MECHANISM OF PROPIONIC ACID FORMATION BY PROPIONIBACTERIUM PENTOSACEUM

EUGENE A. DELWICHE[1]

Laboratory of Bacteriology, Cornell University, Ithaca, New York

Received for publication August 31, 1948

Decarboxylation of succinic acid as a mode of formation of propionic acid in the genus *Propionibacterium* has been suggested (Werkman and Wood, 1942), and evidence has been presented that supports this contention. Numerous observations concerning the ability of the genus to ferment succinic acid with the formation of propionic acid have been made (Shaw and Sherman, 1923; Hitchner, 1934; Fromageot and Bost, 1938). Wood and Werkman (1942) determined the products of the anaerobic breakdown of succinic acid and reported the production of propionic acid and carbon dioxide in approximately equimolar amounts along with small amounts of acetic acid. They reported the production of no other products. The data obtained with isotopic carbon by Carson and Ruben (1940) and Wood *et al.* (1940, 1941) constitute evidence that propionic acid results from the decarboxylation of a symmetrical dicarboxylic acid.

A recent report of investigations by Johns (1948) indicates that bacterial decarboxylation of succinate as a major reaction has been observed with an anaerobic micrococcus isolated from the rumen of sheep. This organism was reported as being strictly anaerobic and differing from the propionic acid bacteria in that it did not ferment sugars. Optimum pH for the decarboxylation was reported as 7.4, which is much higher than the optimum pH of 5.1 to 5.2 of the succinic decarboxylase system described in this paper.

No adequate data, however, have been available showing that the genus *Propionibacterium* can decarboxylate succinic acid at a rate which can account for this pathway as a principal mechanism. Data are herewith presented which demonstrate that succinic acid can be decarboxylated at a rate which can account for all of the propionic acid produced from pyruvic acid under similar conditions.

EXPERIMENTAL METHODS

Cultural methods. Strain E214 of *Propionibacterium pentosaceum* of Professor C. B. van Niel's collection was obtained through the courtesy of Dr. Fritz Lipmann and was used in all experiments.

Cultures were grown for 36 hours at 30 C after a 5 per cent inoculation into a 4-liter Erlenmeyer flask containing 2 liters of a medium composed of 0.5 per cent each of glucose, peptone, and Difco yeast extract. At the end of the incubation period the cells were removed by centrifugation, washed twice in a volume of distilled water equal to the original volume of the fermentation broth, and resuspended in phosphate buffer of pH 5.2 or in distilled water.

Manometric methods. All of the experimental work concerning rates of fer-

[1] Standard Brands Fellow in Bacteriology, Cornell University.

mentation of pyruvate, succinate, and other substrates was conducted by means of the usual Warburg manometric techniques. A total of 3.0 ml was contained in each Warburg vessel and was composed of 1.0 ml of a cell suspension, 0.2 ml of substrate in the side arm, and 0.3 ml of semicarbazide when this latter substance was included. The total volume was made up to 3.0 ml by the addition of phosphate buffer. Specific details concerning cell, buffer, substrate, and inhibitor concentrations and pH are given under "Experimental Results" for each individual experiment. All manometric experiments were conducted at 30 C under an atmosphere of nitrogen.

In earlier experiments when the activities of dried cell preparations were tested, the drying was done by placing the freshly harvested and washed cells in 2- to 3-millimeter layers in petri dishes and drying rapidly *in vacuo* over "drierite." The dried bacteria were stored at 5 C. For use a weighed quantity of cells was resuspended in M/100 phosphate buffer to the desired concentration.

Analytical methods. Volatile acids were determined by pooling several Warburg cups, or by taking an aliquot of the fermentation broth, and proceeding according to the general distillation and partition methods described by Osburn, Wood, and Werkman (1936). When volatile acids were determined from pooled Warburg vessel contents, microtitration methods were employed, which enabled an accuracy of measurement of 0.02 ml of the titration alkali. Molar percentages of acetic and propionic acids were determined from a standard curve carefully worked out with pure acetic and propionic acids of a concentration of the order of the samples to be analyzed.

