ISOTOPIC TRACER TECHNIQUE

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Isotopes were first used as tracers in 1913 when G. Hevesy and F. A. Paneth [1] applied the inseparability of the isotopes lead and radium-*D* to determine the solubility of "insoluble" lead salts. Later, Hevesy and L. Zechmeister [2] demonstrated the lack of exchange between the lead atoms of tetraphenyl-lead and lead ions. The first biological problem studied with this tracer method was the uptake and release of lead (labelled with thorium-*B*) by the roots of bean plants.[3]

Following the discovery of deuterium, and later of the heavy isotopes of nitrogen and carbon, important advances were made in our understanding of metabolic processes. Deuterium was immediately applied to investigate the behaviour of water in biological systems. With the use of D_2O, the rapid exchange of the body water of a goldfish and its surroundings could be observed.[4] Water metabolism in humans was also studied, and the "average life" of water molecules determined as about 14 days.[5]

Synthesis of organic compounds containing stable C–D bonds enabled fat and fatty acid metabolism to be studied.[6] These results, taken with those [7] from studies of the metabolism of amino-acids labelled with ^{15}N, provided the basis for the new concept of the "dynamic state of body constituents".[8]

The introduction of the carbon isotopes ^{11}C and ^{14}C in 1939 and 1940 respectively made possible a new approach to problems of photosynthesis,[9] carbon dioxide fixation,[10] and fatty-acid metabolism.[11] Isotopes of other elements of biological significance (*e.g.*, ^{32}P, ^{131}I) are now readily available and have found extensive and no less important applications.

The field of tracer chemistry is now too broad to be considered in a single Review; this Review will describe practical considerations in the use of stable isotopes, the methods available for the synthesis of compounds containing stable isotopes (except that ^{14}C will be included to give a com-

[1] *Z. anorg. Chem.*, 1913, **82**, 322. [2] *Ber.*, 1920, **53**, 410.
[3] G. Hevesy, *Biochem. J.*, 1923, **17**, 439.
[4] G. Hevesy and E. Hofer, *Z. physiol. Chem.*, 1934, **225**, 28.
[5] *Idem, Klin. Wochenschr.*, 1934, **13**, 1524.
[6] R. Schoenheimer and D. Rittenberg, *Science*, 1935, **82**, 156; *J. Biol. Chem.*, 1935, **111**, 163.
[7] R. Schoenheimer and S. Ratner, *ibid.*, 1939, **127**, 301.
[8] R. Schoenheimer, "The Dynamic State of Body Constituents", Harvard University Press, Cambridge, Massachusetts, 1946.
[9] S. Ruben, W. Z. Hassid, and M. D. Kamen, *J. Amer. Chem. Soc.*, 1939, **61**, 661; S. Ruben and M. D. Kamen, *Proc. Nat. Acad. Sci.*, 1940, **26**, 418; A. W. Frenkel, *Plant Physiol.*, 1941, **16**, 654.
[10] H. G. Wood, C. H. Werkman, A. Hemingway, and A. O. Nier, *J. Biol. Chem.*, 1940, **135**, 789.
[11] H. A. Barker and M. D. Kamen, *Proc. Nat. Acad. Sci.*, 1945, **31**, 219.

plete account of syntheses with isotopic carbon), and a discussion of the limitation of the methods.

Analysis of Stable Isotopes

The detection and analysis of radioactive isotopes are already well described [12] and only D, ^{15}N, ^{13}C, and ^{18}O will be discussed here. In early work with deuterium, analyses were based on the difference in refractive index [13] of D_2O and H_2O, or more conveniently, the difference in density.[14] Of the many densimetric methods available, the most useful is probably the falling-drop method,[15, 16] an accuracy of 1 p.p.m. being possible. In the case of organic compounds, a representative sample of the hydrogen and deuterium must be obtained as water. The dried compound is burnt in hydrogen-free, dry oxygen, over copper oxide. The water is condensed, and rigorously purified by repeated vacuum-distillation.[15]

In the case of ^{15}N and ^{13}C mass-spectrometric analysis is necessary. Aston's mass spectrographs were hardly suited to the routine determination of isotope content, and it was preferable to use an electrical, rather than a photographic, method for the detection of ion beams. Such a spectrometer was first constructed by D. Rittenberg and his colleagues,[17] being modelled after W. M. Bleakney's instrument.[18] Further advances in mass spectrometer design, particularly by A. O. Nier [19] and R. L. Graham *et al.*,[20] have led to commercially available instruments.

In operation, a gas sample at a pressure of a few cm. of mercury is allowed to leak through a fine capillary into the spectrometer tube, which is held at a high vacuum (10^{-7} to 10^{-8} mm. Hg).

The methods used to prepare suitable gas samples will now be described briefly.

Deuterium.—The mass-spectrometric analysis of deuterium has the special advantage that very much smaller samples may be analysed than by the other methods. As little as 5 mg. of an organic compound is burnt in a micro-combustion tube. The water is condensed in a cold trap, and then distilled over hot zinc at 385°. The hydrogen–deuterium mixture is

[12] M. D. Kamen, "Radioactive Tracers in Biology", Academic Press Inc., New York, 1947; G. Hevesy, "Radioactive Tracers, Their Applications in Biochemistry, Animal Physiology and Pathology", Interscience Publishers Inc., New York, 1948; M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert, and P. E. Yankwich, "Isotopic Carbon", J. Wiley & Sons, Inc., New York, 1949.

[13] R. H. Crist, G. M. Murphy, and H. C. Urey, *J. Amer. Chem. Soc.*, 1933, **55**, 5060; *J. Chem. Physics*, 1934, **2**, 112.

[14] D. Rittenberg and R. Schoenheimer, *J. Biol. Chem.*, 1935, **111**, 169.

[15] A. S. Keston, D. Rittenberg, and R. Schoenheimer, *ibid.*, 1937, **122**, 227.

[16] M. Cohn, "Preparation and Measurement of Isotopic Tracers", J. W. Edwards, Ann Arbor, Michigan, 1946, p. 51.

[17] D. Rittenberg, A. S. Keston, F. Rosebury, and R. Schoenheimer, *J. Biol. Chem.*, 1939, **127**, 291.

[18] *Physical Rev.*, 1929, **34**, 157; 1932, **40**, 496.

[19] *Rev. Sci. Instr.*, 1940, **11**, 212; 1947, **18**, 398.

[20] R. L. Graham, A. L. Harkness, and H. G. Thode, *J. Sci. Instr.*, 1947, **24**, 119.

collected in a vacuum system and compressed into a sample bulb with the aid of a Toepler pump.[21]

Some special difficulties in the mass-spectrometric determination of deuterium have been discussed recently by H. W. Washburn.[22] The analyses are made by comparison of the intensities of the ion beams of mass 3 (HD) and 2 (H_2) and are accurate to $\pm$ 0·002 atom % of deuterium.

Nitrogen.—The ideal gas for ^{15}N assay is nitrogen itself. The ratio,

$$R = \frac{\text{intensity of ion beam, mass 28}}{\text{intensity of ion beam, mass 29}} = \frac{[^{14}N^{14}N]}{[^{14}N^{15}N]}$$

is measured. By consideration of the equilibrium

$$^{14}N^{14}N + {}^{15}N^{15}N \rightleftharpoons 2\,{}^{14}N^{15}N$$

(for which $K = 4$ at room temperature), it follows that

$$\text{atom \% } {}^{15}N = 100/(2R + 1)$$

Nitrogen of organic compounds is readily obtained as gaseous nitrogen after a Kjeldahl digestion [23] and subsequent oxidation of the ammonia with hypobromite. The oxidation is carried out *in vacuo* using a two-legged Y-tube, fitted with a ground-glass cap. Nitrogen gas is thus released in the absence of diluting air. Air leakage, giving rise to incorrect ratios, is indicated by ion beams of mass 32 (oxygen) and 40 (argon). Providing the leakage is not more than about 3%, it is possible to correct for this dilution.

Routine ^{15}N analyses are accurate to $\pm$ 0·003 atom-%.

Carbon.—Although several volatile carbon compounds are available, in practice ^{13}C is assayed as carbon dioxide. The ion beams used are those due to $^{12}C^{16}O^{16}O$ (44) and $^{13}C^{16}O^{16}O$ (45). The contribution of $^{12}C^{16}O^{17}O$ to the 45 beam is usually ignored since it is the same in both normal carbon dioxide and the sample. If

$$R = \frac{\text{intensity of ion beam, mass 44}}{\text{intensity of ion beam, mass 45}} = \frac{[^{12}C^{16}O^{16}O]}{[^{13}C^{16}O^{16}O]}$$

it can be shown that atom % $^{13}C = 100/(R + 1)$.

It is more important to obtain a sample of carbon dioxide whose carbon atoms are truly representative of those of the parent compound, than to obtain a quantitative conversion. Either combustion [24] or wet oxidation are suitable methods. By using D. D. Van Slyke and J. Folch's mixture [25] the oxidation may be carried out in simple apparatus [26] or in a vacuum-tube.[27] In either case, the carbon dioxide is collected as barium carbonate

[21] D. Rittenberg, Report of a Symposium on the Use of Isotopes in Biological Research, American Cancer Society, 1947, p. 27; D. B. Sprinson and D. Rittenberg, *U.S. Naval Medical Bull.*, Supplement, March—April, 1948, p. 89.

[22] *Ibid.*, p. 75.

[23] D. Rittenberg, "Preparation and Measurement of Isotopic Tracers", J. W. Edwards, Ann Arbor, Michigan, 1946, p. 31; D. B. Sprinson and D. Rittenberg, *U.S. Naval Medical Bull.*, Supplement, March—April, 1948, p. 82; *J. Biol. Chem.*, 1949, **180**, 707.

[24] D. Rittenberg, *op. cit.*, p. 39. [25] *J. Biol. Chem.*, 1940, **136**, 509.

[26] A. Lindenbaum, J. Schubert, and W. D. Armstrong, *Analyt. Chem.*, 1948, **20**, 1120. [27] H. A. Barker, quoted in "Isotopic Carbon", Calvin *et al.*, 1949, p. 93.

and subsequently decomposed with mineral acid, the two-legged tube being used. A method for purification of carbon dioxide without absorption in baryta has been described.[28]

Apart from these general methods, special techniques are available for some compounds, *e.g.*, the α-carboxyl group of an amino-acid is oxidised to carbon dioxide by the ninhydrin reagent, and fatty acids may be decarboxylated to carbon dioxide by several methods.

In routine work, analyses accurate to ± 0·01 atom % ^{13}C are obtained. Carbon dioxide is pumped away relatively slowly from the spectrometer.

