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# STUDIES ON THE BIOSYNTHESIS OF CHOLESTEROL—7*

## REARRANGEMENT OF METHYL GROUPS DURING ENZYMIC CYCLISATION OF SQUALENE

J. W. CORNFORTH, R. H. CORNFORTH, A. PELTER
National Institute for Medical Research, London

M. G. HORNING and G. POPJÁK
M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London

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**Abstract**—The present state of knowledge of the biosynthesis of cholesterol is summarised. An experiment is described wherein a special application of the carbon isotope $^{13}C$ permits a decision between possible modes of rearrangement during the enzymic cyclisation of squalene.

### INTRODUCTION

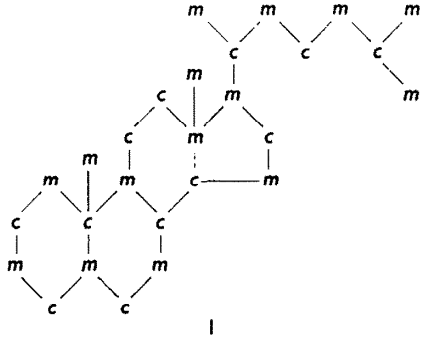
As knowledge is acquired of the manner in which an animal cell synthesises cholesterol, it becomes evident that this sterol, and indeed steroids as a class, belong biogenetically to a much larger family which also includes terpenoids and carotenoids. This family might be termed the *polyprenoids*, since they seem to be built up by association of units each of which contributes a branched chain of five carbon atoms at the oxidation level of isoprene.

Except for the fact that it undoubtedly was synthesised in the animal body, nothing was known for certain about the biosynthesis of cholesterol until isotopically labelled precursors were used. The discovery[1] that deuterioacetic acid is efficiently incorporated by yeast cells into unsaponifiable lipids (mainly ergosterol) was soon followed by demonstrations[2] that acetic acid is an important source of carbon for cholesterol. Konrad Bloch's pioneering work[3] established that acetic acid molecules are incorporated into the structure of cholesterol according to a definite pattern, and this pattern has been completely elucidated[4–9] by the total degradation of cholesterol biosynthesised from radioactive acetic acid, each of the twenty-seven carbon atoms in the structure being identified as coming from the methyl group or from the carboxyl group of acetic acid. The pattern is shown in (I) ($m$ = "methyl carbon",

* Part 6: *Biochem. J.* **69**, 238 (1958). A preliminary account of the present work has already appeared: *Proc. Chem. Soc.* 112 (1958).

[1] R. Sonderhoff and H. Thomas, *Liebigs Ann.* **530**, 195 (1937).
[2] K. Bloch and D. Rittenberg, *J. Biol. Chem.* **159**, 45 (1945); K. Bloch, E. Borek and D. Rittenberg, *J. Biol. Chem.* **162**, 441 (1946).
[3] H. N. Little and K. Bloch, *J. Biol. Chem.* **183**, 33 (1950).
[4] J. Würsch, R. L. Huang and K. Bloch, *J. Biol. Chem.* **195**, 439 (1952).
[5] J. W. Cornforth, G. D. Hunter and G. Popják, *Biochem. J.* **54**, 590, 597 (1953).
[6] R. B. Woodward and K. Bloch, *J. Amer. Chem. Soc.* **75**, 2023 (1953).
[7] K. Bloch, *Helv. Chim. Acta* **36**, 1611 (1953).
[8] W. G. Dauben and K. H. Takemura, *J. Amer. Chem. Soc.* **75**, 6302 (1953).
[9] J. W. Cornforth, I. Youhotsky Gore and G. Popják, *Biochem. J.* **65**, 94 (1957).

$c$ = "carboxyl carbon"). The work was greatly facilitated by the use of slices or cell-free extracts of rat liver to prepare cholesterol from radioactive precursors.



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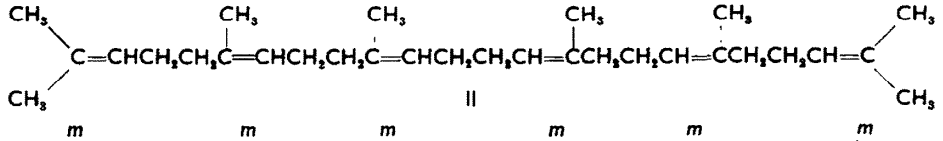

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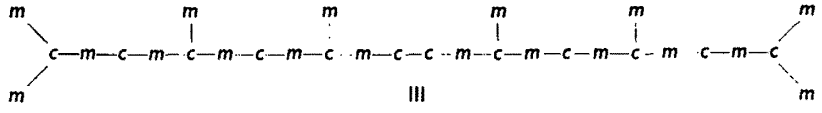

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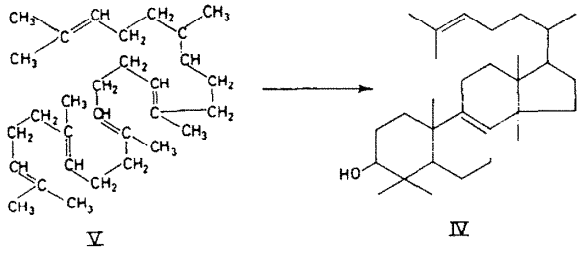


V IV

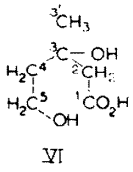
The pattern in the side-chain, which was elucidated first, was related by Bloch to a theory[10] of biosynthesis of the rubber hydrocarbon from acetic acid; and this suggestion of a common origin for terpenes and sterols led him to revive an old hypothesis to which Heilbron,[11] Channon[12] and Robinson[13] had contributed: that squalene (II) might be a precursor of cholesterol. Bloch then demonstrated[14] that squalene is indeed produced from acetic acid, and is converted into cholesterol, in the animal body. By total degradation of squalene biosynthesised from acetic acid we discerned[15] the pattern (III) of "methyl" and "carboxyl" carbons.

The next correlation was made when the structure of lanosterol (IV) suggested to Woodward and Bloch[6] that this substance might be an intermediate between squalene and cholesterol. The hypothesis required that the squalene molecule should be folded for cyclisation in a manner (V) differing from that proposed[13] earlier; and rearrangement of at least one methyl group during cyclisation was a necessary consequence.

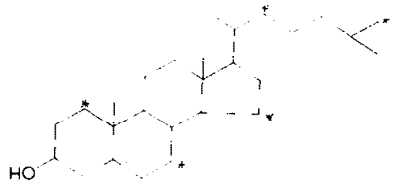
[10] J. Bonner and B. Arreguin, *Arch. Biochem. Biophys.* **21**, 109 (1949).
[11] I. M. Heilbron, E. D. Kamm and W. M. Owens, *J. Chem. Soc.* 1630 (1926).
[12] H. J. Channon, *Biochem. J.* **20**, 400 (1926).
[13] R. Robinson, *J. Soc. Chem. Ind.* **51**, 464 (1932).
[14] R. G. Langdon and K. Bloch, *J. Biol. Chem.* **200**, 129, 135 (1953).
[15] J. W. Cornforth and G. Popják, *Biochem. J.* **58**, 403 (1954).

The pattern (I) of "methyl" and "carboxyl" carbons is completely in harmony with the pattern (III) in squalene when this mode of cyclisation is assumed. Moreover, labelling technique enabled Bloch to show[16,17] that squalene is indeed converted biosynthetically into lanosterol, and lanosterol into cholesterol. Knowledge of the intermediate stages (perhaps ten to twelve in number) between lanosterol and cholesterol is growing rapidly and a complete account may soon be available of the order and manner in which the three superfluous methyl groups are (oxidatively) eliminated and the change from an 8:24-diene to a 5-ene is effected.

The stages of biosynthesis between acetic acid and squalene are less well understood and several gaps remain to be filled; but an important advance was made when mevalonic acid (VI), a growth factor for *Lactobacilli*,[18,19] was discovered[20] to be a highly efficient precursor of cholesterol. When mevalonic acid is incubated with rat-liver homogenate under nitrogen squalene rather than cholesterol is formed,[21] and when squalene biosynthesised in this way from 2-$^{14}$C-mevalonic acid is degraded,[21,22] radioactive carbon is found at only six positions in the molecule (VII).



VI



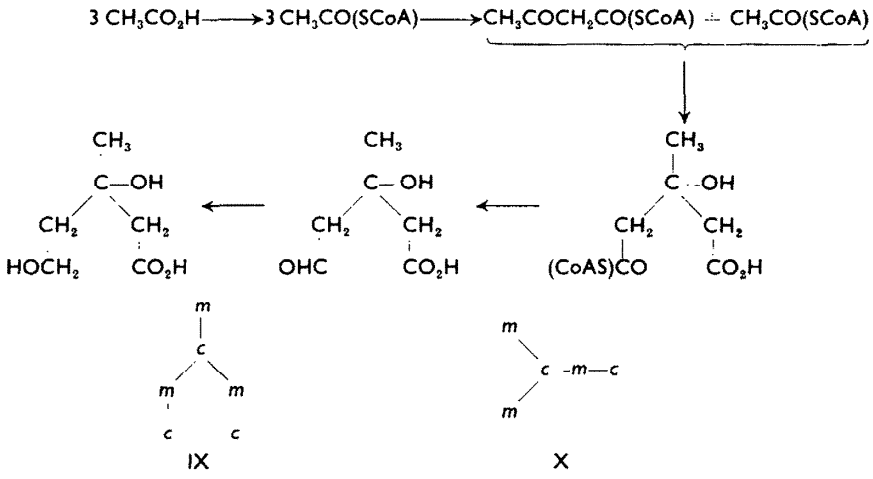
VII

VII

The presumption that the $C_{30}$ squalene (and hence cholesterol) is built up of six $C_5$ units, each contributed by one molecule of mevalonic acid, is well supported by other evidence: the carboxyl group of mevalonic acid is totally lost as carbon dioxide during biosynthesis of cholesterol;[23] mevalonic acid labelled at position 2 with $^{14}$C and at position 5 with tritium is incorporated into squalene without change of the $^{14}$C : T ratio;[24] and cholesterol biosynthesised from 2-$^{14}$C-mevalonic acid has been shown[25] to carry radioactive carbon at five positions only, of which three were identified and found to correspond with the predicted labelling illustrated in (VIII).

[16] R. B. Clayton and K. Bloch, *J. Biol. Chem.* **218**, 305, 319 (1956).
[17] T. T. Tchen and K. Bloch, *J. Biol. Chem.* **226**, 921 (1957).
[18] L. D. Wright, E. L. Cresson, H. R. Skeggs, G. D. E. MacRae, C. H. Hoffman, D. E. Wolf and K. Folkers, *J. Amer. Chem. Soc.* **78**, 5273 (1956).
[19] D. E. Wolf, C. H. Hoffman, P. E. Aldrich, H. R. Skeggs, L. D. Wright and K. Folkers, *J. Amer. Chem. Soc.* **78**, 4499 (1956).
[20] P. A. Tavormina, M. H. Gibbs and J. W. Huff, *J. Amer. Chem. Soc.* **78**, 4498 (1956).
[21] J. W. Cornforth, R. H. Cornforth, G. Popják and I. Youhotsky Gore, *Biochem. J.* **66**, 10P (1957); **69**, 146 (1958).
[22] F. Dituri, S. Gurin and J. L. Rabinowitz, *J. Amer. Chem. Soc.* **79**, 2650 (1957).
[23] P. A. Tavormina and M. H. Gibbs, *J. Amer. Chem. Soc.* **78**, 6210 (1956).
[24] B. H. Amdur, H. Rilling and K. Bloch, *J. Amer. Chem. Soc.* **79**, 2647 (1957).
[25] O. Isler, R. Rüegg, J. Würsch, K. F. Gey and A. Pletscher, *Helv. Chim. Acta* **40**, 2369 (1957).