The partition method of analysis is based upon the distribution of two acids between two immiscible solvents, such as water and diethyl ether, when dilute aqueous solutions of the acids are shaken with acid-free ether in separatory funnels. Each acid of a pair of acids will show a characteristically different distribution, which may be quantitatively determined by titration of the aqueous layer. In the experimental work here reported this principle of distribution between two phases was reduced to an analytical basis as follows: 25 ml of a solution of propionic acid of a known concentration were shaken in a separatory funnel with 50 ml of acid-free ether. The aqueous layer was removed and titrated with 0.01 N NaOH, and this value was designated as N_2. An aliquot of the same acid solution was titrated with no previous ether extraction. This titration value was designated as N_1. The "partition constant," designated as K_1, was obtained by dividing N_2 by N_1 and multiplying by 100. A similar procedure was followed with a solution of acetic acid of equimolar concentration. A "partition constant" for acetic acid was thus obtained. Plotting the K_1 values on the ordinate and molar percentages on the abcissa, a standard curve was established with the K values of pure solutions of acetic and propionic acids providing the minimal two points. Mixtures of the two acids of known concentrations were treated in an identical manner, and it was found that the K values thus obtained fell on the straight line already established with the K values of the 100 molar per cent acids. An identical procedure was followed with different proportions of acid solution and ether. Twenty-five ml of aqueous acid phase and 25 ml of ether

phase were used in this second standardization. "Partition constants" of a different magnitude resulted, and a different standard curve was drawn. Unknown acid solutions were analyzed by obtaining the K_1 and K_2 values by the method described, and reading molar concentrations from the standard curves. The presence of a third acid or of any volatile neutral product would have been reflected by poor agreement of the analytical data from both K_1 and K_2 curves. Analytical data were not accepted as final if poor agreement from K_1 and K_2 values was obtained.

When pyruvate was present in the fermentation mixture, it was removed by shaking for at least 10 hours with 2,4-dinitrophenylhydrazine under acidic conditions.

Of the chemicals used, only the semicarbazide needs special mention. In order to perform the analytical techniques, no volatile acid of a foreign nature could be present; hence the hydrochloride could not be used. Chemically pure semicarbazide was converted to the sulfuric acid salt by digesting with a slight excess of an equivalence of silver sulfate and subsequently removing the excess silver ion by precipitation with hydrogen sulfide. The resulting chloride-free solution was boiled to remove dissolved hydrogen sulfide, adjusted to pH 5.2, and then made up to volume.

EXPERIMENTAL RESULTS

Comparative rates of decomposition of pyruvate, succinate, fumarate, and L-malate were obtained manometrically. Each vessel contained 1.0 ml of a suspension of cells in M/100 phosphate buffer of pH 5.2, and 0.2 ml of M/5 substrate in the side arm. Semicarbazide was included when succinate, fumarate, and L-malate were the substrates, and was added in the amount of 0.3 ml of M/10 semicarbazide sulfate prepared as described under "Experimental Methods." The total volume in all cases was made up to 3.0 ml by the addition of M/100 phosphate buffer of pH 5.2. All solutions were at pH 5.2 before addition to the Warburg cup. Incubation was at 30 C for 120 minutes under an atmosphere of nitrogen. Activity was expressed as μL of CO_2 per mg per hour, hereafter designated as Q_{CO_2}.

Both pyruvate and succinate were found to be decomposed at significant rates (Q_{CO_2} of 11.1 and 4.4, respectively, for the first 60 minutes when 35 mg of cells were present); fumarate was decomposed significantly rapidly (Q_{CO_2} of 2.9); and L-malate was decomposed at a slow rate (see figure 1). Of significance in the case of fumarate decomposition is the observation that with a decreased level of cells present (10 mg per cup) the relative rate of decomposition of fumarate compared to succinate is greatly reduced (figure 2). Making the reasonable assumption that it is the succinate that is being decarboxylated, this difference can be explained on the basis of the greater reducing capacity of the higher concentration of cells. The activity on the L-malate was quite logically found to be lower than the activity of the fumarate and was also decreased by the presence of fewer cells. Semicarbazide was included as a ketone-trapping agent to eliminate the possibility of reversion to pyruvate in the cases of the dicarboxylic acids. This