Oxygen.—Samples of oxygen gas may be directly analysed by comparison of the intensities of the ion beams of mass number 32 ($^{16}O^{16}O$) and 34 ($^{1}O^{16}O$). When large numbers of oxygen analyses are performed, the life of the spectrometer filament is considerably reduced.

Carbon[8] dioxide may also be used in the analysis of ^{18}O, the ion beams of mass 44 ($^{12}C^{16}O^{16}O$) and 46 ($^{12}C^{16}O^{18}O$) being used. As mentioned earlier, carbon dioxide may be obtained from many decarboxylations and analysed directly. It is also employed for the analysis of $H_2{}^{18}O$ samples, by using the exchange reaction [29]

$$C^{16}O_2 \text{ (g.)} + H_2{}^{18}O \text{ (l.)} \rightleftharpoons C^{18}O^{16}O \text{ (g.)} + H_2{}^{16}O \text{ (l.)}$$

The equilibration is conveniently catalysed by the addition of a little carbonic anhydrase, and by using a tube of small volume as little as 5 mg. of $H_2{}^{18}O$ may be successfully analysed. If R is the abundance ratio (mass 44 : mass 46) of the carbon dioxide after equilibration

$$\text{Atom \% } {}^{18}O \text{ in water} = 100/[\tfrac{1}{2}K(2R - 1) + 1]$$

where K is the equilibrium constant of the exchange reaction (= 2·076).[30]

The Synthesis of Labelled Compounds

The incorporation of an isotope into a compound depends largely on the nature and availability of the isotope. The isotopes of carbon and nitrogen are still rare and expensive, and the desired high conversion of isotope can be achieved by appropriate modifications of the usual reaction conditions, such as the use of excess of the non-isotopic reactant, introduction of the isotopic material at the latest possible stage of a synthesis, and recovery of any unused isotopic material.

Isotopic carbon is available as $Na^{13}CN$, containing up to 60 atom % excess of ^{13}C, and $Ba^{14}CO_3$, usually of 1 mc./mM. specific activity. The maximum permissible dilution of ^{13}C is 6000, as 0·01 atom % excess is still detectable with a mass spectrometer.[31] ^{14}C (half-life about 5000 years) [32] emits soft β-rays of approx. 0·15 Mev. maximum energy. $Ba^{14}CO_3$ of a

[28] S. Weinhouse, "Preparation and Measurement of Isotopic Tracers", J. W. Edwards, Ann Arbor, Michigan, 1946, p. 43.

[29] M. Cohn and H. C. Urey, *J. Amer. Chem. Soc.*, 1938, **60**, 679.

[30] R. Bentley, *Nucleonics*, 1948, **2**, (2), 18. [31] *Idem*, *Research*, 1949, **2**, 378.

[32] L. D. Norris and M. G. Inghram, *Physical Rev.*, 1946, **70**, 772; 1948, **73**, 350; A. F. Reid, J. R. Dunning, S. Weinhouse, and A. V. Grosse, *ibid.*, 1946, **70**, 431.

specific activity of 1 mc./mM., giving a counting rate of about 8×10^6 counts per minute on the usual end-window Geiger–Müller counter, can still be accurately counted after 400,000-fold dilution.

^{15}N, available as $^{15}NH_4NO_3$ (containing up to 62 atom % excess in the ammonium radical), $K^{15}NO_3$, and potassium phthalimide can be measured with an accuracy of 0·004 atom % and its maximum detectable dilution [31] is therefore 15,500.

Deuterium is produced as heavy water of almost 100% purity and can be diluted to a greater extent than the other stable isotopes.

The above figures are the maximum dilutions which the isotope can be permitted to undergo during synthesis and subsequent isolation in a chemical or biological experiment. Dilutions in animal experiments are usually considerable (10^3 and greater) and radioactive carbon compounds for such work should have a specific activity greater than 10^{-2} μc./mg., but for chemical investigations much lower activities (10^{-3} to 10^{-4} μc./mg.) will suffice.

Since a hundred-fold dilution of ^{14}C is usually permissible during an organic synthesis from starting material of high specific activity, the carrier technique can be used advantageously. With this technique, intermediates often need not be isolated and manipulations are then reduced to a minimum. Finally, a large excess of non-isotopic end product is added to facilitate the isolation of the labelled compound. Even optical resolutions can be avoided by addition of the appropriate non-isotopic, optically active isomer to the ^{14}C-DL-mixture and crystallisation of the labelled stereoisomer, as shown by the recent preparation of ^{14}C-L-cystine and ^{14}C-D-methionine.[33]

Purity of Isotopic Compounds.—The criteria of purity used in organic chemistry are often sufficient for tracer work, but the result may be in error even when all the conventional criteria of purity are applied. However, additional techniques are available for checking the purity of labelled substances. The molecule may be degraded chemically and the isotope content of the degradation products compared with that of the original substance. A radioactive impurity may be detected by a combination of paper chromatography and radio-autography.[34] If the counter-current distribution method is used, a number of partition coefficients can be determined by measuring the isotope content and by a chemical method. If the values obtained by the two methods are in agreement, the compound can be considered pure. Counter-current extraction has been used to separate ^{14}C-hippuric acid from ^{14}C-α-acetamido-γ-phenylbutyric acid of ten times the specific activity.[35]

Recently, the difference in vapour pressure of solutions containing different amounts of solid has been applied to the determination of the purity

[33] J. L. Wood and H. R. Gutman, *J. Biol. Chem.*, 1949, **179**, 535.

[34] R. M. Fink, C. E. Dent, and K. Fink, *Nature*, 1947, **160**, 801; R. M. Fink and K. Fink, *Science*, 1948, **107**, 253; R. M. Tomarelli and K. Florey, *ibid.*, p. 630; W. Stepka, A. A. Benson, and M. Calvin, *ibid.*, 1948, **108**, 304.

[35] H. R. V. Arnstein and A. Neuberger, *Biochem. J.*, 1949, **45**, iii.

of amino-acids. By means of a simple differential tensimeter it was possible to detect 0·2% of D-glutamic acid in L-glutamic acid.[36]

Location of Labelled Atoms.—Tracer work often necessitates the location of isotopic atoms in a molecule. It is then necessary to degrade the compound by an unequivocal method and to determine the isotope content of the degradation products. Sometimes the isotope content of some atoms can only be determined by difference, but analysis of all the fragments provides a useful check of the purity of the compound and the validity of the degradation procedure. For example, decarboxylation of ^{14}C-methyl-labelled barium acetate:

$$(\overset{2}{C}H_3\cdot\overset{1}{C}O_2)_2Ba \xrightarrow{525^\circ} \overset{1}{B}aCO_3 + \overset{2}{C}H_3\cdot\overset{1}{C}O\cdot\overset{2}{C}H_3 \longrightarrow \overset{2}{C}HI_3 + \overset{2}{C}H_3\cdot\overset{1}{C}O_2H$$

unexpectedly gave barium carbonate containing approximately 1·5—2% of the original isotope, but the iodoform had the same specific activity as the starting material.[37]

Biological as well as chemical methods have been used to degrade glucose obtained from rat-liver glycogen:[38]

$$\overset{1}{C}H(OH)\cdot\overset{2}{C}H(OH)\cdot\overset{3}{C}H(OH)\cdot\overset{4}{C}H(OH)\cdot\overset{5}{C}H\cdot\overset{6}{C}H_2\cdot OH \xrightarrow[\text{fermentation}]{L.\ casei}$$

$$2\overset{1,6}{C}H_3\cdot\overset{2,5}{C}H(OH)\cdot\overset{3,4}{C}O_2H \longrightarrow \overset{1,6}{C}H_3\cdot\overset{2,5}{C}HO + \overset{3,4}{C}O_2 \xrightarrow{NaOI} \overset{1,6}{C}HI_3 + H\cdot\overset{2,5}{C}O_2H$$

$$\xrightarrow[\text{degradation}^{39}]{\text{alternative}} \overset{1,6}{C}H_3\cdot\overset{2,5}{C}O_2H + \overset{3,4}{C}O_2$$

H. G. Wood *et al.*[38] also degraded glucose by purely chemical reactions:

$$\text{Glucose} \xrightarrow{MeOH} MeO\cdot\overset{1}{C}H\cdot\overset{2}{C}H(OH)\cdot\overset{3}{C}H(OH)\cdot\overset{4}{C}H(OH)\cdot\overset{5}{C}H\cdot\overset{6}{C}H_2\cdot OH \xrightarrow{HIO_4}$$

$$MeO\cdot\overset{1}{C}H\cdot\overset{2}{C}HO \quad \overset{4}{C}HO\cdot\overset{5}{C}H\cdot\overset{6}{C}H_2\cdot OH + H\cdot\overset{3}{C}O_2H \xrightarrow{HIO_4}$$

$$\left[\overset{1}{C}HO\cdot\overset{2}{C}HO + \overset{4}{C}HO\cdot\overset{5}{C}H(OH)\cdot\overset{6}{C}O_2H + MeOH\right] \longrightarrow 4H\cdot\overset{1,2,4,5}{C}O_2H + \overset{6}{C}H_2O + MeOH$$

The two procedures permit calculation of the isotopic concentration in positions 1, 6, 3, and 4 separately, as well as the sum of positions 2 and 5.

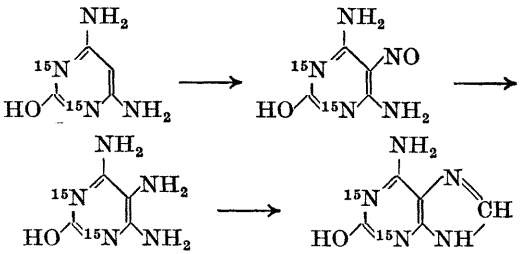
Specific and Non-specific Labelling.—In most cases the isotope is incorporated in a definite position of the molecule, but when the synthesis involves a symmetrical intermediate the final product will be labelled in

[36] I. W. Hughes and G. T. Young, *Nature*, 1949, **164**, 503.
[37] B. A. Fries and M. Calvin, *J. Amer. Chem. Soc.*, 1948, **70**, 2235.
[38] H. G. Wood, N. Lifson, and V. Lorber, *J. Biol. Chem.*, 1945, **159**, 475.
[39] S. Aronoff, H. A. Barker, and M. Calvin, *ibid.*, 1947, **169**, 459.

more than one position; *e.g.*, synthesis of ^{15}N-*iso*guanine: [40]

$$CNBr + {}^{15}NH_3 \longrightarrow CN\cdot{}^{15}NH_2 + {}^{15}NH_4Br$$

$$CN\cdot{}^{15}NH_2 + H_2S \longrightarrow {}^{15}NH_2\cdot CS\cdot{}^{15}NH_2 \xrightarrow{NC\cdot CH_2\cdot CN} \xrightarrow{Cl\cdot CH_2\cdot CO_2H}$$



Biosyntheses.—A number of compounds can be prepared conveniently by biosynthesis, but this procedure usually gives a non-specifically labelled compound.

