Carbon Isotope Effects in Biological Systems

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TRACER STUDIES are subject to error when labeled and unlabeled molecules differ quantitatively in their chemical and physical properties. Such differences in behavior are related to atomic mass and are called isotope effects. This phenomenon has been subjected to rather rigorous theoretical treatment in the case of simple chemical reactions (*1–3*). The application of precise physicochemical theory to biological isotope effects is not likely to be so successful, and for this reason most of the existing knowledge in this field has been obtained empirically. Isotope effects have been studied in biological reactions involving hydrogen (*4, 5*), oxygen (*6*), nitrogen (*7*), potassium (*8*), and carbon (*6, 9–14*).

In the case of carbon-14, kinetic studies have shown very striking differences in the rates of uptake of $C^{14}O_2$ and $C^{12}O_2$ by barley seedlings (*13*) and algae (*13, 14*). In the latter work, Weigl found that when a small inoculum of the microscopic plants had, through growth, converted 70 per cent of the available CO_2 to organic material the specific activity of the plant carbon was 24 per cent less than that of the dissolved inorganic carbon. Mass spectrometric analyses for carbon-13 on the same samples showed a difference of only 4 per cent in the same direction. The latter figure is in satisfactory agreement with results of other workers (*6, 11, 12*) when allowance is made for kinetic effects (*vide infra*), but on theoretical grounds the fractionation factor for carbon-14 is expected to be approximately twice the carbon-13 factor (*3, 15*).

Other investigators have found lesser biological isotope effects with carbon-14 in steady state systems. In studies on the worldwide distribution of this isotope, Libby and his associates have measured the radioactivity of natural carbon sources (*9, 10*). They found that specimens of wood had 7.3 ± 3.0[3] per cent less radioactivity on a carbon basis than sea shell carbonate samples. Very recently Kulp, Feely, and Tryon (*16*) have found modern wood to contain 9.7 ± 1.5[3] per cent less natural radiocarbon than clam shells.

The present work is a brief survey of isotope effects with carbon-14 in biological reactions and, in addition, compares the fractionation factor of carbon-13 with the carbon-14 factor. An attempt has been made to account for some apparently conflicting results of other workers.

"Steady state" experiment. A 25-gallon aquarium was provided with a mercury thermoregulator, a glass cooling coil, and connections of glass tubing, all passing through a Lucite top. Before sealing the tank a 2-inch layer of washed quartz sand was placed uniformly over its bottom, and the fluid from a small open aquarium added to provide microorganisms. Small numbers of several plants, including *Vallisneria* sp. (eel grass), *Ceratophyllum demersum*, and *Lemna minor* (duck weed), were placed in the water, and 5 gallons of a heavy culture of *Scenedesmus obliquus* were added. The medium in which these algae had grown contained $NaHC^{14}O_3$ as the sole carbon source. After the top had been fastened in place with a cemented gasket and clamps, the tank when tested for leakage held a small positive pressure of air for several hours. Tap water was circulated through the cooling coil, and the thermoregulator, set at 22°, was connected to a relay that activated an incandescent flood lamp. The latter served as a heater as well as an illuminator. A small fluorescent lamp was placed adjacent to the tank as a constant source of light. A few days after the tank was sealed, 2 pairs of guppies and about 100 small planorbid snails (*Helisoma* sp.) were added through one of the glass tubes.

During the first year the water in the aquarium was heavily clouded with green algae, and the guppies became quite numerous. During the second year, as the higher plants grew and multiplied, the algae thinned out and the fish population diminished. During the final year the water remained clear, and *Vallisneria* became the predominant plant. The fish disappeared during the middle of the third year. During the second year a filamentous alga, *Oscillatoria* sp., appeared in the vicinity of the fluorescent lamp. After a heavy growth had accumulated, the lamp was moved to the opposite side of the tank, and the alga grew there and diminished at the first site. The snail population remained relatively constant for the first 2 years, but during the final year tended to diminish in both size and number. Dead snail shells accumulated over the 3-year period, and the algal sediment gradually became darker in color until it was almost black. The foregoing indicates that a continuous turnover of carbon was taking place, but that mixing was probably never complete, some material such as snail shells and organic sediment becoming relatively inaccessible to metabolic turnover.

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[3] Standard error.

