception of one experiment, which was conducted in 250 ml suction flasks, the fermentations were carried out in Warburg respirometers. Fermentations in which the substrate concentration was 0.005 - 0.01 millimoles were run in Warburg vessels (20 ml) with two side arms. Small scale experiments were generally performed with 0.2 ml of carbohydrate solution as substrate in one side arm, 0.2 ml of 10 N sulfuric acid in the remaining side arm and 3.0 ml of nonproliferating cell suspensions in the main compartment. When 0.1 - 1.0 mM of carbohydrate were fermented, the fermentations were carried out in 160 ml Warburg flasks. In these large-scale fermentations the following volumes of reactants were employed: 1.0 ml substrate, 1.0 ml 10 N H_2SO_4 and 50 ml of cell suspension.

A suitable concentration of $NaHCO_3$ was generally employed as the buffer in order that the production of organic acids by the cells could be followed manometrically. The gas atmosphere was either 100 per cent CO_2 or 5 per cent CO_2 in N_2, depending on the pH desired for a given fermentation (Umbreit et al, 1948). The buffer was equilibrated with the gas for a minimum of 30 minutes, the cells were then suspended in the buffer and finally the gas equilibration was continued for an additional 10 minutes. The fermentation flasks were filled with the reactants and flushed with gas for at least 30 minutes. The reaction flask and manometer were then closed to the outside air and brought to temperature equilibrium in the water bath. The fermentation reaction was then followed

by the usual Warburg technique (Umbreit et al, 1948). At the conclusion
of the manometric experiment, after the cells had been killed by addition
of mineral acid from the side arm, they were separated from the fermenta-
tion liquid by centrifugation. The cells were washed once with distilled
water. The wash water was combined with the original supernatant and
used for the analyses.

   D. Isotope carbon tracer experiments.

   For the tracer experiments, the radiocarbon tracers ($C^{14}H_3$-COONa,
$C^{14}H_3$-CH_2OH or HC^{14}OONa) were added in 1.0 ml volumes to the side arm con-
taining the substrate. The solutions of C^{14}-labeled compounds were prepared
in such a manner that the specific activity was high, and hence contributed
negligible amounts of carbonaceous material to the fermentation.

   C^{13} (as $C^{13}O_2$) was employed to trace the CO_2 of the gas atmosphere.
$Na_2C^{13}O_3$ was prepared from $BaC^{13}O_3$ containing 51 atom per cent C^{13}. One ml
of the $Na_2C^{13}O_3$ solution, containing about 1 mM, was placed in one side
arm near the end of the final 20 minute gas sweep in order that there would
be only a slight exchange between CO_2 in the sweep gas and the $Na_2C^{13}O_3$.
A partial vacuum was then applied to the Warburg flask and $C^{13}O_2$ was re-
leased from $Na_2C^{13}O_3$ by the addition of 10 N sulfuric acid through the vent
tube of the side arm. Sulfuric acid was always added in excess so that
there would be sufficient free acid remaining in the side arm to release
all CO_2 from the buffer at the end of the fermentation. It was assumed
that good mixing between CO_2 and $C^{13}O_2$ took place while the flasks were
being shaken for temperature equilibration. After temperature equilibrium
was reached, but prior to the transfer of the substrate from the side arm
to the main compartment, a gas sample was removed through the manometer
stopcock by means of an evacuated bulb. The CO_2 contained in the gas sample
was absorbed in 0.5 N NaOH and precipitated as $BaCO_3$ for the carbon isotope

determinations. Also a final gas sample was collected and prepared as $BaCO_3$ after the final sulfuric acid addition. This gas sample was collected by the "vent tube sweep method" used by Foster and Carson (1950).

E. Methods for the separation of fermentation products.

The studies of other investigators (Reynolds and Werkman, 1937; Tasman, 1935; Tikka, 1935) have indicated that the main end products of hexose or pentose fermentation by Escherichia coli were CO_2, H_2, ethanol and formic, acetic, lactic, and succinic acids.

The investigations of Stephenson (1937) and Stokes (1949) have shown that cell suspensions of Escherichia coli did not liberate gas from glucose under anaerobic conditions when the cells were cultivated in the presence of an abundant supply of oxygen. The same phenomenon occurred when xylose was used as substrate in the present investigations. It was, therefore, not necessary to analyze for carbon dioxide and hydrogen. A fractionation procedure for the isolation of a neutral volatile fraction (ethanol), and volatile and nonvolatile acid fractions was employed.

After removing samples for residual pentose and lactic acid determinations, the supernatant was distilled at pH ~9. This distillation served to isolate the ethanol in the volatile fraction, and also reduced the volume of the nonvolatile residue. The excessive foaming so often encountered in alkaline distillations was minimized by uniform heating of the distilling flasks in a silicone bath and also by heating the distilling head with an infra-red lamp. The alkaline nonvolatile portion was then acidified to pH ~1 and steam distilled until a minimum of 250 ml of distillate had been collected. The total volatile acids (formic and acetic) were determined by titration. The residue from the steam distillation was then ether extracted in a Kutscher-Steudel apparatus for at least 20 hours. About 10 ml of water were added to the ether extract, the ether was removed

by evaporation, and the total nonvolatile acids (lactic and succinic) were
titrated.

For nontracer fermentation experiments, in which it was necessary
to know only the amounts of individual end products in the fermentation
supernatant, the complete separation of all products was not required
and the separation of the supernatant into alkaline nonvolatile, acid
volatile, and acid nonvolatile, ether extractable fractions was sufficient.
On the other hand, in some of the isotopic carbon tracer experiments it
was necessary to physically separate lactic and succinic acid in order that
these compounds could subsequently be degraded for isotopic analyses.

These organic acids were isolated by a modification of the parti-
tion chromatography procedure of Peterson and Johnson (1948). The modifi-
cations were (1) the use of ethyl ether as the developing solvent, and
(a) the adsorption of the aqueous sample on dry Celite before transfer to
the chromatogram column. Both modifications were suggested and developed
by E. F. Phares of this laboratory.

The chromatographic separation was carried out in a 1 cm (i.d.)
Pyrex glass tube, with a stopcock sealed to the lower end, and filled with
Celite (Celite 545, a diatomaceous filter aid marketed by Johns-Manville).
Prior to the packing of the column the Celite was moistened with a non-
volatile phase of H_2SO_4 (0.9 ml 0.5 N H_2SO_4 per gram Celite). Carefully
washed ether in equilibrium with 0.5 N H_2SO_4 was used as the developing
solvent. This phase was also used to slurry the Celite during the packing
of the column. The sample to be added to the top of the column was evapor-
ated to about 0.5 ml and thoroughly mixed with sufficient dry Celite to
adsorb any free liquid. The sample mixture was then transferred to the
top of the previously prepared column and the original sample container

was "rinsed" once with an additional small quantity of Celite.

The chromatogram was developed by allowing the mobile phase to descend through the column at the rate of approximately 0.5 ml per minute and the solvent was collected in 2 to 5 ml aliquots. After the addition of a small amount of water to an aliquot, it was titrated and a continuous record of acidity per aliquot was made. The organic acids eluted from such a chromatogram were identified by their "peak effluent volume" in the usual manner. Lactic acid on a column of the above type has a "peak effluent volume" of 120 ml while succinic acid falls at 80 ml. Nonreproducible losses of ether by air evaporation may cause small variation in the "peak effluent volume" from one chromatogram column to another, nevertheless this does not detract from the value of the method when one has previous information concerning the nature of the samples. This H_2SO_4-ether partition chromatogram has a low solvent acidity blank of 0.002 milliquivalent of acid per 5.0 ml developing solvent. Such a chromatogram will adequately separate mixtures containing 0.3 millequivalents of lactic and succinic acids. A similar chromatogram with a mixture of 10 per cent N-butanol in chloroform as the mobile phase will separate mixtures of formic and acetic acids.

After the acids were separated, the aliquots were pooled and the organic solvent removed by evaporation. The pooled sample was then taken to dryness twice under partial vacuum, and finally maintained under a vacuum of about 50 cm of Hg at 75° C. for 30 minutes in order to remove the last traces of organic solvent. Organic solvent carbon lowers the final specific activity measurements (see carbon isotope assay methods). The samples were then redissolved and taken for isotope analyses.

F. Chemical methods for quantitative estimation of end products.

Ethanol. Ethanol was determined by the dichromate oxidation
method of Northrop et al (1919). An aliquot of the volatile fraction from
the alkaline distillation was oxidized in a closed system with acid dichro-
mate, and the residual dichromate determined by iodometric titration. Data
concerning the determination of known ethanol samples are shown in table 1.

TABLE 1

The determination of ethanol by dichromate oxidation

mg ethanol added	mg ethanol found	Per cent ethanol recovered
2.5	2.4	96
2.5	2.4	96
5.0	5.1	102
5.0	5.2	104
5.0	4.9	98
5.0	4.8	96

Xylose. Residual xylose was determined by the orcinol colori-
metric method (Umbreit et al, 1949) employing a Kromatrol photometer with
650 mμ filter for light transmission measurements. Pentoses, when heated
in the presence of $FeCl_3$ and strong HCl, form furfural which, in turn,
complexes with orcinol to give a characteristic blue-green color. All
xylose determinations were run on aliquots of the fermentation superna-
tants. Performance data for the measurement of xylose by the orcinol
method are presented in table 2.