The connexion between acetic acid and mevalonic acid is still being established, but the most probable sequence is



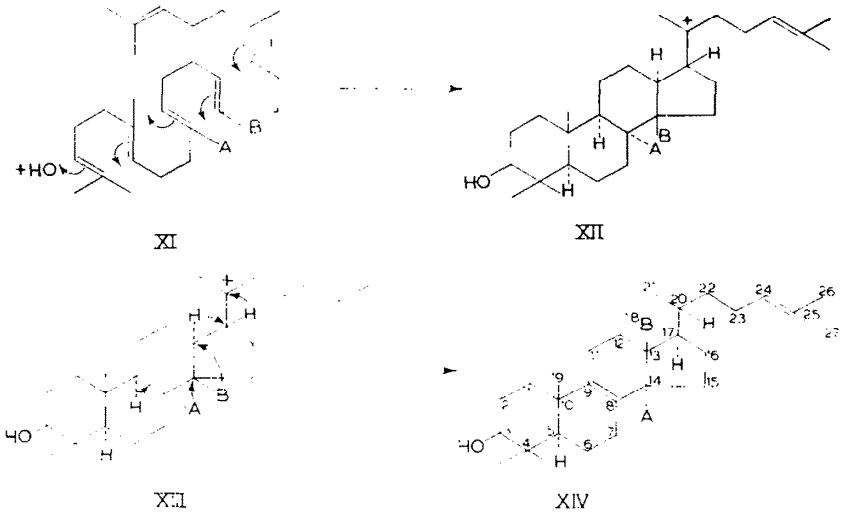

This would make the arrangement of acetic acid "methyl" and "carboxyl" carbons in mevalonic acid as shown in (IX). Consequently the $C_5$ unit, six of which go to make squalene, is (X) in terms of "methyl" and "carboxyl" carbons, and the pattern in squalene formed by association of these units is identical with that found by experiment. Several research groups are studying the role of mevalonic acid as a precursor of other polyprenoids,[26–28] and the evidence is already strong that in mevalonic acid or a closely related substance we have the basis of the "isoprene rule" (mevalonic acid $- CO_2 - 2H_2O =$ isoprene!), though it is still uncertain whether decarboxylation of the acid necessarily precedes the association of units.

## THE CYCLISATION OF SQUALENE

The biogenetic relation of squalene with lanosterol led Ruzicka's school to formulate detailed schemes of biogenesis[29] whereby the carbon skeletons of all known types of triterpenoids could be derived from squalene. All cyclisations and rearrangements were pictured as carbonium-ion reactions, and a few simple and well-founded assumptions were made about the stereochemical course of these changes. The condition was also accepted that each sequence should be "non-stop": once a carbonium ion had been generated in squalene by attack of a cation, the positive charge was not expelled or neutralised except to form the final product. This idea that squalene is cyclised non-stop to lanosterol was strongly supported by the finding[30] that lanosterol biosynthesised from squalene in a heavy water medium contained no excess deuterium attached to carbon. The assumption that a cationic species like $^+OH$ initiated the cyclisation was also supported: experiments with $^{18}O$ showed that the oxygen atom of lanosterol is supplied by molecular oxygen, not by water.

[26] G. D. Braithwaite and T. W. Goodwin, *Biochem. J.* **67**, 13P (1957).
[27] D. Arigoni, *Experientia* **14**, 153 (1958).
[28] A. J. Birch, R. W. Rickards and H. Smith, *Proc. Chem. Soc.* 192 (1958).
[29] A. Eschenmoser, L. Ruzicka, O. Jeger and D. Arigoni, *Helv. Chim. Acta* **38**, 1890 (1955).
[30] T. T. Tchen and K. Bloch, *J. Biol. Chem.* **226**, 931 (1957).

In the proposed mechanism, an hydroxyl cation attacks a terminal double bond in squalene, initiating a concerted reaction which successively closes rings A, B and C. The stereochemistry of lanosterol requires that closure of ring D be accompanied by rearrangements of carbonium ions for which the original paper should be consulted.* Disregarding this refinement, the cyclisation can be pictured as (XI → XII). A series of 1 : 2-shifts, executed by two hydrogen atoms and two methyl groups and ended by expulsion of a proton from $C_{(9)}$, completes the synthesis of lanosterol (XIII → XIV).



To an organic chemist, this scheme is most attractive, for 1 : 2-shifts and cationic cyclisation of dienes are familiar to him. However, 1 : 3-shifts are also not unknown, and the Swiss authors explicitly recognised that the methyl group marked A in (XIII) might move directly to $C_{(13)}$ of lanosterol instead of replacing methyl group B on $C_{(14)}$. Also, it is not *a priori* impossible that a series of squalene molecules might be so arranged upon an enzyme surface that transfer or exchange of methyl groups occurred—an intermolecular rearrangement.

It seemed to us, then, that a valuable experimental test of current theories of biogenesis of cyclic polyprenoids, as well as a contribution to the specific problem of cholesterol biosynthesis, could be made by determining whether a double intramolecular 1 : 2-shift of methyl groups occurs during cyclisation of squalene to lanosterol, or whether the necessary rearrangement takes place by a 1 : 3-shift or by intermolecular transfer. Since the first mechanism is chemically the most plausible, we were inclined to arrange the experiment so that the results would distinguish sharply between this mechanism and the other two. A different procedure would have been required to distinguish between the second and third.

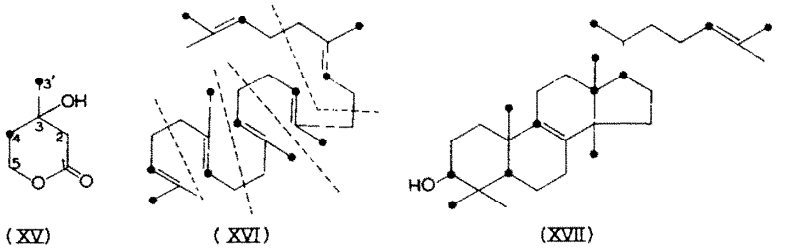
## THE EXPERIMENTAL APPROACH

It might appear that the problem could be resolved fairly easily by labelling the appropriate methyl group in squalene, but this is not so. Squalene is a symmetrical molecule; groups A and B are identical in (XI); neither can be labelled without

* If the double bond marked * in squalene (XI) were isomerised to *cis* geometry before cyclisation, a much more direct cyclisation could be formulated.

effectively labelling the other. A more refined approach is to label both the methyl-group carbon and the carbon atom to which, during cyclisation, it is supposed to migrate; and then to separate from the product (lanosterol or cholesterol) a degradation product containing both these atoms. However, the symmetry of squalene will defeat even this attempt to distinguish between the three mechanisms, so long as the final measurement of isotopic content is expressed as the average for a large number of molecules. Only if it were possible to examine the isotopic content of individual molecules of the degradation product, that is, to count the molecules having two isotopic atoms and those having one and none, could the labelling be so arranged that the mechanisms were distinguishable. This reasoning led to the experiment now to be described. The discovery of mevalonic acid as a precursor of squalene and cholesterol enabled us to replace by a simpler procedure our first schemes requiring a synthesis of squalene.

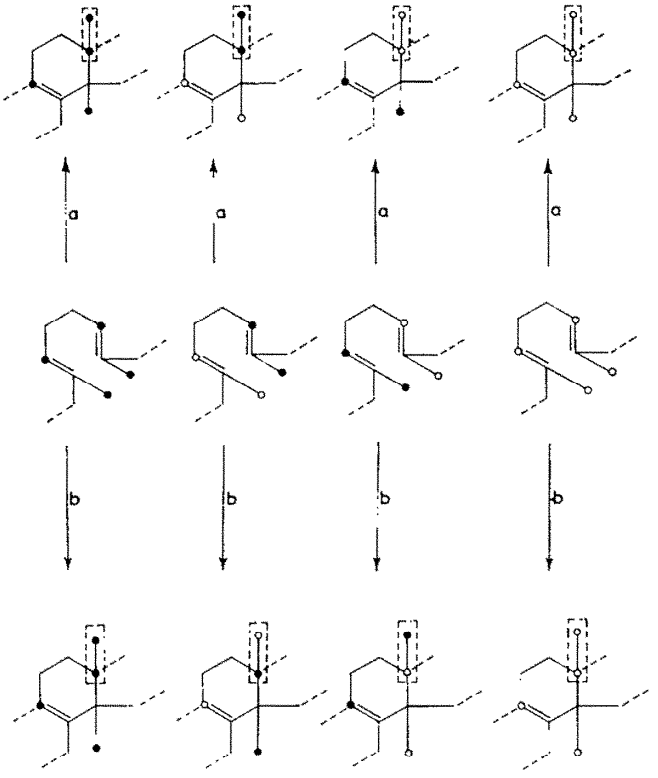
The principle of the experiment can be illustrated by an ideal example. Imagine a specimen of mevalonolactone (XV), which is equivalent to the acid in biosynthesis, having 100% $^{13}C$ at positions 3′ and 4, and 100% $^{12}C$ elsewhere. Let this mevalonolactone be converted biosynthetically into squalene (XVI). Each squalene molecule will contain twelve $^{13}C$ atoms occupying the positions indicated; the division into $C_5$ units is shown by broken lines. When this squalene is converted into lanosterol, the twelve $^{13}C$ atoms occupy the positions shown in (XVII), irrespective of the mechanism of cyclisation.




(XV) (XVI) (XVII)

Now suppose that before biosynthesis the mevalonolactone is diluted by an equal number of molecules of lactone containing only $^{12}C$. Many isotopic types of squalene will be formed, but for the present purpose only the two central $C_5$ units are significant. One-quarter of all squalene molecules would have two labelled units at the central positions; one-quarter would have two unlabelled units; and the remaining half would have one unit of each type. When the squalene is adsorbed on the enzyme surface for cyclisation, the non-symmetric type can be oriented in two equally probable ways, so that the cyclisation occurs with equal numbers of the four types shown on the middle line of scheme 1. If cyclisation is accompanied by a double 1 : 2-shift—mechanism (a)—the four types of lanosterol produced will be as shown on the upper line of Scheme 1; if there is a single 1 : 3-shift—mechanism (b)—the four types on the lower line of Scheme 1 will be formed. The $C_2$ fragment enclosed by broken lines in Scheme 1, when separated from the lanosterol or from the cholesterol derived from it, will be found to contain equal numbers of doubly-isotopic and non-isotopic molecules if mechanism (a) is correct; but by mechanism (b) half the molecules are singly-isotopic and one-quarter each are doubly-isotopic and non-isotopic. If the methyl group is transferred intermolecularly, the distribution is the same as for

mechanism (b). The significant difference between (a) and (b) is that (a) is a migration within a single $C_5$ unit, and no dilution with non-isotopic mevalonolactone can change the certainty of a $^{13}C$ atom becoming attached to another $^{13}C$ atom after migration; but when migration is between different units, the chance of two $^{13}C$




Scheme 1

atoms becoming connected diminishes with the dilution. However, it is still true that if the isotopic content of the $C_2$ fragment were determined in the usual way by combustion and measurement of $CO_2$, the answer (50% $^{13}C$) would be the same for (a) and (b). This would also be true if the isotope were $^{14}C$ and the radioactivity of the fragment had been measured. Therefore, it would be necessary to examine the $C_2$ fragment or a suitable derivative in a mass spectrometer, since this instrument can separate molecules differing in mass and could measure directly the proportions of doubly-isotopic, singly-isotopic and non-isotopic molecules.

The experiment as formulated above would be too difficult in practice. Homogeneous $^{13}C$ is not available; normal carbon contains 1·1% $^{13}C$; the biosynthesis cannot yet be done without dilution of the isotopic material by endogenous end-products or intermediates; and the actual separation of a $C_2$ fragment containing $C_{(13)}$ and $C_{(18)}$ of cholesterol, although it has been done,[9] requires more material than could easily be made. However, it is possible to obtain the same information by less

exacting procedures; and in the experiment as planned, four stages were proposed:

(1) To make mevalonolactone containing a large excess of $^{13}C$ at positions 3′ and 4 (and nowhere else), and to dilute it with a comparable amount of normal mevalonolactone.

(2) To obtain biosynthetic cholesterol, diluted as little as possible with endogenous material, by incubating this mevalonolactone with preparations of rat liver.

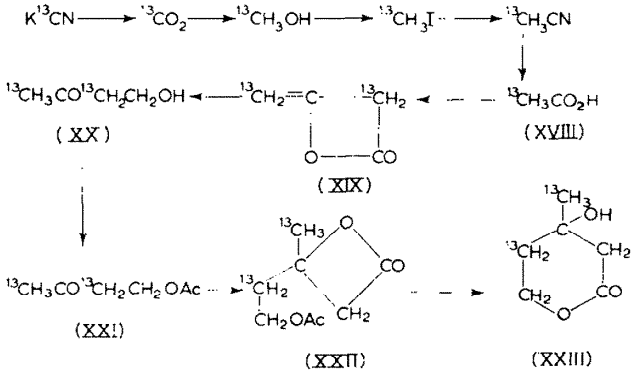
(3) To oxidise this cholesterol to acetic acid, and to determine the proportion of acetic acid molecules containing $C_{(18)} + C_{(13)}$ of cholesterol.

