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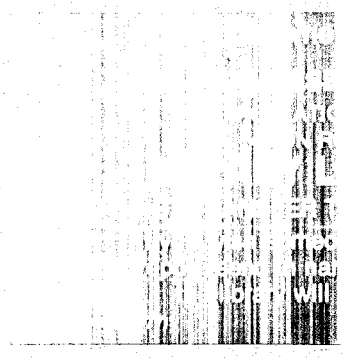

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by

S. F. Carson

Biology Division

OAK RIDGE NATIONAL LABORATORY
division of
CARBIDE AND CARBON CHEMICALS CORPORATION

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DESIGN AND INTERPRETATION OF CARBON ISOTOPE EXPERIMENTS IN BACTERIAL METABOLISM

S. F. Carson

INTRODUCTION

Although carbon isotope experiments in intermediary metabolism had, as a beginning, studies on microbial systems (Wood and cs. (1940, 1941a) with C^{13} and Ruben and cs. (1939, 1940; Carson and Ruben, 1940) with C^{11}, the recent emphasis has been largely on animal systems.

I think it is reasonably clear that a great deal of our present understanding concerning the comparative biochemical outlook in present day biochemistry has really stemmed from the clear thinking and broad point of view of the great Dutch microbiologist Kluyver, who more than twenty years ago brought forth with Donker the concept of "Die Einheit in der Biochemie" (Kluyver and Donker, 1926). The basic principles outlined here in 1926 have many times since been used to find short-cut solutions to rather complex biochemical problems. These principles of hydrogen transfer in coupled oxidation-reduction systems are now firmly impressed on us as every day and commonplace tools of biochemical thinking. Therefore, I think it is perhaps worthwhile to remember that these principles were first worked out in a relatively short time on the basis of studies concerning a number of different microbial processes.

Since a number of the people present at this conference are concerned primarily with the biochemical reactions of animal tissues, it may be worth while to consider a few of the "type experiments" which can rather easily be conducted with microbes. One might call these "model experiments", in the sense that frequently they can be conducted in a relatively uncomplicated manner.

Although it is quite clear that microorganisms must, of necessity, carry out thousands of biochemical reactions in order to build up the constituents of their enzyme systems and cell substance, the microbes have an attitude quite different from that of animals when it comes to deciding what will be their major energy-yielding reactions. In a general sense one might say that animals are rather limited in the possible source of energy-yielding reactions. On the other hand, it is known that the oxidation of any one of several thousand different chemical compounds, organic and inorganic, can serve as energy source for some microbe. In the now famous study of den Dooren de Jong (1926), almost every single organic chemical available in the Delft laboratories was tested and found to serve as both a carbon and energy source for one microbe or another. On the inorganic side, it is clear that such compounds as NH_3, NO_2 and H_2S can serve as energy sources for the chemoautotrophs, and even such compounds as KCN and CO fall into this class.

A second point is the following: microbes are quite independent in regard to the end-products they can produce and during fermentation give off large quantities of a relatively small number of such products.

One can often choose, therefore, not only the starting material he wants to study but the end-product as well. Thus, by a suitable choice of the microbe, one can select a system which will carry out a conversion of a given substrate to a given end-product. It is well to remember that these conversions, especially under anaerobic conditions, go in excellent yield and are largely unaccompanied by serious side reactions.

Finally, it should be brought to attention that rather nice tricks can be played on the redox level of an anerobic process, which make it relatively simple to bring into the limelight certain enzyme processes which would otherwise go entirely unnoticed. A model experiment of this nature will be discussed a little later on.

I would like to discuss with you briefly a few types of reactions which were first found in microorganisms and which led then to an elucidation of general biochemical reactions of considerable importance in many types of living systems.

1. CO_2 Utilization by Heterotrophic Systems

This reaction, which was discovered by H. G. Wood (Wood and Werkman, 1935, 1936), is so well known that I will not take much of your time with it but, rather, point out why it was almost necessary that this important biochemical reaction had to be first found in a microbe.