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

TABLE 2

The determination of xylose by orcinol method

μg xylose added	μg xylose found	Per cent xylose recovered
7.5	7.0	93
7.5	7.2	96
15.0	15.7	105
15.0	15.4	103
30.0	30.8	103
30.0	29.8	99
45.0	44.0	98
45.0	44.2	98

fermentation supernatant, or by the alkaline permanganate oxidation of Ulzer and Seidel modified for micro amounts (Foster and Carson, personal communication) on an aliquot of the nonvolatile acid fraction. The Barker and Summerson method depends upon the oxidation of lactate to acetaldehyde by concentrated sulfuric acid. Acetaldehyde in the presence of p-hydroxy-diphenyl forms a blue-violet complex which is suitable for colorimetric measurements at 565 mμ. This method will readily determine lactic acid present in microgram quantities while the Ulzer and Seidel method can be used for the analysis of 0.2 to 0.5 mg amounts.

The Ulzer and Seidel method is based upon the oxidation of lactate to oxalate with excess alkaline permanganate. The residual permanganate is then destroyed with hydrogen peroxide, and after the decomposition of any excess peroxide by boiling, the oxalate is determined by titration with acid permanganate.

The reliability of these two methods of lactic acid assay were tested by analysing known solutions of pure zinc lactate. In addition, aliquots of the known lactate solutions were ether extracted by the procedure used for unknown examples. A small volume of water was added to the ether extract and the ether evaporated off. Lactate in the aqueous residues were then determined and the results calculated back to the original known lactate solutions. Data concerning the recovery of lactic acid is presented in table 3.

TABLE 3

The determination of zinc lactate with and without ether extraction

Analytical method	Known A 3.38 mg lactate/ml		Known B 5.95 mg lactate/ml	
	mg lactate determined	Per cent recovery	mg lactate determined	Per cent recovery
Barker and Summerson	3.48	103	6.04	102
	3.40	101	6.04	102
	3.34	99	6.05	102
Barker and Summerson after ether extraction	3.42	101	5.90	99
	3.34	99	6.04	102
	3.34	99	5.91	99
Ulzer and Seidel	3.36	99	5.84	98
Ulzer and Seidel after exther extraction	3.43	102	6.05	102

Formic acid. Formic acid was determined in the steam-volatile fraction by the mercuric chloride oxidation method of Fincke (1913). Formic acid reduces mercuric chloride to the insoluble mercurous chloride. The precipitate was filtered, dried and weighed. Recovery data for Fincke's

method shown in table 4.

TABLE 4

The determination of known amounts of formic acid

mg formic acid added	mg formic acid found	Per cent recovery
6.1	6.0	98
6.1	5.9	97
12.2	11.9	98
12.2	11.9	98

Acetic acid. Acetic acid was determined by the difference between the total volatile acid and the formic acid. In some instances formic and acetic acids were determined on the volatile acid fraction by means of a partition chromatogram (see methods for separation of fermentation products).

Succinic acid. Succinic acid was determined manometrically by use of succinoxidase (Umbreit et al, 1949). Succinic acid is oxidized by means of a succinoxidase preparation and the O_2 consumption measured according to the following reaction:

$$COOH-CH_2-CH_2-COOH + 1/2 \ O_2 \longrightarrow COOH-CH=CH-COOH + H_2O$$
$$\text{succinic acid} \hspace{4cm} \text{fumaric acid}$$
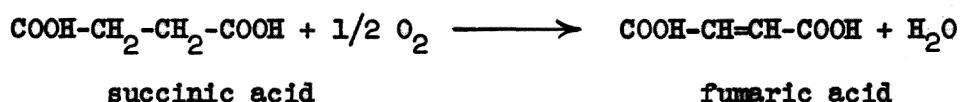
The succinic acid determinations were performed on an aliquot of the ether extractable, steam nonvolatile fraction. Typical data concerning the determination of known amounts of succinic acid are presented in table 5.

G. Combustion methods.

All compounds, except methylamine, which were examined for C^{13} and/or C^{14}, were converted to $BaCO_3$ by a small scale modification of

TABLE 5

Determination of known quantities of succinic acid by succinoxidase

mg succinic acid added	Theoretical O_2 consumption	Observed O_2 consumption	Per cent recovery
0.3	28.5	26.0	91
0.3	28.5	27.0	95
0.5	47.5	45.0	95
0.5	47.5	43.0	91
1.0	95.0	93.0	98
1.0	95.0	90.0	95

the persulfate oxidation procedure of Osburn and Werkman (1932). The modified method was developed and rigorously tested in this laboratory by D. S. Anthony who also assisted the writer in becoming familiar with the method.

The all-glass apparatus consisted essentially of (1) a combustion flask containing the oxidation mixture, (2) a water-cooled condenser to prevent the loss of volatile compounds and (3) a U-tube alkali trap for collecting the CO_2 produced by the combustion. Samples containing about 0.2 millimoles carbon were oxidized to CO_2 in the combustion flask. The CO_2 passed through the condenser and was then absorbed in 0.5 N carbonate-free NaOH contained in the U-tube trap. The trap contents were then rinsed out and the CO_2 precipitated as $BaCO_3$ by the addition of 0.2 M $BaCl_2$. The resulting $BaCO_3$ was digested for 5 minutes at about 90° C., cooled and filtered through a micro porous porcelain filter disk (marketed by Harshaw Scientific Company). The precipitate was then dried, weighed and taken for

radioactivity measurements (see isotope assay methods).

Known amounts of typical compounds encountered in fermentation chemistry were combusted by this method and the results of such determinations are presented in table 6.

TABLE 6

Determination of known quantities of carbon by persulfate oxidation

Substance oxidized	mM	mM C	mg $BaCO_3$ calculated	mg $BaCO_3$ found	Per cent recovery
xylose	0.05	0.25	49.4	49.7	101
zinc lactate	0.07	0.21	41.5	43.0	104
sodium oxalate	0.10	0.20	39.5	41.2	104
sodium oxalate	0.10	0.20	39.5	40.6	103
succinic acid	0.05	0.20	39.5	39.1	99
succinic acid	0.05	0.20	39.5	37.8	96
acetic acid	0.10	0.20	39.5	40.6	103
acetic acid	0.10	0.20	39.5	37.6	95

H. Degradation methods.

During the course of these experiments it was necessary to degrade succinic acid and lactic acid in such a way that the individual carbon atoms could be separated one from the other. The writer performed the initial lactate degradations under the close supervision of E. F. Phares who has developed and tested (Phares) the methods employed.

The lactate was first converted to acetate and CO_2 in 100 per cent yield with acid permanganate. The CO_2 from this initial oxidation step represented the lactate carboxyl group. The acetate from lactate was recovered

by steam distillation and converted to CO_2 and methylamine by the action of NaN_3 in the presence of strong sulfuric acid. The CO_2 from this step was derived from the α-carbon of the original lactate while the methylamine represented the lactate β-carbon. The CO_2 yields from this reaction were from 60-90 per cent. The methylamine was oxidized to CO_2 with alkaline permanganate in yield of from 50-80 per cent. Phares has shown that there is no crossover between carbon atoms in the acetate molecule by this method.

The CO_2 from the individual lactate carbons were precipitated as $BaCO_3$, filtered, weighed and taken for carbon isotope determinations. The degradation of succinic acid was accomplished by converting succinate to lactate and subsequently degrading the lactic acid by the methods presented above. The succinic acid was converted to lactic acid by the procedure developed by Foster and Carson (1950). Though this procedure was originally developed for fumaric acid $\longrightarrow$ lactate it works equally well for succinate $\longrightarrow$ lactate (Carson, 1950).

The reaction: succinate $\longrightarrow$ fumarate $\longrightarrow$ malate, was carried out with a beef heart succinoxidase preparation. The enzyme solids were then removed by centrifugation and the malic acid recovered from the super-natant by ether extraction. Malic acid was decarboxylated by a suspension of _Lactobacillus_ _casei_ yielding lactic acid and CO_2. The CO_2 was collected and precipitated as $BaCO_3$ for radioactivity measurements. The bacterial cells were removed by centrifugation and lactic acid recovered by ether extraction. The lactic acid was subsequently passed through an ether-Celite partition column for final purification. The over-all yield of lactic acid from succinic acid was about 50 per cent.

The degradation methods employed in these investigations are summarized in the following equations:

$$a\ COOH \qquad a\ COOH \qquad\qquad a\ COOH \qquad\qquad a\ CO_2$$

a COOH — succinoxidase 100% → a COOH — fumarase* ⇌ a COOH — L. casei 100% → a CO₂

a COOH		a COOH		a COOH		a CO₂ +
b CH₂	succinoxidase	b CH		b CH₂		b CH₃
c CH₂	100% →	c CH	fumarase* ⇌	c CHOH	L. casei 100% →	c CHOH
d COOH		d COOH		d COOH		d COOH

succinic acid   fumaric acid   malic acid   lactic acid

b CH₃	acid KMnO₄	b CH₃	acetic acid
c CHOH	100% →	c COOH +	
d COOH		d CO₂	

lactic acid

| b CH₃ | NaN₃-H₂SO₄** | b CH₃NH₂ methylamine + |
| c COOH | → | c CO₂ |

acetic acid

| b CH₃NH₂ | alkaline KMnO₄ 100% → | b CO₂ |

methylamine

*Molar ratio fumarate/malate is 1/4

**69-90 per cent yield of CO_2
  50-80 per cent yield of CH_3NH_2

I. Carbon isotope assay methods.

Carbon[14]. All radioactivity measurements were made directly on
$BaCO_3$ contained on the porcelain filter disks. Counts per second were
measured on a thin mica window Geiger-Müller counter. The weight of $BaCO_3$
was as close as possible to "infinite thickness" and counts were corrected
for self-adsorption when necessary (Calvin et al, 1949). All samples
were counted to a "95/100 error" of ± 3.3 per cent (minimum of 1920
individual events) (Calvin et al, 1949). Any count whose magnitude was
less than twice the background count was considered unreliable. Representa-
tive data concerning the precision of specific activity measurements
(counts/second/mg $BaCO_3$) on known labeled compounds are shown in table 7.