(4) To measure this acetic acid by mass spectrometry to obtain the proportions of doubly-isotopic, singly-isotopic and non-isotopic molecules.

## PREPARATION OF LABELLED MEVALONOLACTONES

Two new syntheses of mevalonolactone were devised, the first to obtain $^{13}C$-labelled material for the main experiment and the second to prepare 4-$^{14}C$-mevalonolactone which was required for some confirmatory experiments.

The starting-point for the first synthesis (Scheme 2), $^{13}C$-potassium cyanide, was converted[31] into 2-$^{13}C$-acetic acid (XVIII) and thence to acetyl chloride. An ethereal solution of diketen (XIX), obtained[32] by the action of triethylamine on this acetyl chloride in ether, was added to lithium aluminium hydride, when 2:4-$^{13}C_2$-3-oxobutanol (XX) was formed. An analogous reduction of *cyclo*hexylketen dimer had previously[33] been reported. The 3-oxobutanol was acetylated by keten to 2 : 4-$^{13}C_2$-3-oxobutyl acetate (XXI). Dilution, with a slightly larger amount of normal material, was done for convenience at this stage rather than with mevalonolactone. The diluted product was now treated with keten in the presence of boron trifluoride, and the intermediate product (presumably the $\beta$-lactone (XXII)) was saponified. Acidification, distillation and further purification then gave crystalline 3′:4-$^{13}C_2$-mevalonolactone (XXIII). Normal mevalonolactone can be conveniently prepared in quantity by this method, using 3-oxobutyl acetate prepared from methyl vinyl ketone.




Scheme 2

The isotopic composition of this mevalonolactone follows from the method of synthesis and the composition of the acetic acid (XVIII). The proportion of $^{13}C$ in the methyl group of this acetic acid was obtained by measurement of carbon dioxide

[31] J. D. Cox, H. S. Turner and R. J. Warne, *J. Chem. Soc.* 3167 (1950).
[32] J. C. Sauer, *J. Amer. Chem. Soc.* **69**, 2444 (1947).
[33] A. S. Spriggs, C. M. Hill and G. W. Senter, *J. Amer. Chem. Soc.* **74**, 1555 (1952).

from combustion of the acetic acid and of two specimens obtained from it by dilution with normal acetic acid. The mean value was 0·526 (52·6%).

In the 2:4-$^{13}C_2$-oxobutyl acetate (XXI) four species differing at positions 2 and 4 can be distinguished. The first (XXIV) has $^{13}C$ at both positions and constitutes $(0{\cdot}526)^2 = 0{\cdot}277$ of the whole; the proportion of the second (XXV), with $^{12}C$ at both positions, is $(1 - 0{\cdot}526)^2 = 0{\cdot}225$; the third and fourth (XXVI and XXVII), with $^{13}C$ at one position and $^{12}C$ at the other, each contribute $(0{\cdot}526 \times 0{\cdot}474) = 0{\cdot}249$. The calculation assumes random pairing of $C_2$ units in the formation of diketen. This assumption is not proved, but the normal extent of a $^{13}C$-isotope effect is not sufficient to alter these proportions significantly in a reaction permitted, as this one was, to go to completion.

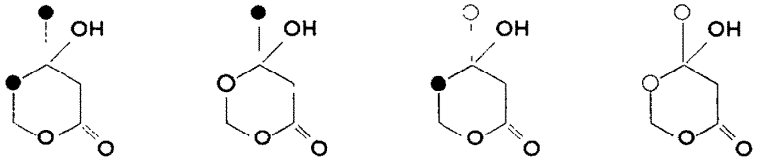
$^{13}CH_3CO^{13}CH_2CH_2OAc$ XXIV

$^{12}CH_3CO^{12}CH_2CH_2OAc$ XXV

$^{13}CH_3CO^{12}CH_2CH_2OAc$ XXVI

$^{12}CH_3CO^{13}CH_2CH_2OAc$ XXVII

The isotopic composition of the 3′:4-$^{13}C_2$-mevalonolactone (XXIII) was also measured by combustion and mass spectrometry of the carbon dioxide: thus the precise degree of dilution at the 3-oxobutyl acetate stage was found. Although dilution was done before mevalonolactone was made, it will be convenient in what follows to speak of "undiluted mevalonolactone", meaning the fraction of lactone originating from the undiluted 2:4-$^{13}C_2$-oxobutyl acetate and therefore containing the four species (XXVIII–XXXI) in the same relative proportions as their progenitors (XXIV–XXVII). The lactone was further diluted with a small, known amount of 2-$^{14}C$-mevalonolactone before the biosynthesis; this facilitated measurement of yields.

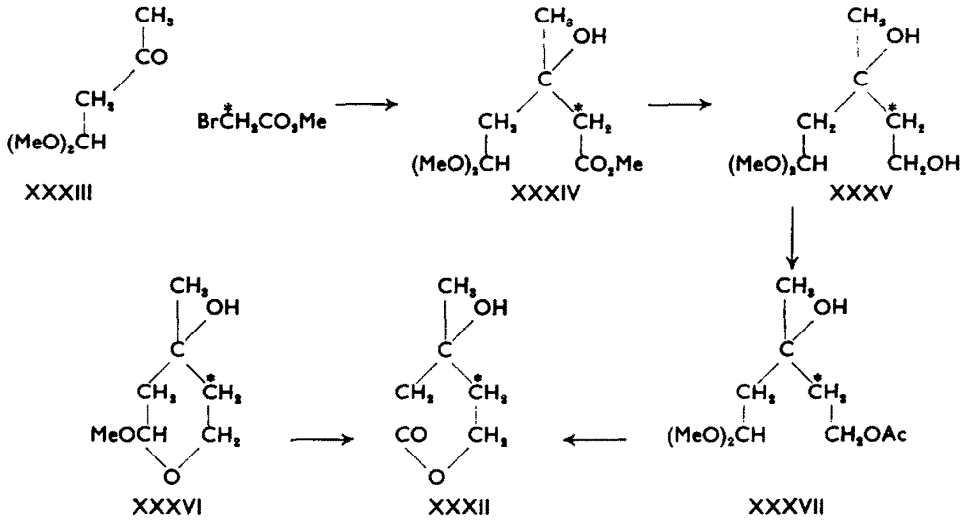


XXVIII XXIX XXX XXXI

● = $^{13}C$ atom, ○ = $^{12}C$ atom

An additional synthesis of mevalonolactone was devised to prepare 4-$^{14}C$ labelled material (XXXII). 4:4-Dimethoxybutanone (XXXIII) with methyl 2-$^{14}C$-bromoacetate and zinc gave, as already reported by Eggerer and Lynen,[34] a hydroxy ester (XXXIV). This was reduced by lithium aluminium hydride; the resulting alcohol (XXXV) formed a pyranoside (XXXVI) rather easily on distillation and was preferably acetylated first. When the product (XXXVII) was heated with hydrogen peroxide in acetic acid containing a little sulphuric acid and the mixture after neutralisation of mineral acid was distilled, 4-$^{14}C$-mevalonolactone was obtained directly. The pyranoside (XXXVI) gave mevalonolactone in somewhat lower yield when treated in the same way.

[34] H. Eggerer and F. Lynen, *Liebigs Ann.* **608**, 71 (1957).

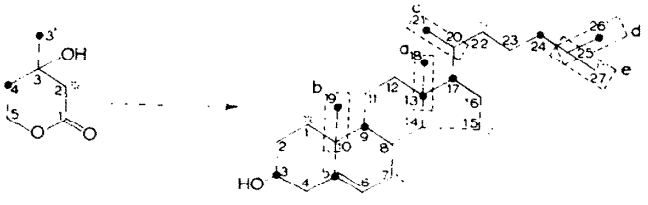



## PREPARATION OF $^{13}$C-CHOLESTEROL

To convert the 3′:4-$^{13}C_2$-mevalonolactone into cholesterol, a cell-free preparation of rat liver was made[35,21] by a modified technique which reduced the proportion of endogenous lipid. To increase further the proportion of newly biosynthesised material the amount of substrate taken was much larger than usual and the enzymes were kept active for as long as possible by repeated addition of co-factors. The cholesterol was separated, after saponification, as the digitonide, and recrystallised from methanol after recovery from the complex. Purification through the dibromide, which is wasteful of material, was not attempted, for the presence of other digitonin-precipitable steroids, even in large amount, could not affect the main calculations. Two specimens of cholesterol, having different degrees of dilution by endogenous material, were obtained.

## DEGRADATION OF $^{13}$C-CHOLESTEROL

Chromic oxidation of cholesterol can give acetic acid from four positions. The carboxyl groups of this acetic acid are supplied by $C_{(10)}$, $C_{(13)}$, $C_{(20)}$, and $C_{(25)}$, and the methyl groups by $C_{(19)}$, $C_{(18)}$, $C_{(21)}$, and $C_{(26)}$ *or* $C_{(27)}$ respectively (see Scheme 3). The positions in cholesterol and in mevalonolactone giving rise to the different types of acetic acid are correlated in Table 1. It can be seen that only one type of acetic acid, that from $C_{(18)} + C_{(13)}$ of cholesterol, has excess $^{13}$C in its carboxyl groups.



• = carbon atom labelled with $^{13}$C ○ = carbon atom labelled with $^{14}$C

Scheme 3

[35] N. L. R. Bucher and K. McGarrahan, *J. Biol. Chem.* **222**, 1 (1956).

TABLE 1. ORIGIN OF CARBON ATOMS IN ACETIC ACID FROM OXIDATION OF CHOLESTEROL

| Type of acetic acid (Scheme 3) | Position in cholesterol providing | | Position in mevalonolactone providing | |
|---|---|---|---|---|
| | methyl | carboxyl | methyl | carboxyl |
| a | 18 | 13 | 3′ | 4 |
| b | 19 | 10 | 3′ | 3 |
| c | 21 | 20 | 3′ | 3 |
| d | 26 | 25 | 3′ | 3 |
| e | 27 | 25 | 2 | 3 |

Thus if the excess $^{13}C$ in the carboxyl groups of the total acetic acid is measured and divided by the excess $^{13}C$ at a labelled position in mevalonolactone (diluted or undiluted), the quotient is the proportion of acetic acid molecules originating from $C_{(3')}$–$C_{(4)}$ of the lactone. If the total $^{13}C$ content of the acetic acid is also known by combustion and measurement of the carbon dioxide, the $^{13}C$ content of the methyl groups, and thence the proportion of acetic acid molecules originating from $C_{(3')}$–$C_{(3)}$ of the lactone, can be calculated. This reasoning dictated the experimental procedure.

To minimize the possibility of rearrangements of methyl groups during oxidation, the ordinary Kuhn–Roth technique[36] using chromic and sulphuric acids was avoided. The isotopic cholesterol was stirred with boiling aqueous chromic acid until it dissolved; phosphoric acid and water were then added and the acetic acid was distilled by repeated evaporation. This is an adaptation of a method described[37] by Kuhn and L'Orsa. Between 2·8 and 3 molecules of acetic acid (70–75% of theory) were obtained from each molecule of cholesterol. The acid was isolated as silver acetate. Normal cholesterol was treated in the same way to provide the bases for determining excess of isotopic species.

Some of the silver acetate was treated with bromine in carbon tetrachloride and the carbon dioxide evolved was isolated as barium carbonate. This method of degradation was preferred since the only oxygen in the reaction mixture is present in the carboxylate groups and no carbon dioxide could possibly be formed from methyl groups.

Another part of the silver acetate was burned to provide carbon dioxide representing the total carbon. The remainder was used for mass spectrometry.

