DETECTION AND COMPUTATION OF ISOTOPE FRACTIONATION IN THE ADSORPTION CHROMATOGRAPHY OF DUAL-LABELLED COMPOUNDS†

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INTRODUCTION

An attractive potentiality of double isotope techniques is the positive identification of an unknown member of a class of compounds by co-chromatography of a tritium-labelled derivative of the unknown with the radiocarbon derivative of the suspected known compound. We have found that the anticipated criterion of a constant isotope ratio across the mixed peak is not fulfilled even when the same compound is used to prepare both derivatives. Specifically, the co-chromatography of cholesterol acetate-1-^{14}C with cholesterol acetate-2-^{3}H on silica gel resulted in two overlapping, but distinctly displaced, radioactivity curves; that is, isotopic fractionation had occurred in the chromatographic process. Such an occurrence is not entirely unexpected in light of previous experiences with inorganic ions[1], small organic molecules[2] and labelled amino-acids[3] on ion-exchange columns, but its presence in the fractionation of molecules of this size by liquid–solid adsorption chromatography with non-polar solvents was surprising. Its appearance is accentuated by the use of two radioisotopes; whereas fractionations of single radioisotope: stable isotope may be unnoticed in the absence of specific activity measurements, the fractionation of one radioisotope from another results in obtrusive changes in the ratio of radioisotopes and is thereby more readily noticed.

Two applications of this effect suggested themselves: first, that the dual-isotope technique might still permit the establishment of identity originally sought, if the magnitude and sign of displacement of one isotope from the other to be expected were known for the two derivatives; and second, that by providing two labels whose relative positions in the molecule could be altered, the technique offered the possibility of mapping the effect of position on the degree of separation and thereby could elucidate the intra-molecular contributions to the adsorption process. Both of these applications, however, required an accurate knowledge of the degree of displacement or separation factor.

This report is concerned with three aspects of the problem: first, the criteria of the authenticity of the phenomenon as an example of isotopic fractionation; second, an analysis of alternative methods for the calculation

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of isotopic fractionation; and third, an exploration of isotopic fractionation and its relation to molecular structure and isotopic location.

EVIDENCE FOR ISOTOPE FRACTIONATION DURING ADSORPTION CHROMATOGRAPHY OF DUAL-LABELLED CHOLESTEROL ACETATES

Cholesterol purified through the dibromide[4], m.p. 148–149°, was acetylated either with acetic anhydride-1-^{14}C or with acetic anhydride-2-^{3}H under nitrogen in the presence of pyridine[5]. The individual labelled acetates were purified and recovered. A mixture of 150,000 cpm of cholesterol acetate-2-^{3}H and 300,000 cpm cholesterol acetate-1-^{14}C was chromatographed on a 100 $\times$ 0·45 cm column of Davison Code 12 silica gel, 200–325 mesh, previously equilibrated at a relative humidity of 7 per cent[6, 7]. Elution was carried out with 16 per cent benzene in pentane, and fractions of 6·5 ml were collected at intervals of 20 minutes. Each fraction was made to 7 ml with pentane and transferred to a scintillation vial, and 10 ml of scintillation fluid [toluene containing 4 per cent 2,5-diphenyloxazole and 0·4 per cent 1,4-bis-2(5-phenyloxazolyl)benzene] was added. Scintillation counting was carried out in Packard Tri-Carb Model 314-EX 2 equipped to drive an IBM 026 card-punch. Channel settings were: lower channel, 50–600 divisions, gain 500; upper channel, 45·5–500 divisions, gain 100; coincidence monitor, A′ setting 100, gain 100; voltage setting, 1105 V.

Computations of cpm ^{3}H, cpm ^{14}C and isotope ratio, ^{3}H/^{14}C, from the counts in each channel were carried out using an established programme for the IBM 1620 computer. In this and subsequent runs, the isotope ratio was accepted as valid only in those regions in which both count rates exceeded 1000 cpm above background. Under these conditions the isotope ratio was found to have a standard error of 0·6 per cent or less in the analysis of known mixtures.

More than 97 per cent of each isotope was recovered in the interval of the chromatogram shown in *Figure 1*. The elution pattern of the ^{3}H-labelled acetate was noticeably displaced with respect to the ^{14}C-acetate as shown by the rising values for the isotope ratio across the peak. These values were 40 per cent higher on the trailing edge than on the leading edge of the mixed chromatographic peak and showed a statistically significant slope.

The results of this and subsequent runs were carefully examined for artefacts that could be responsible for the displacement observed between the two peaks and for the attendant slope in isotope ratio. These potential artefacts may be classified as chromatographic, instrumental, or radiochemical in origin.