^{14}C-Glucose and ^{14}C-fructose have been prepared from $^{14}CO_2$ by photosynthesis using Turkish tobacco leaves,[41] and ^{14}C-labelled sugars have also been obtained from barley seedlings and $^{14}CO_2$. In this case the highest activity was found in the 3 and 4 positions, the lowest in the 1 and 6 positions of the hexose.[39]

^{14}C-Labelled sucrose has been obtained by photosynthesis using the leaves of *Canna indica* [41] and by an ingenious enzymic synthesis, which gives sucrose labelled independently in the glucose or fructose moiety: [42]

$$^{14}\text{C-fructose} + \text{sucrose} \xrightarrow[P.\ saccharophila]{\text{enzyme from}} \text{glucose 1-}^{14}\text{C-fructoside (sucrose)}$$

$$^{14}\text{C-glucose 1-phosphate} + \text{fructose} \xrightarrow{\text{enzyme}} {}^{14}\text{C-glucose 1-fructoside (sucrose)}$$

$^{13}CO_2$ has been used for the biosynthesis of starch by bean leaves,[43] and Geiling *et al.*[44] have prepared labelled nicotine and digitoxin by growing plants in $^{14}CO_2$.

^{14}C-Radioactive silk has also been made biosynthetically,[45] and ^{14}C-bufagin has been obtained [46] from toads which were fed with algæ previously exposed to $^{14}CO_2$.

[40] A. Bendich, J. F. Tinker, and G. B. Brown, *J. Amer. Chem. Soc.*, 1948, **70**, 3109.
[41] E. W. Putnam, W. Z. Hassid, G. Krotkov, and H. A. Barker, *J. Biol. Chem.*, 1948, **173**, 785.
[42] H. Wolochow, E. W. Putnam, M. Doudoroff, W. Z. Hassid, and H. A. Barker, *ibid.*, 1949, **180**, 1237.
[43] L. G. Livingstone and G. Medes, *J. Gen. Physiol.*, 1947, **31**, 75.
[44] E. M. K. Geiling, F. E. Kelsey, B. J. McIntosh, and A. Ganz, *Science*, 1948, **108**, 558; T. E. Kimura and E. M. K. Geiling, *Fed. Proc.*, 1949, **8**, 308.
[45] P. C. Zamecnik, R. B. Loftfield, M. L. Stephenson, and C. M. Williams, *Science*, 1949, **109**, 624.
[46] J. Doull, K. P. DuBois, and E. M. K. Geiling, *Fed. Proc.*, 1949, **8**, 286.

Barker *et al.*[47] showed that *Clostridium acidi-urici* incorporated $^{14}CO_2$ into acetic acid during uric acid fermentation. About 50% of the original radioactivity was found in the acetic acid, two-thirds being located in the methyl group. Another micro-organism, *Clostridium thermoaceticum*, utilised 80% of the $^{14}CO_2$ for acetic acid synthesis, the activity being equally distributed between the two carbon atoms.[11] $^{14}CO_2$ is converted into acetic and butyric acid by *Butyrobacterium rettgeri*,[48] and ^{14}C-acetic acid is incorporated into *n*-butyric and *n*-hexoic acids when ethanol is fermented by *Clostridium kluyveri*.[49]

Recently, yeasts have been used for the biosynthesis of ^{15}N-labelled nucleic acids,[50] and ^{35}S-radioactive penicillin has also been prepared by biosynthesis.[51]

Since the scope of the tracer technique is hindered to a large extent by the limited number of labelled organic compounds available, the synthetic methods which have been developed will now be discussed.

Synthetic ^{13}C and ^{14}C-Compounds.—*One-carbon compounds.* Many one-carbon compounds (see Table I) have been prepared from $^{14}CO_2$, usually as intermediates for the synthesis of more complicated molecules, though some compounds (*e.g.*, urea, guanidine, formic acid) have also been used for biochemical studies. Since ^{13}C is available as cyanide it is usually introduced directly into more complicated molecules.

$^{14}CO_2$ has been generated from $Ba^{14}CO_3$ by treatment with an acid (usually sulphuric acid) and roasting of the salt at high temperatures (1100°).[52] By fusion of barium carbonate with lead chloride or lead chloride–silver chloride (1 : 1) at 400° a quantitative yield of $^{14}CO_2$ has been obtained.[53]

[47] H. A. Barker, S. Ruben, and J. V. Beck, *Proc. Nat. Acad. Sci.*, 1940, **26**, 477.

[48] H. A. Barker, M. D. Kamen, and V. Haas, *ibid.*, 1945, **31**, 355.

[49] H. A. Barker, M. D. Kamen, and B. T. Bornstein, *ibid.*, p. 373.

[50] F. J. DiCarlo, A. J. Schultz, P. M. Roll, and G. B. Brown, *J. Biol. Chem.*, 1949, **180**, 329.

[51] D. Rowley, J. Miller, S. Rowlands, and E. Lester-Smith, *Nature*, 1948, **161**, 1009; S. F. Howell, J. D. Thayer, and L. W. Labaw, *Science*, 1948, **107**, 299.

[52] M. G. Inghram, U.S. Atomic Energy Commission, MDDC 60, June, 1946.

[53] N. Zwiebel, J. Turkevich, and W. W. Miller, *J. Amer. Chem. Soc.*, 1949, **71**, 376.

TABLE I

Compound.	Starting material and method.	Yield, %.	Yield based on CO_2, %.	Ref.
$Na_2{}^{14}CO_3$	$Ba^{14}CO_3$	~ 100	~ 100	54
$NaH^{14}CO_3$	$Ba^{14}CO_3$	~ 100	~ 100	55
$KH^{14}CO_3$	$Ba^{14}CO_3$	~ 100	~ 100	55
^{14}CO	$^{14}CO_2$		~ 99	56
	$^{14}CO_2$ (exchange reaction)			57
	$Ca^{14}CO_3$ (heated with Zn)			58
	$^{14}CO_2$ (heated with Zn)			59
	$H{\cdot}^{14}CO_2H$			60
$^{14}COCl_2$	^{14}CO		95	59, 60
$K^{14}CN$	$^{14}CO_2$ (heated with NH_3–K)		90—96	61
	$^{14}CO_2$		80	62
$Na^{14}CN$	$Ba^{14}CO_3$ (NaN_3 reduction)		78	63
$Ba^{14}CN_2$	$Ba^{14}CO_3$			64
	$Ba^{14}CO_3$ (NH_3 at 850°)		73·5	65
	$Ba^{14}CO_3$			66
$NH_2{\cdot}^{14}CO{\cdot}NH_2$	$^{14}CO_2$		80	67
	$^{14}COCl_2$		90	68
	$Ba^{14}CN_2$	91	67	65
	$Ba^{14}CN_2$		95	66
$NH_2{\cdot}^{14}CS{\cdot}NH_2$	$Ba^{14}CN_2$	61		65
$(NH_2)_2{}^{14}C{:}NH,HCl$	$Ba^{14}CN_2$		61	69
$(NH_2)_2{}^{14}C{:}NH,HNO_3$	$^{14}CN{\cdot}NH{\cdot}^{14}C{:}NH{\cdot}NH_2$	83		65
$^{14}CN{\cdot}NH{\cdot}^{14}C{:}NH{\cdot}NH_2$	$Ba^{14}CN_2$			65
$NH_2{\cdot}^{14}CO_2C_2H_5$	$NH_2{\cdot}^{14}CO{\cdot}NH_2$	40	36	70
$H{\cdot}^{14}CO_2H$	$KH^{14}CO_3$ (catalytic reduction)	98—99	~ 98	55, 60
	$Na^{14}CN$	50—60	~ 50	71
$H{\cdot}^{14}CO_2CH_3$	$H{\cdot}^{14}CO_2H$	90	~ 90	55
$^{14}CH_3{\cdot}OH$	$^{14}CO_2$ (catalytic reduction)		85—90	72
	$H{\cdot}^{14}CO_2CH_3$ (catalytic reduction)			55
$^{14}CH_3I$	$^{14}CH_3{\cdot}OH$		~ 75	55
	$^{14}CH_3{\cdot}OH$	95		72
	$^{14}CH_3{\cdot}OH$	88		73
$^{14}CH_3{\cdot}NO_2$	$^{14}CH_3I$	70		74
$H{\cdot}^{14}CHO$	$^{14}CH_3{\cdot}OH$ (*via* $CH_3{\cdot}CO_2{}^{14}CH_2Cl$)	60		75
	$^{14}CH_3{\cdot}OH$ (catalytic oxidation)	50—60	45—55	76
	$^{14}CH_3{\cdot}OH$ (catalytic oxidation)	60—70		77
$^{14}CH_4$	$^{14}CO_2$ (Ni–ThO_2 catalyst, 330°)		96	78
$^{14}CCl_4$	$^{14}CH_4$		93	78

[54] W. B. Leslie, U.S. Atomic Energy Commission, MDDC 674, June 15, 1947.
[55] D. B. Melville, J. R. Rachele, and E. B. Keller, *J. Biol. Chem.*, 1947, **169**, 419.
[56] R. B. Bernstein and T. I. Taylor, *Science*, 1947, **106**, 498.
[57] J. T. Kummer, *J. Amer. Chem. Soc.*, 1947, **69**, 2239.
[58] S. Weinhouse, *ibid.*, 1948, **70**, 442.
[59] J. L. Huston and T. H. Norris, *ibid.*, p. 1968.
[60] D. B. Melville, J. G. Pierce, and C. W. H. Partridge, *J. Biol. Chem.*, 1949, **180**, 299
[61] "Isotopic Carbon", Calvin *et al.*, 1949, pp. 160, 161.
[62] R. B. Loftfield, *Nucleonics*, 1947, **1**, (3), 54.
[63] A. W. Adamson and W. K. Wilmarth, *J. Amer. Chem. Soc.*, 1947, **69**, 2564.
[64] "Isotopic Carbon", Calvin *et al.*, p. 158.
[65] S. H. Zbarsky and I. Fischer, *Canadian J. Res.*, 1949, **27**, 81.
[66] A. Murray and A. R. Ronzio, *J. Amer. Chem. Soc.*, 1949, **71**, 2245.
[67] "Isotopic Carbon", Calvin *et al.*, 1949, p. 157.
[68] *Ibid.*, p. 158.
[69] *Ibid.*, p. 159.