A characteristic of heterotrophs, whether they be microbes or animals, is the production of CO_2. For example, it is now known that reactions such as in equation 1 go on simultaneously with the second one predominating, hence a net production of CO_2 is always observed (equation 1).

$$A + CO_2 \longrightarrow B$$

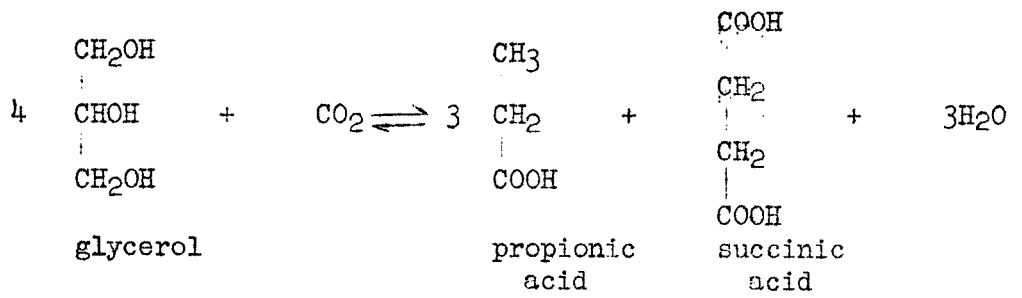
$$C \longrightarrow CO_2 + H_2O$$

Therefore it is not surprising that until 1934 a clear-cut case of CO_2 utilization had not been discovered. That this was accomplished was due only to the fact that Wood was studying the propionic acid fermentation, which gives propionic acid in almost 100% yield from glycerol in a medium containing phosphate as a buffer (van Niel, 1928) (equation 2).

$$\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array} \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ CH_2 \\ | \\ COOH \end{array} + H_2O$$

glycerol — propionic acid

When Wood used large amounts of $CaCO_3$ as a buffer (in the presence of phosphate), a new end product appeared, namely succinic acid (equation 3).



For every mole of succinate formed, a mole of CO_2 disappeared. Hard as this was to believe, the analyses were quite clear-cut and Wood had the first evidence of CO_2 utilization by a typical heterotroph. It is reasonably clear, then, that the synthesis of the 4 carbon succinate was due to a CO_2 pick-up. Subsequent isotope experiments with labelled CO_2 not only checked this result, but indicated a possible mechanism and turned up an entirely unsuspected result. The succinate was found to be carboxyl-labelled; but the real surprise was that the propionate was labelled, and in the carboxyl group. Some further consideration of this fermentation will be made a little later.

These experiments led to the finding by both the Berkeley group (Barker and Kamen, 1945; Barker, Kamen and Haas, 1945; Carson and Ruben, 1940; Ruben and Kamen, 1940) and by Wood and cs. (1940, 1941a) that all heterotrophic systems tested utilized CO_2, whether they were microbial or animal; and we then had the fine quantitative studies by Wood and colleagues (1940, 1941a, b,c,d) on many heterotrophic systems. Subsequently it was found that CO_2 pick-up is involved in such an important system as the Krebs cycle (Wood, Werkman, Hemingway and Nier, 1941c), and eventually we have the truly magnificent studies of Ochoa and his group (Ochoa 1945, 1948; Ochoa and Weisz-Tabori, 1945) on a number of reactions in this cycle. In all fairness it should be pointed out that Ochoa apparently does not need isotopes.

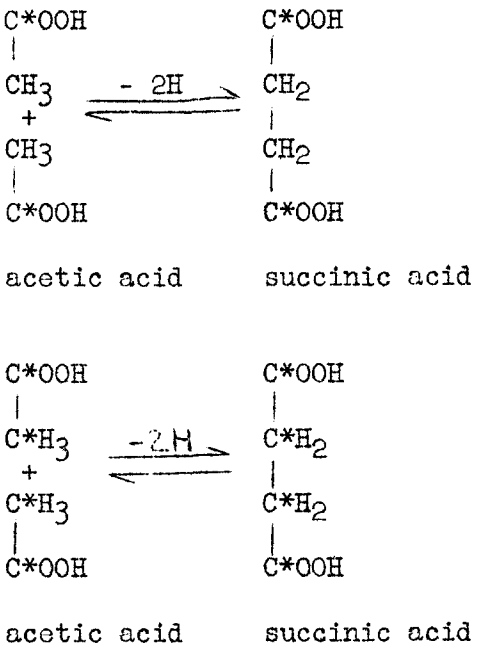
2. Thunberg - Wieland Acetate Condensation