With respect to chromatographic artefacts, one might conceive that despite the non-polar environment a small but significant degree of hydrolysis and re-esterification takes place during the chromatographic process. On this basis, a slower rate of re-esterification by the ^{3}H-acetic acid radical would produce the displacement noted. Such a mechanism would be effective only if the hydrolysis products had approximately the same chromatographic mobility as the intact ester. This condition is clearly unfulfilled: on these columns free cholesterol and acetic acid are

essentially stationary and require elution with pure benzene and with 10 per cent ether in pentane respectively. Furthermore, the sharp rise in polarity produced by hydrolysis would be accompanied by an increase in adsorptive interaction of the products with the surface. This would shift the equilibrium in such a way as to make the re-esterification process thermodynamically unfavourable.

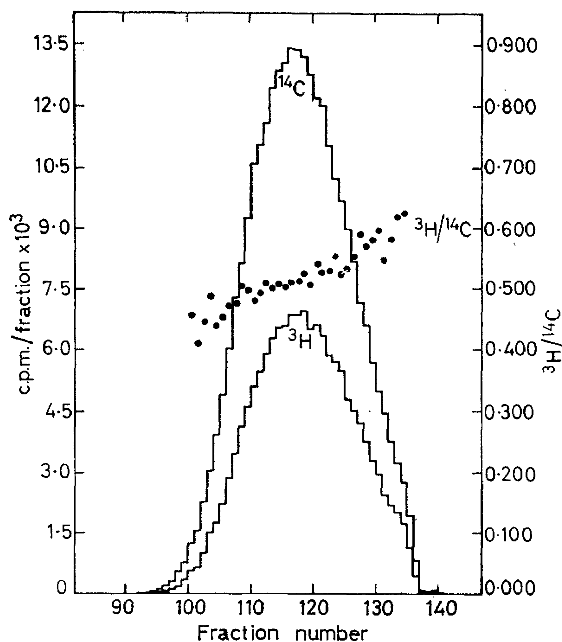

Figure 1. Chromatogram of cholesterol acetate-1-^{14}C mixed with cholesterol acetate-2-^{3}H; upper histogram, ^{14}C; lower histogram, ^{3}H; solid circles, ^{3}H/^{14}C

Although the scintillation counting equipment had given satisfactory results with either isotopic acetate alone, tests were made for quenching. When radioactivity of either type was added to the chromatographic fractions it was fully recovered in all regions of the peak, thus eliminating the possibility of a selective quenching process. The possibility of any bizarre dependence of isotope ratio on count rate was eliminated by subjecting the plot of isotope ratio *v.* count rate for both ^{3}H and ^{14}C to a least squares analysis. The slope obtained did not differ significantly from zero.

The synthesis used by the manufacturer to prepare the acetic anhydride appeared to preclude any radiochemical artefacts. ^{14}C was incorporated into acetic acid by reaction of methyl magnesium iodide with $^{14}CO_2$, fully tritiated malonic acid was decarboxylated to yield sodium acetate, and both labelled sodium acetates were converted to acetic anhydride by reaction with *p*-toluene-sulphonic acid followed by distillation. Paper chromatography of the sodium acetate starting material showed no higher homologues than acetic acid, and gas chromatography of the acetic anhydride revealed a maximum of 1 per cent contamination by acetic acid.

The radiochemical purity is emphasized by the plot of cumulative per cent elution for each isotope in *Figure 2*. Each ^{3}H value falls below the corresponding ^{14}C value, and the probit least squares analysis (shown in expanded form on the right side) indicates a clear and significant displacement between the two plots over their entire length. Any contaminant would have a mobility different from the major constituent and if present in quantity would introduce a distorting curvature into the plot.

It therefore appears that the most plausible alternatives to actual isotope fractionation can be eliminated from consideration and that fractionation occurs during the chromatographic process. Attention can then be devoted to the measurement and estimation of actual displacement.

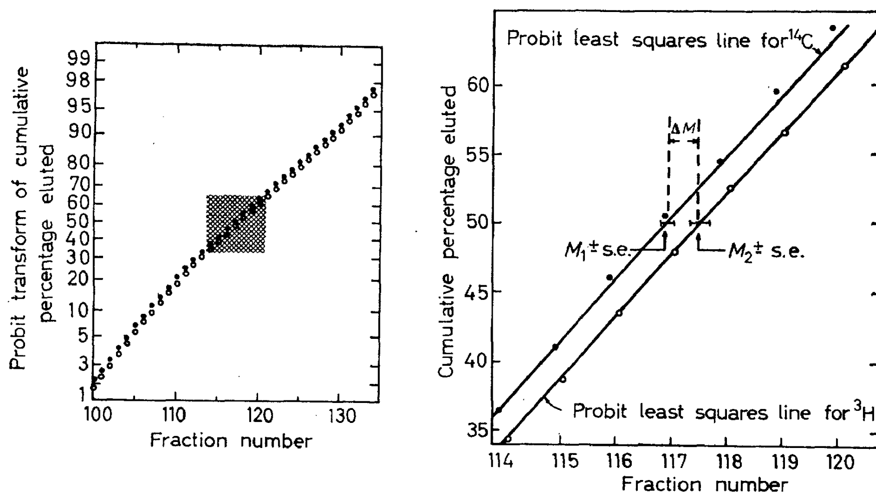

Figure 2. Cumulative percentage elution of ^{14}C and ^{3}H in *Figure 1*; left-hand graph: total range, plotted on probability coordinate *v.* fraction number; right-hand graph: expansion of centre portion of left-hand graph, showing probit least squares lines for ^{14}C and ^{3}H, the mid-points of each, M_1 and M_2 and the associated standard errors, and the net displacement ΔM