Functionally labelled compounds related to carboxylic acids. Carboxylic acids (see Table II) are usually prepared by carbonation of a Grignard reagent with isotopic carbon dioxide (side reactions being avoided by modifying the reaction conditions),[79] or by reaction of a suitable halide with isotopic cyanide. Carbonation of organo-metallic compounds has also been used.[80] ^{13}C-Phenylacetic acid has been prepared by the Arndt–Eistert reaction :[81]

$$C_6H_5\cdot{}^{13}COCl + CH_2N_2 \longrightarrow C_6H_5\cdot{}^{13}CO\cdot CHN_2 \xrightarrow{Ag} C_6H_5\cdot CH_2\cdot{}^{13}CO_2H$$

TABLE II

Functionally labelled compounds

Compound.	Method.*	Ref.	Compound.	Method.*	Ref.
$CH_3\cdot{}^{13}CO_2H$	*a*	82, 83	^{14}C-2-Naphthoic acid	*a*	93
$CH_3\cdot{}^{14}CO_2H$		84	n-$C_7H_{15}\cdot{}^{13}CO_2H$	*a*	94
$CH_3\cdot{}^{13}CO\cdot CH_2\cdot{}^{13}CO_2H$		82	n-$C_{10}H_{21}\cdot{}^{14}CO_2H$	*a*	95
$CH_3\cdot CO\cdot CH_2\cdot{}^{13}CO_2H$		82	n-$C_{11}H_{23}\cdot{}^{14}CO_2H$	*a*	96
$CH_3\cdot CH_2\cdot{}^{13}CO_2H$		85	n-$C_{11}H_{23}\cdot{}^{14}CN$		96
$CH_3\cdot CO\cdot{}^{13}CO_2H$		82	n-$C_{15}H_{31}\cdot{}^{14}CO_2H$	*a*	95
iso-$C_4H_9\cdot{}^{14}CO_2H$	*a*	54	n-$C_{15}H_{31}\cdot{}^{14}CO_2Me$		95
$C_6H_5\cdot{}^{14}CO_2H$	*a*	80	^{14}C-Tripalmitin		95
	c	86	n-$C_{29}H_{59}\cdot{}^{14}CO_2H$	*a*	97
p-$MeO\cdot C_6H_4\cdot{}^{14}CO_2H$	*a*	87, 88	Fluorene-9-^{14}C-carboxylic acid	*b*	98
3 : 4-$(MeO)_2C_6H_3\cdot{}^{14}CO_2H$	*b*	89	^{14}C-Nicotinic acid	*b*	80
p-$NH_2\cdot C_6H_4\cdot{}^{14}CO_2H$	*b*	80	$^{14}CO_2H\cdot{}^{14}CO_2H$		99
$C_6H_5\cdot CH_2\cdot{}^{14}CO_2H$	*d*	81	$^{14}CO_2H\cdot CH_2\cdot{}^{14}CO_2H$	*e*	100
	a	90	$^{14}CO_2H\cdot C{:}C\cdot{}^{14}CO_2H$	*b*	101
$C_6H_5\cdot CH_2\cdot{}^{14}CO\cdot NH_2$		91	$^{14}CO_2H\cdot CH_2\cdot CH_2\cdot{}^{14}CO_2H$	*b*	101
^{14}C-1-Naphthoic acid	*a*	92			

* *a*, By carbonation of Grignard reagent. *b*, By carbonation of organometallic compound. *c*, By oxidation of ketone. *d*, By Arndt–Eistert reaction. *e*, From nitrile.

[70] H. E. Skipper, C. E. Bryan, and O. S. Hutchinson, U.S. Atomic Energy Commission, Circular C-8, September, 1947.

[71] " Isotopic Carbon ", Calvin *et al.*, 1949, p. 165.

[72] B. M. Tolbert, *J. Amer. Chem. Soc.*, 1947, **69**, 1529.

[73] R. B. Regier and R. W. Blue, *J. Org. Chem.*, 1949, **14**, 505.

[74] J. C. Sowden, *J. Biol. Chem.*, 1949, **180**, 55.

[75] A. R. Jones and W. J. Skraba, *Science*, 1949, **110**, 332.

[76] C. Heidelberger, *J. Biol. Chem.*, 1949, **179**, 139.

[77] H. R. V. Arnstein, *Nature*, 1949, **164**, 361.

[78] W. H. Beamer, *J. Amer. Chem. Soc.*, 1948, **70**, 3900.

[79] W. G. Dauben, J. C. Reid, and P. E. Yankwich, *Analyt. Chem.*, 1947, **19**, 828.

[80] A. Murray, W. W. Foreman, and W. Langham, *J. Amer. Chem. Soc.*, 1948, **70**, 1037.

[81] C. Huggett, R. T. Arnold, and T. I. Taylor, *ibid.*, 1942, **64**, 3043.

[82] W. Sakami, W. E. Evans, and S. Gurin, *ibid.*, 1947, **69**, 1110.

[83] M. E. Swendseid, R. H. Barnes, A. Hemingway, and A. O. Nier, *J. Biol. Chem.*, 1942, **142**, 47.

[84] L. B. Spector, U.S. Atomic Energy Commission, MDDC 532.

[85] H. G. Wood, C. H. Werkman, A. Hemingway, A. O. Nier, and C. G. Stuckwisch, *J. Amer. Chem. Soc.*, 1941, **63**, 2140.

[86] O. K. Neville, *ibid.*, 1948, **70**, 3499.

[87] J. C. Reid, *Science*, 1947, **105**, 208.

[88] J. C. Reid and H. B. Jones, *J. Biol. Chem.*, 1948, **174**, 427.

[89] " Isotopic Carbon ", Calvin *et al.*, 1949, p. 183.

[90] *Ibid.*, p. 180.

[91] *Ibid.*, p. 181.

Non-functionally labelled carboxylic acids. These compounds can often be prepared by carbonation of labelled Grignard reagents with non-isotopic carbon dioxide, *e.g.*, methyl-labelled ^{14}C-sodium acetate from methyl iodide in 70—80% yield (see Table III). Methyl-labelled ^{13}C-acetic acid has been made from cyanide by an ingenious synthesis : [103]

$$Na^{13}CN \longrightarrow Zn(^{13}CN)_2 \xrightarrow{\text{phenol}} HO\cdot C_6H_4\cdot{}^{13}CH{:}NH,HCl \xrightarrow{Zn}$$

$$HO\cdot C_6H_4\cdot{}^{13}CH_3 \xrightarrow{CrO_3} {}^{13}CH_3\cdot CO_2H$$

Doubly-labelled ^{14}C-acetic acid is available by the following series of reactions : [104]

$$Ba^{14}CO_3 \xrightarrow[\text{at } 700—750°]{Mg-H_2} Ba^{14}C_2 \xrightarrow{H_2O} {}^{14}CH{:}{}^{14}CH \xrightarrow[\text{asbestos}]{KOH \text{ on}}$$

$$^{14}CH_3\cdot{}^{14}CO_2K \xrightarrow{H_2SO_4} {}^{14}CH_3\cdot{}^{14}CO_2H \text{ (60—80\% yield)}$$

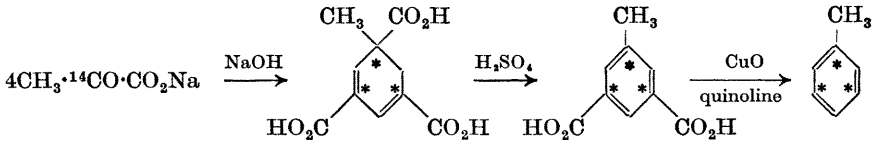
TABLE III

Non-functionally labelled compounds

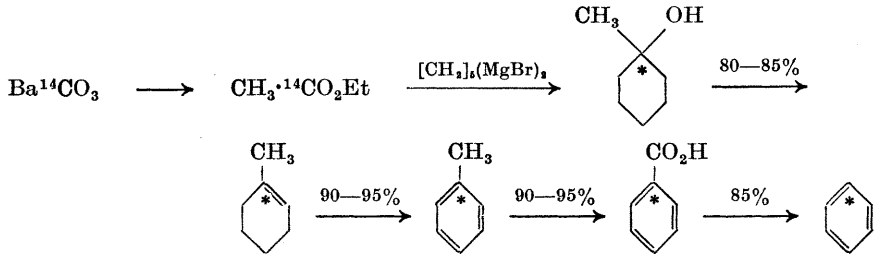
Compound.	Ref.	Compound.	Ref.
$^{14}CH_3\cdot CO_2H$	102	$^{14}CH_3\cdot{}^{14}CH_2I$	113
$^{13}CH_3\cdot CO_2H$	103	$^{14}CH_3\cdot{}^{14}CH_2MgI$	113
$^{14}CH_3\cdot{}^{14}CO_2H$	104	$CH_3\cdot CH_2\cdot{}^{14}CH_2\cdot OH$	110
$CH_3\cdot{}^{13}CH_2\cdot CO_2H$	105		
$^{13}CH_3\cdot CH_2\cdot CO_2H$	105	$C_6H_5\cdot{}^{14}CO\cdot CH_3$	86
$CH_3\cdot CH_2\cdot{}^{13}CH_2\cdot CO_2H$	105	$C_6H_5\cdot{}^{14}CO\cdot CHO$	86
$^{13}CH_3\cdot{}^{13}CO\cdot CO_2H$	82	$C_6H_5\cdot{}^{14}CO\cdot CHBr_2$	86
$CH_3\cdot{}^{14}CO\cdot CO_2H$	99, 106, 107	$C_6H_5\cdot{}^{14}CH(OH)\cdot CO_2H$	86
$C_{10}H_{21}\cdot{}^{14}CH_2\cdot[CH_2]_4\cdot CO_2H$	95		
		^{14}C-Biotin	60
$^{14}CH{:}{}^{14}CH$	108	16-^{13}C-Dehydro*iso*androsterone acetate	114
$^{14}CH_2{:}{}^{14}CH_2$	109	21-^{14}C-Progesterone	115, 116
$CH_3\cdot CH{:}{}^{14}CH_2$	110	17-(^{14}C-Methyl)-testosterone	116
$CH_3\cdot CH_2\cdot{}^{13}CH_3$	111	3-^{14}C-Testosterone	117
		1-^{14}C-D-Glucose	74
$CH_3\cdot{}^{14}CH_2\cdot OH$	112	1-^{14}C-D-Mannose	74
$^{14}CH_3\cdot CH_2\cdot OH$	112	1-^{14}C-Methadone	112
$CH_3\cdot{}^{14}CH_2Br$	112		
$^{14}CH_3\cdot CH_2Br$	112		

[92] W. G. Dauben, *J. Org. Chem.*, 1948, **13**, 313.
[93] C. Heidelberger, P. Brewer, and W. G. Dauben, *J. Amer. Chem. Soc.*, 1947, **69**, 1389.
[94] S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, 1944, **155**, 143.
[95] W. G. Dauben, *J. Amer. Chem. Soc.*, 1948, **70**, 1376.
[96] H. J. Harwood and A. W. Ralston, *J. Org. Chem.*, 1947, **12**, 740.
[97] E. Hines and A. Gemant, *Science*, 1949, **110**, 19.
[98] C. J. Collins, *J. Amer. Chem. Soc.*, 1948, **70**, 2418.
[99] D. M. Hughes and J. C. Reid, *J. Org. Chem.*, 1949, **14**, 516.
[100] " Isotopic Carbon ", Calvin *et al.*, 1949, p. 191.
[101] Y. J. Topper, *J. Biol. Chem.*, 1949, **177**, 303.
[102] B. M. Tolbert, *ibid.*, 1948, **173**, 205.
[103] H. S. Anker, *ibid.*, 1946, **166**, 219.