TABLE 7

Duplicate specific activity measurements on known labeled compounds.

Labeled compound combusted	Weight $BaCO_3$	Counts/second	Specific activity (c/s/mg)	Per cent deviation from mean value
formate[14]	41.8	19.1	0.457	-5.2
formate[14]	41.3	20.9	0.506	+5.2
acetate[14]	39.1	55.6	1.42	-3.4
acetate[14]	40.3	61.4	1.52	+3.4
lactate[14]	42.4	19.7	0.464	+0.6
lactate[14]	42.2	19.4	0.459	-0.6

Carbon[13] . After the radioactivity measurements were obtained from
the $BaCO_3$ sample, it was decomposed by the addition of H_3PO_4 and the dried

CO_2 was trapped by freezing in a break-seal tube. The CO_2 samples were then analysed for C^{13} in a mass spectrometer. The C^{13} analyses were performed by R. F. Hibbs, J. L. Heatherly and C. R. Fultz in the Assay Laboratory of the Y-12 plant of Carbide and Carbon Chemicals Division, Union Carbide and Carbon Company at Oak Ridge, Tennessee. All values are the average of 6 to 12 determinations, per gas sample, plus or minus the limit of error of the mean at the 95/100 confidence level.

IV. EXPERIMENTAL

A. Nongaseous fermentation by Escherichia coli K-12.

It appeared that worthwhile information concerning the fermentation of pentoses could be obtained from investigations employing a bacterial system as the "working model". Preliminary investigations indicated that Escherichia coli K-12 would be a suitable organism for this type of study. Escherchia coli K-12 could be adapted to grow well on xylose as the sole carbon source and a nonproliferating cell suspension was capable of fermenting xylose. An initial suggestion by I. C. Gunsalus (1949) and the work of Stokes (1949), on the fermentation of glucose by Escherichia coli, made it appear feasible that one could obtain a fermentation of xylose in which there were no gaseous end products. A fermentation of this type was desirable because it would eliminate the need for specific gas analyses and it would provide a metabolic reaction suitable for studying the intermediary steps of pentose decomposition.

Experiment 1. In order to test the possibility that a nongaseous fermentation of glucose and xylose by Escherichia coli K-12 could be achieved in this laboratory, a series of small scale fermentations were run in small Warburg flasks and followed manometrically. The fermentations were conducted in 2.0 ml M/100 $NaHCO_3$ buffer plus cells under an atmosphere of 5 per cent CO_2, 95 per cent N_2 with 0.005 mM substrate present. The endogenous fermentation rate was negligible. The results of these experiments are presented in table 8.

The close agreement between the initial HCO_3^- carbon dioxide (column 2) and the sum of the residual HCO_3^- carbon dioxide and the carbon dioxide released during the fermentation (column 5) showed that little, if any, gaseous CO_2 was produced by the cells. If "metabolic CO_2" or other

TABLE 8

Experiment 1

Carbon dioxide balances for the fermentation

of glucose and xylose by E. coli at pH 7.1

Substrate	Initial $HCO_3^- - CO_2$ mm^3	CO_2 liberated mm^3	Residual $HCO_3^- - CO_2$ mm^3	Total CO_2 mm^3
none	384	18	374	392
none	388	19	360	379
glucose	384	261	108	369
glucose	384	262	105	367
glucose	388	287	106	393
xylose	388	255	121	376
xylose	388	252	127	379

gas had been produced, the values in column 5 would have exceeded the values of column 2. The results confirmed the findings of Stokes (1949) regarding the nongaseous fermentation of glucose by E. coli and further showed that E. coli cells, grown in aerated liquid medium containing xylose as the carbon source, fermented xylose without the accumulation of gaseous end products. This finding led to the conclusion that one should be able to make a fermentation balance on the fermentation of xylose by E. coli without specific gas analyses. This was found to be the case in the large scale fermentations which were carried out later (see fermentation balances).

B. Xylose fermentations

1. Non tracer experiments

a. Influence of pH on lactate production

The next series of experiments were designed to ascertain the

experimental conditions necessary to produce lactic acid from xylose in a molar ratio of 1:1. Reynolds and Werkman (1937) studied the fermentation of xylose by $\underline{E}$. $\underline{coli}$ in growing cultures and found that lactic acid was formed from xylose in this ratio. In the interpretation of their findings, these investigators quite logically suggested that the lactic acid accounted for the C_3 portion of the xylose molecule and that end products other than lactic acid originated from the C_2 fragment. A fermentation of this type would be a valuable tool in studies devoted to the intermediary metabolism of the C_2 moiety. Since it is known that pH has a profound influence on microbial fermentations (Gunsalus and Niven, 1942; Stokes, 1949), it appeared reasonable that a xylose fermentation by $\underline{E}$. $\underline{coli}$ at the proper pH level might give a lactate/xylose ratio of 1:1.

Experiment 2. In order to test this hypothesis, a series of small scale xylose fermentations in either bicarbonate or phosphate buffer and at various pH levels were carried out. The fermentation supernatants were analysed colorimetrically for residual xylose and for lactic acid. The residual xylose in all cases was less than 2 per cent of the added substrate and was considered negligible. The moles of lactic acid produced per mole of xylose fermented in this experiment are listed in table 9.

These findings were comparable to those of Stokes (1949) in that larger quantities of lactic acid were produced at low pH levels. However, the production of considerably more than one mole of lactic acid per mole of xylose fermented at pH ~ 5.5 was a completely unexpected and highly important finding. Since at pH ~ 5.5 there were more moles of lactate formed per mole of xylose fermented than could be accounted for by the C_3 piece of the C_5 molecule, this phenomenon was interpreted as good evidence that some of the lactic acid originated from the C_2 fragment. The import-

TABLE 9

Experiment 2

Effects of pH on the quantities of lactic acid

formed in the fermentation of xylose by E.coli

Buffer Used	pH	Moles lactate formed per mole xylose fermented
M/15 phosphate	8.0	0.41
M/15 phosphate	7.5	0.41
M/100 bicarbonate	7.1	0.81
M/15 phosphate	7.1	0.43
M/15 phosphate	6.6	0.89
M/100 bicarbonate	6.5	0.76
M/100 bicarbonate	6.5	0.60
M/15 phosphate	6.5	0.61
M/15 phosphate	5.8	1.02
M/100 bicarbonate	5.7	1.20
M/15 phosphate	5.5	1.43

ance of these findings made it mandatory to perform more experiments similar to those reported in table 8. These confirmatory experiments are described in the next section.

b. Influence of pH on CO_2 fixation

Experiment 3. These fermentations were carried out in the presence of bicarbonate buffer so that manometric data, as well as the xylose and lactic acid determinations, could be secured. The results of this experiment are presented in table 10.

The results of experiment 3 demonstrated that more than 1 mole

TABLE 10

Experiment 3

Effect of pH on the quantities of lactic acid produced

and CO_2 fixed in the fermentation of 0.01mM xylose by

E. coli

pH	Initial HCO_3^--CO_2	CO_2 liberated from HCO_3^- during fermentation	Residual HCO_3^--CO_2	Net CO_2 Uptake	Moles lactate formed per xylose fermented
	mm^3	mm^3	mm^3	mm^3	
7.7	851	478	349	24	0.14
7.7	851	468	341	42	0.13
6.5	744	408	273	63	0.61
6.5	744	404	262	78	0.72
5.7	708	327	288	107	1.59
5.7	708	340	314	68	1.60

of lactic acid is produced per mole of xylose at low pH. Equally important

is the striking observation that a net CO_2 uptake occurs, and increases

at the same time lactate production increases, i.e., as the pH is lowered.

It is most important to note that the quantity of the CO_2 fixed appeared

to parallel the increased production of lactic acid. This was taken as

presumptive evidence that CO_2 fixation was related, in some unknown manner,

to quantity of lactate which was produced in excess of 1 mole lactate per

mole of xylose.

c. Fermentation balances

At this point, it was considered desirable to investigate the

quantities of all end products of the fermentation of xylose by E. coli at different pH levels.

Experiment 4. A large scale (1 mM) xylose fermentation was carried out at pH 7.9. The nonproliferating cell suspension was prepared from cells harvested from 2 liters of growth medium and the fermentation time was 5 hours. The results of this experiment are presented in table 11.

Experiment 5. A large scale (1 mM) xylose fermentation was also conducted at pH 6.5. Cells from 2 liters of growth medium were allowed to ferment for 6 hours. The results of this experiment are shown in table 12.