CALCULATION OF ISOTOPE FRACTIONATION IN CHROMATOGRAPHIC PROCESSES INVOLVING DUAL-LABELLED COMPOUNDS

The probit analysis is a valuable though infrequently utilized method for obtaining the difference between the mid-points of two Gaussian distributions. It suffers from the disadvantage that as the means approach each other, the standard error of each mid-point becomes an appreciable portion of the difference between the two mid-points. On the other hand, the specific activity, or isotope ratio of each fraction constitutes an extremely sensitive parameter for measuring this displacement.

Several treatments have been reported of the relationship between specific activity and fraction number. For example, the equation of Piez and Eagle[3] predicts the specific activity curve in the region of overlap between the two peaks if the distributions are assumed to be identical.

In this equation

$$\ln S = \frac{(m_1 - m_2)\,x}{\sigma^2} + \frac{m_2{}^2 - m_1{}^2}{2\sigma^2} \tag{1}$$

S is the specific activity, m_1 is the mean of the activity curve, m_2 is the mean of the colorimetric curve, σ is the standard deviation and x is the fraction number.

Glueckauf[8] has provided a specific treatment for chromatographic columns by considering the proportion of material eluted in a given segment of the elution curve. From this, he obtains for the ratio of isotopes

$$C_1/C_2 = (m_1/m_2)/(\bar{v}_1/\bar{v}_2) \exp \frac{N}{2}\left[\frac{(\bar{v}_2 - v)^2}{\bar{v}_2\, v} - \frac{(\bar{v}_1 - v)^2}{\bar{v}_1\, v}\right] \tag{2}$$

where C_1 and C_2 are the concentrations of isotopes 1 and 2 in the fraction, m_1 and m_2 are the amounts of material applied to the column, $\bar{v}_1$ and $\bar{v}_2$ are the peak elution volumes for isotopes 1 and 2, N is the number of plates generated in the column, and v is the elapsed volume of eluent.

The local separation factor δ is defined as the separation factor minus one; the separation factor is given by the ratio of $\bar{v}_2/\bar{v}_1$; $\tilde{v}$, the centre of the mixed peak, is given by

$$\tilde{v} = (\bar{v}_1\, \bar{v}_2)^{\frac{1}{2}} \tag{3}$$

which leads to the transformation

$$\ln\,(C_1/C_2)/(m_1/m_2) = \ln\,(C_1/C_2)/(C_2{}^0/C_1{}^0) \cong N\delta\, \frac{\tilde{v} - v}{(v\tilde{v})^{\frac{1}{2}}}$$

N has been calculated by Glueckauf according to the formula

$$N = 8(\bar{v}/\beta)^2 \tag{5}$$

where β is the bandwidth at the point where C_{max} has fallen to $1/e$ of its original value. The gradient $N\delta$ is obtained from a plot of $\ln\,(C_1/C_2)/(C_2{}^0/C_1{}^0)$ against $(\tilde{v} - v)/(v\tilde{v})^{\frac{1}{2}}$ or against the approximation $(\tilde{v} - v)/\tilde{v}$ and from knowledge of N, δ is obtained.

Although this treatment represents an elegant and accurate means of obtaining the local separation coefficient, it leaves undefined any error in the estimation of the plate number, and moreover requires the assumption that the dispersion of the two isotopes is identical or nearly so. While this assumption is met in most cases, particularly those involving a single radioisotope, the calculation is more useful when one can recognize differences in dispersion as well as correct for them. Equations for this purpose will be presented below.

We have found it convenient to use both synthetic and analytical approaches to the problem in a complementary fashion through the use of an IBM 1620, (*a*) to construct synthetic chromatograms and isotope ratio curves from known parameters and (*b*) to compute the derived analytical relationships using the same parameters for comparison. In all instances, complete agreement was attained.

The synthetic chromatogram programme has the following properties: given the number of counts of each isotope, n_1 and n_2, their respective mean elution volumes M_1 and M_2, and dispersions σ_1, σ_2, as well as the fraction size Δx, the programme calculates an ideal (*i.e.* Gaussian) elution curve for each isotope, its concentration in each fraction and the isotope ratio ϕ of the fraction. A complete description of the programme and its applicability to three-component systems is available upon request.