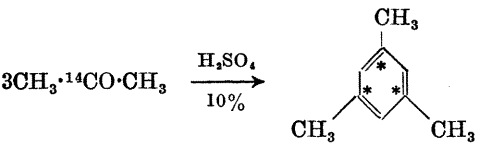
Ring-labelled aromatic compounds. Benzene derivatives labelled in the ring have been made by three procedures. 1 : 3 : 5-^{14}C-Toluene has been synthesised from sodium pyruvate *via* uvitic acid : [99]



M. Fields *et al.* have labelled toluene in the 1-position and also prepared benzoic acid and benzene : [118]



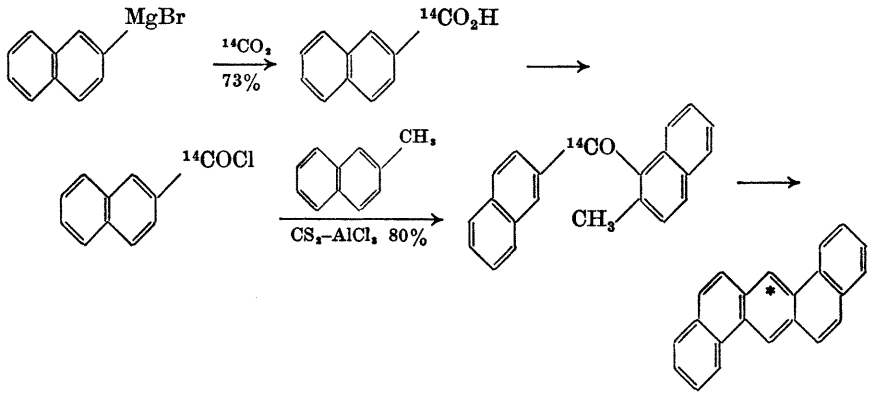
1 : 3 : 5-^{14}C-Mesitylene has been made from acetone : [119]



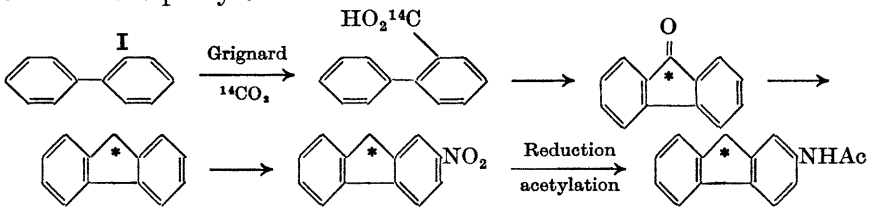
Most other aromatic compounds have been prepared by cyclisation of labelled carboxylic acids. Thus 9-^{14}C-1 : 2-5 : 6-dibenzanthracene and 20-methyl-11-^{14}C-cholanthrene have been made [92, 93] from the ^{14}C-carboxyl-

[104] R. Abrams, *Experientia*, 1947, **3**, 488.
[105] " Isotopic Carbon ", Calvin *et al.*, pp. 192, 193.
[106] H. S. Anker, *J. Biol. Chem.*, 1948, **176**, 1333.
[107] M. Calvin and R. Lemmon, *J. Amer. Chem. Soc.*, 1947, **69**, 1232.
[108] W. J. Arrol and R. Glascock, *Nature*, 1947, **159**, 810.
[109] *Idem, ibid.*, 1948, **161**, 932.
[110] B. A. Fries and M. Calvin, *J. Amer. Chem. Soc.*, 1948, **70**, 2235.
[111] O. Beeck, J. W. Otvos, D. P. Stevenson, and C. D. Wagner, *J. Chem. Physics*, 1948, **16**, 255.
[113] B. M. Tolbert, F. Christenson, F. Nai-Hsuan Chang, and P. P. T. Sah, *J. Org. Chem.*, 1949, **14**, 525.
[113] W. J. Arrol and R. Glascock, *Nature*, 1949, **163**, 61.
[114] E. B. Hershberg, E. Schwenk, and E. Stahl, *Arch. Biochem.*, 1948, **19**, 300.
[115] B. Riegel and F. S. Prout, *J. Org. Chem.*, 1948, **13**, 933.
[116] H. B. MacPhillamy and C. R. Scholz, *J. Biol. Chem.*, 1949, **178**, 37.
[117] R. B. Turner, *Science*, 1947, **106**, 248.
[118] M. Fields, M. A. Leaffer, and J. Rohan, *ibid.*, 1949, **109**, 35.
[119] A. V. Grosse and S. Weinhouse, *ibid.*, 1946, **104**, 402.

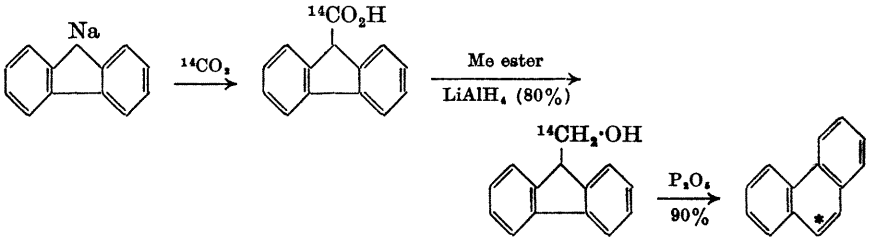
labelled 1- and 2-naphthoic acids, *e.g.*:



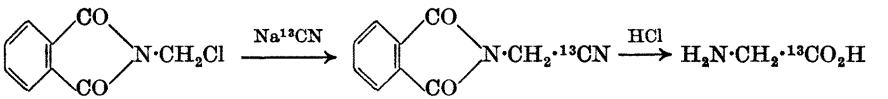
2-Acetamido-9-^{14}C-fluorene was made by a similar series of reactions from 2-iododiphenyl: [120]



9-^{14}C-Phenanthrene has been prepared by the Wagner rearrangement of 9-^{14}C-hydroxymethylfluorene: [98]



Amino-acids (see Table IV). Carboxyl- or α-labelled glycine has been prepared by reaction of chloro- [121] or bromo-acetic acid [122] with ammonia. Other syntheses of carboxyl-labelled glycine have been carried out with ^{13}C or ^{14}C cyanide: [82, 62]



$$2H{\cdot}CHO + K^{14}CN \longrightarrow CH_2{:}N{\cdot}CH_2{\cdot}{}^{14}CN \longrightarrow H_2N{\cdot}CH_2{\cdot}{}^{14}CN \longrightarrow H_2N{\cdot}CH_2{\cdot}{}^{14}CO_2H$$

[120] F. E. Ray and C. R. Geiser, *Science*, 1949, **109**, 200.
[121] R. Ostwald, *J. Biol. Chem.*, 1948, **173**, 207.
[122] N. Olsen, A. Hemingway, and A. O. Nier, *ibid.*, 1943, **148**, 611.

Carboxyl-labelled alanine has been made from isotopic cyanide: [62, 123]

$$CH_3{\cdot}CHO + H^{14}CN + NH_3 \longrightarrow CH_3{\cdot}\underset{\displaystyle NH_2}{\underset{|}{CH}}{\cdot}{}^{14}CN \longrightarrow CH_3{\cdot}\underset{\displaystyle NH_2}{\underset{|}{CH}}{\cdot}{}^{14}CO_2H$$

Cyanide has also been used in a novel synthesis of α-labelled alanine: [124]

$$C_6H_5{\cdot}CHO + Cl{\cdot}CH_2{\cdot}CO_2Na + Na^{13}CN \longrightarrow C_6H_5{\cdot}CH{:}\underset{\displaystyle CO_2H}{\underset{|}{C}}{\cdot}{}^{13}CN \longrightarrow$$

$$C_6H_5{\cdot}CH{:}CH{\cdot}{}^{13}CN \longrightarrow C_6H_5{\cdot}CH{:}CH{\cdot}{}^{13}CO_2H \xrightarrow[\text{LiMe}]{\text{Li salt}}$$

$$C_6H_5{\cdot}CH{:}CH{\cdot}{}^{13}CO{\cdot}CH_3 \longrightarrow \text{oxime} \longrightarrow C_6H_5{\cdot}CH{:}CH{\cdot}{}^{13}\underset{\displaystyle NH{\cdot}COPh}{\underset{|}{CH}}{\cdot}CH_3 \xrightarrow{Ca(MnO_4)_2}$$

$$CH_3{\cdot}{}^{13}\underset{\displaystyle NH{\cdot}COPh}{\underset{|}{CH}}{\cdot}CO_2H \longrightarrow CH_3{\cdot}{}^{13}\underset{\displaystyle NH_2}{\underset{|}{CH}}{\cdot}CO_2H$$

TABLE IV

Carbon-labelled amino-acids

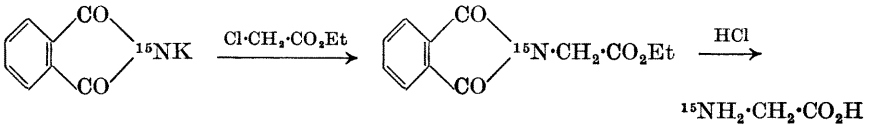
Amino-acid.	Position of label.	Ref.	Amino-acid.	Position of label.	Ref.
Glycine . . .	1-^{14}C, 2-^{14}C	121	DL-Tyrosine .	β-^{14}C	87, 88
	1-^{13}C	82, 122	DL-3 : 4-Dihy-		
	1-^{14}C	62	droxyphenyl-		
DL-Alanine . .	1-^{14}C	62	alanine . .	β-^{14}C	129
	1-^{13}C	123	DL-Tryptophan	β-^{14}C	130, 76
	2-^{13}C	124		Carboxyl-^{13}C	131
DL-Serine . .	^{15}N-1-^{13}C	125		^{15}N-β-^{14}C	132
L-Serine. . .	3-^{14}C	77	DL-Lysine . .	ε-^{14}C	133
DL-Methionine .	^{34}S-3 : 4-^{13}C	126	L-Lysine . .	ε-^{14}C	134
L-Methionine .	Methyl-^{14}C	55	DL-Leucine .	β-^{14}C	135
DL-Phenyl-	α-Carboxyl-^{14}C	127		γ-^{14}C	135
alanine	α-Carboxyl-^{14}C	128	Anthranilic acid	Carboxyl-^{14}C	136
	1 : 3 : 5-^{14}C	128			

