EXPOSURE OF AN ISOTOPE EFFECT BY ^{3}H-LABELED FATTY ACIDS ON SILICA–SILVER NITRATE CHROMATOGRAPHY

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When tritium-labeled methyl linoleate[1] was eluted from a silica–silver nitrate column in our laboratory, it was apparent that the radioactive peak in the effluent did not precisely coincide with the methyl ester peak as located by spectrophotometric measurements but followed it closely. The two curves were identically shaped; the difference in resolution seemed to be due to a slower movement of the tritiated molecules. Similar isotope fractionation effects were observed upon chromatography of methyl esters of oleic or linoleic acid doubly labeled with tritium and carbon-14. Molecules labeled with tritium at the active centers of unsaturation showed a higher retention time on silica–silver nitrate columns than molecules labeled with carbon-14 at the carboxyl group.

EXPERIMENTAL

The chromatographic method of DE VRIES[2] was adapted. Silicic acid (100 g) was mixed with a silver nitrate solution (100 g in 200 ml water) dried in a tray at 120° for 16 h and passed through a 140 mesh sieve. For most experiments a column 150 mm in length and 12 mm in diameter was packed with 10 g of adsorbent. On one occasion a longer column under pressure was employed and in another a series of columns with varying amounts of silver nitrate per g of adsorbent was used. In each experiment a charge of approximately 70 mg of methyl ester was applied to the column, the eluting solvent was always benzene in light petroleum ether (b.p. 40–60°). The flow rate was about 1 ml/2 min and fractions of 1 ml were collected. The methyl linoleate concentration was determined spectrophotometrically[3] with the aid of a Cary Model 11 M recording spectrophotometer.

For radioactivity measurements 0.1 to 0.5 ml from each fraction was evaporated under nitrogen and taken up in 15 ml of a solution consisting of 3 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(phenyloxazolyl)-benzene per liter of toluene. A Packard Tricarb, automatic Model 314-EX scintillation spectrometer was used. Samples containing the two radioisotopes, ^{3}H and ^{14}C were counted by the discriminator ratio method[4] as modified by KABARA *et al.*[5]. The level of both isotopes was so chosen that count rates exceeded a 20:1 count/background ratio in order to eliminate the necessity of reckoning with the background in the $^{3}H/^{14}C$ ratio determination.

Linoleic acid labeled with tritium at the 9, 10, 12 and 13 positions was prepared as reported earlier[1]. [1-^{14}C]-Linoleic acid was purchased from California Corporation of Biochemical Research. In contrast to our tritium labeled linoleic acid the purchased

[1-^{14}C]-linoleic acid contained a considerable amount of *trans* isomers. For the purpose of this study, removal of *trans* isomers was necessary because homogeneity (regarding the geometry of the double bonds) of the labeled compounds was imperative. Since silica impregnated with silver nitrate resolves compounds which differ either in the number or geometry of double bonds, the presence of *trans* isomers would result in additional peaks. Thus, methyl [1-^{14}C]-linoleate was purified by chromatography on a silica-silver nitrate column using 20 ml of the eluting solvents in the following sequence: 35, 40, 45, 50 and 55 % benzene in petroleum ether and finally 70 ml benzene. Aliquots of the eluant fractions were counted and alternatively characterized by thin-layer chromatography on silicic acid–silver nitrate plates[1] and by infrared analysis*. The elution curve gave three components; the last to elute was almost 98 % *cis, cis* 18-diene and represented 86 % of the total material.

A mixture of [9, 10, 12, 13-^{3}H]-linoleic and [1-^{14}C]-linoleic acids was completely hydrogenated[6] to give stearic acid labeled in an identical manner as the original linoleic acids. Preparative gas phase chromatography was used for the purification of the methyl stearate[7].

Oleic acid labeled with ^{14}C at the carboxyl group and with ^{3}H at 9, 10, 12 and 13 positions was similarly prepared by controlled hydrogenation[8]. The product of this hydrogenation was subjected to preparative gas phase chromatography and the 18-monoene peak was isolated. Geometric and positional isomers of the 18-monoene formed during the hydrogenation were removed by silica–silver nitrate column chromatography[2]. The eluting solvent was 28 % benzene in petroleum ether. Periodate permanganate oxidation at the double bond indicated that 90 % of the 18-monoene had the double bond at the 9 position. Methyl esters of all the fatty acids were formed by reaction with diazomethane[9].