Behaviour of the isotope ratio ϕ with simple displacement

A series of synthetic chromatograms were obtained for constant n_1, n_2, σ_1, σ_2 and M_1, varying only M_2. The behaviour of the isotope ratio ϕ is shown in *Figure 3*. With small displacements, (ΔM), ϕ is approximately

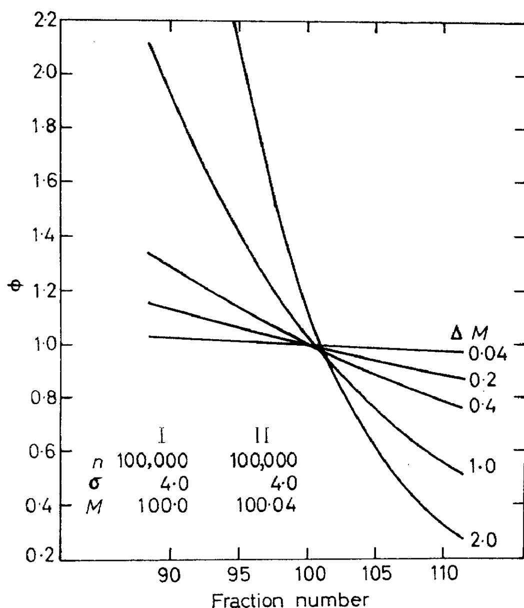

Figure 3. Behaviour of the isotope ratio ϕ (Isotope I/Isotope II) for various displacements of M_2 from M_1 for two chromatographic peaks having the characteristics of n counts, dispersion and elution volume M shown

linear with x, the fraction number, but soon displays a curvature as ΔM reaches 1 per cent of M_1. Progressive analysis at constant M_2 for various σ_1, σ_2 and for various n_1, n_2, resulted in the equation:

$$\phi = (n_1/n_2) \exp \frac{(M_2 - M_1)}{\sigma^2} (\overline{M} - x) \tag{6}$$

where σ^2 is the product $\sigma_1\sigma_2$ when $\sigma_1 = \sigma_2$, $\overline{M}$ is the arithmetic average of M_1 and M_2 and x is the fraction number.

Behaviour of the isotope ratio ϕ with changes in dispersion at zero displacement

Figure 4 displays the effect of a difference in dispersion (σ) on the isotope ratio for two chromatographic peaks with identical retention volumes

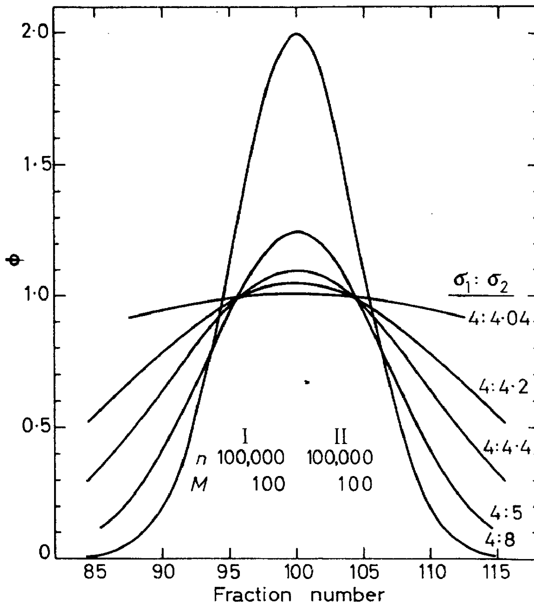

Figure 4. Behaviour of the isotope ratio ϕ for two chromatographic peaks having zero displacement from one another but different dispersions as listed

($M_1 = M_2$). This can be predicted from the individual equations for the distribution function:

$$f(x) = \frac{1}{(2\pi)^{\frac{1}{2}}\,\sigma_1} \exp \frac{-(x-\mu_1)^2}{2\sigma_1^2}\,dx \tag{7}$$

$$f(x) = \frac{1}{(2\pi)^{\frac{1}{2}}\,\sigma_2} \exp \frac{-(x-\mu_2)^2}{2\sigma_2^2}\,dx$$

where μ_1 and μ_2 are the respective means (equivalent to M_1, M_2 or retention volumes) and x is the fraction number.