[123] S. Gurin and D. W. Wilson, *Fed. Proc.*, 1942, **1**, 114.
[124] J. Baddiley, G. Ehrensvärd, and H. Nilsson, *J. Biol. Chem.*, 1949, **178**, 399.
[125] D. Shemin, *ibid.*, 1946, **162**, 297.
[126] G. W. Kilmer and V. du Vigneaud, *ibid.*, 1944, **154**, 247.
[127] S. Gurin and A. M. Delluva, *ibid.*, 1947, **170**, 545.
[128] B. Schepartz and S. Gurin, *ibid.*, 1949, **180**, 663.
[129] "Isotopic Carbon", Calvin *et al.*, 1949, p. 225.
[130] C. Heidelberger, *J. Biol. Chem.*, 1948, **175**, 471.
[131] H. W. Bond, *ibid.*, p. 531.
[132] R. W. Schayer, G. L. Foster, and D. Shemin, *Fed. Proc.*, 1949, **8**, 248.
[133] P. Olynyk, D. B. Camp, A. M. Griffith, S. Woislowski, and R. W. Helmkamp, *J. Org. Chem.*, 1948, **13**, 465.
[134] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.*, 1948, **176**, 1383.
[135] M. J. Coon, S. Gurin, and D. W. Wilson, *Fed. Proc.*, 1949, **8**, 192; M. J. Coon and S. Gurin, *J. Biol. Chem.*, 1949, **180**, 1159.
[136] J. F. Nye, H. K. Mitchell, E. Leifer, and W. H. Langham, *ibid.*, 1949, **179**, 783.

Syntheses with Heavy Nitrogen

The introduction of ^{15}N from available starting materials (see p. 176) involves the formation of a carbon–nitrogen linkage, and the synthetic reactions used for this purpose are relatively few:

(*a*) *Gabriel phthalimide synthesis: e.g.*, preparation of ^{15}N-glycine:[137]



(*b*) *Knoop–Oesterlin reduction of keto-acids.* Two equivalents (three in the case of dicarboxylic acids) of $^{15}NH_3$ are used and the isotope which does not react is recovered.[137]

(*c*) *Cyanamide syntheses.* Labelled cyanamide has been prepared from ammonia and cyanogen bromide:[138]

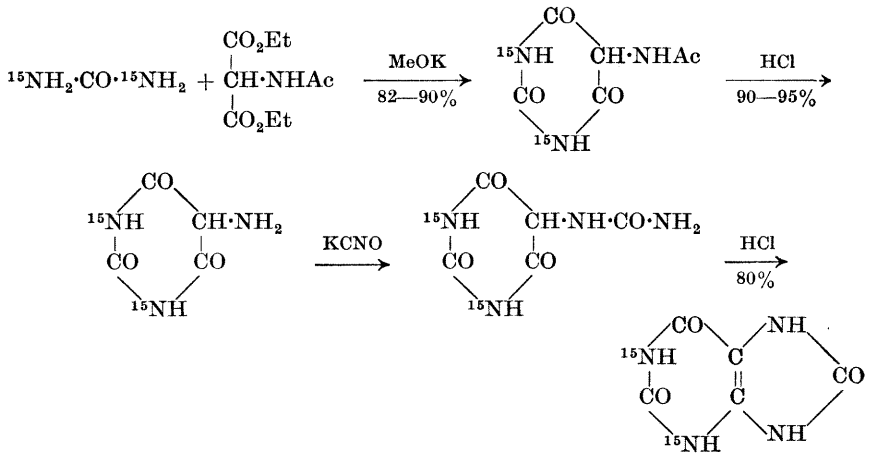
$$2^{15}NH_3 + CNBr \longrightarrow {}^{15}NH_2{\cdot}CN + {}^{15}NH_4Br$$

Condensation of ^{15}N-cyanamide with ^{15}N-sarcosine has been used to prepare creatine:[138]

$$^{15}NH_2{\cdot}CN + CH_3{\cdot}{}^{15}NH{\cdot}CH_2{\cdot}CO_2H \longrightarrow {}^{15}NH{:}C(NH_2){\cdot}{}^{15}N(CH_3){\cdot}CH_2{\cdot}CO_2H$$

Cyanamide has also been converted into *iso*guanine[40] and guanidine.[139]

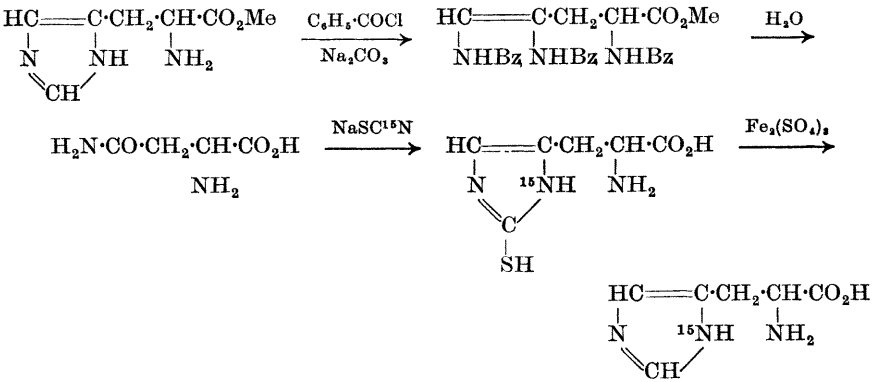
(*d*) *Syntheses with urea.* Ring-labelled uric acid has been synthesised from ^{15}N-urea,[140, 141] *e.g.*:



(*e*) ^{15}N-Sodium thiocyanate, prepared in good yield from ammonia,[142]

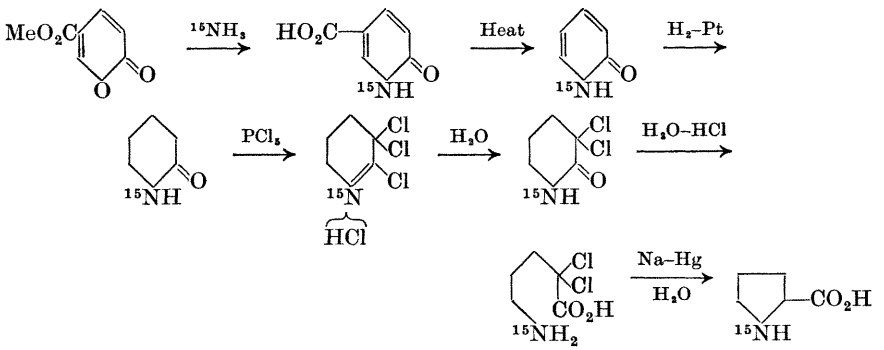
[137] R. Schoenheimer and S. Ratner, *J. Biol. Chem.*, 1939, **127**, 301.
[138] K. Bloch, R. Schoenheimer, and D. Rittenberg, *ibid.*, 1941, **138**, 155.
[139] G. B. Brown, P. M. Roll, and A. A. Plentl, *Fed. Proc.*, 1949, **6**, 517.
[140] K. Bloch and R. Schoenheimer, *J. Biol. Chem.*, 1941, **138**, 167.
[141] L. F. Cavalieri, V. E. Blair, and G. B. Brown, *J. Amer. Chem. Soc.*, 1948, **70**, 1240.
[142] C. Tesar and D. Rittenberg, *J. Biol. Chem.*, 1947, **170**, 35.

has been used to label histidine by an interesting synthesis from natural, optically active histidine: [142]



(*f*) ^{15}N-Formamidine has been prepared [139] and converted into ^{15}N-adenine.[143]

(*g*) Proline has been labelled by an interesting method involving substitution of a heterocyclic oxygen with heavy ammonia: [144]



Other similar syntheses are tabulated in Table V, page 188.

Syntheses with Deuterium

In recent years ^{13}C and ^{14}C have to some extent superseded deuterium in biological tracer work. However, its low cost and ready incorporation into organic compounds partly outweigh its chief disadvantage, *viz.*, loss of label by exchange, and its importance in the study of reaction mechanisms is undiminished.

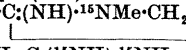
The hydrogen atoms of organic compounds can be broadly divided into three groups with respect to their stability to exchange under normal conditions: (1) Stable, *e.g.*, hydrogen atoms of paraffin hydrocarbons;

[143] L. F. Cavalieri, J. F. Tinker, and G. B. Brown, *J. Amer. Chem. Soc.*, 1949, **71**, 533.

[144] M. R. Stetten and R. Schoenheimer, *J. Biol. Chem.*, 1944, **153**, 113.

TABLE V

^{15}N-*Labelled compounds*

Amino-acid.	Ref.	Compound.	Ref.
DL-Alanine	137, 125	$^{15}NH_2 \cdot CN$	138, 140
L(+)-Alanine	125	$NaSC^{15}N$	142
DL-Arginine	140	$^{15}NH_2 \cdot CH{:}^{15}NH,HCl$	139, 143
L(+)-Arginine	140	$^{15}NH_2 \cdot CO \cdot ^{15}NH_2$	140, 141
DL-Aspartic acid	137	$^{15}NH{:}C(OMe) \cdot ^{15}NH_2$	140
DL-Glutamic acid	137	$^{15}NH_2 \cdot CS \cdot ^{15}NH_2$	156
L(+)-Glutamic acid	125	$^{15}NH_2 \cdot C{:}(^{15}NH) \cdot ^{15}NMe \cdot CH_2 \cdot CO_2H$	138
Glycine	125, 145	$NH_2 \cdot C{:}(NH) \cdot ^{15}NMe \cdot CH_2 \cdot CO_2H$	152
L(+)-Histidine	142		152
DL-Hydroxyproline	146	$^{15}NH_2 \cdot C{:}(^{15}NH) \cdot ^{15}NH_2$	156
DL-Leucine	137	$NH_2 \cdot C{:}(NH) \cdot ^{15}NH \cdot CH_2 \cdot CO_2H$	140
L(−)-Leucine	147	$NH_2 \cdot CO \cdot ^{15}NH \cdot CH_2 \cdot CO_2H$	140
D(+)-Leucine	147, 148	$NH_2 \cdot CO \cdot ^{15}NMe \cdot CH_2 \cdot CO_2H$	140
DL-Lysine (α-^{15}N)	149	Betaine	157, 145
L(+)-Lysine (α-^{15}N)	149	Choline	140, 158
D(−)-Lysine (α-^{15}N)	149	Ethanolamine	140
D(−)-Lysine (α-^{15}N)	150	Aniline	159
DL-Norleucine	137	2-Phenylindole	159
DL-Phenylalanine	137	1 : 3-^{15}N-Thymine	156
DL-Phenylaminobutyric acid	151	1 : 3-^{15}N-Uracil	156
L(+)-Phenylaminobutyric acid	151	1 : 3-^{15}N-Uric acid	141
D(−)-Phenylaminobutyric acid	151	9-^{15}N-Uric acid	141
DL-Proline	144	1 : 3-^{15}N-Adenine	143
L(−)-Proline	144, 125	1 : 3-^{15}N-Xanthine	156
Sarcosine	152, 153	1 : 2 : 3-^{15}N-Guanine	156
DL-Serine	154	1 : 3-^{15}N-*iso*Guanine	53
L(−)-Serine	154	Orotic acid	160
D(+)-Serine	154	1 : 3-^{15}N-2 : 6-Diaminopurine	40
DL-Tyrosine	137		

[145] V. du Vigneaud, S. Simmonds, J. P. Chandler, and M. Cohn, *J. Biol. Chem.*, 1946, **165**, 639.