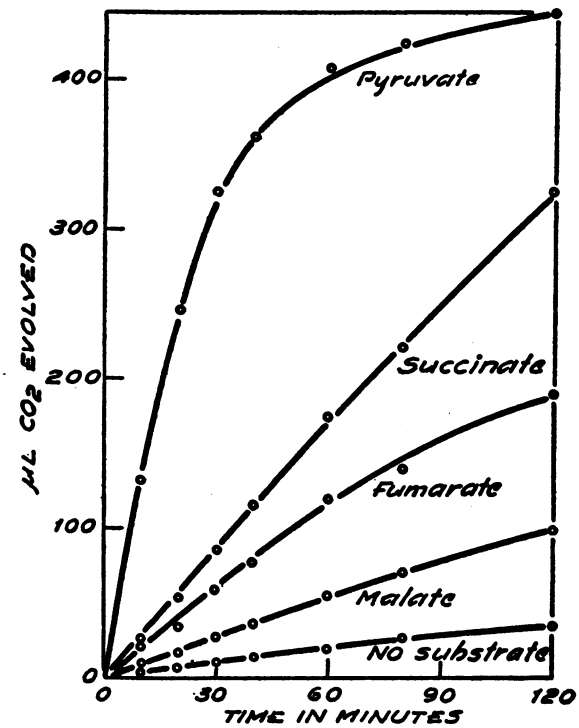

Figure 1. Activity of heavy cell suspension (35 mg per vessel).

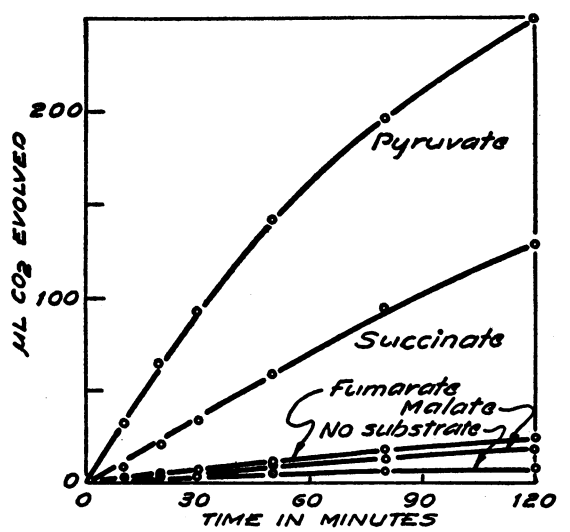

Figure 2. Activity of light cell suspension (10 mg per vessel).

addition can be considered as a mere precautionary measure, since separate experiment established that the rate of decomposition of succinate, fumarate, and malate remained unchanged upon the addition of this inhibitor.

A similar experiment was conducted in order to determine the amounts and proportions of volatile acids produced and the carbon dioxide evolved from pyruvate as compared to succinate. The experiment was identical to the one concerning rates with the exception that the incubation period was increased to 240 minutes and the only vessels utilized were six with pyruvate as a substrate and six with succinate as a substrate. In each case six control cups were run for the purpose of making endogenous corrections of the analysis data. At the end of the incubation period 0.5 ml of 5 N H_2SO_4 were added to each cup to stop the fermentation reactions, and the six cups were pooled for analysis. They were distilled and analyzed for volatile acids by the partition methods described. For amounts and proportions of volatile acids and CO_2 produced, refer to table 1.

In the experimental work which resulted in the data presented in table 1, the same crop of cells was used in all fermentations, and identical experimental conditions were maintained; hence, the data may be considered completely comparable. Of primary significance is the observation that in the decomposition of succinate,

TABLE 1

Volatile acids and carbon dioxide produced from pyruvate and succinate under identical conditions

	FROM PYRUVATE*	FROM SUCCINATE*
Acetic acid	114 μ Moles	6 μ Moles
Propionic acid	46 μ Moles	96 μ Moles
Carbon dioxide	129 μ Moles	108 μ Moles

* Values are corrected for the following endogenous production: 73 μM acetic acid, 38 μM propionic acid, and 34 μM CO_2. Endogenous values remained the same in the presence of semicarbazide.

propionate, appearing quite logically in approximately equimolar amounts with carbon dioxide, is produced in significantly greater amounts than in the case of pyruvate decomposition.

Preliminary studies of the succinic decarboxylase system have shown it to be most active at pH 5.1 to 5.2 (figure 3), as previously reported by Werkman and Wood (1942). In determining the optimum pH, manometric measurements of carbon dioxide evolution were made. Each Warburg cup contained 1.0 ml of a suspension of 10 mg of washed cells suspended in distilled water, 0.2 ml of M/5 succinate in the side arm, and 1.8 ml of M/100 phosphate buffer. The latter two constituents were adjusted to the desired pH values before addition to the cup. A vessel without substrate was included for each pH value at which utilization was tested. Each of the points indicated on the curve in figure 3 is properly corrected for the endogenous fermentation. Incubation was made at 30 C for 1 hour under an atmosphere of nitrogen. Activity in terms of Q_{CO_2} was determined and plotted against pH values (figure 3).