Dividing and rearranging, one obtains:

$$\phi = \sigma_2/\sigma_1 \exp \frac{-\sigma_2^2(\mu_1 - x)^2 + \sigma_1^2(\mu_2 - x)^2}{2\sigma_1^2\,\sigma_2^2} \tag{8}$$

For small differences between μ_1 and μ_2, $(\mu_1 - x)$ and $(\mu_2 - x)$ may be replaced by $(\overline{M} - x)$:

$$\phi = \sigma_2/\sigma_1 \exp \frac{(\sigma_1^2 - \sigma_2^2)(\overline{M} - x)^2}{2\sigma_1^2\,\sigma_2^2} \tag{9}$$

Behaviour of the isotope ratio ϕ for simultaneous variations in retention volume, dispersion and counts

Combining equations (6) and (9), one obtains the over-all equation for any two compounds with retention volumes M_1 and M_2, counts n_1, n_2 and dispersions σ_1, σ_2:

$$\phi = (n_1/n_2)/(\sigma_2/\sigma_1) \exp \left[\frac{(M_2 - M_1)(\overline{M} - x)}{\sigma_1 \sigma_2} + \frac{(\sigma_1^2 - \sigma_2^2)(\overline{M} - x)^2}{2\sigma_1^2 \sigma_2^2} \right] \quad (10)$$

An example of the synthetic and analytical values for a particular set of parameters is given in *Figure 5.*

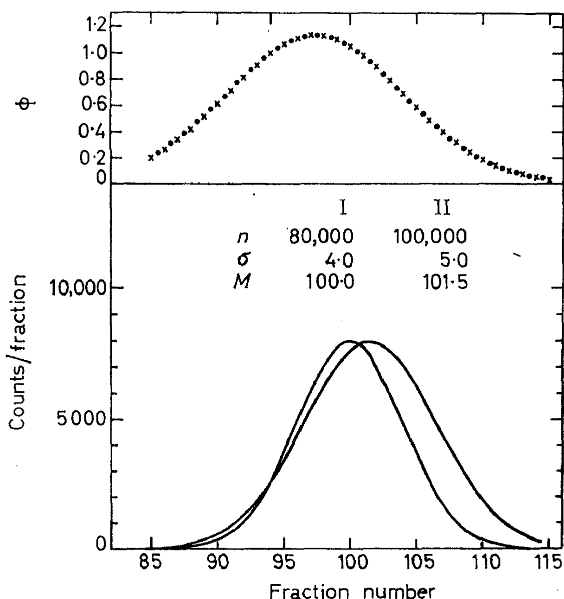

Figure 5. Lower graph: synthetic chromatogram for two compounds having different counts (n_1, n_2), dispersions (σ_1, σ_2) and retention volumes (M_1, M_2); upper graph: isotope ratio, ϕ; circles, synthetic values from chromatogram, crosses, computed from equation (10).

Calculation of ΔM and its standard error—If the cumulative percentage of each isotope eluted is calculated for each fraction of the peak, a probit analysis[9] will yield the following quantities: $x_{50\%}$ ($\equiv M_1$, M_2), the fraction at which 50 per cent of the isotope has been eluted; s.e.$_M$, the standard error of the retention volume M_1 or M_2; σ, the dispersion or standard deviation of the distribution; and s.e.$_\sigma$, the standard error of the dispersion. These quantities, together with the ϕ values for the corresponding fractions enable three estimates to be made of ΔM and its standard error:

(*i*) *Probit value*

$$\Delta M = M_2 - M_1, \quad \text{s.e.}_{\Delta M} = [(\text{s.e.}_{M_1})^2 + (\text{s.e.}_{M_2})^2]^{\frac{1}{2}} \quad (11)$$

(*ii*) Ln-ϕ

If the dispersions are identical, then equation (6) may be used, in the form:

$$\ln \phi = \frac{(M_2 - M_1)(\overline{M})}{\sigma_1 \sigma_2} - \frac{(M_2 - M_1)\,x}{\sigma_1 \sigma_2} \quad (12)$$

A linear regression analysis of Ln-ϕ *v.* x yields the slope b and its standard error s.e.$_b$ and represents the quantity $-(M_2 - M_1)/\sigma_1\sigma_2$, whence multiplication by $\sigma_1\sigma_2$ yields ΔM.

The standard error of ΔM is given by:

$$\text{s.e.}_{M} = \Delta M\,[(\text{s.e. }\sigma_1/\sigma_1)^2 + (\text{s.e. }\sigma_2/\sigma_2)^2 + (\text{s.e. }b/b)^2]^{\frac{1}{2}} \quad (13)$$

(*iii*) MODI-ϕ

For two chromatographic peaks of different dispersions, equation (10) may be used in the form:

$$\left[\ln \frac{(n_2\,\sigma_1)}{(n_1\,\sigma_2)}\phi - \frac{(\sigma_1^2 - \sigma_2^2)(\overline{M} - x)^2}{2\sigma_1^2\,\sigma_2^2}\right] = \frac{(M_2 - M_1)(\overline{M})}{\sigma_1\,\sigma_2} - \frac{(M_2 - M_1)(x)}{\sigma_1\,\sigma_2} \quad (14)$$

The method of calculating ΔM and its standard error is then identical to that for Ln-ϕ.