Cholesterol from 4-$^{14}C$-mevalonolactone was also oxidised and the silver acetate was treated as above to obtain barium carbonate representing (a) carboxyl groups and (b) total carbon. The radioactivity of (b) was, within experimental error, one-half that of (a). Specimens of cholesterol from 3′:4-$^{13}C_2$-mevalonolactone and from 4-$^{14}C$-mevalonolactone were also burned, and the carbon dioxide was assayed for $^{13}C$ and $^{14}C$ respectively. Hence the excess of isotope at each "labelled" position in the cholesterol could be calculated, assuming 10 labelled positions for cholesterol from the $^{13}C$-lactone (cf. Scheme 3) and 6 for the $^{14}C$-lactone. The proportion of the total

[36] R. Kuhn and H. Roth, *Ber. Dtsch. Chem. Ges.* **66**, 1274 (1933).
[37] R. Kuhn and F. L'Orsa, *Z. Angew. Chem.* **44**, 847 (1931).

acetic acid which contained labelled carboxyl groups could then be calculated and was found to be essentially the same (ca. 20%) for the $^{13}C$ and $^{14}C$ specimens. These measurements support the conclusions (i) that the methyl groups of the acetic acid from oxidation contain no carbon originating from $C_{(4)}$ of mevalonolactone, and (ii) that the excess $^{13}C$ in the carboxyl groups of the acetic acid originates entirely from $C_{(4)}$ of mevalonolactone.

## MASS SPECTROMETRY OF ACETIC ACID

Published mass spectra of acetic acid[38] show a strong peak at mass 60 ($CH_3CO_2H^+$) and a minor peak at 61 (principally $^{13}CH_3CO_2H^+$ and $CH_3{}^{13}CO_2H^+$); we also found a still smaller peak at 62, attributable to $CH_3C^{18}OOH^+$ and minor proportions of other isotopic species. There were no background peaks at 60, 61 or 62, and no peaks in the acetic acid spectrum at 59 or 58; this simplified the analysis. Fragmentation of the acetic acid accompanies ionisation and is a possible source of error if the $^{13}C$ species are markedly less easily disintegrated than the $^{12}C$ species. We were able to measure this effect by comparing the values obtained for the mass 61 content of synthetic $^{13}CH_3CO_2H$ with the values deduced from measurement of carbon dioxide after combustion. The small difference between these two sets of values was taken as a measure of the isotope effect on fragmentation of acetic acid, and a corresponding correction was applied to the results obtained by direct measurement of the acetic acid from cholesterol. A further small correction must be made for the slightly altered distribution of $^{18}O$, $^{17}O$ and D among the three peaks 60, 61 and 62.

Wood[39] reported unsuccessful attempts to measure directly the proportions of $^{13}C$ species in acetic acid, which he eventually converted into ethylene before measurement.

The difficulties were (i) preparation of pure acetic acid in small quantities, (ii) introduction into the spectrometer, and (iii) strong absorption on the instrument's walls. The first of these difficulties was overcome by preparing acetic acid in a closed system from dry silver acetate and dry hydrogen chloride, the second by arranging a pinhole leak fed by acetic acid vapour from a liquid sample held at constant temperature. Since the inlet system had a very small surface area, absorption on its walls was negligible. Absorption on the spectrometer walls still caused difficulty, for a new sample had to be run for some time before the contribution of the previous sample had been reduced to insignificance; but this could be accepted since relatively few samples were to be measured.

## CONCLUSION

The calculation of the expected excess above normal of $^{13}C_1$- and $^{13}C_2$-acetic acids, in the acetic acid from oxidation of cholesterol, according to mechanisms (a) and (b), is detailed and explained in the experimental section. The calculated values for mechanism (a) are derived from three experimentally determined figures: the proportion of $^{13}C$ in the methyl group of the synthetic $^{13}CH_3CO_2H$, the excess $^{13}C$ in the carboxyl group of the acetic acid from oxidation of cholesterol, and the excess $^{13}C$ in the total carbon of the acetic acid from oxidation of cholesterol. For calculation by mechanism (b), the degree of dilution of the isotopic lactone before biosynthesis is also needed. Both calculations assume, as indicated above, random combination of

[38] G. P. Happ and D. W. Stewart, *J. Amer. Chem. Soc.* **74**, 4404 (1952).
[39] H. G. Wood, *J. Biol. Chem.* **194**, 905 (1952).

isotopic $C_2$ units during synthesis of mevalonolactone; there is also an implicit assumption that isotopic and normal molecules are used at random for biosynthesis of squalene. In addition, the calculation for mechanism (b) assumes that no further dilution of the mevalonolactone by endogenous intermediates occurs during biosynthesis of squalene; this assumption is not necessarily true. If such dilution does take place, the calculated value for $^{13}C_2$-acetic acid would be still lower and the value for $^{13}C_1$-acetic acid higher. The calculated values for mechanism (a) would be completely unaffected.

TABLE 2. OBSERVED AND CALCULATED EXCESS OF $^{13}C_1$- AND $^{13}C_2$-ACETIC ACID IN PRODUCT FROM OXIDATION OF CHOLESTEROL

| Experiment | Quantity | Calc. for (a) | Observed | Calc. for (b) |
|---|---|---|---|---|
| I | % excess $^{13}C_1$-acetic | 1·37 | 1·40 | 1·57 |
| | % excess $^{13}C_2$-acetic | 0·206 | 0·210 | 0·107 |
| | Ratio $^{13}C_1/^{13}C_2$ | 6·65 | 6·67 | 14·7 |
| II | % excess $^{13}C_1$-acetic | 2·09 | 2·08 | 2·37 |
| | % excess $^{13}C_2$-acetic | 0·294 | 0·285 | 0·153 |
| | Ratio $^{13}C_1/^{13}C_2$ | 7·11 | 7·30 | 15·5 |

The experimental and calculated results are set out in Table 2; the agreement between observed values and those calculated for mechanism (a) is excellent. The conclusion following unquestionably from the results is that during biosynthesis of cholesterol from mevalonolactone and subsequent chemical degradation to acetic acid, a carbon atom originating from position 3′ of the lactone migrates to a carbon atom originating from position 4 of the same molecule of lactone. This means that the migration, whenever it took place, was an intramolecular 1:2-shift.

If the rearrangement which undoubtedly occurs during enzymic cyclisation of squalene had taken place by mechanism (b), or intermolecularly, and was followed by further rearrangement during oxidation, it does not seem possible to explain the experimental figures by except assuming that a total migration of $C_{(19)}$ to $C_{(5)}$ during oxidation is accompanied by a total migration of $C_{(18)}$ to $C_{(14)}$ or $C_{(12)}$ (cf. Scheme 3). An oxidation of this sort could not be defended on chemical grounds. Rearrangement after biosynthesis therefore seems to be excluded.

A second possibility is that rearrangement during cyclisation of squalene to lanosterol takes place by mechanism (b) but is followed by intramolecular rearrangement during demethylation of lanosterol to cholesterol, the methyl group on $C_{(14)}$ migrating to $C_{(13)}$. But this methyl group would have to cross from the $\alpha$-side to the $\beta$-side of the molecule during an intramolecular rearrangement, which seems most improbable. The same objection applies to an exchange of methyl groups in lanosterol itself.

A third possibility is that squalene cyclises by a mechanism of type (b) to give not lanosterol but a 13$\alpha$:14$\beta$ isomer, and that this rearranges, with exchange of methyl groups, to lanosterol. However, it is difficult to imagine a mechanism for this exchange; nor is there evidence to suggest that any intermediate substance intervenes between squalene and lanosterol.

Thus an intramolecular 1:2 shift according to mechanism (a) during cyclisation of squalene to lanosterol appears to be the only mechanism which needs no improbable assumptions to explain the results.

In an independent research simultaneous with ours, Maugdal, Tchen and Bloch[40] established, by studying the cyclisation of squalene labelled in a special manner with $^{13}C$, that the methyl group attached to $C_{(14)}$ of lanosterol has also arrived there by migration. The results, though they do not exclude an intermolecular rearrangement, are entirely consistent with mechanism (a) and not with mechanism (b); and the two experimental approaches, dealing as they do with different methyl groups, are complementary. We are much indebted to Professor Bloch for communicating these results before publication.

## EXPERIMENTAL

### *Preparation of isotopic mevalonolactones*

*Preparation of* 2:4-$^{13}C_2$-3-*oxobutanol.* Sodium 2-$^{13}C$-acetate (6·584 g), prepared from K$^{13}CN$ by the method of Cox *et al.*,[31] was deposited evenly by freeze-drying on the walls of a flask and dehydrated at 220°/0·07 mm for 4 hr. Redistilled phthalyl chloride (55 ml) was added and the mixture was heated (bath 170°), the acetyl chloride distilling into a 3-necked flask cooled to −70° and equipped with an efficient stirrer and a dry-ice condenser. The last traces of acetyl chloride were driven over by brief heating with a free flame.

The acetyl chloride generator was disconnected and ether (80 ml) was distilled from lithium aluminium hydride into the 3-necked flask, which was then brought to room temperature. Dry, redistilled triethylamine (11·5 ml) was added during 1 hr to the vigorously stirred mixture; the temp. rose until the ether was refluxing. Finally the mixture was stirred for a further 2¼ hr, a tube fitted with a filter plug of glass wool was introduced, and the ethereal filtrate was pressed into another flask by means of dry nitrogen. The residue of triethylamine hydrochloride was washed twice with 50 ml portions of ether (dried by $LiAlH_4$).

The total filtrate was added in four portions to well-stirred ethereal lithium aluminium hydride (59 ml of 0·9 M) cooled at −70°. A yellow solid was formed immediately. The mixture was stirred for 2 hr and excess of hydride was decomposed by addition of ethereal ethyl acetate. Saturated aqueous $KHCO_3$ was then added until the solid coagulated. The mixture was filtered and the solid washed twice with ether. Distillation of the filtrate then gave 2:4-$^{13}C_2$-3-oxobutanol (0·711 g) as a colourless liquid, b.p. 76–78°/16 mm.

The identity of this product, though not its purity, had been previously established by submitting normal sodium acetate to identical procedures, when the product after redistillation gave an infra-red spectrum identical with that of 3-oxobutanol prepared from acetone and formaldehyde. When normal diketen was reduced as above, the maximum yield of 3-oxobutanol obtained was 48%. When normal acetyl chloride was condensed by triethylamine as above and the mixture treated with ethanolic sulphuric acid, ethyl acetoacetate was obtained in 70% yield.

2:4-$^{13}C_2$-3-*Oxobutyl acetate.* 2:4-$^{13}C_2$-3-oxobutanol (0·711 g) and dry ether (0·7 ml) were cooled to −15° and treated with a slight excess over one equivalent of keten. After 20 min the product was distilled to give 2:4-$^{13}C_2$-3-oxobutyl acetate (0·937 g)

[40] R. K. Maugdal, T. T. Tchen and K. Bloch, *J. Amer. Chem. Soc.* **80**, 2589 (1958).

b.p. 40°/0·1 mm. A product obtained similarly from normal 3-oxobutanol had an infra-red spectrum almost identical with that of 3-oxobutyl acetate prepared as described below: the spectrum in the carbonyl region indicated a small amount of impurity (diketen?).

*3-Hydroxy-3-methylpentano-5-lactone (mevalonolactone).* Normal 3-oxobutyl acetate was prepared by heating methyl vinyl ketone (35 g), acetic acid (150 ml) and water (1 drop) overnight under reflux. Fractionation gave recovered ketone and acid and 3-oxobutyl acetate (28·6 g; 44%), b.p. 78–84°/15 mm, which was freed from traces of acetic acid as described previously.[21] The infra-red spectrum was identical with that of an authentic specimen (made by acetylation of 3-oxobutanol with acetic anhydride). This process is an improvement on a patented procedure.[41]

3-Oxobutyl acetate (26 g) and dry ether (20 ml) were cooled (bath at −30°) and boron trifluoride etherate (0·6 ml) was added. Keten (slightly over 1 equiv.) was passed and the mixture was then left at −25° for 2 hr; some crystallisation usually occurred. The mixture was added, with the aid of more ether, to N methanolic potassium hydroxide (600 ml) cooled to −15°. The solution was kept overnight at room temperature, then neutralised (phenol red) by methanolic hydrogen chloride and evaporated at low pressure. The residue after washing with ether and chloroform was treated with methanol and then with sufficient methanolic hydrogen chloride to bring the total added to the equivalent of 592·4 ml of N (7·6 ml of N alkali is consumed by 0·6 ml boron trifluoride etherate). Potassium chloride was removed by filtration and the product, recovered by evaporation at low pressure, was extracted by chloroform. The filtered chloroform solution was distilled to give 24·9 g; b.p. 110°/0·01 mm. This contained a small amount of β-hydroxy*iso*valeric acid, since the keten was prepared as usual by pyrolysis of acetone and some acetone remained in the gas stream even though it had passed through a trap at −30° before entering the reaction vessel. The product was therefore treated with ethereal diazomethane until the yellow colour of the reagent persisted; redistillation then gave mevalonolactone (23·3 g; 90%), b.p. 114°/0·01 mm. (Found: C, 55·7; H, 7·7; Calc. for $C_6H_{10}O_3$: C, 55·4; H, 7·7%).