The condensation of acetate to succinate with concommitant dehydrogenation had been suggested by Wieland more than twenty-five years ago (Wieland, 1922, 1933) as a possible pathway for acetate oxidation in animal tissues. Until very recently there had never been a good indication that any biological system can carry out such a reaction.

There now has been a fairly strong suggestion, with both an *Aerobacter* sp. and *E coli*, that the following reversible reaction may take place in biological systems (equation 4).

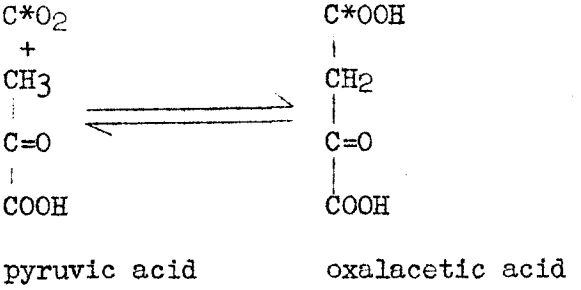
Aerobacter sp. --- Slade

E. coli --- Kalnitsky



3. Oxalacetate Formation from Pyruvate and CO_2

This exceedingly important carboxylation reaction was first suspected from the isotope work on the propionic acid fermentation, and the first real proof came from the studies of Krampitz, *et al.* (1941, 1943) on enzyme preparations from *M. lysodeikticus* (equation 5).



These studies led directly to experiments on animal tissues and, more recently, on green plants. Since this carboxylation reaction is a key point in maintaining one of the components of the Krebs cycle, it was important to have the initial unequivocal evidence obtained by Krampitz on the bacterial "model system".

4. Oxidative Decarboxylation of Pyruvate

The formation of acetyl phosphate as a probable intermediate on the path to acetic acid was first demonstrated by Lipmann on dried cell preparations of *L. delbrückii* (Lipmann, 1937, 1940). The reversal of this type of reaction was demonstrated by Lipmann *et al.* (Utter, Lipmann and Werkman, 1945) using

$CH_3C^{13}OOH$ and $HC^{13}OOH$ on active juices prepared from E. coli (equation 6).

$$CH_3.C{=}O.C^*OOH \rightleftharpoons CH_3.COOH + HC^*OOH$$

pyruvic acid acetic acid formic acid

$$CH_3.C^*{=}O.COOH \rightleftharpoons CH_3.C^*OOH + HCOOH$$

(In presence of adenyl pyrophosphate)

It has recently become somewhat uncertain that acetyl phosphate is the actual intermediate involved and most people prefer now to speak of an "active C_2 fragment".

The splendid work of Barker and cs. on acetate metabolism and synthesis of butyric and caproic acids (Barker, 1947; Barker and cs., 1945) was discussed at some length by Barker at the Wisconsin Symposium last fall, so it is hardly necessary to describe this again here.

Rather, the examples given above have been brought up in order to emphasize the point that one or another microbial system can usually be found which will serve as a model system for the study of certain fundamental biochemical reactions. These results have almost invariably led to similar or equivalent findings in the more complicated animal systems.

I would like now to discuss some research on the propionic acid fermentation which will perhaps illustrate some principles of possibilities of design and interpretation. In this work we have had the collaboration of Martin Kuna and D. S. Anthony, and the assistance of Misses Long and Bachmann. The studies were conducted on the propionic acid fermentation with Propionibacterium pentosaceum using C^{14} as a tracer.

It has been mentioned that the mechanism of formation of propionic acid in biological systems has always been a difficult problem. On the other hand, a biological mechanism for the formation of some of the even-numbered saturated fatty acids has been worked out.