Experiment 6. Large scale fermentations at pH 5.3 were carried out in duplicate with 0.1mM xylose per fermentations. The nonproliferating cell suspension was prepared with cells taken from 2 liters of growth medium and the fermentations were followed manometrically for 6 hours. The results of the fermentations at pH 5.3 are shown in tables 13 and 14. The manometric data for the 0.1mM xylose fermentations at pH 5.3 (table 14) substantiated the results presented in table 10; i.e., at low pH there was a net fixation of CO_2.

For sake of clarity, the main results presented in tables 11, 12 and 13 are summarized in table 15.

TABLE 11

Experiment 4

The fermentation of xylose by $\underline{E}$. $\underline{coli}$ at pH 7.9

Xylose fermented: 1 mM
Fermentation medium: 0.1 M $NaHCO_3$
Gas atmosphere: 5% CO_2 in N_2

End product	Moles end product formed per mole xylose fermented	Moles carbon	Moles* "available hydrogen"
Lactic acid	0.24	0.72	2.88
Succinic acid	0.13	0.52	1.56
Formic acid	1.17	1.17	2.34
Acetic acid	0.59	1.18	4.72
Ethanol	0.61	1.22	7.32
Totals		4.81	18.82

% carbon recovered = $\frac{4.81}{5.00}$ = 96   % "available hydrogen" = $\frac{18.82}{20.00}$ = 94

* As used by Barker (1936)

TABLE 12

Experiment 5

The fermentation of xylose by E. coli at pH 6.5

Xylose fermented: 0.93 mM
Fermentation medium: 0.06M $NaHCO_3$
Gas atmosphere: 100% CO_2

End product	Moles end product formed per mole xylose fermented	Moles carbon	Moles * "available hydrogen"
Lactic acid	0.73	2.19	8.76
Succinic acid	0.06	0.24	2.88
Formic acid	0.91	0.91	1.82
Acetic acid	0.47	0.94	3.76
Ethanol	0.31	0.62	3.72
Totals		4.90	20.94

% carbon recovered $= \dfrac{4.90}{5.00} = 98$

% "available hydrogen" recovered $= \dfrac{20.94}{20.00} = 105$

TABLE 13

Experiment 6

The fermentation of xylose by E. coli at pH 5.3;

Xylose fermented: 0.1mM
Fermentation medium: 0.0025 M NaHCO$_3$
Gas atmosphere: 100% CO$_2$

End product	Moles end product formed per mole xylose fermented		Moles carbon		Moles * "available hydrogen"	
	(1)	(2)	(1)	(2)	(1)	(2)
Lactic acid	1.40	1.42	4.20	4.26	16.80	17.04
Succinic acid	0.00	0.00	0.00	0.00	0.00	0.00
Formic acid	0.21	0.19	0.21	0.19	0.42	0.38
Acetic acid	0.15	0.25	0.30	0.50	1.20	2.00
Ethanol	0.00	0.00	0.00	0.00	0.00	0.00
Totals			4.71	4.95	18.42	19.42

(1) % carbon recovered $= \dfrac{4.71}{5.00} = 94$

% "available hydrogen" recovered $= \dfrac{18.40}{20.00} = 92$

(2) % carbon recovered $= \dfrac{4.95}{5.00} = 99$

% "available hydrogen" $= \dfrac{19.42}{20.00} = 97$

TABLE 14

Experiment 6

Manometric data for the fermentation of xylose by E. coli

at pH 5.3: manometric data

1	2	3	4	5	6
Duplicate	Initial $HCO_3^--CO_2$	CO_2 liberated	Residual $HCO_3^--CO_2$	Total CO_2 (3 + 4)	CO_2 fixed
	mm^3	mm^3	mm^3	mm^3	mm^3
(1)	6072	3822	1688	5510	562
(2)	6072	4047	1690	5737	335

TABLE 15

Experiments 3, 4 and 5

The fermentation of xylose by E. coli at different pH levels

End product	Moles end product per mole xylose fermented			
	pH 7.9	pH 6.5	pH 5.3*	
Lactic acid	0.24	0.73	1.40	1.42
Succinic acid	0.13	0.06	0.00	0.00
Formic acid	1.17	0.91	0.21	0.19
Acetic acid	0.59	0.47	0.15	0.25
Ethanol	0.61	0.31	0.00	0.00

* Duplicate fermentations

The results of these large scale experiments were similar to the results from the small scale experiments (tables 9 and 10) and demonstrated the production of more than 1 mole of lactic acid per mole of xylose fermented at low pH. Since all of the lactate produced at low pH could not have come from the C_3 piece of the pentose molecule, some of the lactate must have come from the C_2 portion of the pentose. Furthermore, it was apparent from the manometric data that C_1 (CO_2) was involved. The possibility that a C_2 and a C_1 moiety were involved in the production of lactic acid was so intriguing that the remainder of these investigations were devoted toward the elucidation of the pathway of lactate formation.

2. Tracer experiments

a. Lactate produced with $C^{14}H_3COOH$ and $C^{13}O_2$ as tracers.

It seemed probable that additional information concerning production of lactate from C_2 could be obtained from isotopic carbon tracer experiments. The most direct approach would have been through the use of radioactive xylose labeled exclusively in one or two carbon atoms. This type of labeled xylose was not readily available and, therefore, more indirect types of tracer experiments were used.

It appeared reasonable that one could trace the C_2 piece of the xylose molecule with C^{14}-labeled acetate. Also, since CO_2 fixation appeared to be associated with the high lactate yields, it seemed desirable to trace the carbon dioxide with $C^{13}O_2$.

Previous nontracer xylose fermentations at pH 5.3 demonstrated that the nonvolatile acid fraction consisted entirely of lactic acid. Due to the small quantity of lactate isolated it was usually necessary to add carrier lactate before degradation in order to obtain reasonable amounts of $BaCO_3$. The C^{14} counts per second were calculated back to the original

lactate present in the fermentation supernatant; results are expressed as specific activity (counts per second per mg $BaCO_3$) of the original lactate. The specific activity of the final CO_2 gas sample was zero. The C^{13} data was also expressed in terms of the original lactate.

Experiments 7 and 8. Xylose (0.1 mM) was fermented at low pH with $C^{14}H_3COOH$ and $C^{13}O_2$ as tracers. The activity of the $C^{14}H_3COOH$ (added as 0.01mM $C^{14}H_3COONa$) was 6000 c/s (counts/second). The $C^{13}O_2$ was added as 1mM $Na_2C^{13}O_3$ and the initial CO_2 gas was 8 atom per cent C^{13} (see MATERIALS and METHODS). This was a duplicate experiment and the results of the isotope determinations on lactate produced are shown in tables 16 and 17.

The relatively high specific activity of the lactate β carbon (tables 16 and 17) clearly indicated that a C_2 moiety was converted into the CH_3-CHOH- portion of the lactate molecule. Likewise, the relatively high atom per cent excess C^{13} of the lactate carboxyl indicated that a C_1 piece was converted into the carboxyl group of the lactate. This means that the following over-all reaction must have taken place:

$$C_2 + C_1 \longrightarrow CH_3\text{-CHOH-COOH}$$
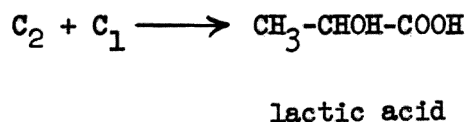
<div align="center">lactic acid</div>

TABLE 16

Experiment 7

Lactate produced with $C^{14}H_3COOH$ and $C^{13}O_2$ as tracers

Xylose fermented:	0.1 mM
Fermentation medium:	0.0025 M $NaHCO_3$
Gas atmosphere:	100% CO_2
Lactate produced:	0.12 mM
Lactate isolated:	0.11 mM

Labeled lactic acid	C^{14} c/s/mg $BaCO_3$	C^{13} atom per cent excess
CH_3-CHOH-COOH	1.01	not determined
CH_3-CHOH-	1.13	not determined
CH_3-	2.59	0.000 ± 0.006
-CHOH-	0.00	0.000 ± 0.018
-COOH	0.25	0.147 ± 0.008

TABLE 17

Experiment 8

Lactate produced with $C^{14}H_3COOH$ and $C^{13}O_2$ as tracers

Xylose fermented:　　0.1 mM
Fermentation medium: 0.0025 M $NaHCO_3$
Gas atmosphere:　　　100% CO_2
Lactate produced:　　0.14 mM
Lactate isolated:　　0.13 mM

Labeled lactic acid	C^{14} c/s/mg $BaCO_3$	C^{13} atom per cent excess
CH_3-CHOH-COOH	0.93	not determined
CH_3-CHOH-	1.67	not determined
CH_3	3.07	0.000 ± 0.008
-CHOH-	0.00	0.000 ± 0.010
-COOH	0.21	0.030 ± 0.01

Experiment 9. As a further check on the validity of the findings presented in tables 16 and 17, the same type experiment was conducted on a large scale. This large scale tracer experiment was carried out in two 250 suction flasks and the fermentation supernatants were pooled for analyses. Each flask contained cells from 2 liters of growth medium, 0.5 mM xylose substrate, 250 ml bicarbonate buffer, 6000 c/s $C^{14}H_3COONa$ and 7.6 mM $C^{13}O_2$. The $C^{13}O_2$ was generated externally by the addition of concentrated H_3PO_4 to a $BaC^{13}O_3$ slurry and sucked into the fermentation flask with partial vacuum. The CO_2 was about 12 atom per cent C^{13}. The fermentations were agitated with magnet stirrers for a period of 6 hours.