*3′-4:$^{13}C_2$-3-hydroxy-3-methylpentano-5-lactone.* 2:4-$^{13}C_2$-3-oxobutyl acetate (937 mg) was diluted with pure, normal 3-oxobutyl acetate (937 mg), and treated in ether with boron trifluoride and keten as described above. The product was isolated, also as described above; after second distillation the mevalonolactone (1·14 g) was dissolved in dry ether (20 ml) under reflux in a weighed conical flask. The solution was cooled, seeded and set aside at −5° for 24 hr. Large crystals separated and were isolated by decanting the mother liquor, washing rapidly with a little chilled ether and transferring the flask immediately to a vacuum desiccator. The crystalline hygroscopic 3′:4-$^{13}C_2$-mevalonolactone (813 mg) was the specimen used for biosynthesis and $^{13}C$ assay. A second crop was recovered from the mother liquor. A normal specimen, similarly prepared, had m.p. 28°.

*Methyl 2-$^{14}$C-3-hydroxy-5:5-dimethoxy-3-methylpentanoate*(XXXIV). Five portions of 2-$^{14}$C-bromoacetic acid (12·9 mg and 0·1 mC each) were esterified by ethereal diazomethane and normal methyl bromoacetate (1·223 g) was added. The whole was pipetted with the aid of ether (8 ml) into the reaction flask which contained activated zinc (1 g), ether (1·7 ml) and 4:4-dimethoxybutan-2-one (1·1 g). The

[41] du Pont, *U.S. Patent* 2,010,828 (1935).

mixture was stirred vigorously for 2½ hr. Reaction then set in suddenly; when it subsided the mixture was stirred and refluxed for 1½ hr and left for 18 hr.

A chilled mixture of acetic acid (0·5 ml) and water (3·5 ml) was added. The aqueous layer was saturated with ammonium chloride and extracted four times with ether which was then united with the ethereal layer, washed with a little saturated aqueous $KHCO_3$ (which was subsequently re-extracted with ether), dried ($MgSO_4$) and distilled. The acetal ester (1·09 g; 55%) had b.p. 60–70°/0·05 mm. Redistillation gave 0·986 g, b.p. 64–66°/0·05 mm.

*4-Hydroxy-2-methoxy-4-methyltetrahydropyran* (XXXVI). Lithium aluminium hydride (1·5 g) was stirred with tetrahydrofuran (30 ml) for 1 hr, cooled in ice water, and treated dropwise with normal methyl 3-hydroxy-5:5-dimethoxy-3-methylpentanoate (3 g) in tetrahydrofuran (20 ml). The mixture was stirred overnight at room temp., the hydride was decomposed by addition of ethyl acetate (9 ml), saturated aqueous ammonium chloride (9 ml) was added and the product was isolated in the usual manner. Distillation gave *4-hydroxy-2-methoxy-4-methyltetrahydropyran* (1·22 g; 1·1 g after redistillation), b.p. 60–62°/0·1 mm, as a colourless liquid. (Found: C, 57·4; H, 9·5; $C_7H_{14}O_3$ requires C, 57·5; H, 9·6%).

*1-Acetoxy-2-*$^{14}$*C-5:5-dimethoxy-3-methylpentan-3-ol* (XXXVII). Methyl 2-$^{14}$C-3-hydroxy-5:5-dimethoxy-3-methylpentanoate (0·968 g) in ether (5 ml) was stirred and cooled in ice during dropwise addition of 0·95 M ethereal lithium aluminium hydride (11 ml). After 30 min additional ether (2 ml) was added and the mixture was stirred at room temp. for 4 hr and refluxed for 1 hr. Ethereal ethyl acetate was added slowly until reaction ceased, then saturated aqueous $KHCO_3$ was added until the solid coagulated. The solid was washed on a filter with dry ether and the ethereal solutions were evaporated at low pressure. To the residue, pyridine (4 ml) and acetic anhydride (1 ml) were added. After 2½ hr the mixture was warmed for 20 min on a steam-bath and then distilled. The product (0·807 g, 80%) had b.p. 80–84°/0·003 mm. A non-isotopic specimen of the *1-acetoxy-5:5-dimethoxy-3-methylpentan-3-ol*, prepared similarly, was analysed (Found: C, 54·7; H, 8·9; $C_{10}H_{20}O_5$ requires C, 54·6; H, 9·1%).

*4-*$^{14}$*C-3-hydroxy-3-methylpentano-5-lactone* (*mevalonolactone*). 1-Acetoxy-2-$^{14}$C-5:5-dimethoxy-3-methylpentan-3-ol (0·807 g) was added to a mixture of acetic acid (2 ml), 30% hydrogen peroxide (1 ml), water (2 ml) and sulphuric acid (0·02 ml; d 1·84). The mixture was refluxed for 1 hr, anhydrous potassium carbonate (100 mg) was added and the solution was evaporated at low pressure. The residue was extracted with chloroform; distillation of the extract gave 4-$^{14}$C-mevalonolactone (322 mg; 68%), which was crystallised from ether (6 ml) as described earlier to give a first crop (0·143 g) used for biosynthesis. A non-isotopic specimen was prepared in the same way in 77% yield.

Mevalonolactone was also obtained in 59% yield by oxidising the pyranoside (XXXVI) in the same way.

*Biosynthesis of cholesterol from isotopic mevalonolactones*

*Experiment with 2-*$^{14}$*C-mevalonolactone.* This preliminary run was made to determine the advantage gained by adding co-factors at intervals during incubation and to try whether free cholesterol formed during incubation would be less diluted by endogenous material than total (free and esterified) cholesterol.

Rat liver (25 g; from 6 rats) was homogenised by the method of Bucher and McGarrahan[35] in 0·1 M phosphate buffer (62·5 ml; pH 7·4–7·5) containing nicotinamide (0·03 M) and magnesium chloride (0·004 M). The homogenate was centrifuged at 700 g for 25 min at 0° and decanted through several layers of gauze to remove a fatty layer. The supernatant was centrifuged at 10,000 g for 25 min at 0°, and again decanted through gauze from the residue of mitochondria. The supernatant fraction containing microsomes and soluble enzymes[21] is referred to as $S_{10}$.

Three flasks each containing $S_{10}$ (10 ml) and 2-$^{14}$C-mevalonolactone (21 $\mu$moles; 0·04 $\mu$C/$\mu$mole) were incubated aerobically at 37°. After 1 hr, DPNH (5 $\mu$moles), TPN (2·5 $\mu$moles), ATP 25 ($\mu$moles) and ascorbate (75 $\mu$moles) were added to flasks 2 and 3. After 2 hr, the same additions were made to flask 3 alone. After 3 hr, each flask was emptied into alcohol–ether (3:1 v/v; 100 ml + 50 ml for rinsing). The solutions were boiled for 1 min, filtered (Whatman No. 43 paper) and evaporated at low pressure (bath 40–50°). The residue was extracted with light petroleum (b.p. 40–60°); the extract after being clarified by centrifugation was brought to a volume of 1·3 ml in another centrifuge tube. Acetone (5 vol) and magnesium chloride (2 drops of saturated solution in ethanol) were added and after 1 hr at 0° the precipitate of phospholipids was centrifuged. The supernatant and two washings (acetone) were evaporated to 1 ml in another centrifuge tube and digitonin (5 ml of 0·5% in 90% ethanol) and N hydrochloric acid (6 drops) were added. The precipitate was centrifuged, washed with 1:1 alcohol-ether, 1:1 acetone-ether, and acetone (2 ml of each), suspended in acetone and collected on a weighed disk, area 2 cm$^2$, of Whatman No. 54 paper for counting.[42] The results are shown in Table 3.

TABLE 3. INCORPORATION OF 2-$^{14}$C-MEVALONOLACTONE INTO FREE AND ESTERIFIED CHOLESTEROL

| Flask | Digitonides (mg) | Corrected counts/min at inf. thickness | Total $^{14}$C in sterol, $\mu$C × $10^{-3}$ | Mevalonolactone incorporated, $\mu$M |
|---|---|---|---|---|
| 1 (no cofactors added) | 6·7 (free) | 17,300 | 92·6 | 2·64 |
| | 1·3 (esters) | 10,911 | 11·3 | 0·34 |
| 2 (cofactors added after 1 hr) | 6·9 (free) | 29,400 | 162 | 4·88 |
| | 1·4 (esters) | 28,300 | 31·7 | 0·96 |
| 3 (cofactors added after 1 and 2 hr) | 7·4 (free) | 32,530 | 193 | 5·78 |
| | 1·7 (esters) | 34,400 | 46.8 | 1·4 |

The supernatant and washings from the digitonide were reduced to 1·5 ml and ether (5 vol) was added to precipitate digitonin. The supernatant was then evaporated and the residue dissolved in light petroleum (b.p. 40–60°). This was clarified by centrifuging, reduced to 0·5 ml and treated with ethanol (1 ml) and 4 N KOH (0·5 ml).

[42] G. Popják, *Biochem. J.* **46**, 560 (1950).

The mixture was heated (bath 80°) for 1½ hr then extracted with light petroleum. The extracted material in acetone (0·5 ml) was treated with digitonin solution (2 ml) and N HCl (6 drops). The digitonides were prepared for counting as before (Table 3).

Thus a total of 7·2 μmoles of mevalonolactone had been incorporated into cholesterol in flask 3 which had received two additions of cofactors. Since the theoretical maximum from 21 μmoles of DL-lactone is probably 10·5 μmoles, this procedure was considered satisfactory and was used in the $^{13}C$ experiments.

*Experiments with* 2-$^{14}$C-3′:4-$^{13}C_2$-*mevalonolactone.* $S_{10}$ was prepared from ca. 130 g of liver pulp from 25 white rats. The 3′:4-$^{13}C_2$-mevalonolactone (524 mg) described above was mixed with 2-$^{14}$C-mevalonolactone (18 mg; 0·2 μC/μmole).

Ten flasks each containing $S_{10}$ (20 ml) and the labelled lactone (40 μmoles) were incubated aerobically at 37°. After 1 hr and 2 hr, each flask received DPNH (10 μmoles), TPN (5 μmoles), ATP (50 μmoles) and ascorbate (150 μmoles). After 3½ hr, potassium hydroxide (2 g pellets) and ethanol (20 ml) were added to each flask. After being heated overnight on a steam-bath the contents of the flasks were pooled and extracted thrice with light petroleum (b.p. 40–60°) which was then washed with water, dried ($MgSO_4$) and evaporated at low pressure (bath 50°). An aliquot (0·5 ml) of a solution (10 ml) of the residue in acetone was taken and a digitonide prepared from this as described above.

Five such experiments were run; the weights and radioactivities of the digitonides are given in Table 4.

TABLE 4. YIELDS AND RADIOACTIVITIES OF DIGITONIDES FROM 2-$^{14}$C-3′:4-$^{13}C_2$-MEVALONOLACTONE

| Experiment | Digitonide (mg) | Counts/min at inf. thickness |
|---|---|---|
| 1 | 9·5 | 2840 |
| 2 | 13·5 | 3290 |
| 3 | 17·9 | 2347 |
| 4 | 12·3 | 4560 |
| 5 | 14·1 | 4400 |

In the first three experiments the liver homogenate was spun at 700 g for 10 min, instead of 25 min. Although the reason for the superiority of a 25 min period was not rigorously established we believe that more endogenous sterol (as a lipoprotein complex?) is carried down when spinning is prolonged.

From the combined product of Experiments 1, 2 and 3, 680 mg of digitonide were isolated. This was dissolved in acetic acid (60 ml), boiled for 5 min, cooled and treated with ether (300 ml). The precipitate was centrifuged and washed twice with ether. The ethereal solution after washing with water (4 × 200 ml), and with M sodium hydrogen carbonate until neutral, was dried ($MgSO_4$) and evaporated to leave crude cholesterol (139 mg). Recrystallisation from methanol gave colourless plates (114·5 mg), m.p. 143–144°. This was the "more dilute" cholesterol used in Exp. I.