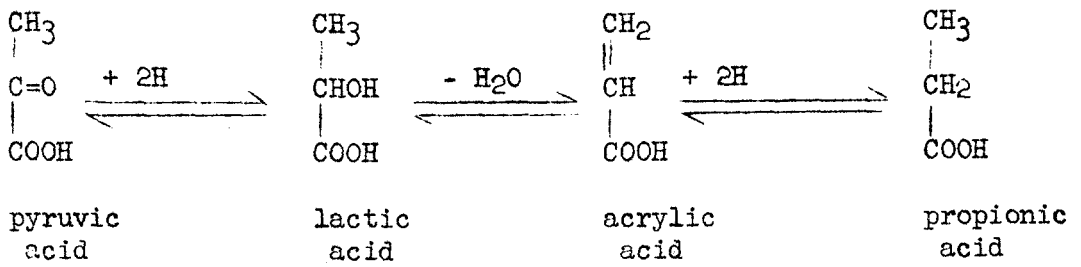
Therefore, it was felt that it would be worthwhile to reinvestigate this problem of the formation of propionic acid as a continuation of our initial studies made a number of years ago at Berkeley (Carson, Foster, Ruben and Barker, 1941; Carson and Ruben, 1940), and those of Wood and cs. (1941 b,d) at Iowa. The general overall reactions of the propionic acid fermentation, showing the dependence of the direction of the reactions upon the hydrogen balance (Barker and Lipmann, 1944; Chaix-Audemard, 1940; van Niel, 1928), or the state of oxidation of the substrates, are (equation 7):

Propionic Acid Fermentation

Redox State													
(+1)	4	Glycerol	+	CO_2	$\rightleftharpoons$	3 Propionate	+	Succinate	+	H_2O			
(0)	$1\frac{1}{2}$	Glycerol	+	$1\frac{1}{2}$ pyruvate	$\rightleftharpoons$	2 Propionate	+	Acetate	+	CO_2	+	H_2O	
(-1)	3	Pyruvate	+	H_2O	$\rightleftharpoons$	Propionate	+	2 Acetate	+	2 CO_2			

One of the older schemes proposed for the formation of propionic acid from pyruvic acid suggested lactic acid and acrylic acid as intermediates in the conversion (equation 8).

Early Scheme for Conversion of Pyruvate to Propionate



This scheme was discarded when it was not possible to show conversion of acrylate to pyruvate with dried preparations of the propionic acid bacteria and when it was clearly shown by Barker and Lipmann that NaF inhibited the conversion of lactate to propionate but did not inhibit the conversion of pyruvate to propionate (originally observed by Chaix-Audemard, 1940).

Earlier experiments by Wood, Stone and Werkman (Stone and Werkman, 1936; Wood, Stone and Werkman, 1937) have indicated that acetate can be metabolized during this fermentation. In one set of experiments acetate reduced methylene blue, and in the analyses of some fermentations there was a reasonable indication that some of the initial acetate which was formed disappeared during later stages. The isotope experiments described below give a rather definite indication that acetate is metabolized at a somewhat considerable rate. Previous tracer experiments by Wood and cs. (1941 b,d) and the Berkeley group (Carson, Foster, Ruben and Barker, 1941; Carson and Ruben, 1940) have indicated that propionate is formed by the decarboxylation of C_4 compounds which arise by the process of CO_2 fixation or by condensation of intermediate products. Therefore it seemed desirable to set up an experiment which would show the probable equilibrium relationships between substrate-acetate-propionate and to test whether or not simple conversion of pyruvate to propionate and acetate occurs reversibly with the C_3 skeleton remaining intact between pyruvate and propionate. Using substrates at three stages of oxidation, the shift in equilibrium was traced with acetate* (* indicates compound labelled with C^{14}) and CO_2* as the amount of available hydrogen varied. Thus, reactions which might appear obvious with one state of available hydrogen, but not with another, may be made clearly obvious by the shift in equilibrium.

It is realized by all of you, I think, that the complete separation of all the end products of a biological reaction and the subsequent degradation of each compound into individual carbon fragments is a very necessary prelude to an interpretation of the most probable path of the reaction. Hence, some preliminary tracer experiments should be made in order that one can be more certain that such difficult and time consuming experiments will not be largely wasted effort.

