CARBON-ISOTOPE EFFECTS IN ENZYME SYSTEMS

II. STUDIES WITH FORMIC ACID DEHYDROGENASE*

JOSEPH L. RABINOWITZ, J. S. LAFAIR, H. D. STRAUSS AND HERBERT C. ALLEN, JR.

Radioisotope Department, Methodist Hospital, Baylor University College of Medicine, Houston, Texas, and Radioisotope Service, Veterans Administration Hospital, Philadelphia, Pa. (U.S.A.)

INTRODUCTION

Carbon-isotope effects have been reported in different biological systems by this and other laboratories. The present study was undertaken to ascertain the percentage error in rate determinations due to isotope effects in the formic acid–formic dehydrogenase system. In our previous publication[1] the average maximum error due to an isotope effect was measured at 10 ± 2% for the urease system. It was therefore of interest to determine if for other molecules containing only one carbon, the average maximum isotope effect would be in the same range. The data obtained during the course of this study indicated that the variations in the specific activity of the carbon dioxide obtained from the reaction at different time intervals and the change in reaction-rate constants for ^{12}C and for ^{14}C fell within the previously determined range. The data obtained was for a formic acid–formic acid dehydrogenase enzyme system at 37° C in a pH 7 phosphate buffer.

$$\begin{array}{lcl} H^{14}COOH & \text{Formic acid dehydrogenase} & {}^{14}CO_2 \\ & \xrightarrow[(KNO_3 \text{ and DPN})]{37^\circ\text{ C} \quad 0.1\ M \text{ Phosphate}} & \\ H^{12}COOH & & {}^{12}CO_2 \end{array}$$

The effects of varying $^{14}C/^{12}C$ concentration on the substrate were also studied.

Many studies concerning the mode of action of formic acid dehydrogenase on formic acid have been reported, and some studies have recently reported differences due to the presence of ^{2}H in the formate[2]. Very large amounts of partially purified extracts of a vegetable enzyme system were used in this study for each run. This was necessary since our assay technique was dependent upon the collection of the CO_2 as $BaCO_3$ and a minimum of 16 mg of $BaCO_3$ was required for plating purposes. The reactions were carried out as described in the experimental part and were shown to be reproducible.

EXPERIMENTAL

Labeled formic ^{14}C acid in the form of sodium formate was dehydrogenated by formic dehydrogenase in the presence of sodium nitrate and diphosphopyridine nucleotide. The resulting CO_2 freed from the formic acid was flushed with the aid of oxygen (CO_2 free) at a rate of 100 ml/min from the reaction flask into a manifold and bubbled through a centrifuge tube filled with barium hydroxide. The CO_2 obtained as the precipitate $BaCO_3$ was then centrifuged and the precipitate

* All radioactive materials were obtained under allocation of the U.S. Atomic Energy Commission.

References p. 548.

washed several times in order to remove the excess $Ba(OH)_2$. The $BaCO_3$ was dried at 115° C in an oven overnight and the yield by weight was determined for the dried samples. The samples were then plated on special aluminum planchets by means of a pilling machine[1].

The apparatus used in this study has already been described in previous publications[1,3]. Formic acid labeled with ^{14}C was obtained from a commercial source. The formic acid dehydrogenase was prepared from peas according to the method of NASON AND LITTLE[4]. Cell-free extracts were prepared from 100 g of green split-peas which were soaked overnight in water. After decanting the water, the peas were blended with 200 ml of 0.1 *M* Na_2HPO_4 in a Waring blendor for two minutes. The mixture was allowed to stand at room temperature for two hours and then pressed through cheese cloth in order to remove coarse material. Upon centrifuging at 2000 r.p.m. for 30 min, a light green supernatant containing the enzyme was obtained.