From the combined products of experiments 4 and 5 digitonide (504 mg), crude sterol (115 mg), and recrystallised sterol (75 mg; m.p. 142–143°) were obtained in

the same way. The recrystallised material ("less dilute" cholesterol) was used in Exp. II.

Cholesterol was prepared from 4-$^{14}$C-mevalonolactone in the same way. A portion (10·5 mg) of the product was mixed with purified cholesterol (76·9 mg) and the whole recrystallised from methanol to give 69 mg.

*Degradation of cholesterol to silver acetate*

*Experiment* I. The "more dilute" cholesterol (96·5 mg) was melted on the walls of a 50 ml flask fitted with a side tubulature. A solution of chromium trioxide (5 g) in water (12·5 ml) was added, a platinum Hershberg-type stirrer operating through a reflux condenser was fitted and the mixture was boiled gently with stirring for 11 hr, when very little insoluble material remained. The condenser and stirrer were removed and rinsed with a little water, a spray-trap and condenser were fitted, phosphoric acid (3 ml; d 1·75) was added and the mixture was distilled until the residue began to foam. Successive quantities of water (ca. 10 ml) and fresh porous pot were added through the tubulature after each distillation. After six distillations the total distillate was heated to boiling and neutralised to phenol red with 0·1 N NaOH (7·40 ml). The liquid was concentrated at low pressure to ca. 2 ml, treated with aqueous silver nitrate (2·5 ml of 5%), filtered hot from traces of silver chromate, concentrated and treated with a little ethanol. Silver acetate crystallised and was collected. Two crops (85 + 20 mg) were obtained (Found: Ag, 64·5; Calc. for $C_2H_3O_2Ag$: 64·65%).

*Experiment* II. The "less dilute" cholesterol (72 mg) was oxidised as above to give silver acetate (77 mg) (Found: Ag, 64·8%). Cholesterol (50·4 mg) from 4-$^{14}$C-mevalonolactone was oxidised as above to give silver acetate (49 mg).

A normal specimen of cholesterol was also oxidised to acetic acid as above.

*Preparation of Samples for Measurement of Isotopes*

*Mass spectrometry of acetic acid*

*Combustions.* Weighed samples were burned in oxygen in a micro-combustion furnace (quartz tube filled with copper oxide, 700°). The issuing gases passed through two bubblers each charged with half-saturated barium hydroxide solution (4 ml) and light petroleum (b.p. 100–120°; 4 ml). A significant amount of barium carbonate was never found in the second bubbler, provided that the bubbles produced in the first bubbler were small. Each bubbler had a detachable head consisting of a B24 socket carrying inlet and outlet tubes; the lower part was a heavy-walled tube surmounted by a B24 cone. After the combustion the heads were removed and rinsed with $CO_2$-free water. The tubes were then centrifuged and the aqueous layer withdrawn. The barium carbonate was stirred and centrifuged three times with $CO_2$-free water, then twice with $CO_2$-free ethanol, and finally dried at 100°.

The following samples were burned in this way:

(i) Silver acetate from synthetic $^{13}CH_3CO_2Na$ (Found: Ag 64·3; Calc. for $C_2H_3O_2Ag$ with 0·52 atom excess $^{13}C$ per molecule: 64·4%).

(ii) Silver acetate prepared by recrystallising a mixture of (i) (5·85 mg) and normal silver acetate (82·2 mg). This is a dilution of 1 in 15·1 (Found: Ag 64·5%).

(iii) Silver acetate prepared by recrystallising a mixture of (i) (3·61 mg) and normal silver acetate (86·63 mg). This is a dilution of 1 in 25 (Found: Ag 64·7%).

(iv) Silver acetate from oxidation of cholesterol from 4-$^{14}$C-mevalonolactone (Found: Ag 64·3%).

(v) Silver acetate from oxidation of cholesterol from 2-$^{14}$C-3′:4-$^{13}C_2$-mevalonolactone (Exp. I).

(vi) As (v) (Exp. II).

(vii) Cholesterol from 4-$^{14}$C-mevalonolactone.

(viii) Cholesterol from 2-$^{14}$C-3′:4-$^{13}C_2$-mevalonolactone (Exp. I).

(ix) As (viii) (Exp. II).

(x) 3′:4-$^{13}C_2$-mevalonolactone (as synthesised).

(xi) Normal cholesterol.

*Degradation of silver acetate.* A small flask fitted with a bubbler tube, side-tube, dry-ice condenser and magnetic stirrer was charged with silver acetate (ca. 20 mg), swept out with dry, $CO_2$-free nitrogen, and connected to two baryta bubblers prepared as above. The gas stream was regulated, the condenser was cooled to −70° and bromine (0·03 ml) in carbon tetrachloride (1 ml), both dried over phosphoric oxide, was introduced quickly through the side-tube. The mixture was then stirred and heated at 40–50° for 2 hr. The barium carbonate was collected as described above: in general its weight was slightly less than that of the silver acetate (70–80% yields).

The specimens degraded in this way were:

(i) Silver acetate from cholesterol from 4-$^{14}$C-mevalonolactone.

(ii) Silver acetate from $^{13}$C-$^{14}$C-cholesterol (Exp. I).

(iii) As (ii) (Exp. II).

*Assay of $^{14}$C in barium carbonate specimens.* The specimens were counted as "infinitely thick" layers on 1 cm$^2$ polythene disks by means of a thin-mica-window Geiger–Müller counter, and counting was continued sufficiently long to reduce the standard error of the counts below 2%.

*Assay of $^{13}$C in barium carbonate specimens.* The specimens were decomposed in Rittenberg tubes by $CO_2$-free 4% hydrochloric acid and the carbon dioxide was measured in the normal manner.

*Preparation of acetic acid for mass spectrometry.* The apparatus is shown in Fig. 1, A and B. Silver acetate (44–45 mg) was introduced into the flask A which was then evacuated and dried. The tube B, which contained a little phosphorus pentoxide, was filled with dry hydrogen chloride at atmospheric pressure by means of a glass probe inserted through the stopcock to the bottom of the tube. Tube B then contained slightly less than 1 equivalent of hydrogen chloride. The hydrogen chloride was transferred by vacuum-line technique, using liquid air, to the flask A, which was sealed and detached at the constriction. The flask was then heated in boiling water, when acetic acid distilled into the capillary side-tube. Heating was continued for 2 hr, the acetic acid being occasionally driven back into the flask. Finally the capillary tube was sealed. The melting-point of the acetic acid was then determined.

Six specimens were so prepared:

(i) Acetic acid from oxidation of "more dilute" cholesterol (Exp. I); m.p. 15·5–16°.

(ii) Acetic acid from oxidation of "less dilute" cholesterol (Exp. II); m.p. 14·5–15·5°.

(iii) Acetic acid from normal cholesterol; m.p. 15·5–16°.

(iv) Synthetic $^{13}$C acetic acid (undiluted).

(v) (iv), diluted 1 : 14·1 with normal material.

(vi), (iv), diluted 1 : 24 with normal material.

*Mass spectrometry of acetic acid.* The spectrometer used was a 60° instrument with Nier type ion source. Ionising potential was 70 V and accelerating potential 1620 V for mass 60. The special inlet is shown in Fig. 1, C. A capillary tube containing acetic acid, prepared as above, was opened at both ends and the liquid was

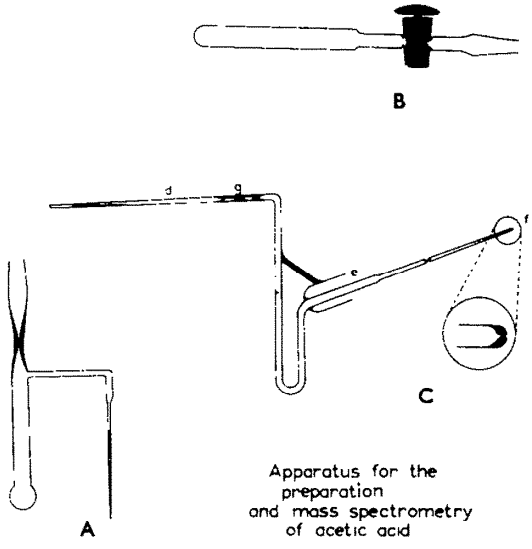



FIG. 1.

touched with a finer capillary until a little acetic acid (1–2 mg) had been transferred. The reservoir of acetic acid was resealed. The smaller capillary was sealed at one end and introduced into the thin horizontal tube d, which was connected to the spectrometer at e. The thin tube was sealed and a cooling bath was placed around the U-tube. As air was removed into the spectrometer through the pinhole leak at f, which projected as far as possible into the spectrometer, acetic acid distilled into the U-tube. When evacuation was complete (2–3 hr), the cooling bath was replaced by a Dewar flask containing water at a temperature which allowed acetic acid vapour to enter the spectrometer at a convenient rate (in this apparatus, ca. 10°). Measurements were then made at masses 60, 61 and 62 and were continued at intervals during that day and the next day. When the previous sample was not too different in composition, values became constant after several hours; but only the second day's readings were used. After a determination, the whole inlet was warmed in hot air to expel adsorbed acetic acid. The horizontal tube was then opened, the constrictions at g preventing dust and fragments of glass from reaching the pinhole.

The first inlet designed used a sintered glass valve sealed by mercury.[43] This was found unsatisfactory because of the tendency of acetic acid to accumulate (perhaps partly as metallic acetates) in the sintered glass.

The six acetic acid specimens prepared as above were measured.

[43] R. C. Taylor and W. S. Young, *Industr. Engng. Chem. (Anal.)* **17**, 811 (1945).

## CALCULATIONS

The normal isotopic abundances assumed in the following calculations are: $^{13}C$, 1·11%; $^{18}O$, 0·20%; $^{17}O$, 0·037%; D, 0·016%.

### (1) $^{13}C$ *Content of* $CO_2$ *from mass-spectrometric readings*

If $x$ is the proportion of $^{13}C$ in the sample, and $(1 - x)$ the proportion of $^{12}C$, the strength of signal at 44 relative to that at 45 (ratio R) differs slightly from $(1 - x)/x$ for several reasons: overlap of peaks, differential pumping of isotopes, differential speed through pinhole, differential cracking on ionisation, and finally the presence of $^{17}O$ in the sample, which increases peak 45 at the expense of 44. Since the R value found for carbon dioxide from "normal" cholesterol (see Table 6) does not differ

TABLE 5. MEASUREMENTS OF RADIOACTIVITY OF $BaCO_3$ SPECIMENS

| No. | Origin of sample | Method of prepn. | Corrected counts/min |
|---|---|---|---|
| (i) | $^{13}C$-$^{14}C$-cholesterol (Exp. I) | Combustion | 377 |
| (ii) | $^{13}C$-$^{14}C$-cholesterol (Exp. II) | Combustion | 533·5 |
| (iii) | Silver acetate from oxidn. of (i) | Combustion | 165 |
| (iv) | Silver acetate from oxidn. of (ii) | Combustion | 257 |
| (v) | Cholesterol from 4-$^{14}C$-mevalonolactone | Combustion | 264 |
| (vi) | Silver acetate from oxidn. of (v) | Combustion | 128 |
| (vii) | Silver acetate from oxidn. of (v) | $Br_2$–$CCl_4$ | 249 |

TABLE 6. MEASUREMENT OF $^{13}C$ CONTENT OF $BaCO_3$ SPECIMENS

| No. | Origin of sample | Method of prepn. | Ratio 44/45 (R) | Uncorrected $^{13}C$ content ($x$) | Corrected $^{13}C$ content | Atom % excess $^{13}C$ |
|---|---|---|---|---|---|---|
| (i) | Normal cholesterol | Combustion | 80·6 | 0·01225 | 0·0111 | — |
| (ii) | $^{13}CH_3CO_2Ag$ (undiluted) | Combustion | 2·70 | 0·2703 | 0·2692 | 25·81 |
| (iii) | $^{13}CH_3CO_2Ag$ (diluted 1 : 14·1) | Combustion | 33·3 | 0·02915 | 0·02800 | 1·690 |
| (iv) | $^{13}CH_3CO_2Ag$ (diluted 1 : 24) | Combustion | 43·2 | 0·02262 | 0·02147 | 1·037 |
| (v) | 3′:4-$^{13}C_2$-Mevalonolactone | Combustion | 9·53 | 0·09497 | 0·09382 | 8·272 |
| (vi) | $^{13}C$-cholesterol (Exp. I) | Combustion | 52·9 | 0·01855 | 0·01740 | 0·630 |
| (vii) | $^{13}C$-cholesterol (Exp. II) | Combustion | 45·6 | 0·02146 | 0·02031 | 0·921 |
| (viii) | $CH_3CO_2Ag$ from oxidn. (Exp. I) | Combustion | 46·3 | 0·02114 | 0·01999 | 0·889 |
| (ix) | $CH_3CO_2Ag$ from oxidn. (Exp. II) | Combustion | 38·0 | 0·02564 | 0·02449 | 1·339 |
| *(x) | As (viii) | $Br_2$–$CCl_4$ | 62·0 | 0·01587 | 0·01472 | 0·362 |
| *(xi) | As (ix) | $Br_2$–$CCl_4$ | 56·5 | 0·01739 | 0·01624 | 0·514 |

* A duplicate determination gave an identical result.

materially from that found for other "normal" specimens of carbon dioxide in this spectrometer, we set the $^{13}C$ content of this carbon dioxide at 1·11% instead of the value 1·225% found by applying the formula $R = (1 - x)/x$. The correction, 0·115%, assumed to represent the sources of error mentioned above, is applied to the other values of $x$ calculated from the same formula. Thence the $^{13}C$ contents and atom % excess $^{13}C$ of the last two columns in Table 6 are derived.