At the end of the third year various samples were removed for analysis. The determinations were carried out by means of proportional gas counting, as described elsewhere (*17*). To prevent contamination of specimens with air CO_2, certain precautions were taken. Water samples were withdrawn through one of the sampling tubes into evacuated flasks before the tank was opened. The other samples were withdrawn with forceps, hooks, and siphons fitted with diaphragms that covered the tank orifice during manipulations. The samples were quickly placed in vials, sealed, and then frozen before drying. In addition to these precautions, tests were conducted to determine whether the specific activity of the plants changed with time when left exposed to room air. No change was noticed in 2 hours, and only a slight diminution was found after 2 days.

Multiple samples of the more abundant organisms were taken separately and analyzed separately. The data obtained are summarized in Table 1. Each of the recorded standard errors was calculated from the results of a replicated series of analyses and was not derived by the application of the statistics of counting, as is customary in reporting radioactivity measurements. The error estimates, therefore, include all random manipulative error as well as genuine sample variation, if any.

TABLE 1

SPECIFIC ACTIVITIES OF SPECIMENS IN AN ISOTOPICALLY LABELED AQUARIUM AFTER PROLONGED ISOLATION FROM EXTRANEOUS CARBON SOURCES

Sample	No. of samples assayed	Specific Activity	
		(cts/min/mM)	Relative (% of dissolved CO_2)
CO_2 dissolved in water	8	204,300 ± 500*	100 ± 0.3*
Plants			
Vallisneria			
Tops	7	192,300 ± 900	94.0 ± 0.5
Middle leaf sections	5	193,000 ± 800	94.5 ± 0.4
Roots	3	194,600 ± 400	95.3 ± 0.2
Stalk bases	4	196,900 ± 900	96.4 ± 0.5
Whole young leaves	2	194,500 ± 1000	95.2 ± 0.5
Ceratophyllum	1	198,500	97.2
Lemna minor	1	195,600	95.7
Oscillatoria	5	197,300 ± 500	96.6 ± 0.3
Helisoma (living snails)			
Shell carbonate	8	208,600 ± 300	102.1 ± 0.2
Organic matter	4	196,100 ± 1000	96.0 ± 0.5
Dead snail shells		220,000†	
		234,100	
		221,100	
		234,400	
Organic sediment		245,600†	
		240,400	
		250,500	

* Standard error of mean.

† Separate analyses.

Consistent with all the published data discussed above, these results show that plants tend to reject the heavier isotope in favor of carbon-12. The results with *Vallisneria* show that the heavy stalks at the base of the plants had a slightly higher specific radioactivity than the tops, and the central leaf section was intermediate. This is presumptive evidence that the isotope content of available CO_2 was slowly diminishing during the growth of these plants. This may have been partly the result of slow turnover in the original unlabeled organisms and partly the result of the acquisition of unlabeled CO_2 by the system from undetected sources, e.g., leakage or through bacterial action on the aquarium cement or gasket. The analyses on the dead snail shells and organic sediment definitely show that a true steady state had not been attained during the 3-year period. However, it seems likely that the rate of change was very slow at the end of this time, and that a comparison between the CO_2 in solution and recently growing organisms gives a close estimate of the actual isotope effect in a steady state.

Although plants and the organic matter of snails appeared to reject the heavier isotope, the carbonate of the shell tended to concentrate it to some extent. In living snails the difference in specific activity between the organic material and the shell carbonate was 6 per cent, and the difference between recently grown plant tissue and shell carbonate was about 8 per cent. These differences in isotope concentration are approximately twice those found with carbon-13 (*6, 11, 12*) in similar materials and are in agreement with the observations of Anderson and Libby (*9*), and of Kulp and associates (*16*) on the natural abundance of carbon-14 in material of this type.

None of these data shows as spectacular differences as were found by Weigl and Calvin (*13*) and Weigl (*14*). Since this type of discrepancy could have resulted from differences in kinetics, the isotope dynamics in a sealed system of growing algae were studied in a second experiment.

Isotope kinetics during algal growth. A 12-gallon Pyrex carboy was fitted with a rubber stopper holding a glass cooling coil, thermoregulator, thermometer, and 3 lengths of glass tubing for sampling. A magnetic stirrer was inserted, and 30 liters of C^{14}-labeled inorganic culture medium were added to the bottle. The stopper assembly was tightly wired on the carboy, and the thermoregulator set at 22°. Refrigerated water was circulated through the cooling coil, and 2 reflector flood lamps activated by the thermoregulator were used to maintain the temperature as well as to furnish light. Before algae were added, analyses of the CO_2 present in solution on 3 successive days showed the mean specific activity to be $50{,}530 \pm 10^3$ cts/min/mM of carbon. The medium was then inoculated with 90 mg (dry weight basis) of radioactive *Scenedesmus obliquus*. The specific radioactivity of this inoculum was within 2 per cent of the activity of the medium, thus minimizing isotope dilution error during early sampling. Samples of the culture were taken at intervals during this period and analyzed for concentration and radioactivity of both CO_2 and algae. The pH of each sample was measured and 50 ml of *N* HCl were added each time the pH rose above 7.0.