RESULTS

The greatest variations in the specific activity of the recovered carbon dioxide were observed during the early phases of the reaction. With time the specific activity of the obtained carbon dioxide approached an asymptote and then maintained a constant value (Fig. 1). Each experience was repeated at least once and the variations in total activity observed are attributed to the variable enzyme content of the different preparations. The curves plotted for the observed data were all of the same general shape and the reaction rate constants calculated for similar runs showed minor deviations.

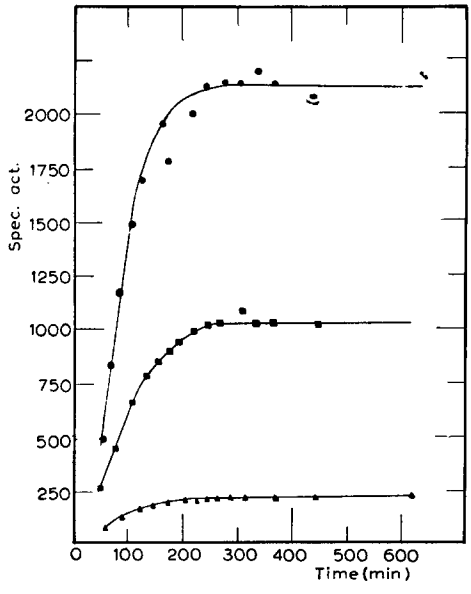

Fig. 1.
● 100 μc/mmole · 0.66 scale
■ 4 μc/mmole
▲ 8 μc/mmole

Due to the ease of denaturation of this enzyme system, it was necessary to prepare a new "enzyme batch" for each individual experiment. The "enzyme batches" in all cases contained endogenous formic acid, and therefore, the added ^{14}C-formate activity was diluted and an additional mixing period was required. In order to determine whether some of the carbon dioxide obtained was due to the action of the formic dehydrogenase enzyme on a large amount of endogenous formic acid, or to a generalized non-specific decarboxylating mechanism of the enzyme on other substrates present, an experiment was performed in which ^{14}C-labelled acetate was

References p. 548.

substituted for the ^{14}C-formate in the reaction. Although the amount of carbon dioxide which was collected as barium carbonate was appreciable in two hours, there was very little radioactivity associated with each of the samples collected. This run gave evidence for the presence of endogenous formate, and helped evaluate the content for each experiment. The added radioactive formate was in all cases diluted by a factor, approximately 3 to 4 fold of the $^{14}C/^{12}C$ ratio.

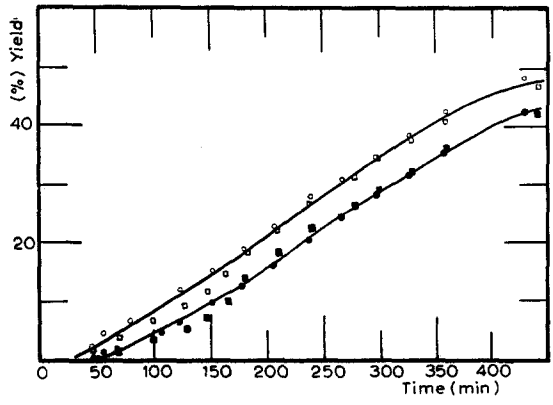

Fig. 2.

	^{12}C	^{14}C
Exp. 1	○	●
Exp. 2	□	■

(^{12}C by weight) (^{14}C by activity)

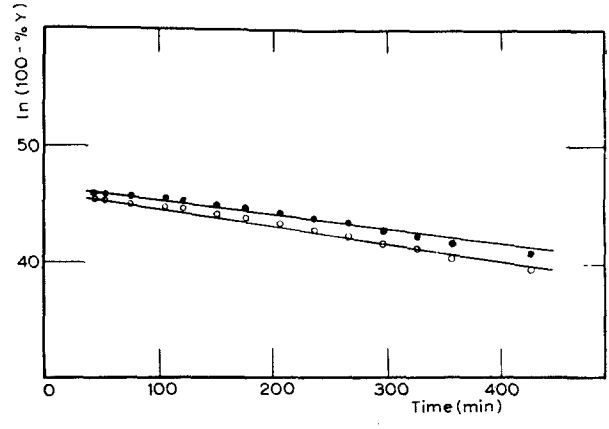

Fig. 3.