(2) $^{13}C$ *Content of the methyl group in synthetic* $^{13}CH_3CO_2H$

(a) *By combustion.* In specimens (ii), (iii) and (iv) of Table 6, the labelled methyl group is associated with a normal carboxyl group, so that atom % excess in the methyl group is twice that of the $CO_2$ from combustion. Atom % excess $^{13}C$ in methyl group:

From (ii): $2 \times 25{\cdot}8 = 51{\cdot}6\%$; $\therefore$ $^{13}C$ content $= 0{\cdot}516 + 0{\cdot}011 = 0{\cdot}527$

From (iii): $2 \times 1{\cdot}69 \times 15{\cdot}1 = 51{\cdot}1\%$; $\therefore$ $^{13}C$ content $= 0{\cdot}511 + 0{\cdot}011 = 0{\cdot}522$

From (iv): $2 \times 1{\cdot}037 \times 25 = 51{\cdot}9\%$; $\therefore$ $^{13}C$ content $= 0{\cdot}519 + 0{\cdot}011 = 0{\cdot}530$

(b) *By direct measurement.* The data in Table 7, nos. (i), (ii) and (iii), were

TABLE 7. MEASUREMENT OF ACETIC ACID SPECIMENS IN THE MASS SPECTROMETER

(Results are expressed as percentages of the sum of the three peaks [in No. (i), two peaks] measured)

| No. | Sample | Mass 60 | Mass 61 | Mass 62 |
|---|---|---|---|---|
| (i) | $^{13}CH_3CO_2H$ (undiluted) | 45·9 | 54·1 | Not included |
| (ii) | $^{13}CH_3CO_2H$ (diluted 1 : 14·1) | 93·66 | 5·85 | 0·486 |
| (iii) | $^{13}CH_3CO_2H$ (diluted 1 : 24) | 94·96 | 4·55 | 0·487 |
| (iv) | $CH_3CO_2H$ from Exp. I | 95·43 | 3·91 | 0·665 |
| (v) | $CH_3CO_2H$ from Exp. II | 94·66 | 4·60 | 0·742 |
| (vi) | $CH_3CO_2H$ from normal cholesterol | 97·06 | 2·49 | 0·451 |

obtained by direct measurement. To derive the $^{13}C$ content of the methyl group from (ii) and (iii), the following procedure is used. The % excess of mass 61 above normal in (ii) is 3·36 and in (iii) is 2·06. By a calculation similar to that given later [Section (8)] the effects of redistribution of $^{18}O$, $^{17}O$ and D are determined; thus, the % excess of $^{13}C_1$-acetic acid in (ii) is found to be $3{\cdot}36 + 0{\cdot}02 = 3{\cdot}38$, and in (iii) $2{\cdot}06 + 0{\cdot}015 = 2{\cdot}075$. Then from (ii), the undiluted acetic acid has

$$3{\cdot}38 \times 15{\cdot}1 = 51{\cdot}0\%,$$

and from (iii), $2{\cdot}075 \times 25 = 51{\cdot}9\%$ $^{13}C_1$-acetic acid above normal.

Now if $x$ be the proportion of $^{13}C$ in the methyl group of the undiluted acetic acid, then the proportion of $^{13}CH_3{}^{12}CO_2H$ molecules is

$$x(1 - 0{\cdot}0111),$$

and of $^{12}CH_3{}^{13}CO_2H$ molecules,

$$0{\cdot}0111(1 - x).$$

The normal abundance of each of these species is

$$0{\cdot}0111(1 - 0{\cdot}0111)$$

Thus % excess of $^{13}C_1$-acetic acid is

$$x(1 - 0{\cdot}0111) + 0{\cdot}0111(1 - x) - 2 \times 0{\cdot}0111(1 - 0{\cdot}0111) \times 100 = 97{\cdot}78x - 1{\cdot}09$$

$$\text{When } 97{\cdot}78x - 1{\cdot}09 = 51{\cdot}0$$
$$x = 0{\cdot}533.$$
$$\text{When } 97{\cdot}78x - 1{\cdot}09 = 51{\cdot}9$$
$$x = 0{\cdot}542.$$

For calculations from (i), "% excess mass 61 above normal" is not a reliable basis since peaks 60 and 61 are of comparable size and the effect of overlap is largely neutralised.

The experimental ratio of the heights of the 61 and 60 peaks in (i) is 1·18. Isotopes besides $^{13}C$ affecting this ratio are $^{17}O$ and D, since introduction of one of either of these atoms converts a molecule of mass 60 to mass 61.

Let $x$ be the $^{13}C$ content of the methyl group. Then a proportion $x$ of the acetic acid molecules have a $^{13}C$-methyl group and of these $(0{\cdot}0111 + 2 \times 0{\cdot}00037 + 4 \times 0{\cdot}00016)x = 0{\cdot}01248x$ have also a $^{13}C$-carboxyl group, a $^{17}O$ or a D atom and therefore do not contribute to the 61 peak. The remaining proportion $(1 - x)$ forms the 60 peak except for 0·01248 $(1 - x)$ which is added to the 61 peak.

$$\text{Thus } \frac{x + 0{\cdot}01248(1 - x) - 0{\cdot}01248x}{0{\cdot}98752(1 - x)} = 1{\cdot}18$$

whence $x = 0{\cdot}539$.

The six values obtained for the $^{13}C$ content of the methyl group are therefore 0·522, 0·527, 0·530 (from $CO_2$ measurements), 0·533, 0·539, 0·542 (from acetic acid measurements).

As indicated earlier, the second measurements are probably higher because of an isotope effect in the extensive fragmentation of acetic acid molecules occurring during ionisation, a $^{12}C$–$^{13}C$ bond being slightly less easily broken than a $^{12}C$–$^{12}C$ bond. Since this effect is not large, we have previously[44] taken the mean of all six figures as the basis for further calculations. Another method is to use the difference between the two set of figures as a measure of the $^{13}C$ isotope effect in the mass spectrometry of acetic acid, and this method will be used here. Thus the mean value from the carbon dioxide measurements is 0·526, and the mean value from the acetic acid measurements is 0·538. We therefore assume that in mass spectrometry of acetic acid, $^{13}C_1$-acetic acid is over-represented to the extent of 2%; it follows that $^{13}C_2$-acetic acid is over-represented to the extent of 4%. These corrections will be applied to the results of mass spectrometry of the acetic acid from oxidation of cholesterol.

The proportion of $^{13}C$ in the methyl group of the synthetic acetic acid is therefore taken as 0·526; the atom % excess $^{13}C$ is 52·6 − 1·1 = 51·5.

(3) *Degree of dilution of* 3-*oxobutyl acetate before synthesis of* 3′:4-$^{13}C_2$-*mevalonolactone*

Since the 3′:4-$^{13}C_2$-mevalonolactone has 8·272% excess $^{13}C$ [Table 6; (v)] and 2/6 of its carbon atoms are labelled, a labelled position has $8{\cdot}272 \times 3 = 24{\cdot}816$

[44] J. W. Cornforth, R. H. Cornforth, A. Pelter, M. G. Horning and G. Popják, *Proc. Chem. Soc.* 112 (1958).

atom % excess $^{13}C$. Since the acetic acid from which the 3-oxobutyl acetate was made contained 51·5 atom % excess $^{13}C$ in its methyl group, this is also the degree of labelling at positions 2 and 4 of the oxobutyl acetate, which was therefore diluted with $[(51{\cdot}5/24{\cdot}816) - 1] = 1{\cdot}075$ parts of normal material before conversion into mevalonolactone.

The further dilution of mevalonolactone with 2-$^{14}C$-mevalonolactone before biosynthesis (18 mg, normal in $^{13}C$, added to 524 mg) gives the mevalonolactone used in biosynthesis the following composition:

1 part "undiluted mevalonolactone" (i.e. derived from undiluted 3-oxobutyl acetate)

1·15 parts "normal mevalonolactone".

(4) *Proportion of endogenous material in cholesterol biosynthesised from* 2-$^{14}C$-3′:4-$^{13}C_2$-*mevalonolactone*

The mevalonolactone used in biosynthesis had (allowing for dilution by radioactive material) 24·0 atom % excess $^{13}C$ at its labelled positions. The cholesterol is presumed (see Scheme 3) to have 10 labelled positions out of 27. Now the total carbon of cholesterol used in Exp. I had 0·630 atom % excess $^{13}C$, and in Exp. II 0·921% (Table 6; (vi) and (vii)); hence the dilution factors are

Exp. I: $$24{\cdot}0 \Big/ \left(0{\cdot}630 \times \frac{27}{10}\right) = 14{\cdot}1$$

Exp. II: $$24{\cdot}0 \Big/ \left(0{\cdot}921 \times \frac{27}{10}\right) = 9{\cdot}65$$

(5) *Proportions of labelled molecules in "undiluted mevalonolactone"*

Since the isotopic acetic acid contained 52·6 atom % $^{13}C$ in its methyl group, the "undiluted mevalonolactone" contains the following proportions of molecules differing at $C_{(3')}$ and $C_{(4')}$:

(i) $^{13}C$ at $C_{(3')}$ and $C_{(4)}$: $0{\cdot}526 \times 0{\cdot}526 \times 100 = 27{\cdot}67\%$

(ii) $^{13}C$ at $C_{(3')}$, $^{12}C$ at $C_{(4)}$: $0{\cdot}526 \times 0{\cdot}474 \times 100 = 24{\cdot}93\%$

(iii) $^{12}C$ at $C_{(3')}$, $^{13}C$ at $C_{(4)}$: $0{\cdot}474 \times 0{\cdot}526 \times 100 = 24{\cdot}93\%$

(iv) $^{12}C$ at $C_{(3')}$ and $C_{(4)}$: $0{\cdot}474 \times 0{\cdot}474 \times 100 = 22{\cdot}47\%$

(6) *Relative proportions of molecules, in the acetic acid from oxidation of cholesterol, originating from* $C_{(3')}$–$C_{(4)}$ *and from* $C_{(3')}$–$C_{(3)}$ *of mevalonolactone*

All excess $^{13}C$ in the *carboxyl* group of this acetic acid is provided by molecules originating from $C_{(18)}$–$C_{(13)}$ of cholesterol, *i.e.*, from $C_{(3')}$–$C_{(4)}$ of mevalonolactone (see Scheme 3). The excess $^{13}C$ in the *methyl* group is partly provided from the same source and partly from other molecules having a labelled methyl group ($C_{(3')}$ of mevalonolactone) attached to a normal carboxyl group ($C_{(3)}$ of mevalonolactone). These other molecules originate from $C_{(14)}$–$C_{(10)}$, $C_{(21)}$–$C_{(20)}$, and $C_{(26 \text{ or } 27)}$–$C_{(25)}$ of cholesterol.