A basic principle of fermentation chemistry which has seldom been used, but which lends itself readily to studies of anaerobic processes, is the use of substrates of varying redox value. It is quite apparent, from the large qualitative and quantitative differences in end products from the three different

substrates shown before, that some possible enzyme reactions are utterly and completely hidden under one set of redox conditions but are able to show their effect under other conditions.

In the actual experiment, substrates of several different states of oxidation (or available H) were used: glycerol, a highly reduced substrate; pyruvate, a highly oxidized substrate; and a 1:1 mixture of glycerol and pyruvate. Pyruvate is very probably an intermediate in the conversion of glycerol to propionate and acetate. Two fermentations were carried out with each of the three substrates; one with high specific activity carboxyl labelled acetic acid* as a tracer and one with CO_2* in the form of $NaHCO_3$* as a tracer (table 1).

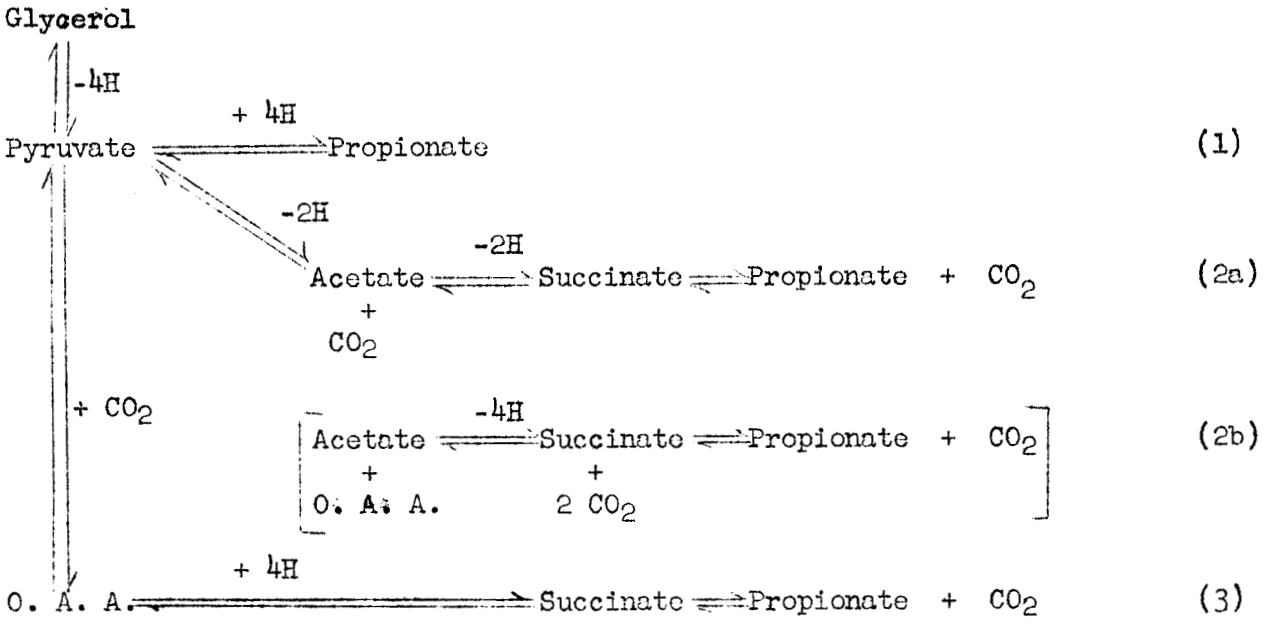
TABLE 1

Substrate	Glycerol		Glycerol + Pyruvate		Pyruvate	
Reduction Value	14	(+1)	12	(0)	10	(-1)
Tracer Added	H.Ac*	CO_2*	H.Ac*	CO_2*	H.Ac*	CO_2*
% initial C* found as CO_2*	2	66	6	73	33	58
Ratio Specific Act. $\frac{c/s}{mM}$ $\frac{\text{Propionic}}{\text{Acetic}}$	$\frac{1}{100}$	$\frac{1}{3}$	$\frac{1}{33}$	$\frac{1}{1.7}$	$\frac{1}{4}$	$\frac{1}{0.5}$

With glycerol, a reduced substance, as substrate the ratio of the specific activity of propionate* formed to acetate* remaining is 1:100; with a mixture of glycerol and pyruvate the proportion was 1:33; while with pyruvate as substrate the ratio was 1:4. Thus the ratio of conversion of acetate* to propionate* was 25 times as great in the presence of an oxidized substrate.