The total amount of xylose fermented was 0.55 mM and total lactate produced was 0.71 mM. The fermentation balance for this experiment is shown in table 18.

In experiment 9 the formate and acetate, as well as the lactate, were isolated and analysed for C^{14} content. The specific activity of the acetate carboxyl was zero, whereas the α carbon contained 147 c/s/mg $BaCO_3$; hence, no crossover occurred between the acetate carbons. The specific activity of the formate was 0.10 c/s/mg $BaCO_3$ which indicated that the formate in equilibrium with the carboxyl of the C_3 (intermediate) precursor of lactate. The lactate carboxyl exhibited a specific activity of 0.19 c/s/mg $BaCO_3$. The results of the isotope determinations on lactate from experiment 9 are shown in table 19.

TABLE 18

Experiment 9

Large scale fermentation of xylose by E. coli at pH 5.3

Xylose fermented: 0.55 mM
Fermentation medium: 0.0025 M $NaHCO_3$
Gas atmosphere: 100% CO_2

End product	Moles end product formed per mole xylose fermented	Moles carbon	Moles "available hydrogen"
Lactic acid	1.29	3.87	15.48
Succinic acid	0.00	0.00	0.00
Formic acid	0.07	0.07	0.14
Acetic acid	0.73	1.46	5.84
Ethanol	not determined	n.d.	n.d.
Totals		5.40	21.46

% carbon recovered $= \dfrac{5.40}{5.00} = 108$

% "available hydrogen $= \dfrac{21.39}{20.00} = 108$

TABLE 19

Experiment 9

Lactate produced with $C^{14}H_3COOH$ and $C^{13}O_2$ as tracers

Xylose fermented: 0.55 mM
Fermentation medium: 0.0025 M $NaHCO_3$
Gas atmosphere: 100% CO_2
Lactate produced: 0.71 mM
Lactate isolated: 0.68 mM

Labeled Lactic acid	C^{14} c/s/mg $BaCO_3$	C^{13} atom per cent excess
CH₃-CHOH-COOH	0.81	not determined
CH₃-CHOH-	1.09	not determined
CH₃-	2.19	0.000 ± 0.006
-CHOH-	0.00	0.000 ± 0.003
-COOH	0.19	0.287 ± 0.002

Hence, the results from the large scale xylose fermentation at pH 5.3 confirmed the findings from the small-scaled experiments, namely,

$$C_2 + C_1 \longrightarrow CH_3\text{-CHOH-COOH}$$
$$\text{lactic acid}$$
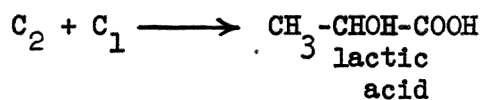
Since there was good evidence that lactate could be produced from a $C_2 + C_1$ condensation at low pH, it was desirable to know if this reaction also occurred at higher pH, where lactate yields are much lower; i.e., ~0.2 moles lactate per mole xylose fermented.

Experiment 10. A xylose fermentation was conducted at pH 7.5 with $C^{14}H_3COOH$ as tracer. The fermentation was carried in two Warburg flasks with 0.1 mM substrate per flask and the flask contents were pooled for analysis. The lactic acid was finally isolated by use of an ether-celite partition column. The specific activity measurements expressed in terms of the lactic acid produced in the fermentation are presented in table 20.

The results (table 20) clearly show that the α carbon of CH_3COOH was found in the lactate β carbon and it was thus concluded that $C_2 + C_1$, to give lactate, took place at pH 7.4 as well as at pH 5.3.

b. Succinate produced with $C^{14}H_3COOH$ as tracer

The succinic acid produced in experiment 10 was also analysed for C^{14} content and the results of these determinations are presented in table 21.

TABLE 20

Experiment 10

Lactate produced at pH 7.4 with $C^{14}H_3COOH$ as tracer

Xylose fermented: 0.2mM
Fermentation medium: 0.02 M $NaHCO_3$
Gas atmosphere: 5% CO_2 in N_2
Lactate produced: 0.27 mM
Lactate isolated: 0.12 mM

Labeled lactic acid	C^{14} c/s/mg $BaCO_3$
CH_3-CHOH-COOH	3.72
CH_3-	12.89
-CHOH-	0.00
-COOH	0.00

٤

TABLE 21

Experiment 10

Succinate produced at pH 7.4 with $C^{14}H_3COOH$ as tracer

Xylose fermented: 0.2mM
Fermentation medium: 0.02 M $NaHCO_3$
Gas atmosphere: 5% CO_2 in N_2
Succinate produced: 0.09 mM
Succinate isolated: 0.06 mM

Labeled lactic acid	C^{14} c/s/mg $BaCO_3$
$HOOC-CH_2-CH_2-COOH$	1.92
$HOOC-$	0.00
$-CH_2-CH_2-$	3.16
$-COOH$	0.00

The specific activity of the fragments of the succinate molecule were lower than would be expected on the basis of the specific activity of the whole molecule. Some dead carbon was evidently picked up during the many steps of the degradation procedure; a blank set of reactions was not run in this instance, hence no proper correction could be applied.

In $C^{14}H_3COOH$ tracer experiments the lactate contained 1 to 3 per cent of the counts added. Foster et al, (1949) have shown that the ethanol C_2 is more active than the acetate C_2 in the formation of fumaric acid by Rhizopus nigricans. It, therefore, seemed possible that $C^{14}H_3CH_2OH$ might be a better tracer than $C^{14}H_3COOH$ for the $C_2 + C_1$ condensation and an experiment was conducted to test this possibility.

C. Lactate produced with $C^{14}H_3CH_2OH$ as tracer

Experiment 11. Lactate was produced from 0.1 mM xylose at pH 5.3 in the usual manner except that $C^{14}H_3CH_2OH$ (5400 c/s) was added as the tracer in place of $C^{14}H_3COOH$. The results are presented in table 22.

The results obtained with $C^{14}H_3CH_2OH$ tracer (table 22) showed that, as in the case of $C^{14}H_3COOH$ tracer, the α carbon of the C_2 tracer was converted, in part, to the lactic β carbon. This was added evidence that lactate could be produced by a $C_2 + C_1$ condensation. The conversion of $C^{14}H_3CH_2OH$ activity to lactate activity was about 0.2 per cent compared to the 1 to 3 per cent converstion of $C^{14}H_3COOH$; indicating that, under these experimental condotions, there was better converstion of $C^{14}H_3COOH$ than $C^{14}H_3CH_2OH$ into the active C_2 fragment.

Previous experiments (7, 8 and 9) performed with $C^{13}O_2$ as a tracer indicated that a C_1 piece was definitely involved in lactate production from xylose. In view of the fact that formate may function as a methyl donor (du Vigneaud, Ressler, C., and Rachale, J. R., 1950) it seemed desirable to trace the $C_2 + C_1$ condensation with $HC^{14}OOH$.

TABLE 22

Experiment 11

Lactate produced at pH 5.3 with $C^{14}H_3CH_2OH$ as tracer

Xylose fermented: 0.1 mM
Fermentation medium: 0.0025 M $NaHCO_3$
Gas atmosphere: 5% CO_2 in N_2
Lactate produced: 0.12 mM
Lactate isolated: 0.12 mM

Labeled lactic acid	C^{14} c/s/mg $BaCO_3$
CH_3-CHOH-COOH	0.14
CH_3-	0.34
-CHOH	0.00
-COOH	0.00

D. Lactate produced with $HC^{14}OOH$ as tracer

Experiment 12. The usual type of 0.1mM xylose fermentation at pH 5.3 was carried out in duplicate with $HC^{14}OOH$ ($\sim$10,000 c/ɛ) as the tracer. The duplicate supernatants were pooled for analysis. The lactic acid was isolated from a partition column and there was no evidence that acids other than lactic were present in the nonvolatile acid fraction. The results of the C^{14} determinations on the lactate isolated are shown in table 23.

TABLE 23

Experiment 12

Lactate produced at pH 5.3 with $HC^{14}OOH$ as tracer

Xylose fermented: 0.17 mM
Fermentation medium: 0.0025 M $NaHCO_3$
Gas atmosphere: 5% CO_2 in N_2
Lactate produced: 0.22 mM
Lactate isolated: 0.19 mM

Labeled lactic acid	C^{14} c/s/mg $BaCO_3$
CH_3-CHOH-COOH	2.51
CH_3-CHOH-	0.00
-COOH	6.97

These results demonstrated beyond any reasonable doubt that formate was converted to the lactate carboxyl group and did enter the α or β positions. This was taken as further evidence that part of the
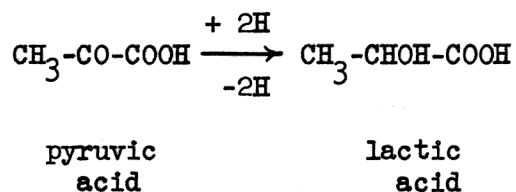
lactate was formed via a $C_2 + C_1$ condensation. The $C_2 + C_1$ condensation and its relationship to other metabolic mechanisms are more fully discussed in the next section.