RESULTS AND DISCUSSION

The isotope effect is shown in Fig. 1A and B. In this experiment, the sample of methyl linoleate (75 mg) had a total activity of 0.35 μC. The column was 150 mm long (Fig. 1A) and the elution was carried out with 20 ml each of 40, 45, 50 and 55 % benzene in petroleum ether and finally with 70 ml benzene. It is apparent that the presence of tritium in the olefinic positions caused a slower movement of the methyl linoleate through the column. If it is assumed that both curves representing the concentration and the radioactivity distribution were gaussian with the same standard deviation σ and different means (m_1 and m_2) a semilogarithmic plot of the specific activity S *versus* fraction number n would result in a straight line and the relationship could be described by the formula[10]:

$$\ln S = \frac{m_1 - m_2}{\sigma^2} n + \frac{m_2^2 - m_1^2}{2\sigma^2}$$

Our plot shows a linear relationship between the logarithm of specific activity and the fraction number for that part of the chromatogram where the two curves overlap each other. A rapid increase of the specific activity with decreasing fraction number

* For the determination of *trans* double bonds by infrared spectroscopy, samples were prepared by grinding 30 mg KBr with 0.1 ml of ester solution in a mortar. Discs of 5 mm diameter were pressed in an evacuated die and examined in a Beckman IR-7 infrared spectrophotometer.

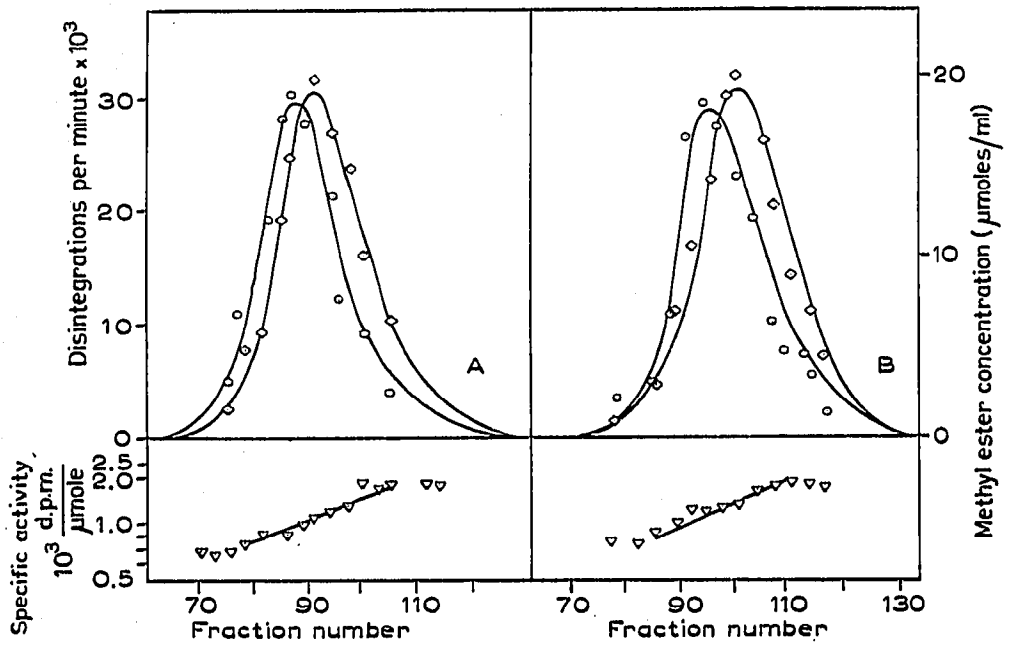

Fig. 1. Effluent curves from the column chromatography of tritium labeled methyl linoleate on silica–silver nitrate column (A = 150 × 12 mm; B = 230 × 12 mm); ⊙, methyl ester concentration as determined by spectrophotometric measurement; ◇, methyl ester activity as determined by ^{3}H counting; ▽, calculated specific activity.

made it difficult to extrapolate the specific activities of earlier fractions for which spectrophotometric measurements gave a relatively larger error. The slope of the line is a measure of the resolution of the labeled and the unlabeled compound. When a 230 mm long column was employed, the slope was increased indicating a higher resolution (Fig. 1B). On the other hand, the region of the linear relationship between ln S and n became shorter and the error at the leading and trailing edges of the peak concentration became larger.

When methyl [1-^{14}C]-linoleate was passed through a similar column under identical experimental conditions, the activity change was only 1.8 %, no greater than the limits of errors of the experiment. However, when a mixture of methyl [1-^{14}C]-linoleate and methyl [9, 10, 12, 13-^{3}H]-linoleate (ratio of $^{3}H/^{14}C$ = 3.25) was chromatographed and the ratio of the two radioisotopes was determined in each fraction of the collected effluent a steady increase of the ratio in favor of tritium was observed indicating a faster movement of the carbon-14 labeled molecules. The logarithm of the ratio of the isotopes plotted against the fraction number resulted in a slope identical with that in Fig. 1.