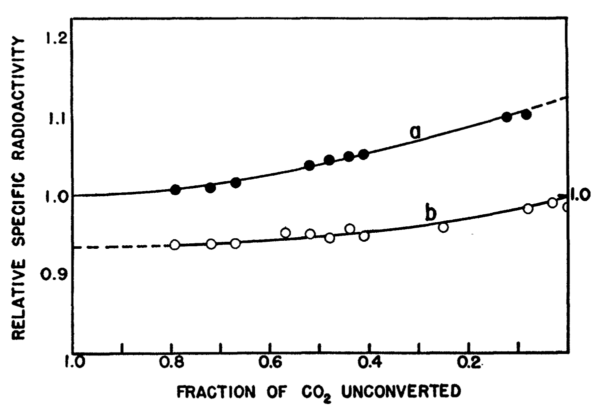

FIG. 1. Change in specific radioactivities of dissolved CO_2-bicarbonate (curve a) and of algal carbon (curve b) during growth. (Relative specific radioactivity 1.0 equals 59,530 cts/min/mM of carbon.)

Figure 1 shows the specific radioactivity of inorganic carbon and of algae relative to the initial level as a function of the fraction of CO_2 unconverted to algal carbon. The first samples, taken after 21 per cent of the total CO_2 had been converted, showed that the radioactivity in the organic carbon was 7 per cent lower than in dissolved CO_2 and bicarbonate. At this stage of growth the original inoculum was diluted 30-fold, and its contribution could not have altered the algal specific radioactivity by more than 0.1 per cent. As growth progressed, the difference gradually increased and was 11 per cent when 92 per cent of the CO_2 had been converted. Allowing for experimental error, the final algal specific radioactivity was equal to that of the CO_2 initially.

The gradual increase with growth of the difference in isotope content makes it necessary to extrapolate the data to the point where conversion first began in order to estimate the true difference between the conversion rates of $C^{14}O_2$ and unlabeled CO_2. A plot of the logarithm of the specific radioactivity ratios of algal to inorganic carbon against the fractional conversion was found to be approximately linear. A regression analysis of these data showed the initial ratio to be 0.935 ± 0.005[3]. This ratio is in satisfactory agreement with the most reliable value of Table 1, that of recently grown *Vallisneria* shoots, 0.940 ± 0.005[3] (relative to dissolved CO_2).

Comparison of the isotopes effects with carbon-13 and carbon-14. For this comparison the algal growth experiment was repeated. Samples of CO_2 and algae were taken on two successive days when more than half of the inorganic carbon had been incorporated into the plants. The samples were converted to CO_2, and the gas was divided and analyzed for specific radioactivity by gas counting (*17*) and for carbon-13 abundance by mass spectrometry. The mass spectrometer was one of those used by Urey (*18*) and his group for the measurement of small changes in isotope abundance ratios in which the maximum experimental deviation is within ± 0.01% of the absolute abundance. A correction was applied for the presence of $C^{12}O^{16}O^{17}$. For each pair of CO_2 samples the one which was originally inorganic was used as a standard against which the mass abundance of the algal CO_2 was measured. To ensure that no systematic error was present in the mass spectrometer measurements, one pair of carbon dioxide samples was referred to A. O. Nier at the University of Minnesota. The absolute abundance values he obtained confirmed the results by the direct difference method.

TABLE 2

COMPARISON OF ISOTOPE EFFECTS WITH CARBON-14 AND CARBON-13

Sample No.	C^{14} abundance			C^{13} abundance	C^{14} abundance difference
	CO_2 c/m/mM	Algal carbon c/m/mM	Difference % of CO_2	Difference % of CO_2	C^{13} abundance difference
1A	73,510	65,730	10.6	4.20	2.52
		65,990	10.2		2.43
1B	73,240	66,580	9.1	4.25	2.14
		65,830*	10.1		2.38
2A	74,030	66,400	10.3	4.39	2.35
	73,660*	66,160*	10.4		2.37
2B	74,070	66,320	10.5	4.37	2.40
2C	74,280	65,920	11.3	4.46	2.53
	74,210*	66,160*	10.8		2.42
				Av	2.39 ± 0.04†

* CO_2 absorbed in ethylenediamine and liberated with H_2SO_4.
† Standard error.