V. DISCUSSION

A consideration of the structure of the pentose molecule suggested that one of the initial reactions in the metabolic decomposition of pentose was an aldolase type cleavage which would yield a C_2 piece and a C_3 piece. Furthermore, the findings of other investigators (Fred, Peterson, and Anderson, 1921; Lampen, 1950) strongly supported this concept. In addition to their general value, investigations concerned with bacterial fermentations of pentoses are of particular significance in that they are a potential source of information concerning a C_2 intermediate. The important role of C_2 pieces in intermediary carbohydrate metabolism is well established (Gurin, 1940; Foster, et al, 1949; Lipmann, 1946; Wood, 1946).

The results of these investigations have re-emphasized the metabolic versatility of the C_2 moiety. The striking finding that, under certain conditions, lactic acid is produced from xylose in a ratio of considerably more than one mole per mole of xylose fermented, was taken as a priori evidence that the C_2 portion of the C_5 molecule was involved in the formation of lactic acid. Furthermore, the concurrent CO_2 utilization indicated that the lactate was formed by a direct participation of CO_2.

The main pathway of lactic acid formation in E. coli is probably through the reduction of pyruvic acid (C_3):

$$CH_3\text{-CO-COOH} \xrightarrow[-2H]{+2H} CH_3\text{-CHOH-COOH}$$

$$\text{pyruvic} \qquad\qquad \text{lactic}$$
$$\text{acid} \qquad\qquad\qquad \text{acid}$$

With pentose as substrate the maximum quantity of lactate which could have

come from the C_3 portion was one mole lactate per mole xylose. Any lactic produced in excess of one mole lactate per mole xylose must have been derived at least in part from the C_2 moiety of the C_5 molecule.

There are a number of pathways by which the C_2 moiety could have been incorporated in the carbon skeleton of lactic acid (C_3), for example:

Scheme 1.

$$2C_2 \longrightarrow C_4 \longrightarrow C_3 \ + \ C_1$$
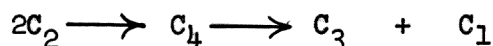
The specific steps of the over-all scheme shown above can be visualized as follows:

a COOH   a COOH   a COOH   a COOH
b $C^{14}H_3$ $\xrightarrow[- 2H]{+ 2H}$ b $C^{14}H_2$ $\xrightarrow[- 2H]{+ 2H}$ b $C^{14}H$ $\xrightarrow[+ HOH]{- HOH}$ b $C^{14}H_2$
c $C^{14}H_3$ c $C^{14}H_2$ c $C^{14}H$ c $C^{14}HOH$
d COOH   d COOH   d COOH   d COOH

2 acetic   succinic   fumaric   malic
   acid   acid   acid   acid

a CO_2
   $+$
b $C^{14}H_3$

c $C^{14}HOH$

d COOH

   lactic
   acid
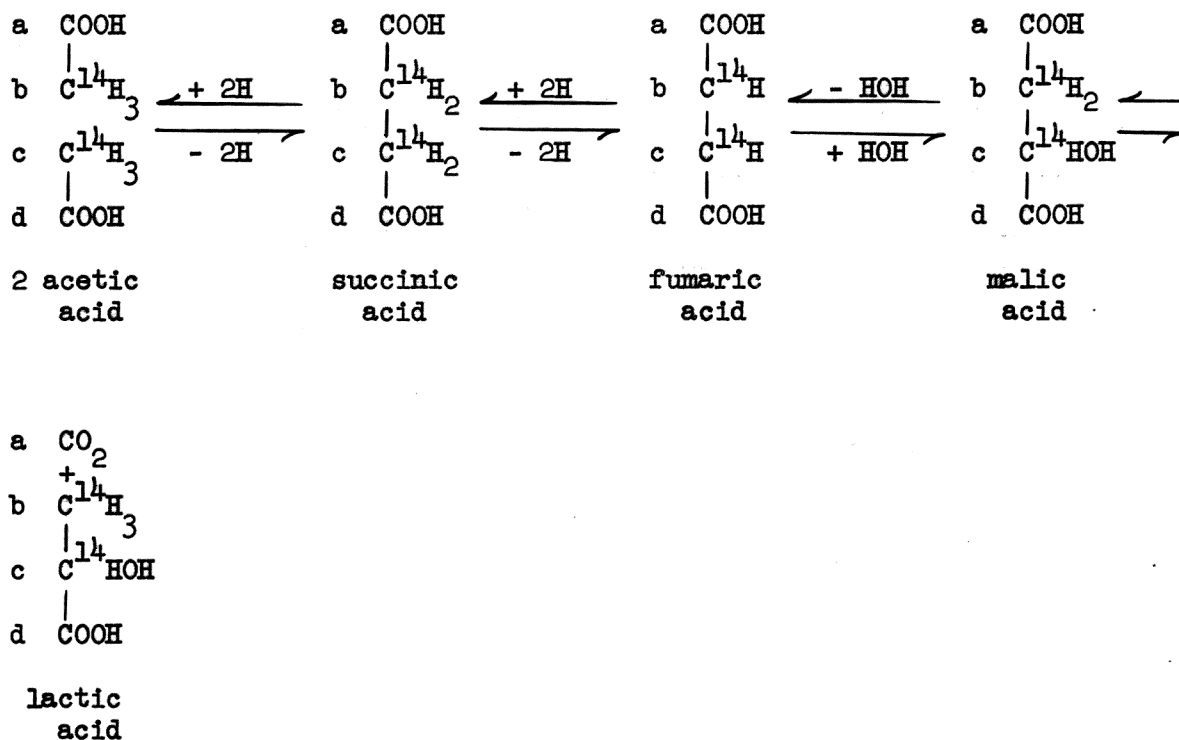
~~Convincing~~ evidence that the reaction: 2 acetate $\longrightarrow$ succinic takes place in **E. coli** has been presented by Swim and Krampitz (1950) while the reactions: succinate $\longrightarrow$ fumarate and fumarate $\longrightarrow$ malate takes place in several types of metabolic systems (Wood, 1946). The decarboxylation

of malate (probably through oxaloacetic acid) to yield lactate and CO_2 has been demonstrated in <u>Lactobacilli</u> (Korkes and Ochoa, 1948). It is to be noted that any lactic acid produced by this mechanism would have contained equal amounts of C^{14} in the α and β carbons. In the present investigations, however, the lactate produced in the presence of $C^{14}H_3COOH$ was labeled almost exclusively in the β carbon. Hence the major portion of the lactate derived from C_2 must have been produced by means of another mechanism. The inside labeled succinate produced in these investigations could have been formed via Scheme 1, however, the lactate could not have been in close equilibrium with this succinate.

Scheme 2.

$$C_2 + C_4 \longrightarrow C_6 \longrightarrow \underset{+\ C_1}{C_5} \longrightarrow \underset{+\ C_1}{C_4} \longrightarrow \underset{+\ C_1}{C_3}$$
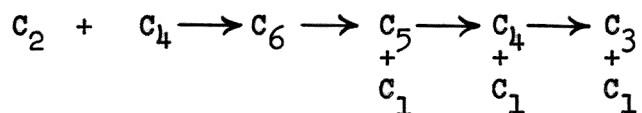
Although the existence of a "tricarboxylic acid" cycle (Wood, 1946) has not been demonstrated in <u>E. coli</u>, it is possible that lactic acid could be produced from C_2 by the following pathway:

acetic acid

$C^{14}H_3\text{-COOH}$
$+$
$C^{14}OH\text{-COOH}$
$\|^{14}$
$C^{14}H\text{-COOH}$

$\xleftarrow{\;+HOH\;}$
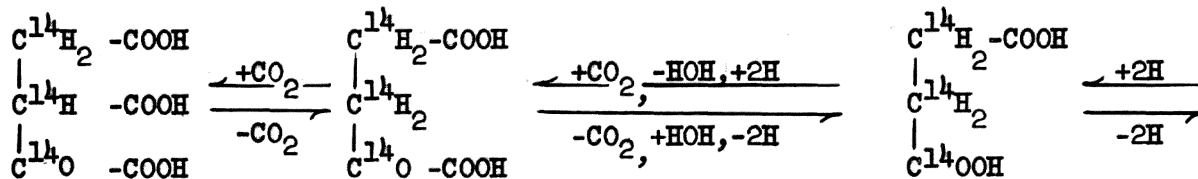$\xrightarrow{\;-HOH\;}$

$C^{14}H_2\text{-COOH}$
$|^{14}$
$C^{14}\text{-COOH}$
$\|^{14}$
$C^{14}H\text{-COOH}$

$\xleftarrow{\;-HOH\;}$
$\xrightarrow{\;+HOH\;}$

$C^{14}H_2\text{-COOH}$
$|^{14}$
$C^{14}H\text{-COOH}$
$|^{14}$
$C^{14}HOH\text{-COOH}$