With glycerol as a substrate and CO_2* as a tracer, the ratio of the specific activity of propionate* produced to that of acetate* produced was 1:3. With a mixture of glycerol and pyruvate the ratio was 1:1.7, and with pyruvate as a substrate the ratio was 1:0.5. Thus six times as much CO_2* is converted to propionate* with an oxidized substrate. The amount of the initial C^{14} added as acetate* which was converted to CO_2* was 2% with glycerol as substrate, 6% with a mixture of glycerol and pyruvate, and 33% with pyruvate. This is an important indication that acetate* is metabolized through a condensation reaction which subsequently yields CO_2*. Such a reaction is not apparent with glycerol as the substrate, but with the use of an oxidized substrate (pyruvate) it obviously assumes great importance in the equilibrium scheme.

On the basis of pathways suggested by earlier as well as the present tracer experiments, we have proposed a working scheme around which one can design a more complete tracer experiment (equation 9).



HA_c* ⟶ Propionate*	Highest conversion with pyruvate	
HA_c* ⟶ CO_2*	" " "	"
CO_2* ⟶ Propionate*	" " "	"
CO_2* ⟶ Acetate*	" " "	glycerol

If reaction (1) occurs: it would yield propionate* from acetate* and propionate* from CO_2* only by reversal of the oxidative decarboxylation of pyruvate, would therefore <u>require</u> hydrogen, and would be expected to occur to the greatest extent with glycerol as the substrate.

However, the largest production of propionate* from acetate* occurred with pyruvate as the substrate with simultaneous production of CO_2*. In addition, the production of propionate* from CO_2* was favored by pyruvate. Finally, the pyruvate remaining in these fermentations (1/3 of the initial amount) did not contain any measurable amount of labelled C*.

If reaction (3) goes, it would yield propionate* from CO_2*, and would be favored by glycerol as a substrate. The production of propionate from CO_2* was favored by pyruvate which is not what one would expect.

However, if reactions (2) and (3) were occurring simultaneously this would be possible. Reaction (2a) or (2b) would yield propionate* and CO_2* from acetate* and would be favored by pyruvate. Actually the largest production of propionate* and CO_2* from acetate* occurred in the presence of pyruvate, indicating that reactions (2) were possible. Reactions (2) would proceed with the evolution of pairs of hydrogen atoms which would allow reaction (3) to proceed. This would account for the greater production of propionate* from CO_2* in the presence of pyruvate, since the hydrogen necessary to allow reaction (3) to proceed can come from reaction (2).

This may seem like a very round-about method of turning up the possible intermediates between pyruvate and propionate; however, it now begins to become somewhat clear why the experiments of Barker and Lipmann were unsuccessful in demonstrating lactate or acrylate as intermediates. One of the redox conditions under which the present experiments were conducted demonstrated that acetate can be converted to propionate and CO_2; therefore, one is almost forced to look for an acetate condensation. In addition, there is now excellent comparative biochemical evidence for a reaction involving the decarboxylation of succinate to propionate and CO_2.

SUMMARY

One might say, first, that microorganisms can be, and have been, used as model systems to test certain fundamental types of biochemical reactions. Secondly, that advantage can be taken under anerobic conditions to change the state of the redox level at will in order to make evident reactions which otherwise might be completely hidden, and that this can be accomplished without recourse to addition of foreign substances such as redox dyes. This advantage may then be used to design complex tracer experiments by looking over the possible reactions and then choosing the most ideal conditions under which to carry out the difficult and time consuming radiochemical separations and degradations.

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