The results appear in Table 2 and show the carbon-14 effect to be distinctly more than double the carbon-13 effect. The mean ratio of the separate isotope effects is 2.39 ± 0.04[3]. The value is not in agreement with the theoretical separation factor of 1.98 based on mass difference alone (*2, 15*). This deviation might be partially accounted for by the necessarily different methods of preparation of CO_2 from the inorganic and organic samples, but the analysis of several compounds with this method has given a recovery error much lower than would account for this variation from the theoretical value. Furthermore, absorption of some of the samples in carbonate-free alkali (*19*) with subsequent evacuation and collection of neutral impurities gave entirely negative results (< 0.1%). Subsequent acidification and collection of the gas evolved gave CO_2 with no significant change in activity except in sample 1B, where the second analysis was *lower* and more consistent with its duplicate 1A. This deviation from the theoretical value has been noted recently by Stevens *et al.* (*20*), who have carried out experiments specifically designed to evaluate this factor. These workers chose the partial decarboxylation of mesitoic acid as a fractionating system and obtained a value of 2.66 ± 0.15^4 for the ratio. In this work both isotopes were measured mass spectrometrically.

It is implied in the discussion by Bigeleisen (*1*) that the apparent isotope effect at any stage of a reaction is influenced by the degree of reversibility. In biological reactions such as the ones studied here the extent of reversibility, a quantity difficult to estimate, probably depends to some extent upon the overall rate of the process. Since the rate may vary with the experimental conditions, separate experiments may give different results. A theoretical consideration of the algae experiment allows an evaluation of the extent to which reversibility could have influenced the data. To simplify the mathematical consideration the conversion of inorganic to algal carbon will first be regarded as irreversible and then as completely reversible.

The irreversible conversion is satisfactorily dealt with by the Rayleigh equation (*21*), originally applied to batch distillations. As employed in the present situation the equation may be written:

$$\ln C = \int_{x_0}^{x} \frac{dx}{y - x}, \qquad (1)$$

where C is the fraction of the original CO_2 that remains in the inorganic form, x is the specific radioactivity of the CO_2, and y is the specific radioactivity of carbon *as it enters the algae.* Because the mole fraction of $C^{14}O_2$ remains exceedingly low, the radioactivity of carbon transferred to the algae may be represented by the equation:

$$y = bx, \qquad (2)$$

where b is a constant. Substituting in equation (1) and integrating:

$$\ln C = \frac{1}{b-1} \ln \frac{x}{x_0}; \qquad (3)$$

$$\frac{x}{x_0} = C^{b-1}. \qquad (4)$$

By material balance:

$$\bar{y}(1 - C) + xC = x_0, \qquad (5)$$

where $\bar{y}$ is the specific radioactivity of the *total* algae present. By substitution from equation (4) this gives:

$$\frac{\bar{y}}{x_0} = \frac{1 - C^b}{1 - C}. \qquad (6)$$

Regarding the synthetic processes of algal growth to be irreversible, equations (4) and (6) give the respective specific radioactivities of CO_2 and the total algal carbon as a function of the fractional quantity of CO_2 remaining in the system.

If the conversions were totally reversible, the specific radioactivity of the *total* algal carbon should remain a constant fraction of the specific radioactivity of the remaining CO_2. When bx is substituted for $\bar{y}$ in (5), the specific radioactivity of CO_2 is defined by:

$$\frac{x}{x_0} = [b(1 - C) + C]^{-1}; \qquad (7)$$

and similarly:

$$\frac{\bar{y}}{x_0} = b[b(1 - C) + C]^{-1}. \qquad (8)$$

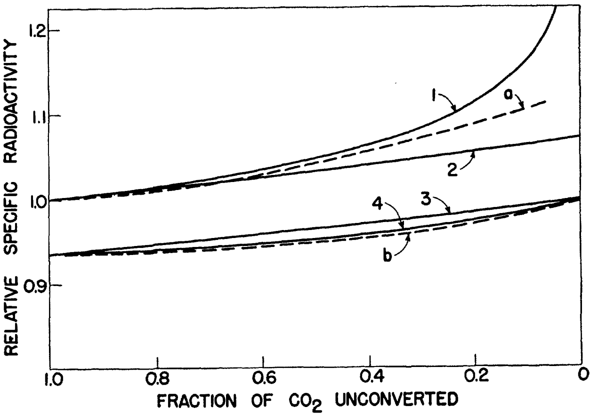

FIG. 2. Change in theoretical specific radioactivities of dissolved CO_2-bicarbonate and algal carbon with growth: 1, CO_2-bicarbonate during "irreversible" growth (equation 4); 2, CO_2-bicarbonate during reversible growth (equation 7); 3, algal carbon during "reversible" growth (equation 8); 4, algal carbon during "irreversible" growth (equation 6); a, CO_2-bicarbonate, experimental; b, algal carbon, experimental (see Fig. 1).