$\xleftarrow{\;+2H\;}$
$\xrightarrow{\;-2H\;}$
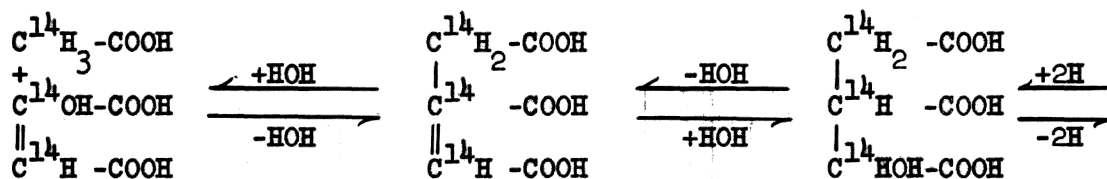
enol-oxaloacetic
acid; from $2C_2$
condensation
via succinic acid

cis-aconitic
acid

iso-citric
acid

$C^{14}H_2\text{-COOH}$
$|$
$C^{14}H\text{-COOH}$
$|^{14}$
$C^{14}O\text{-COOH}$

$\xleftarrow{\;+CO_2\;}$
$\xrightarrow{\;-CO_2\;}$

$C^{14}H_2\text{-COOH}$
$|$
$C^{14}H_2$
$|^{14}$
$C^{14}O\text{-COOH}$

$\xleftarrow{\;+CO_2,\ -HOH,+2H\;}$
$\xrightarrow{\;-CO_2,\ +HOH,-2H\;}$

$C^{14}H_2\text{-COOH}$
$|$
$C^{14}H_2$
$|$
$C^{14}OOH$

$\xleftarrow{\;+2H\;}$
$\xrightarrow{\;-2H\;}$

oxalo-
succinic acid

α-keto
glutaric

succinic
acid

$C^{14}H\text{-COOH}$
$\|^{14}$
$C^{14}H$
$|^{14}$
$C^{14}OOH$

$\xleftarrow{\;-HOH\;}$
$\xrightarrow{\;+HOH\;}$

$C^{14}H_2\text{-COOH}$
$|$
$C^{14}HOH\text{-}$
$|^{14}$
$C^{14}OOH$

$\xleftarrow{\;+CO_2\;}$
$\xrightarrow{\;-CO_2\;}$

$C^{14}H_3$
$|^{14}$
$C^{14}HOH$
$|^{14}$
$C^{14}OOH$
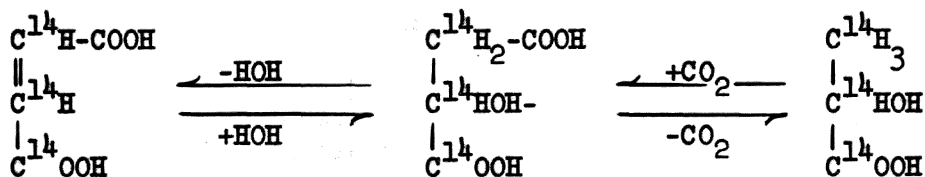
fumaric
acid

malic
acid

lactic
acid

Lactic acid produced by this series of reactions would be labeled in all three carbon atoms and would be accompanied by a net yield of 2 moles CO_2 per mole lactate. The findings of the present investigations are not in agreement with the requirements of Scheme 2, namely (1) the lactate was labeled almost exclusively in the β carbon, and (2) a net utilization of CO_2 occurred.

Scheme 3.
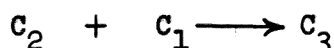
$$C_2 \quad + \quad C_1 \longrightarrow C_3$$

During the past decade a number of investigations on microbial metabolism have suggested the participation of a C_2 + C_1 condensation (see reviews by Wood, 1946; Gurin, 1948). The condensation may be paraphrased by the following mechanism:

acetic acid — unknown C_2 — formic acid — pyruvic acid — lactic acid
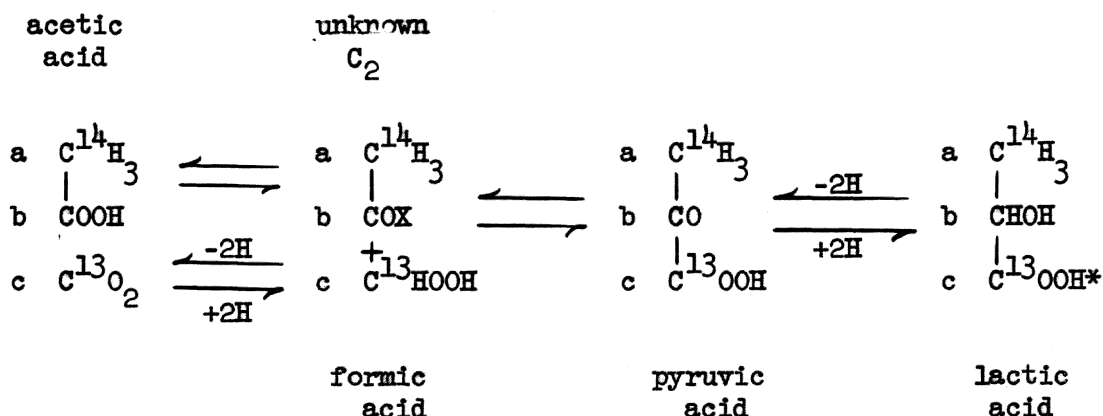
* With $HC^{14}OOH$ as tracer C^{14} found in lactate carboxyl

Recently Streckler, Wood and Krampitz (1950) have shown the fixation of $HC^{14}OOH$ in the carboxyl carbon of pyruvate by E. coli. In the case of Micrococcus pyogenes var. aureus $HC^{14}OOH$ was fixed in the carboxyl carbons of both pyruvate and lactate. Acetyl phosphate was not the C_2 moiety involved in the condensation. Watt and Werkman (1950)

have also investigated the $C_2 + C_1$ condensation in <u>Micrococcus</u> <u>pyogenes</u> var. <u>aureus</u> and concluded that CO_2 but not acetate could be fixed in pyruvate.
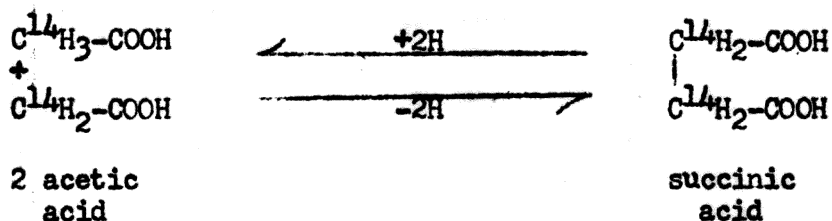
The data presented in the present investigation have established the following points:

1. At low pH, more lactic acid was produced than could be accounted for by the C_3 portion of the pentose.

2. At low pH, there was a net fixation of carbon dioxide.

3. With $C^{13}O_2$ as tracer, the lactate produced was labeled exclusively in the carboxyl carbon.

4. With $HC^{14}OOH$ as tracer, the lactate produced was labeled predominently in the carboxyl carbon.

5. With $C^{14}H_3COOH$ or $C^{14}H_3CH_2OH$ as tracer, the lactate produced as labeled only in the β carbon.

6. With $C^{14}H_3COOH$ as tracer, the acetate produced is labeled exclusively in the α carbon; i.e., there is no cross-over between acetate carbons.

7. With $C^{14}H_3COOH$ as tracer, the formate specific activity approximated the specific activity of the lactate carboxyl; i.e., the formate was probably in equilibrium with the C_3 carboxyl.

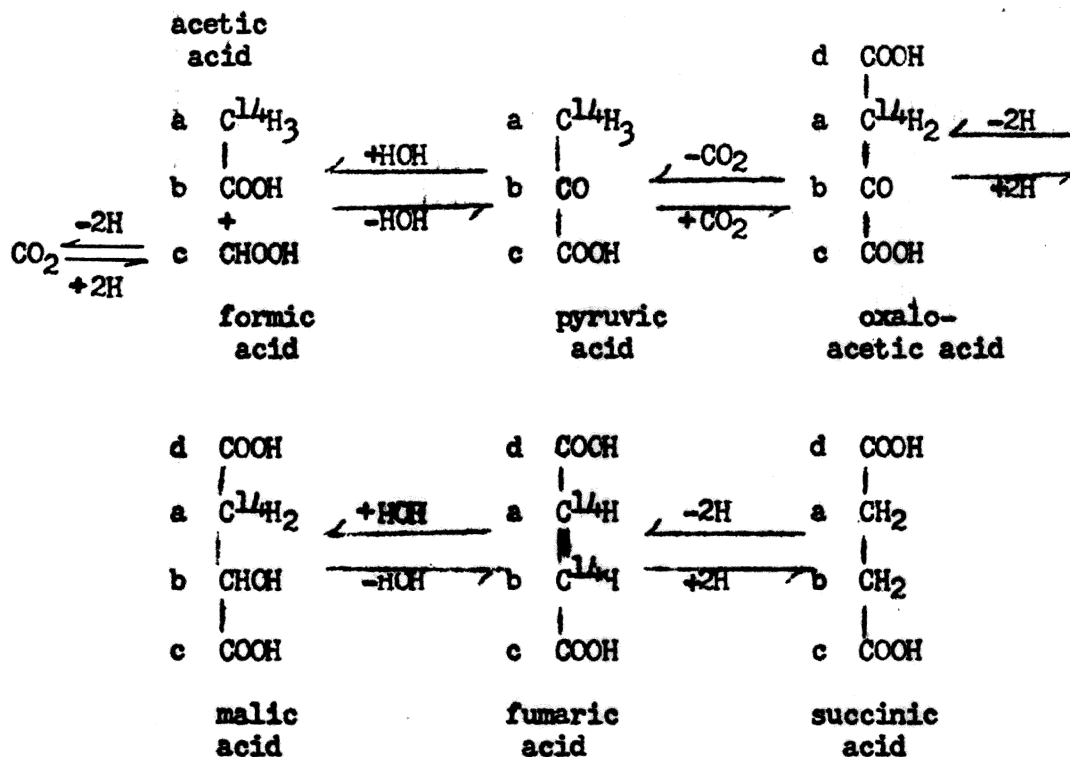These findings constitute good evidence that part of the lactic acid produced from the fermentation of xylose by E. coli at low pH arose from a $C_2 + C_1$ condensation; the C_2 piece was probably derived from a $C_2 - C_3$ split of the pentose. The data further indicated that a $C_2 + C_1$ condensation functioned at pH 7.4 as well as at pH 5.3.