In comparing one chromatographic run with another, or one pair of retention volumes with another, it is preferable to express ΔM as a percentage of M_1. The relationship between ΔM per cent and the conventional separation factor is:

$$\text{Separation factor} = 1 + \frac{\Delta M\%}{100} \quad (15)$$

$$\text{Glueckauf's local separation factor } \delta = \frac{\Delta M\%}{100} \quad (16)$$

Some examples of the application of these calculations are shown in *Table 1*. The input data were obtained from three chromatographic runs of a mixture of cholesterol acetate-2-^{14}C and cholesterol acetate-2-^{3}H on silica gel columns eluted by 16 per cent benzene in pentane. The percentage displacement (ΔM per cent) was calculated by each of the three methods. It is evident that both Ln-ϕ and MODI-ϕ represent a more precise form of computation than the probit method, since their standard errors are much smaller. On the other hand, for the average ΔM per cent of the three runs, the standard errors for the three methods are similar, as are the average ΔM per cent values themselves. This indicates that the experimental variation from run to run is as yet larger than the error in computation.

Comparison of the Ln-ϕ and MODI-ϕ values and their standard errors does not, in these instances, demonstrate the benefits to be expected from the improvement in computation method when MODI-ϕ is used. This is not unexpected, however, in light of the extremely close agreement between the dispersion values (σ_1 and σ_2), which for all practical purposes are identical.

Table 1. Comparison of results from probit, Ln-ϕ and MODI-ϕ calculations using data from three chromatographic runs of a mixture of cholesterol acetate-2-^{14}C and cholesterol acetate-2-^{3}H; all values are accompanied by their standard errors

Run			*Probit*	Ln-ϕ	MODI-ϕ
122·1	M_1†	123·382 ± 0·114			
	M_2	122·961 ± 0·126			
	σ_1	6·485 ± 0·119			
	σ_2	6·464 ± 0·132			
	b			0·01038 ± 0·00164	0·01167 ± 0·00188
	ΔM		−0·421 ± 0·170	−0·435 ± 0·070	−0·489 ± 0·065
	ΔM%§		0·341 ± 0·138	0·352 ± 0·057	0·396 ± 0·053
122·2	M_1	108·986 ± 0·105			
	M_2	108·492 ± 0·101			
	σ_1	5·941 ± 0·108			
	σ_2	5·921 ± 0·104			
	b			0·01355 ± 0·00071	0·01403 ± 0·00089
	ΔM		−0·494 ± 0·137	−0·477 ± 0·028	−0·494 ± 0·034
	ΔM%§		0·453 ± 0·137	0·438 ± 0·026	0·453 ± 0·031
122·3	M_1	116·812 ± 0·032			
	M_2	116·106 ± 0·039			
	σ_1	5·458 ± 0·031			
	σ_2	5·464 ± 0·038			
	b			0·02203 ± 0·00078	0·02415 ± 0·00065
	ΔM		−0·706 ± 0·051	−0·657 ± 0·024	−0·720 ± 0·020
	ΔM%§		0·604 ± 0·043	0·562 ± 0·020	0·616 ± 0·017
Average of the three runs	ΔM%		0·466 ± 0·076	0·451 ± 0·060	0·488 ± 0·066

† M_1, retention volume, and σ_1, dispersion of cholesterol acetate-2-^{3}H
M_2, retention volume, and σ_2, dispersion of cholesterol acetate-2-^{14}C.
b, slope.
§ When the isotope ratio is expressed as ^{3}H/^{14}C, the displacement of M_2 (^{14}C) from M_1 (^{3}H) is negative. The sign is artificially reversed in the presentation of ΔM% to indicate the displacement of ^{3}H from ^{14}C in the conventional manner.

In the work reported below, values for ΔM per cent were calculated from the MODI-ϕ equations.

ISOTOPE FRACTIONATION AND ITS RELATION TO COMPOUND STRUCTURE AND ISOTOPE LOCATION

Table 2 lists the per cent displacement of the ^{3}H isotope from the ^{14}C isotope for the 2-^{14}C and 2-^{3}H acetates of cholesterol, the 1-^{14}C and 2-^{3}H acetates of cholesterol and benzyl alcohol and 1-^{14}C and 2-^{3}H labelled acetic acid. Within the accuracy of the method, no distinction can be made between the displacements of the two pairs of cholesterol acetate. Reducing the molecular weight of the alcohol from 385 to 108 (cholesterol *versus* benzyl alcohol) resulted in a smaller displacement between the isotopes. Adsorption chromatography of the free acetic acid on the same type of columns yielded a displacement which was twice as large as that of the cholesterol acetates. These results suggested that the isotope displacement between the acetates was relatively insensitive to the presence

of an alcohol or its structure and, accordingly, it was of interest to investigate the displacement when both labels were in the cholesterol portion of the ester.