● ln (100 — % $Y_{^{14}C}$)
$k_{^{14}C} = 1.25 \cdot 10^{-3}$ min^{-1}

○ ln (100 — % $Y_{^{12}C}$)
$k_{^{12}C} = 1.50 \cdot 10^{-3}$ min^{-1}

Figs 1, 2, 3 and Tables I, II, and III list the data from some of the experiments. The observed deviations for the ^{14}C-formate rate activity were negative compared to the ^{12}C-formate and in all the studied cases the approximate value of the 21 $\pm$ 3% was found. This effect included all estimated errors due to randomness in the counting and timing of the samples, efficiency, etc. The atom-percentage excess of ^{14}C over ^{12}C in these studies involved variation of up to two orders of magnitude. The full activity level of the stock solution (100 μc/mmole) and levels 1/25 (4 μc/mmole) and 1/125 (0.8 μc/mmole), of the full level were used. In all experiments 12.5 ml of the labelled formate which had a concentration of 13.6 mg/ml was used. The isotope effect remained approximately constant through different concentrations and the variations in the rate constants were minimal.

The rate constants were determined by both graphical and analytical methods

References p. 548.

TABLE I

OBSERVED VALUES OF THE YIELDS BY WEIGHT (^{12}C) AND TOTAL ACTIVITY (^{14}C) OF THE BARIUM CARBONATE COLLECTED AS A FUNCTION OF TIME AND THE CORRESPONDING VALUES OF THE SPECIFIC ACTIVITY OF THE SAMPLES

Time min	*Weight mg*	*% Yield ^{12}C*	*S.A. counts/min/mg*	*T.A. 10^3 counts/min*	*% Yield ^{14}C*
45	46.2	2.3	700	32.3	0.5
55	89.6	4.5	1230	85.7	1.4
78	131.6	6.7	1740	158.6	2.5
108	181.1	9.2	2230	269.2	4.3
123	239.6	12.2	2530	417.2	6.6
153	309.6	15.7	2940	623.0	9.9
178	384.6	19.5	2690	824.6	13.1
208	453.2	23.0	2997	1030.2	16.3
238	539.4	27.4	3240	1309.7	20.8
268	615.6	31.2	3220	1554.7	24.6
298	696.4	35.3	3330	1815.7	28.8
328	769.2	39.0	3240	2059.1	32.7
358	855.1	43.4	3210	2334.7	37.0
428	869.6	49.0	3110	2690.9	42.6
∞	1972.0	100.0	3230	6310.4	100.0

The above data is for the reaction utilizing 12.5 ml of the formate solution which had a concentration of 100 μc/mmole. S.A. is the specific activity and T.A. is the total activity.

TABLE II

RESULTS FROM A RUN USING 12.5 ml OF 4 μc/mmole FORMATE

Time min	*Weight mg*	*% Yield ^{12}C*	*S.A. counts/min/mg*	*T.A. 10^3 counts/min*	*% Yield ^{14}C*
50	41.0	2.1	253	10.4	0.5
70	75.1	3.8	451	25.8	1.3
100	121.4	6.2	666	56.7	2.8
130	174.8	8.9	777	98.0	4.9
150	219.4	11.1	847	135.7	6.7
165	280.7	14.2	901	190.9	9.5
180	374.5	19.0	952	280.2	13.9
210	482.7	24.5	997	388.1	19.3
240	568.2	28.8	1024	475.7	23.6
270	621.0	31.5	1020	529.6	26.3
300	691.8	35.1	1096	607.2	30.1
330	744.5	37.8	1008	660.3	32.8
360	807.3	40.9	1009	723.7	35.9
445	938.1	47.6	999	854.3	42.4
∞	1972	100	1022	2,015.4	100

for the first order reaction. The rate constants for various time intervals were calculated and the mean values were found to be $k_{^{12}C} = 1.51 \cdot 10^{-3}$ min^{-1} and $k_{^{14}C} = 1.19 \cdot 10^{-3}$ min^{-1}. These values gave an approximate carbon isotope effect of 21%. The analytical expression used to evaluate the rate constants was:

$$k = (1/t) \ln (100 - \% Y)/100 = 1/t \ln (R_\infty - R_t)/R_\infty$$

t = time from start of the reaction.