The activities of cell preparations in the decarboxylation of succinate from cultures ranging in age from 12 hours to 16 days were determined by the manometric

method described in the rate studies. It was found that activity was greatest after 32 to 38 hours of incubation. After 6 days the activity had dropped to one-tenth, and it remained at about the same low value for cultures ranging in age up to 11 days. After 15 days of incubation activity was insignificant.

A dried preparation of *Propionibacterium pentosaceum* was examined for activity on succinate. No significant activity was obtained with this preparation.

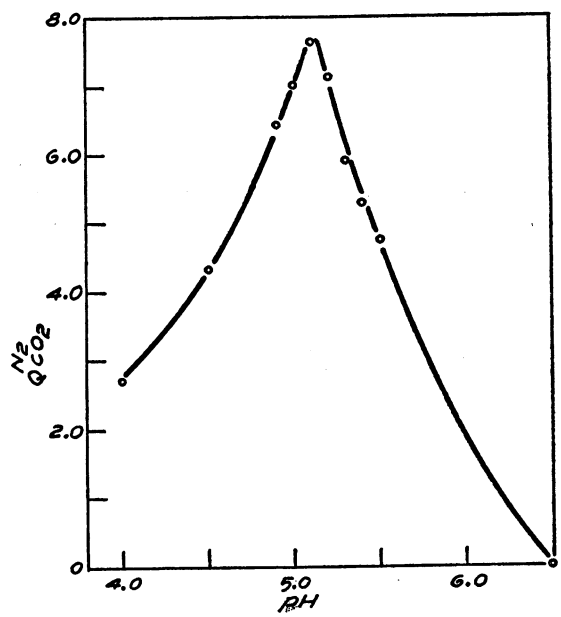

Figure 3. Effect of pH upon the activity of the succinic decarboxylase system.

TABLE 2

The production of volatile acids from glucose as related to the pH of a buffered culture medium

TIME OF INCUBATION	pH	μM ACETIC ACID	μM PROPIONIC ACID
19 hours	7.12	39	0
23 hours	6.76	160	111
25 hours	6.45	168	201
27 hours	5.93	225	219
29 hours	5.21	234	365
31 hours	4.80	244	524
41 hours	4.75	269	634

A study of the production of acetic and propionic acids as related to pH, and therefore to the activity of the succinic decarboxylase system, was made by the analysis of an ordinary fermentation broth for volatile acids at varying time and pH intervals throughout the fermentation. Aliquots of an ordinary culture medium composed of 0.5 per cent each of glucose, peptone, Difco yeast extract, and K_2HPO_4 were analyzed at such intervals as to cover the critical pH range of an assumed succinic decarboxylase system. In general, it was apparent that

significant amounts of propionic acid did not appear until the pH had dropped below 6.5, and that peak production did not appear until the pH was well below 6.0. Careful examination of table 2, however, will show that somewhat contradictory to the general tenets already set forward is the appearance of appreciable amounts of propionic acid at a pH of 6.76, whereas studies concerning optimum activity of the succinic decarboxylase system indicate only slight activity at pH 6.5. This apparent discrepancy is not necessarily evidence for an alternate system of propionate formation. All experimental work was conducted with intact cells in which cell permeability is a decided factor in the utilization of any substrate. It is not unreasonable to visualize that at higher pH ranges the cell membranes may not be permeable to succinate, a situation which, with growing cells, would not be a factor since the succinate would necessarily be produced within the boundaries of the cell.

Studies concerning the optimum pH of the succinic decarboxylase system show greatest activity at the general pH range of 4.5 to 5.5, with slight activity as high as pH 6.5. Thus it is evident that in ordinary culture media the production of propionic acid occurs at a pH range not incompatible with the functioning of a succinic decarboxylase system as at least an important mechanism.