Use of the empirically derived value of b, 0.935, in equations (4), (6), (7), and (8), respectively, results in the 4 unbroken curves of Fig. 2 for inorganic and algal carbon predicted from the limiting conditions. The experimental data are repeated in Fig. 2 as broken curves. A comparison of the hypothetical curves with the measured specific activity of CO_2 during the final half of the growth period seems to show that, under these conditions, some of the conversion is reversible and some is not. However, the difference between the CO_2 in solution and the algal carbon from our theoretical curves for irreversible growth after 70 per cent conversion is only 11 per cent, as contrasted with the value of 24 per cent computed from Weigl's data (*14*). In 12 separate comparisons of CO_2 from the gas phase with CO_2 in the medium, we found the isotope level to be only 1.58 ± 0.03^3 per cent lower in the gas phase, whereas Weigl found this difference to be 7 per cent. In Weigl's experiment the large isotope effect with carbon-14 was accompanied by a carbon-13 effect similar to ours. For these reasons it must be concluded that our carbon-14 results are in substantial disagreement with his.

Data such as these cannot be interpreted so as to allow a prediction of the biological isotope effect in other experiments. The need for evaluation of the isotope effect depends upon the type and goal of the research. In many investigations where carbon-14 is

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[4] Error estimates not specified.

used as a tracer, the effect may be neglected because small differences do not influence the interpretation of results. In other types of experiments, such as those involving retention and excretion of potentially radiotoxic carbon compounds, interest is primarily centered on the behavior of the isotope per se and, since isotope effects contribute to this behavior, it is unnecessary to evaluate them. In a few types of experiments, however, especially those in which kinetic tracer data are employed for a quantitative interpretation of a natural process, isotope effects should be experimentally evaluated and considered in the interpretation.

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Forest Ray Moulton: 1872–1952

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Dr. F. R. Moulton was born in 1872 on a farm in the clearing forest of southern Michigan, now the village of LeRoy. He was the first child in the family, followed by four brothers, all five brothers being recorded in *Who's Who in America.* F. R. Moulton graduated from Albion College in 1894. He entered the University of Chicago for his graduate training, and received his Ph.D. in astronomy and mathematics in that University in 1899. But three years earlier (1896) he was appointed an Assistant in that University, and he continued to serve for 30 years on that faculty, from Assistant to full Professor, until he resigned in 1926. Dr. Moulton was an outstanding teacher, both to undergraduates and graduate students.

Even sixty years ago there were a number of outstanding scientists on the faculty of the University of Chicago, none greater than the geologist T. C. Chamberlin. At the turn of the century Dr. Moulton collaborated with Dr. Chamberlin in developing the challenging *Planetesimal Hypothesis* of solar system evolution.

In 1923 sixteen members of the faculties in the natural sciences (including psychology) collaborated in planning and giving a six-month elective course for college freshmen, called *The Nature of the World and of Man.* Dr. Moulton, as one of the sixteen, presented astronomy. After two years experience with this new type of college course, each of the sixteen faculty participants agreed to prepare a chapter on his specific subject for a book. When the sixteen drafts were ready, all the participants spent one evening a week for four months in commenting and criticizing each chapter. Dr. Moulton proved himself a very competent critic, revealing his basic understanding of all phases of the natural sciences, even though his own specialty was mathematical astronomy. This may be called a *freshman experience,* very useful for Dr. Moulton's later significant service as Secretary of our Association. In 1937 Dr. Moulton became the editor of the revised edition of the book, *The Nature of the World and of Man,* now issued under the title, *The World and Man as Science Sees Them.*

When Dr. Moulton resigned from the University of Chicago faculty in 1926, he became a business man, as the financial director of the Utilities Power and Light Corporation of Chicago. In 1932 he became the Director of Concessions of the Chicago World's Fair. The concessions were the main source of income to meet the great expenses of the Fair, and the financial depression added to Dr. Moulton's burdens. He came out financially a victor, but with a serious coronary heart injury, from which he made a very good recovery. Evidently there was more to that man, F. R. Moulton, than a superior cerebrum.

When Dr. Moulton became the permanent secretary