In order to exclude the possibility that the observed isotope effect was due to some radioactive contamination, we tested many fractions by gas chromatography and thin-layer chromatography using the isotope dilution technique. In all cases the radioactivity coincided with the methyl linoleate peak or spot and there was no indication that artifacts could have been responsible for the differences. There are two possible explanations which should be considered for this phenomenon. The first involves the possibility of an increase in mass by the tritium, which appears to be unlikely, since theory predicts that replacement of one or two hydrogen atoms by tritium causes only a change of 2 or 3 % in mass. This increase in mass would not seem sufficient to cause

the observed differences in chromatographic behavior. The second involves the possibility that tritium exerts an effect upon the ability of the olefinic linkage to form coordination complexes with the silver ion.

It seems relevant to speculate whether the presence of tritium at the olefinic bonds may relate to changes in the affinity of the olefinic bonds to form complexes. This possibility seems to be quite reasonable in view of general agreement that resolution on a silica–silver nitrate column is attributable to the formation of these complexes. Indeed, if the effect reflects a difference in the coordination products, the slope which is a measure of the degree of resolution should depend upon the number of double bonds affiliated with tritium and present in the vehicle molecule. In addition the magnitude of the isotope effect should be related to the concentration of silver ions in the adsorbent. In order to test these points the double-label isotope ratio technique was employed. The assumption was made that the carbon-14 labeled methyl esters carrying the label in a position remote from the reaction center have a similar behavior on the column to the unlabeled esters and thus, the carbon-14 could serve as an internal reference standard. This assumption was supported by the fact previously established that methyl [1-^{14}C]-linoleate behaved identically to the unlabeled compound.

Methyl esters of the following specifically labeled fatty acids were chromatographed in the amounts indicated (the figures showing μmoles, μC of ^{3}H and the ratio of ^{3}H/^{14}C respectively): [1-^{14}C]-stearic and [9, 10-^{3}H]-stearic acid 200, 0.38, 1.20; [1-^{14}C]-oleic and [9, 10, 12, 13-^{3}H]-oleic acid 210, 0.41, 1.24. For each methyl ester two effluent radioactivity curves were determined, one based on carbon-14 and the other on tritium.

If we consider the carbon-14 curve as representing the actual concentration, the tritium activity curve shows an apparent concentration in those areas where partial resolution occurred. The ratio of the apparent concentration to the actual concen-

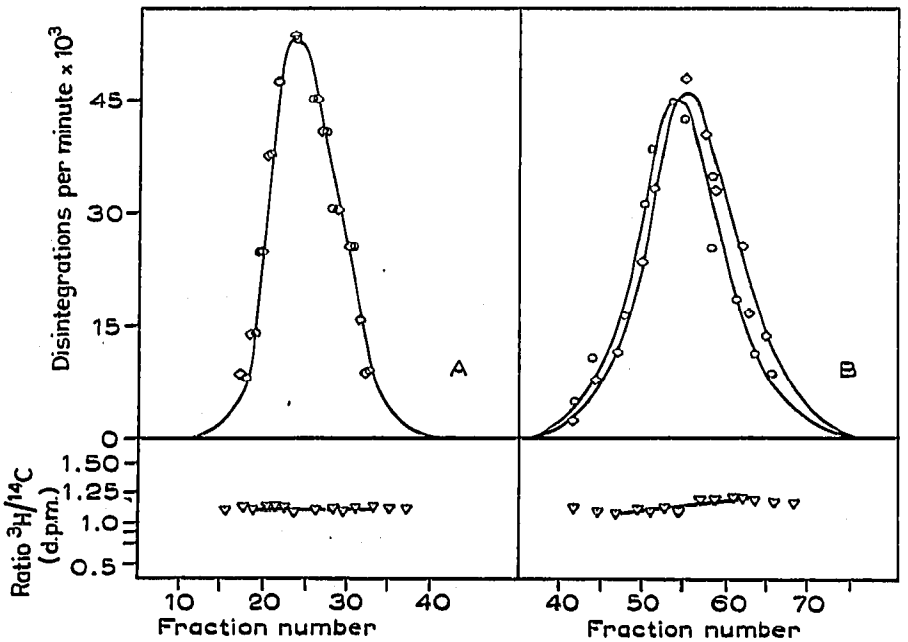

Fig. 2. Effluent curves from the column chromatography of double labeled methyl stearate (A) and methyl oleate (B): ⊙, ^{14}C activity as determined by ^{14}C counting; ◇, ^{3}H activity as determined by ^{3}H counting; ▽, ratio of ^{3}H/^{14}C.

tration which is identical to the ratio of $^{3}H/^{14}C$ provided a measure of relative specific activity in successive fractions. Fig. 2 represents the pairs of curves. As anticipated, there was no effect in the case of methyl stearate while resolution of doubly labeled methyl oleate was apparent. The value obtained for the slope of methyl oleate was approximately one third of the observed value for methyl linoleate. These slopes can be used only for qualitative comparisons. Further evidence in support of our interpretation of the observed phenomenon is given in Table I. Here, the slope constant representing the degree of resolution of methyl linoleate varied directly with the concentration of the silver nitrate solution used for the preparation of the adsorbent.