[146] M. R. Stetten, *Fed. Proc.*, 1949, **8**, 256.

[147] R. Schoenheimer, S. Ratner, and D. Rittenberg, *J. Biol. Chem.*, 1939, **130**, 703.

[148] S. Ratner, R. Schoenheimer, and D. Rittenberg, *ibid.*, 1940, **134**, 653.

[149] N. Weissman and R. Schoenheimer, *ibid.*, 1941, **140**, 779.

[150] R. M. Fink, T. Enns, C. P. Kimball, H. E. Silberstein, W. F. Bale, S. C. Maddens, and G. H. Whipple, *J. Exp. Med.*, 1944, **80**, 455.

[151] V. du Vigneaud, M. Cohn, G. B. Brown, O. J. Irish, R. Schoenheimer, and D. Rittenberg, *J. Biol. Chem.*, 1939, **131**, 273.

[152] K. Bloch and R. Schoenheimer, *ibid.*, p. 111.

[153] V. du Vigneaud, S. Simmonds, and M. Cohn, *ibid.*, 1946, **166**, 47.

[154] D. Stetten, *ibid.*, 1942, **144**, 501.

[156] A. A. Plentl and R. Schoenheimer, *ibid.*, 1944, **153**, 203.

[157] D. Stetten, *ibid.*, 1941, **140**, 143.

[158] G. E. Boxer and D. Stetten, *ibid.*, 1944, **153**, 617.

[159] C. F. H. Allan and C. V. Wilson, *J. Amer. Chem. Soc.*, 1943, **65**, 611.

[160] S. Bergström, H. Arvidson, E. Hammersten, N. A. Eliasson, P. Reichard, and H. v. Ubisch, *J. Biol. Chem.*, 1949, **177**, 495.

(2) semi-labile, *e.g.*, the methylene hydrogen atoms of glycine,[161] and the enolisable hydrogen atoms of a ketone, whose exchange with water is catalysed by alkali;[162] (3) labile, *e.g.*, the carboxyl hydrogen of a carboxylic acid. The lability of a hydrogen atom is increased by an "activating" factor (such as unsaturation or a high concentration of positive charges) on the adjacent carbon atom and it must be ascertained in each case. Since exchange may occur during the isolation procedure, as well as in a biochemical reaction, the effect of the isolation technique should be checked independently.

Exchange Reactions.—Under sufficiently vigorous conditions ordinarily stable hydrogen atoms become labile or semi-labile. Aromatic compounds exchange ring hydrogen atoms when heated with DCl and aluminium chloride [163] or with concentrated D_2SO_4.[164] When heated with concentrated D_2SO_4, several amino-acids [161] and fatty acids [165] exchange the α-hydrogen atoms, but under different conditions (D_2O and platinum at 130°) fatty acids take up more deuterium, which is evenly distributed throughout the chain.[165] Cholesterol has been labelled by heating it with heavy water, acetic acid, and platinum; [166] more deuterium was introduced into the side chain than the rings.

The chief disadvantages of this method are the unavoidable dilution of the heavy isotope, which can be minimised by using a large excess of D_2O and the difficulty of labelling specific positions in some cases, *e.g.*, cholesterol.

Addition Reactions.—Deuterium gas can be added to C≡C, C=C, C≡N, C=N, or C=O bonds, but only the carbon-bound atoms are resistant to subsequent exchange. Reductions are carried out catalytically with D_2, or chemically with D_2O, or reagents such as lithium aluminium deuteride.

Amino-acids have been labelled by reductive amination [137, 167] and by reduction of the oximes and phenylhydrazones of α-keto-acids.[168]

Hydrogenation reactions give compounds labelled in at least two positions; if one of the positions is "activated" the label can be "washed out" by exchange and a specifically labelled compound is obtained. Another method of labelling only one position is by addition of DBr to a double bond. Dry DBr is made by heating deuterium with bromine [169] or reaction of D_2O with thionyl bromide.[170]

[161] D. Rittenberg, A. S. Keston, R. Schoenheimer, and G. L. Foster, *J. Biol. Chem.*, 1938, **125**, 1.

[162] M. Anchel and R. Schoenheimer, *ibid.*, p. 23.

[163] A. Kilt and A. Langseth, *Z. physikal. Chem.*, 1936, **176**, *A*, 65.

[164] C. K. Ingold, C. G. Raisin, and C. L. Wilson, *J.*, 1936, 915.

[165] W. E. van Heyningen and D. Rittenberg, *J. Biol. Chem.*, 1938, **125**, 495.

[166] K. Bloch and D. Rittenberg, *ibid.*, 1943, **149**, 505.

[167] D. Rittenberg, S. Ratner, and H. D. Hoberman, *J. Amer. Chem. Soc.*, 1940, **62**, 2249.

[168] D. Shemin and R. M. Herbst, *ibid.*, 1938, **60**, 1951.

[169] C. L. Wilson and A. W. Wylies, *J.*, 1941, 596.

[170] R. C. Elderfield, W. J. Gensler, F. Brody, J. D. Head, S. C. Dickerman, L. Wiederholt, C. B. Kremer, H. A. Hageman, F. J. Kreysa, J. M. Griffing, S. M. Kupchan, B. Newman, and J. T. Maynard, *J. Amer. Chem. Soc.*, 1946, **68**, 1579.

Sometimes water can be added to an unsaturated system, *e.g.*, preparation of acetaldehyde from acetylene and D_2O.[171, 172]

Replacement Reactions.—Decarboxylation of an acid, labelled with deuterium in the carboxyl group, results in the transfer of the label to the α-carbon atom. For example, acetaldehyde labelled with deuterium in the aldehyde group can be prepared by decarboxylation of $CH_3 \cdot CO \cdot CO_2D$, and deuterobenzene has been made by decarboxylating calcium mellitate in the presence of $Ca(OD)_2$.[173]

Halogen atoms are easily replaced with deuterium by decomposing the Grignard reagent with heavy water, by reduction with deuterium and palladium-black, or by reaction with Raney nickel containing deuterium. Desulphurisation with Raney nickel containing deuterium [174] results in the replacement of a thiol or thio-ether group with deuterium. This method has recently been used to prepare 7-deuterocholesterol from 7-bromocholesterol and 7 : 7-dideuterocholesterol from 7-ketocholesterol [175] (after conversion into the mercaptol by reaction with ethanedithiol) as well as deuterodethiopenicillin.[174]

Limitations of the Tracer Technique

Interpretation of Tracer Experiments.—The outlines of the tracer technique may be summarised as follows : A compound X, containing an excess above normal of one or more isotopic atoms, is converted into Y. If, after purification, Y also contains an excess of the isotope, one or more atoms of Y must have been derived from X. It would seem, therefore, easy to decide whether X is a direct precursor of another substance, Y, simply by a determination of the isotopic content of Y. There are, however, several factors which may affect more or less seriously the interpretation of the results, especially of experiments *in vivo*. For example, the labelled substance, though a normal intermediate in an animal, may be unstable under the conditions of administration or may be destroyed before absorption, or may not be transferred across cell membranes. If the labelled compound is readily oxidised *in vivo*, the labelled atom may also be incorporated into compound Y by an alternative pathway often involving fixation of carbon dioxide. *In vitro* experiments may be used advantageously in such cases, as one of the pathways may be selectively blocked by suitable enzyme inhibitors. It is often difficult to make valid quantitative, or even qualitative, interpretations of *in vivo* experiments and some effects peculiar to isotopic compounds and their reactions are discussed below.

The isotope effect. Since isotopes differ in mass and zero-point energy, physicochemical differences may be expected to become marked in a continuous process where repeated fractionation can occur. In 1939, A. O. Nier and E. A. Gulbranson [176] found that the ^{12}C-isotope was enriched in

[171] K. Bloch and D. Rittenberg, *J. Biol. Chem.*, 1944, **155**, 243.
[172] J. E. Zanetti and D. V. Sickman, *J. Amer. Chem. Soc.*, 1936, **58**, 2034.
[173] H. Erlenmeyer and H. Lobeck, *Helv. Chim. Acta*, 1935, **18**, 1464.
[174] " The Chemistry of Penicillin ", Princeton University Press, 1949, p. 267.
[175] D. K. Fukushima and S. Lieberman, *Fed. Proc.*, 1949, **8**, 200.
[176] *J. Amer. Chem. Soc.*, 1939, **61**, 697.

plants; the same effect has recently been explained by the faster utilisation of $^{12}CO_2$, compared with $^{14}CO_2$, for photosynthesis by barley seedlings.[177] Since the enhanced ^{18}O content of the atmosphere could not be due to photosynthetic reactions,[178] a possible explanation was fractionation by bacteria. Fractionation by soil bacteria was observed but it was insufficient to explain the enrichment quantitatively.[179] Recently it was found that in the hydrolysis of ^{14}C-labelled urea with the enzyme urease, the carbon dioxide produced early in the reaction had a higher specific activity than the later fractions.[179a]

An isotope effect has also been observed in chemical reactions. With carbon, the ^{12}C–^{12}C bond seems to be less stable than either the ^{12}C–^{13}C or the ^{12}C–^{14}C bond. When 1-^{13}C-propane was pyrolysed it was found that the ^{12}C–^{12}C bonds were ruptured 8% more often than the ^{12}C–^{13}C bonds.[180] During the decarboxylation of malonic and bromomalonic acid the ^{12}C–^{12}C bond was ruptured 1·12 and 1·4 times, respectively, as frequently as the ^{12}C–^{14}C bond.[181] A similar effect has now been observed in the alkaline hydrolysis of ^{14}C-labelled ethyl benzoate: the rate of hydrolysis of the ^{14}C-labelled compound was slower than that of the normal ester.[181a]

Isotopic asymmetry. Attempts to prepare compounds possessing optical activity as a result of isotopic asymmetry have usually been unsuccessful. In 1936, G. R. Clemo and A. McQuillen [182] claimed to have synthesised and resolved α-pentadeuterophenylbenzylamine,

$$C_6H_5{\cdot}CO{\cdot}C_6D_5 \longrightarrow \begin{matrix} C_6H_5 \\ C_6D_5 \end{matrix}\!\!>\!C{:}NOH \longrightarrow \begin{matrix} C_6H_5 \\ C_6D_5 \end{matrix}\!\!>\!CH{\cdot}NH_2$$

but the observed rotation was very small. Attempts by H. Erlenmeyer and H. Schenkel [183] to resolve a similar compound $C_6D_5{\cdot}CHPh{\cdot}CO_2H$ were unsuccessful. Recent experiments on the preparation of an optically active acid $R{\cdot}CHD{\cdot}CO_2H$ (R = benzyl, ethyl, or *n*-butyl) by the Marckwald asymmetric synthesis also failed in spite of elaborate precautions to prevent racemisation during the reaction.[184] Nevertheless, it is believed that failure to obtain an isotopically asymmetric compound is due to practical rather than theoretical reasons, especially as L. E. Young and C. W. Porter [185] have shown that replacement of hydrogen by deuterium in an optically active compound changes the specific rotation.*

[177] J. W. Weigl and M. Calvin, *J. Chem. Physics*, 1949, **17**, 210.