$\% Y$ = per cent yield of the product by weight (^{12}C) or by total activity (^{14}C).

R_t and R_∞ = actual value of the readings of weight or activity at time t and at theoretical completion of experiment.

References p. 548.

TABLE III

RESULTS FROM USING A 0.8 μc/mmole SOLUTION OF FORMATE

Time min	*Weight mg*	% *Yield* ^{12}C	*S.A. counts/min/mg*	*T.A. counts/min*	% *Yield* ^{14}C
60	54.2	2.8	73	3.5	0.87
90	124.7	6.3	112	11.4	2.9
120	172.3	8.7	144	18.3	4.6
145	208.4	10.6	159	24.0	6.0
175	249.9	12.7	171	31.1	7.8
205	292.4	14.8	180	38.8	9.7
225	329.0	16.7	179	45.4	11.3
245	380.8	19.3	192	55.4	13.8
260	455.0	23.1	202	70.4	17.6
285	522.3	26.5	201	83.9	21.0
310	584.3	27.6	202	96.4	24.1
370	698.2	35.4	204	119.6	30.0
440	810.2	41.1	212	143.3	35.8
705	942.6	47.8	215	171.8	43.0
720	1042.5	52.9	193	191.1	47.7
∞	1972.0	100	203	400.3	100

DISCUSSION

The variation in specific activity of the formed CO_2 and the difference in the observed reaction rate constants for the formic acid–formic dehydrogenase system indicates a more rapid utilization of the ^{12}C-formic acid. The values determined were close to those that have been previously reported. It may be suggested that this difference in reaction rate between the ^{12}C- and ^{14}C-formates was in reality more of a result of the absorption–desorption characteristics of formate or of CO_2 on the enzyme rather than due to a different rate of utilization. On the other hand, it may be assumed that the total observed effect is the summation of the various mass effects due to the $^{12}C/^{14}C$ atomic-weight ratio. Of course, fortuitous cancellations may have occurred that could have reduced this overall result to the values found rather than the theoretical one[5].

ACKNOWLEDGEMENTS

The authors are happy to acknowledge the help from a grant-in-aid of the Smith, Kline and French Foundation and the technical assistance of O. OLEKSYSHYN. The advice and encouragement of Dr. JOHN MILLER and of Dr. CLARK GRIFFIN is deeply appreciated.

SUMMARY

It has been shown that formic acid ^{12}C is utilized more rapidly by the formic acid dehydrogenase enzyme system than is formic acid ^{14}C. Regardless of the $^{14}C/^{12}C$ concentration ratios of the material, the observed reaction rate constants were recorded as $k^{12}C = 1.51 \cdot 10^{-3}$ and $k^{14}C = 1.19 \cdot 10^{-3}$ at 37° C at pH 7 in phosphate buffer.

REFERENCES

1 J. L. RABINOWITZ, T. SALL, J. N. BIERLY, JR. AND O. OLEKSYSHYN, *Arch. Biochem. Biophys.*, 63 (1956) 437.
2 H. AEBI, EVA FREI AND MARLIES SCHWENDIMANN, *Helv. Chim. Acta*, 39 (1956) 1765.
3 J. L. RABINOWITZ, *Anal. Chem.*, 29 (1957) 982.
4 A. NASON AND H. N. LITTLE, in S. A. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press Inc., New York, 1955, p. 536.
5 J. BIGELEISEN, *Science*, 110 (1949) 14.

Received October 11th, 1957