Succinic acid produced from xylose with $C^{14}H_3COOH$ as tracer

was labeled exclusively in the methylene carbons. Methylene-labeled

succinate could have been formed from $C^{14}H_3COOH$ via the $2C_2$ condensation:

$$
\begin{array}{ccc}
C^{14}H_3{-}COOH & & \\
+ & \xrightleftharpoons[-2H]{+2H} & C^{14}H_2{-}COOH \\
C^{14}H_2{-}COOH & & C^{14}H_2{-}COOH \\
\end{array}
$$

$$
\begin{array}{cc}
\text{2 acetic} & \text{succinic} \\
\text{acid} & \text{acid} \\
\end{array}
$$

Although the succinate produced in the present investigations may have

originated from a $2C_2$ condensation, the possibility that it was formed

by a $C_2 + C_1$ condensation followed by the Wood-Werkman reaction (Wood-

Werkman, 1938) cannot be ruled out. This scheme may be represented as

follows:

acetic
acid

a $C^{14}H_3$
b COOH
+
c CHOOH
formic
acid

a $C^{14}H_3$
b CO
c COOH
pyruvic
acid

d COOH
a $C^{14}H_2$
b CO
c COOH
oxalo-
acetic acid

d COOH
a $C^{14}H_2$
b CHOH
c COOH
malic
acid

d COOH
a $C^{14}H$
b $C^{14}H$
c COOH
fumaric
acid

d COOH
a CH_2
b CH_2
c COOH
succinic
acid

Consideration of the scheme presented above indicates that the C_2 + C_1 condensation could have been involved in the formation of succinate, though a $2C_2$ condensation would also explain the inside labeling of succinate.

In any event, the results from the present investigations lead to the striking conclusion that, under certain conditions, there was a net synthesis of lactic acid via the C_2 + C_1 condensation. This finding is highly significant in view of the recent concept that autotrophic carboxylation functions through a cycle involving C_2 + C_1 condensation (Ochoa, 1950). In addition, this condensation provides a mechanism for the utilization of C_2 derived from the aldolase split of pentoses. The utilization of this C_2 fragment to form C_3 is of general significance in that it suggests a pathway for the interconversion of pentoses and hexoses.

VI. SUMMARY

The ubiquitous distribution of the pentose molecule in nature and particularly its presence in certain enzymes and in nucleic acids emphasizes the metabolic significance of these carbohydrates. In living systems the pentoses are undergoing continuous metabolic changes. It thus appeared that investigations concerning the metabolic decomposition of the pentose molecule would be important from a comparative biochemical point of view. The advantages of a microbial system as a working model for biochemical investigations are well known. The present investigations were, therefore, carried out with a washed bacterial cell suspension utilizing xylose as the sole substrate.

Previous investigators have obtained good evidence that one of the first reactions in the fermentation of pentoses was a carbon bond cleavage resulting in the production of a C_3 and a C_2 fragment.

The importance of the C_2 fragment in enzymatic systems is well recognized and it thus seemed plausible that investigations on bacterial pentose fermentations would be of significant value to the field of intermediary metabolism. Preliminary investigations revealed that cells of Escherichia coli K-12 grown in the presence of pentose possessed the ability to ferment pentoses in the nonproliferating cell state.

The results of the present investigations have re-emphasized the metabolic importance of the C_2 fragment. In fermentations conducted at low pH, lactic acid was produced in a ratio of approximately 1.3 moles per mole of xylose fermented. This was taken as a priori evidence that the C_2 portion of the C_5 molecule was involved in the formation of lactic acid. Furthermore, at low pH, there was a net fixation of CO_2 which indicated that a direct participation of CO_2 was involved in the production of lactate. There were a number of pathways by which lactate could have

been formed from C_2 and carbon tracer experiments were conducted in order to determine the main mechanism of $C_2 \longrightarrow C_3$ in this system. These experiments demonstrated that C_2 tracers ($C^{14}H_3COOH$ and $C^{14}H_3CH_2OH$) were converted to the CH_3-CHOH- portion of lactate while C_1 tracers ($C^{13}O_2$ and $HC^{14}OOH$) appeared in the lactate carboxyl. This latter piece of evidence was a further indication that lactate was formed via a $C_2 + C_1$ condensation. This condensation functioned at pH 7.4 as well as at pH 5.3. With $C^{14}H_3COOH$ as tracer succinate was labeled exclusively in the methylene carbons and it was concluded that the lactate was not in close equilibrium with succinate.

The production of lactate via $C_2 + C_1$ condensation further emphasizes the general role of this reaction in intermediary metabolism. The fact that C_2 produced from pentoses apparently can be converted to C_3 also provides a mechanism for the conversion of pentoses into hexoses and vice versa.

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LACTIC ACID FERMENTATION OF XYLOSE BY ESCHERICHIA COLI;

CARBON TRACER STUDIES ON THE C_2 + C_1

CONDENSATION REACTION

by

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The ubiquitous distribution of the pentose molecule in nature
and particularly its presence in certain enzymes and in nucleic acids
emphasizes the metabolic significance of these carbohydrates. In living
systems the pentoses are undergoing continuous metabolic changes. It thus
appeared that investigations concerning the metabolic decomposition of the
pentose molecule would be important from a comparative biochemical point
of view. The advantages of a microbial system as a working model for bio-
chemical investigations are well known. Investigations concerning pentose
metabolism were, therefore, carried out with a washed bacterial cell sus-
pension utilizing xylose as a sole substrate.

Previous investigators have obtained evidence that one of the
first reactions in the fermentation of pentoses was a carbon bond cleavage
resulting in the production of a C_3 and a C_2 fragment. The importance of
the C_2 fragment in enzymatic systems is well recognized and it thus seemed
plausible that investigations on bacterial pentose fermentations would be
of significant value to the field of intermediary metabolism.

Preliminary investigations revealed that cells of Escherichia
coli K-12 grown in the presence of pentose possessed the ability to fer-
ment pentoses in the nonproliferating cell state. Additional experiments
concerning the anaerobic decomposition of xylose re-emphasized the meta-
bolic importance of the C_2 fragment. In fermentations conducted at low
pH, lactic acid was produced in a ratio of approximately 1.3 moles per
mole of xylose fermented. Since a maximum of only 1.0 moles of lactic
acid could have been derived from the C_3 portion of the xylose molecule,
this was taken as a priori evidence that the C_2 portion of the C_5 molecule
was also involved in the formation of lactic acid. Furthermore, at low
pH, there was a net fixation of CO_2 which indicated that a direct partici-

pation of CO_2 was involved in the production of lactate. There were a number of pathways by which lactate could have been formed from C_2 and carbon tracer experiments were conducted in order to determine the main mechanism of $C_2 \longrightarrow C_3$ in this system. These experiments demonstrated that C_2 tracers ($C^{14}H_3COOH$ and $C^{14}H_3CH_2OH$) were converted to the $CH_3-CHOH-$ portion of lactate while C_1 tracers ($C^{13}O_2$ and $HC^{14}OOH$) appeared in the lactate carboxyl. This latter piece of evidence was a further indication that lactate was formed via a $C_2 + C_1$ condensation. This condensation functioned at pH 7.4 as well as at pH 5.3. With $C^{14}H_3COOH$ as tracer succinate was labeled exclusively in the methylene carbons and it was concluded that the lactate was not in close equilibrium with succinate.

The production of lactate via $C_2 + C_1$ condensation further emphasizes the general role of this reaction in intermediary metabolism. The fact that C_2 produced from pentoses apparently can be converted to C_3 also provides a mechanism for the conversion of pentoses into hexoses and vice versa.

BIOGRAPHICAL SKETCH

Leighton Adams Nutting was born in Salem, New Hampshire on September 21, 1919, son of Marguerite Adams Nutting and Clarence Leighton Nutting. Mr. Nutting's boyhood was spent in the state of his birth and he completed his undergraduate studies with the B.S. degree in Bacteriology from the University of New Hampshire in June, 1941. His graduate studies, initiated in 1941, at Virginia Polytechnic Institute were interrupted from 1942 to 1946 by service with the U. S. Army; the major portion of service was with an engineering detachment assigned to the Manhattan Project at Oak Ridge, Tennessee. Upon discharge he resumed his graduate studies and in June, 1948 received an M.S. degree in Bacteriology from Virginia Polytechnic Institute and completed the residence requirements towards the Ph.D. degree. During the period 1948 to 1950 Mr. Nutting completed his doctoral thesis studies as a Graduate Fellow of the Oak Ridge Institute of Nuclear Studies at Oak Ridge, Tennessee. He will receive the Ph.D. degree from Virginia Polytechnic Institute in 1951 under a cooperative arrangement between this institution and the Oak Ridge Institute of Nuclear Studies.

Mr. Nutting has accepted a position as Research Microbiologist with the National Dairy Research Laboratories, Inc., Oakdale, New York.