In studies of this nature it is desirable to separate and delineate critical reactions insofar as possible. The use of malonate in a concentration of 0.3 M in the Warburg cup was found to cause an approximately 90 per cent inhibition of the functioning of the decarboxylase on succinate. If succinate decarboxylation was the main pathway to propionic acid, it would then have to follow that, in a system inhibited by malonate, propionate production from pyruvate would be essentially eliminated. Using the methods already described in the work concerning the amounts and proportions of volatile acids produced, this hypothesis was tested for experimental validity. When the contents of the six pooled cups were analyzed, it was found that in the presence of 0.3 M malonate only traces of propionate were produced from pyruvate.

DISCUSSION

The formation of propionate from pyruvate can be postulated as including the following major reactions resulting in the over-all transformation indicated by reaction (7):

(1) $2CH_3COCOOH + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H$

(2) $CH_3COCOOH + CO_2 \rightarrow COOHCH_2COCOOH$

(3) $COOHCH_2COCOOH + 2H \rightarrow COOHCH_2CHOHCOOH$

(4) $COOHCH_2CHOHCOOH \rightarrow COOHCH{=}CHCOOH + H_2O$

(5) $COOHCH{=}CHCOOH + 2H \rightarrow COOHCH_2CH_2COOH$

(6) $COOHCH_2CH_2COOH \rightarrow CH_3CH_2COOH + CO_2$

---

(7) $3CH_3COCOOH + H_2O \rightarrow 2CH_3COOH + CH_3CH_2COOH + 2CO_2$

Evidence in favor of such a scheme is not lacking. The carbon dioxide fixation step is supported by the original studies of Wood and Werkman (1936*a*) on the general problem of heterotrophic carbon dioxide fixation and by their more specific investigations (1936*b*, 1938, 1940). The investigations with carbon isotopes conducted by Carson and Ruben (1940) and Wood *et al.* (1940, 1941) support a theory of random decarboxylation of a symmetrical dicarboxylic acid in the formation of propionate from glycerol and isotope-labeled carbon dioxide. The series of reactions from oxalacetate to succinate have been shown by Krebs and Eggleston (1941) to be reversible in the case of the propionic acid bacteria.

The possibility of the decarboxylation of succinic acid as a major reaction in the formation of propionic acid was considered by Werkman and Wood (1942). In their review concerning heterotrophic assimilation of carbon dioxide, they present an excellent analysis of the isotope data previously mentioned, and in their treatment of the subject they state: "This is further evidence that the propionic acid is formed in the glycerol fermentations exclusively by decarboxylation of a symmetrical dicarboxylic acid. It is the mechanism of the decarboxylation that particularly is uncertain." Also: "There is some evidence that the propionic acid bacteria can decarboxylate succinate anaerobically ($COOHCH_2CH_2COOH \rightarrow CO_2 + CH_3CH_2COOH$) but it is questionable whether or not the rate of this reaction is high enough to be of any considerable importance." They describe unpublished experiments in their laboratory in which *Propionibacterium arabinosum* fermented succinate to propionic acid, carbon dioxide, and small amounts of acetic acid, but a large conversion was not obtained.

Noteworthy are the earlier investigations of Shaw and Sherman (1923), Hitchner (1934), and Wood *et al.* (1937) in which the fermentation of succinate to propionate is reported. Although in all cases these investigations were in the nature of fermentation studies and the more controlled conditions of resting cell techniques were not employed, their data in the light of the more recent isotope studies and the findings reported in this paper become more meaningful.

Fromageot and Bost (1938) made the interesting observation that their culture of *Propionibacterium pentosaceum* could produce propionic acid from succinate only in the presence of glucose. They worked with 48-hour wet cell suspensions in a pH 6.4 bicarbonate buffer. An explanation of their failure to show activity on succinate alone could be the unfavorable pH. When glucose was present, acid production could occur to an extent that could easily lower the pH sufficiently to permit activity of a succinate decarboxylase of the type described in this paper.

Acetate condensation as a mode of formation of succinate is under investigation by Carson *et al.* (1948). In abstracted form they suggest that a C_2 condensation may occur. The analytical data of table 1 do not support a theory of acetate condensation as a major mechanism.

The possibility of the direct reduction of pyruvate with lactate as an intermediate has been well eliminated by the fluoride inhibition studies of Chaix-Audemand as cited by Barker and Lipmann (1944), and also by the more detailed investigations of the latter. With the revision of the hypothetical scheme of Barker and Lipmann (1944) to include a carbon dioxide fixation step and the

necessary reductions, succinate can be included as the hypothetical compound "X." The fluoride sensitivity they discuss is probably concerned in a lactate to pyruvate transformation and is possibly a matter of inhibition of a hydrogen transport mechanism.