Table 2. The effect of alcohol structure on isotopic fractionation of acetates

Compounds	*Elution*	$\Delta M\%$	s.e.
Cholesterol acetate-1-^{14}C Cholesterol acetate-2-^{3}H	16% Benzene in pentane	0·443	0·046
Cholesterol acetate-2-^{14}C Cholesterol acetate-2-^{3}H	16% Benzene in pentane	0·488	0·066
Benzyl acetate-1-^{14}C Benzyl acetate-2-^{3}H	16% Benzene in pentane	0·325	0·028
Acetic acid-1-^{14}C Acetic acid-2-^{3}H	10% Ether in pentane	1·065	0·090

Cholesterol was doubly labelled with ^{3}H and ^{14}C in the following manner. A rat was injected with a mixture of 50μc of mevalonic acid-5-^{3}H and 10 μc of mevalonic acid-2-^{14}C and killed after 30 minutes. The liver sterols were extracted and the free sterols were precipitated with digitonin. The digitonides were cleaved with pyridine and the recovered sterols were acetylated with unlabelled acetic anhydride. An aliquot containing $5\cdot0 \times 10^{5}$ cpm ^{3}H and $2\cdot0 \times 10^{5}$ cpm ^{14}C was chromatographed on Code 12 silica gel as described previously. The chromatogram is shown in *Figure 6*. Two peaks

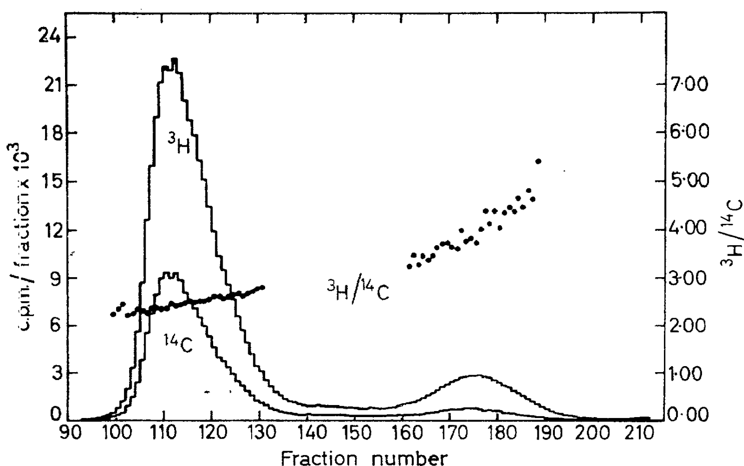

Figure 6. Chromatogram of sterol acetates prepared from the free liver sterols of an animal which had received a mixture of mevalonic acid-2-^{14}C and mevalonic acid-5-^{3}H, 30 minutes before being killed

can be distinguished: the first and largest, cholesterol, is followed by a secondary sterol whose structure has not yet been determined but which is believed to be a partially methylated sterol (C_{28} or C_{29}). The mass of the secondary sterol is negligible in comparison to cholesterol. Both sterols give evidence of isotope displacement, indicating that ^{14}C-rich

sterol is being eluted at the front end of the peak and ^{3}H-rich sterol at the back of the peak. A quantitative difference in displacement in the secondary sterol is evident from the steeper slope of the isotope ratio values. The ΔM per cent values for the two chromatographic peaks (*Table 3*, Experiment I) confirm these impressions: The ΔM per cent of the secondary sterol is

Table 3. Isotopic fractionation of sterols labelled with ^{3}H and ^{14}C *in vivo*. In Experiment I, a mixture of two labelled mevalonic acids was injected into a rat; in Experiment II, the mevalonic acids were injected into different rats; the sterols were isolated and mixed before chromatography

Experiment I			*Experiment II*		
	$\Delta M\%$	s.e.		$\Delta M\%$	s.e.
Cholesterol-x-^{14}C-y-^{3}H acetate†	0·228	0·010	Cholesterol-x-^{14}C acetate Cholesterol-y-^{3}H acetate	0·214	0·028
Secondary sterol-x-^{14}C-y-^{3}H acetate	0·540	0·020	Secondary sterol-x-^{14}C acetate Secondary sterol-y-^{3}H acetate	0·296	0·049

† Biosynthetic labelling of cholesterol from mevalonic acid has the following patterns[10]: from mevalonic acid-2-^{14}C, positions (1), (7), (14), (22), (28); from mevalonic acid-5-^{3}H, positions (2), (6), (11), (12), (15), 23). Because each position has equal probability, the generic notation of -x-^{14}C and -y-^{3}H is used to indicate the presence of multiple isotope sites.

twice that of cholesterol. This effect appeared to be due, at least in part, to an enrichment of ^{3}H relative to ^{14}C in the biosynthetic process, since the larger ΔM per cent was accompanied by a higher *absolute* isotope ratio. Accordingly, two rats were used to prepare ^{3}H-labelled sterol and ^{14}C-labelled sterol separately and the mixed sterol acetates were chromatographed. In this instance, (*Table 3*, Experiment II), the displacement of the individually labelled cholesterols was indistinguishable from that observed with the doubly-labelled cholesterol; the displacement for the secondary sterols was much closer to that for cholesterol, and about half that for the doubly-labelled secondary sterol.