*Exp.* I. The carboxyl group of the acetic acid contained 0·362 atom % excess

$^{13}C$, and the total carbon (methyl + carboxyl) contained 0·889 atom % excess. [Table 6; (viii) and (x)]. Then if $x$ is the % excess $^{13}C$ in the methyl group,

$$\frac{0{\cdot}362 + x}{2} = 0{\cdot}889,$$

$$\text{whence } x = 1{\cdot}416$$

Now since $C_{(13)}$ of cholesterol contributes 0·362% excess $^{13}C$ to the carboxyl group of the acetic acid, $C_{(18)}$, which is attached to it and is also a labelled position, contributes 0·362% excess $^{13}C$ to the methyl group. The remaining 1·416 − 0·362 = 1·054% is supplied by acetic acid originating from other positions, i.e., from $C_{(3')}$–$C_{(3)}$ of mevalonolactone. Hence 1·054/0·362 = 2·912 times as many molecules of acetic acid originate from $C_{(3')}$–$C_{(3)}$ of mevalonolactone as from $C_{(3')}$–$C_{(4)}$.

*Exp.* II. Here

$$\frac{0{\cdot}514 + x}{2} = 1{\cdot}339$$

$$x = 2{\cdot}164,$$

and the ratio sought is

$$\frac{2{\cdot}164 - 0{\cdot}514}{0{\cdot}514} = 3{\cdot}210$$

(7) *Calculation of % excess $^{13}C_2$-acetic acid and $^{13}C_1$-acetic acid, expected in the acetic acid from oxidation of cholesterol, according to mechanisms* (a) *and* (b)

This acetic acid contained 0·362 atom % excess $^{13}C$ (Exp. I) and 0·514 atom % excess $^{13}C$ (Exp. II) in its carboxyl group. Since the undiluted mevalonolactone contained 51·5 atom % excess $^{13}C$ at a labelled position it follows that a fraction 0·362/51·5 (Exp. I) or 0·514/51·5 (Exp. II) of the total acetic acid had a carboxyl group derived from $C_{(4)}$ of this undiluted lactone. That is, out of each 1,422,700 (Exp. I) or 1,001,900 (Exp. II) molecules of the total acetic acid, 10,000 originate from $C_{(3')}$–$C_{(4)}$ of the undiluted lactone. Out of these totals another 10,000 × 2·912 = 29,120 (Exp. I), or 10,000 × 3·210 = 32,100 (Exp. II) molecules originate from $C_{(3')}$–$C_{(3)}$ of the undiluted lactone.

*Mechanism* (a). By this mechanism, each of the 10,000 molecules of acetic acid originating from $C_{(3')}$–$C_{(4)}$ of undiluted lactone has methyl and carboxyl groups originating from the same molecule of lactone, and the proportions given in Section (5) apply: that is, 2767 of the 10,000 are $^{13}CH_3{}^{13}CO_2H$, 2493 are $^{13}CH_3{}^{12}CO_2H$ and 2493 are $^{12}CH_3{}^{13}CO_2H$. The acetic acid originating from $C_{(3')}$–$C_{(3)}$ of the undiluted mevalonolactone contains a labelled carbon (52·6 atom % $^{13}C$) and a normal carbon (1·11 atom % $^{13}C$); it therefore contains a proportion (0·526 × 0·0111) of $^{13}C_2$-acetic acid and [0·526(1 − 0·0111) + 0·0111(1 − 0·526)] of $^{13}C_1$-acetic acid. That is, of the 29,120 molecules in Exp. I, 170 are $^{13}C_2$-acetic acid and 15,300 are $^{13}C_1$-acetic acid; of the 32,100 molecules in Exp. II, 187 are $^{13}C_2$-acetic and 16,865 are $^{13}C_1$-acetic acid.

Then in Exp. I, a total of 39,120 molecules contains 2937 molecules of $^{13}C_2$-acetic acid and 20,286 molecules of $^{13}C_1$-acetic, against a normal abundance of 5 and 859 molecules respectively. In Exp. II, a total of 42,100 molecules contains 2954

molecules of $^{13}C_2$-acetic acid and 21,851 of $^{13}C_1$-acetic acid, against a normal abundance of 5 and 924 respectively. The rest of the acetic acid in each specimen is normal with respect to $^{13}C$. Thus in the total acetic acid of Exp. I, 1,422,700 molecules contain 2932 molecules of $^{13}C_2$-acetic acid and 19,427 molecules of $^{13}C_1$ acetic acid above normal; in Exp. II, 1,001,900 molecules contain 2949 molecules of $^{13}C_2$-acetic and 20,927 molecules of $^{13}C_1$-acetic acid above normal. Thus the expected proportions by mechanism (a) are:

| | % excess $^{13}C_1$-acetic acid | % excess $^{13}C_2$-acetic acid |
|---|---|---|
| Exp. I | 1·37 | 0·206 |
| Exp. II | 2·09 | 0·294 |

*Mechanism* (b). If the rearrangement of methyl groups is by mechanism (b) or by intermolecular transfer, the calculation must be based on the diluted mevalonolactone used in biosynthesis. Since each sample of 1,422,700 molecules (Exp. I) or 1,001,900 molecules (Exp. II) of the acetic acid contains 10,000 molecules containing $C_{(4)}$ of the undiluted lactone, then each contains 21,500 [Section (3)] molecules containing $C_{(4)}$ of the diluted lactone. Since the $^{13}C$ content at a labelled position in this lactone is 24·0 + 1·1 = 25·1% [Section (4)], these 21,500 molecules contain 5397 molecules with a $^{13}C$-carboxyl group. Now these 21,500 molecules have been supplied with methyl groups from 21,500 other molecules of diluted lactone, including 5397 with a $^{13}C$-methyl group. Thus the number of $^{13}C_2$-acetic acid molecules present is $5397 \times (5397/21{,}500) = 1355$, and of $^{13}C_1$-acetic acid molecules $2 \times 5397 \times [(21{,}500 - 5397)/21{,}500] = 8084$. The numbers of molecules of acetic acid from $C_{(3')}$–$C_{(3)}$ of mevalonolactone can be taken as calculated from mechanism (a), since for them dilution before biosynthesis is immaterial. Thus in totals of 21,500 + 29,120 = 50,620 molecules (Exp. I) or 21,500 + 32,100 = 53,600 molecules (Exp. II) of labelled acetic acid there are 1355 + 170 = 1525 molecules of $^{13}C_2$-acetic acid and 8084 + 15,300 = 23,384 molecules of $^{13}C_1$-acetic acid (Exp. I), or 1355 + 187 = 1542 molecules of $^{13}C_2$-acetic acid and 8084 + 16,865 = 24,949 molecules of $^{13}C_1$-acetic acid (Exp. II). Normal abundances for the 50,620 molecules (Exp. I) are 6 molecules of $^{13}C_2$-acetic acid and 1111 molecules of $^{13}C_1$-acetic acid; for the 53,600 molecules (Exp. II), 6 molecules of $^{13}C_2$-acetic acid and 1176 of $^{13}C_1$-acetic acid. Thus the whole sample of 1,422,700 molecules (Exp. I) has 1519 molecules of $^{13}C_2$-acetic acid and 22,273 of $^{13}C_1$-acetic acid above normal; the sample of 1,001,900 molecules (Exp. II) has 1536 molecules of $^{13}C_2$-acetic acid and 23,773 of $^{13}C_1$-acetic acid above normal. Thus the expected proportions by mechanism (b) are

| | % excess $^{13}C_1$-acetic acid | % excess $^{13}C_2$-acetic acid |
|---|---|---|
| Exp. I | 1·57 | 0·107 |
| Exp. II | 2·37 | 0·153 |

(8) *Proportions of $^{13}C_2$-acetic acid and $^{13}C_1$-acetic acid, calculated from proportions of mass* 61 *and mass* 62 *found by mass spectrometry*

It has already been deduced (Section (2)) that isotope effects during fragmentation cause $^{13}C_1$-acetic acid to be over-represented to the extent of 2% and $^{13}C_2$-acetic acid to twice this extent. In addition, a correction must be made for the altered distribution of oxygen and hydrogen isotopes in acetic acid containing excess $^{13}C$. Most of

this correction is due to $^{18}O$. In normal acetic acid, nearly all the 62 peak is formed by $CH_3C^{18}OOH$ ions, but if the acetic acid contains extra $^{13}C$ some of the $^{18}O$ is present in molecules of mass 63.

Consider $10^6$ molecules of normal acetic acid. These contain 21,954 molecules ($2 \times 0{\cdot}0111 \times 0{\cdot}9889 \times 10^6$) with one $^{13}C$ atom, 123 ($0{\cdot}0111 \times 0{\cdot}0111 \times 10^6$) with two and 977,923 with none. The oxygen and hydrogen isotopes will be distributed among these three groups. Of the 4000 $^{18}O$ atoms, 89 are in $^{13}C_1$-acetic acid and 3899 in $^{12}CH_3{}^{12}C^{18}O^{16}OH$. Of the 740 $^{17}O$ atoms, 16 are in $^{13}C_1$-acetic acid and 723 are in $^{12}CH_3{}^{12}C^{17}O^{16}OH$. Of the 640 D atoms, 14 are in $^{13}C_1$-acetic acid and 621 in $^{12}CH_2D{}^{12}C^{16}{}_2O_2H$ or $^{12}CH_3{}^{12}C^{16}{}_2O_2D$. Other species are negligible. The number of molecules of mass 60, i.e., $^{12}CH_3{}^{12}C^{16}{}_2O_2H$, is

$$977{,}923 - 3899 - 723 - 621 = 972{,}680$$

The number of molecules of mass 61, i.e., $^{13}CH_3{}^{12}C^{16}{}_2O_2H + {}^{12}CH_3{}^{13}C^{16}{}_2O_2H + {}^{12}CH_3{}^{12}C^{17}O^{16}OH + {}^{12}CH_2D{}^{12}C^{16}{}_2O_2H + {}^{12}CH_3{}^{12}C^{16}{}_2O_2D$, is

$$21954 + 621 + 723 - 89 - 16 - 14 = 23179.$$

The number of molecules of mass 62, $^{13}CH_3{}^{13}C^{16}{}_2O_2H + {}^{12}CH_3{}^{12}C^{18}O^{16}OH + {}^{13}CH_3{}^{12}C^{17}O^{16}OH + {}^{12}CH_3{}^{13}C^{17}O^{16}OH + {}^{12}CH_2D{}^{13}C^{16}{}_2O_2H + {}^{13}CH_2D{}^{12}C^{16}{}_2O_2H + {}^{12}CH_3{}^{13}C^{16}{}_2O_2D + {}^{13}CH_3{}^{12}C^{16}{}_2O_2D$, is

$$123 + 3899 + 16 + 14 = 4052.$$

Total 999,911 molecules of mass 60, 61 or 62

$$\% \; 61 = 2{\cdot}318 \qquad \% \; 62 = 0{\cdot}405$$

If the acetic acid contains 1·4% $^{13}C_1$-acetic acid above normal and 0·2% $^{13}C_2$-acetic acid above normal (the approximate figures for Exp. I), there are in $10^6$ molecules 35954 molecules of $^{13}C_1$-acetic acid and 2123 of $^{13}C_2$ acetic acid. The numbers of molecules of masses 60, 61 and 62, calculated as above, are

Mass 60 : 956,778
Mass 61 : 37,063 Total 999,849
Mass 62 : 6008
% 61 = 3·707 % 62 = 0·601.

Thus a specimen containing 0·200% excess $^{13}C_2$-acetic acid and 1·40% excess $^{13}C_1$-acetic acid would show in the mass spectrometer 1·39% excess mass 61 and 0·196% excess mass 62, provided that relative peak heights accurately represented relative numbers of molecules. But actually, $^{13}C_1$-acetic acid would be over-represented by 2% and $^{13}C_2$-acetic acid by 4%, so that the observed percentages would be 1·42 and 0·204 respectively. Similarly it can be shown that for a specimen containing 0·300% excess $^{13}C_2$-acetic acid and 2·10% $^{13}C_1$-acetic acid (the approximate figures

for Exp. II) the observed percentage excess for mass 61 would be 2·14 and for mass 62 0·306.

These corrections are now applied to the experimental values for acetic acid from Exp. I and II (Table 8).

TABLE 8. RELATION OF PEAK HEIGHT TO PROPORTION OF $^{13}C$ SPECIES IN MASS SPECTROMETRY OF ACETIC ACID

| Exp. | Observed % excess mass 62 | Calculated % excess $^{13}C_2$-acetic acid | Observed % excess mass 61 | Calculated % excess $^{13}C_1$-acetic acid |
|---|---|---|---|---|
| I | 0·214 | 0·210 | 1·42 | 1·40 |
| II | 0·291 | 0·285 | 2·11 | 2·08 |

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