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. W. W. Umbreit of the Merck Institute and to Professor James M. Sherman for their many invaluable suggestions throughout the course of this investigation.

SUMMARY

Data are presented which demonstrate that *Propionibacterium pentosaceum* possesses a succinic decarboxylase system sufficiently active to produce propionic acid from succinic acid at a rate comparable to the rate of production of propionic acid from pyruvic acid.

The succinic decarboxylase system is most active after 36 to 38 hours' incubation under the conditions stated, and shows greatest activity at pH 5.1 to 5.2. It is over 90 per cent destroyed by drying, and is approximately 90 per cent inhibited by 0.3 M malonate.

The production of propionic acid from pyruvic acid is almost completely inhibited by the presence of 0.3 M malonate.

REFERENCES

BARKER, H. A., AND LIPMANN, F. 1944 On lactic acid metabolism in propionic acid bacteria and the problem of oxido-reduction in the system fatty-hydroxy-keto acid. Arch. Biochem., **4**, 361–369.

CARSON, S. F., ANTHONY, D. S., KUNA, B., BACHMANN, B. J., AND LONG, M. 1948 Studies on the mechanism of the propionic acid fermentation. Proc. Meetings Soc. Am. Bact., 1948, 12.

CARSON, S. F., AND RUBEN, S. 1940 CO_2 assimilation by propionic acid bacteria studied by the use of radioactive carbon. Proc. Natl. Acad. Sci. U. S., **26**, 422–426.

FROMAGEOT, C., AND BOST, G. 1938 La fermentation du glucose par quelques bactéries propionique croissant en "milieux définis." Enzymologia, **4**, 225–240.

HITCHNER, E. R. 1934 Characteristics of propionic acid bacteria. J. Bact., **28**, 473–479.

JOHNS, A. T. 1948 The production of propionic acid by decarboxylation of succinic acid in a bacterial fermentation. Biochem. J., **42**, Proc. Biochem. Soc., ii–iii.

KREBS, H. A., AND EGGLESTON, L. V. 1941 Biological synthesis of oxaloacetic acid from pyruvic acid and CO_2; the mechanism of carbon dioxide fixation in propionic acid bacteria. Biochem. J., **35**, 676–687.

OSBURN, O. L., WOOD, H. G., AND WERKMAN, C. H. 1936 Determination of volatile fatty acids by the partition method. Ind. Eng. Chem., Anal. Ed., **8**, 270–275.

SHAW, R. H., AND SHERMAN, J. M. 1923 The production of volatile fatty acids and carbon dioxide by propionic acid bacteria with special reference to their action in cheese. J. Dairy Sci., **6**, 303–309.

WERKMAN, C. H., AND WOOD, H. G. 1942 Heterotrophic assimilation of carbon dioxide. Advances in Enzymol., **2**, 135–182.

WOOD, H. G., STONE, R. W., AND WERKMAN, C. H. 1937 The intermediate metabolism of propionic acid bacteria. Biochem. J., **31**, 349–359.

WOOD, H. G., AND WERKMAN, C. H. 1936*a* The utilization of CO_2 in the dissimilation of glycerol by the propionic acid bacteria. Biochem J., **30**, 48–53.

WOOD, H. G., AND WERKMAN, C. H. 1936*b* Mechanism of glucose dissimilation by the propionic acid bacteria. Biochem. J., **30**, 618–623.

WOOD, H. G., AND WERKMAN, C. H. 1938 The utilization of CO_2 by the propionic acid bacteria. Biochem. J., **32**, 1262–1271.

WOOD, H. G., AND WERKMAN, C. H. 1940 The relationship of bacterial utilization of CO_2 to succinic acid formation. Biochem. J., **34**, 129–137.

WOOD, H. G., WERKMAN, C. H., HEMINGWAY, A., AND NIER, A. O. 1940 Heavy carbon as a tracer in bacterial fixation of carbon dioxide. J. Biol. Chem., **135**, 789–790.

WOOD, H. G., WERKMAN, C. H., HEMINGWAY, A., AND NIER, A. O. 1941 Heavy carbon as a tracer in heterotrophic carbon dioxide assimilation. J. Biol. Chem., **139**, 365–376.