[178] S. Ruben, M. Randall, M. D. Kamen, and J. L. Hyde, *J. Amer. Chem. Soc.*, 1941, **61**, 877. [179] M. Dole, R. C. Hawkings, and H. A. Barker, *ibid.*, 1947, **69**, 226.

[179a] F. Daniels and A. L. Meyerson, *Science*, 1948, **108**, 676.

[180] D. P. Stevenson, C. D. Wagner, O. Beeck, and J. W. Otvos, *J. Chem. Physics*, 1948, **16**, 993. [181] P. E. Yankwich and M. Calvin, *ibid.*, 1949, **17**, 109.

[181a] W. H. Stevens and R. W. Attree, *Canadian J. Res.*, 1949, **27**, *B*, 807.

[182] *J.*, 1936, 808. [183] *Helv. Chim. Acta*, 1936, **19**, 1169.

[184] D. J. G. Ives and M. R. Nettleton, *J.*, 1948, 1085.

[185] *J. Amer. Chem. Soc.*, 1937, **59**, 328, 437.

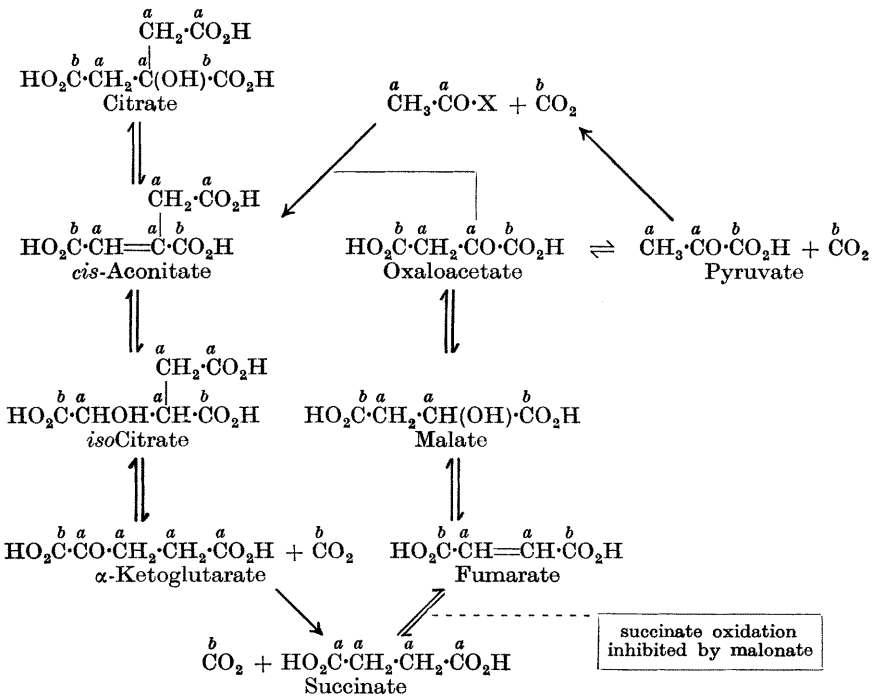
* [Added in proof.] E. L. Eliel (*J. Amer. Chem. Soc.*, 1949, **71**, 3970) has recently prepared optically active $Ph{\cdot}CHD{\cdot}CH_3$ by reduction of optically active $Ph{\cdot}CHCl{\cdot}CH_3$ with lithium aluminium deuteride.

In the interpretation of biochemical tracer experiments it has been assumed that if the administration of a compound (X) labelled in a specific position gave rise to a compound (Y) in which the labelled atoms are equally distributed in two or more positions, the conversion of X into Y must have taken place through a symmetrical intermediate (Z):

$$\underset{\text{(X.)}}{R_1\cdot\overset{*}{C}H_2\cdot CH_2R_2} \longrightarrow \underset{\text{(Z.)}}{R_2\cdot\overset{*}{C}H_2\cdot\overset{*}{C}H_2R_2} \longrightarrow \underset{\text{(Y.)}}{R_3\cdot\overset{*}{C}H_2\cdot\overset{*}{C}H_2R_2}$$

conversely, if the distribution of the labelled atoms in Y was unequal, it was concluded that a symmetrical compound (Z) could not have been an intermediate. This argument has been applied to the biochemical conversion of serine into glycine,[186] where the glycine isolated after administration of serine labelled with ^{15}N and ^{13}C (in the carboxyl group) had the same $^{15}N : {}^{13}C$ ratio; it was concluded, therefore, that aminomalonic acid could

The tricarboxylic acid cycle (after H. G. Wood[187])

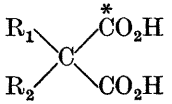
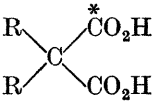


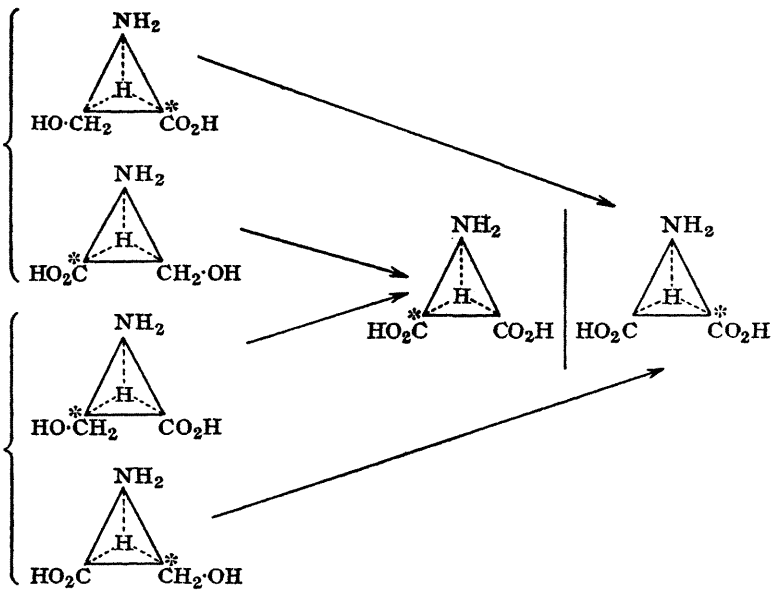
Starting with fumarate, carbon atoms which are equivalent (because of symmetry or origin from a symmetrical compound) are designated by the same letter. On completion of a cycle any label originally in a position *a* becomes generally distributed throughout the carbon atoms of succinate; conversion of succinate into the other participants of the cycle then results in a completely random distribution of the isotopic tracer. On the other hand any label originally in *b* will be found only in the positions marked *b*.

[186] D. Shemin, *J. Biol. Chem.*, 1946, **162**, 297. [187] *Physiol. Rev.*, 1946, **26**, 198.

not have been an intermediate, for in this case the expected ^{15}N : ^{13}C ratio would be twice that of the starting material.

A more important example of this type is the relationship of citric acid and the tricarboxylic acid cycle. The α-ketoglutaric acid isolated during studies on the fixation, *in vitro*, of $^{11}CO_2$ by pigeon liver contained ^{11}C almost exclusively in the α-carboxyl group.[188] In identical experiments with $^{13}CO_2$, the addition of non-isotopic citric acid caused no decrease in the isotope content of the α-ketoglutaric acid.[189] On the basis of the distribution of isotope in α-ketoglutaric acid, citric acid (a symmetrical compound) was excluded as a direct participant of the tricarboxylic acid cycle, as shown on previous page.

However, it has been pointed out by A. G. Ogston [190] that some symmetrical molecules might behave like an asymmetric compound if three-point attachment to an asymmetrical enzyme were a pre-requisite for reaction. The main condition for the recognition of such a complex by the tracer technique is the possibility of " isotopic asymmetry " in the " symmetrical " compound ; thus  fulfils this requirement whereas  does not. Both aminomalonic acid (labelled in one carboxyl group) and citric acid (labelled in one of the terminal carboxyl groups) could exist in two " isotopic modifications ", which can, in theory at least, be derived from D- and L-forms, *e.g.* :



[188] E. A. Evans and L. Slotin, *J. Biol. Chem.*, 1941, **141**, 439.

[189] H. G. Wood, C. H. Werkman, A. Hemingway, and A. O. Nier, *ibid.*, 1941, **139**, 483 ; 1942, **142**, 31.

[190] *Nature*, 1948, **162**, 963.

These " isotopic modifications " have not yet been resolved chemically ; however, in a biological system containing a presumably asymmetrical enzyme, such stereochemical differences have been observed, as, for example, in the following experiment.[191] Citric acid, biosynthesised *in vitro* from oxaloacetate, pyruvate, and $^{14}CO_2$, was found to be isotopically asymmetrical, *i.e.*, only one of the terminal carboxyl groups contained ^{14}C (as expected from the tricarboxylic acid cycle). After isolation and purification by the carrier technique, the labelled citric acid was converted enzymically into α-ketoglutarate. The α-ketoglutaric acid was found to contain ^{14}C almost exclusively in the α-carboxyl carbon atom, thus providing experimental proof for Ogston's views.

Ogston's original concept concerning biosynthesis through an enzyme substrate complex has recently been extended [192] to include any partial asymmetrical synthesis involving the reaction of a symmetrical compound, labelled with an isotope in one position, with an asymmetric reagent.

[191] V. R. Potter and C. Heidelberger, *Nature*, 1949, **164**, 180.
[192] P. E. Wilcox, *ibid.*, p. 757.