TABLE I

SLOPE OF CURVE RELATING THE LOGARITHM OF SPECIFIC ACTIVITY OF TRITIUM LABELED METHYL LINOLEATE TO FRACTION NUMBER

Grams of silver nitrate per 100 ml water per 100 g silicic acid	*Slope constant value*
0	—
25	0.32
50	0.40
75	0.42
100	0.46

It therefore appears certain that tritium exerts its effect on the formation and breaking of the silver ion double bond complexes which comprise the transition structure during sorption and desorption.

Isotope effects based on chromatographic behavior have been reported for a number of chemical systems. The use of column chromatography[10–12] and gas chromatography[13] in a repeated cycling operation has been proposed for the separation and enrichment of isotope mixtures. In most cases it was shown that the effect depended upon the isotope occupying a particular position in the molecule[12,14]. From that particular position the isotope could affect electronic effects (inductive effects, resonance, ionization) which had a direct bearing upon the chromatographic behaviour of the entire molecule. For tritium-labeled linoleic acid, tritium could exist in a stable form at either the 9, 10, 12 or 13 (or any combination) position and its presence in those positions would exert an effect on the formation of the silver–olefin equilibrium constants. Recently, CVETANOVIC, DUNCAN AND FALCONER[15] have published the gas chromatographic results of the effect of progressive deuteration on the retention volumes of olefinic hydrocarbons on silver nitrate–ethylene glycol firebrick columns. They have shown a reasonably additive effect of considerable magnitude attributable to deuterium isotope effects on the silver–olefin equilibrium constants.

Such phenomena may not be readily evident and it is the excellent resolving power of modern chromatographic techniques that reveals them. Nevertheless, if overlooked or ignored it may result in a serious flaw in the evaluation and interpretation of experimental data, whenever radioactive tracers are used in combination with chromatography.

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SUMMARY

A tritium isotope effect was observed during the chromatography of tritiated unsaturated fatty acids as their methyl esters on silica–silver nitrate columns. The results are interpreted in terms of the coordination complexes formed between olefinic bonds and silver ions.

REFERENCES

1 D. S. SGOUTAS AND F. A. KUMMEROW, *Biochemistry*, 3 (1964) 406.
2 B. DE VRIES, *J. Am. Oil Chemists' Soc.*, 40 (1963) 184.
3 S. F. HERB AND R. W. RIEMENSCHNEIDER, *Anal. Chem.*, 25 (1953) 953.
4 G. T. OKITA, J. J. KABARA, F. RICHARDSON AND G. V. LEROY, *Nucleonics*, 15 (1957) 111.
5 J. J. KABARA, N. R. SPAFFORD, M. A. MCKENDRY AND N. L. FREEMAN, in SEYMOUR ROTHCHILD, (Editor), *Advances in Tracer Methodology*, Vol. 1, Plenum Press, New York, 1963, p. 76.
6 W. J. GENSLER AND J. J. BRUNO, *J. Org. Chem.*, 28 (1963) 1254.
7 A. K. HAJRA AND N. S. RADIN, *J. Lipid Res.*, 3 (1962) 131.
8 R. O. FEUGE, E. R. COUSINS, S. P. FORE, E. F. DUPRE AND R. T. O'CONNOR, *J. Am. Oil Chemists' Soc.*, 30 (1953) 454.
9 H. SCHLENK AND J. L. GELLERMAN, *Anal. Chem.*, 32 (1962) 1412.
10 K. A. PIEZ AND H. EAGLE, *Science*, 122 (1955) 968.
11 F. H. SPEDDING, J. E. POWELL AND H. J. SVEC, *J. Am. Chem. Soc.*, 77 (1955) 1393.
12 H. GOTTSCHLING AND E. FREESE, *Nature*, 196 (1962) 829.
13 J. W. ROOT, E. K. C. LEE AND F. S. ROWLAND, *Science*, 143 (1964) 676.
14 K. A. PIEZ AND H. EAGLE, *J. Am. Chem. Soc.*, 78 (1956) 5285.
15 R. J. CVETANOVIC, E. J. DUNCAN AND W. E. FALCONER, *Can. J. Chem.*, 41 (1963) 2095.