Both the experiments with the labelled acetates (*Table 2*) and those with the labelled sterols (*Table 3*) indicated that isotopic displacement could occur when the isotopes were present in the same regions of the molecule. To assess the effect of spatial separation between the isotopes the displacements of three combinations were tested: ^{14}C-labelled cholesterol (from mevalonic acid-2-^{14}C, *in vivo*) was acetylated with acetic anhydride -2-^{3}H; the reverse combination: ^{3}H-labelled cholesterol acetylated with acetic anhydride-1-^{14}C; and dihydrolanosterol-24,25-^{3}H acetylated with acetic anhydride-1-^{14}C. The latter sterol was prepared from lanosterol by catalytic reduction of the 24,25 double bond with tritium gas over Raney nickel catalyst.

The ΔM per cent values for these three combinations are shown in *Table 4*. The mixed sterol acetate with ^{3}H in the acetate position showed a

displacement which, though significant, was greatly reduced in comparison to the dually labelled sterol in *Table 3.* Moving the ^{14}C from the sterol to the acetate position abolished the displacement in the case of both cholesterol-*y*-^{3}H and dihydrolanosterol-24,24-^{3}H. This striking disappearance of displacement indicates that relative location of the two isotopes is a

Table 4. Isotope fractionation and its relation to isotope location

Compounds	$\Delta M\%$	s.e.	*Fractionation*
Cholesterol-*x*-^{14}C acetate† § Cholesterol-acetate-2-^{3}H	0·089	0·036	Present
Cholesterol-*y*-^{3}H acetate† § Cholesterol acetate-1-^{14}C	0·061	0·064	Absent
Dihydrolanosterol-24,25-^{3}H acetate Dihydrolanosterol acetate-1-^{14}C	0·034	0·037	Absent

† See footnote to *Table 3.*
§ Although the labelled sterols were acetylated with the oppositely labelled acetic anhydride, the probability of a doubly labelled molecule is so low as to make the mixture equivalent to two individual mixtures of isotope.

strong determinant of the displacement observed in this chromatographic process.

DISCUSSION

It appears reasonable that isotopic fractionation occurs during the chromatography of compounds labelled with tritium and ^{14}C. It has been shown that easily recognizable sources of artefacts can be ruled out under these circumstances; while the possibility of more exotic sources cannot be excluded, the reproducibility of the phenomenon and its appearance in a variety of situations makes this possibility appear quite small. Bolstering this contention is the fact that three forms of computation, *i.e.*, probit analysis, based on the cumulative percentage, fraction by fraction, of each isotope eluted, Ln-ϕ and MODI-ϕ, based on the isotope ratio of successive fractions, all yield significant and similar values for the displacement of ^{3}H from ^{14}C in each instance.

On the other hand, while the existence of isotopic fractionation seems certain, the mechanism by which it occurs does not. At present it seems likely that the greater portion of the isotopic effect is due to the presence of tritium, but it has not been established that the ^{14}C is simply an indicator of the migration rate of the non-isotopic component. The displacement of tritium from ^{14}C is actually the difference between the displacement of ^{3}H from ^{1}H and the displacement of ^{14}C from ^{12}C, and the value of the former gives no indication of the absolute magnitude of the two contributory displacements. Work currently in progress is concerned with measuring the migration rate of the unlabelled molecules so that the two individual fractionation processes can be determined. It should be noted that although isotopic displacements are usually expected to be additive in all combinations this expectation is not met in those instances where the isotopes are

spatially separated in the molecule by the ester group (*Table 4*). Further investigation of this seeming discrepancy is under way to determine whether or not the individual isotope displacements are preserved in these combinations.

The experiments reported here indicate the necessity for caution in the use and interpretation of doubly-labelled tracer systems, particularly where a chemical component is subjected to purification in a system with good resolution. Under these conditions, isotopic fractionation becomes obtrusive and may in fact obscure the underlying processes being studied.

To the biochemist, perhaps the most disconcerting point is that isotopic fractionation is not only present but visible during the analytical separation of molecular species of this size; to adjust to the idea that one tritium atom can exert a measurable effect in the presence of 47 hydrogen atoms (and a total molecular weight of 430) is not easy. On the other hand, this same high visibility is a potentially valuable tool for measuring the increments of adsorptive interaction which each region of the molecule contributes to the total interaction with the surface. Such studies on the prototype surfaces of adsorbents such as silica may yield further insight into the physical parameters of orientation and interaction with the surface of the enzyme.

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