ENHANCEMENT AND MODIFICATION OF ISOTOPE FRACTIONATION DURING THE PARTITION CHROMATOGRAPHY OF ^{3}H AND ^{14}C LABELED STEROIDS*

VACLAV CEJKA**, ELIZABETH M. VENNEMAN AND NEL BELT-VAN DEN BOSCH
*Department of Internal Medicine***, University of Amsterdam, Binnengasthuis, Amsterdam (The Netherlands)*
AND
PETER D. KLEIN
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill. (U.S.A.)
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There is a growing awareness of the occurrence of isotope fractionation during preparative or analytical resolution of labeled steroids by chromatographic means. Such instances have been observed by JENSEN AND JACOBSEN[1] for estradiol-4-^{14}C and estradiol-1,2-^{3}H in paper chromatographic systems; by TAIT[2], CEJKA AND VENNEMAN[3] and by LARAGH, SEALEY AND KLEIN[4] for aldosterone-1,2-^{3}H diacetate-1-^{14}C on celite columns and paper chromatograms, and by KIRSCHNER AND LIPSETT[5] for testosterone-1,2-^{3}H acetate-1-^{14}C on gas–liquid chromatograms. The general occurrence of such fractionation in analytical separations has recently been reviewed by KLEIN[6].

Isotope fractionation of doubly labeled steroids is a particularly vexing consequence in the separation processes when isotope dilution measurements are being carried out, since the anticipated criterion of purity (a constant isotope ratio) is not to be found. LARAGH, SEALEY AND KLEIN[4] have described the mathematical basis for determining the isotope dilution when isotope fractionation is present, but an alternative approach is to ascertain whether the degree of fractionation itself can be altered. Such an approach, if fruitful, might also yield information on the mechanism(s) whereby one isotopically substituted steroid exhibits a different mobility from the same steroid with a different label.

One indication that the isotope fractionation of aldosterone-1,2-^{3}H diacetate from aldosterone diacetate-1-^{14}C might be modified by experimental circumstances came to light in an exchange of data between the Binnengasthuis and Argonne Laboratories. In the system used by CEJKA AND VENNEMAN, the displacement between the ^{3}H and ^{14}C forms was 1.85 % whereas LARAGH AND SEALEY (at Columbia University College of Physicians and Surgeons) had found the displacement to be less than half as much: 0.65 %. A point-by-point comparison of procedures suggested that the composition of the stationary phase influenced the degree of separation and that further modifications might be possible. The present report is a verification of this possibility.

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** Present address: Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill., U.S.A.
*** Head: Prof. Dr. J. G. G. BORST.

The data to be presented indicate that the degree of fractionation can be varied over more than a twofold range for doubly labeled aldosterone and over a tenfold range for dually labeled cortisone. These appear to be the largest changes in isotope fractionation in response to a procedural variation in the fractionation system that have been reported to date.

METHODS

Aldosterone-1,2-^{3}H (specific activity 31.3 C/mM), cortisone-4-^{14}C (specific activity 42 mC/mM), and cortisone-1,2-^{3}H (specific activity 721 mC/mM) were purchased from New England Nuclear Corp., Boston, U.S.A., and freed from radioactive impurities by column chromatography prior to use. Aldosterone-4-^{14}C (specific activity 46 mC/mM) was obtained through the courtesy of Dr. MORRIS M. GRAFF from the Endocrinology Study Section of the U.S.N.I.H.

The mobile phase of the solvent systems consisted of a mixture of four parts toluene (Analar, British Drug House) and one part ligroin (b.p. 80–100°; Brocades and Stheeman Pharmacia, Amsterdam). The stationary phase consisted of various aqueous methanol concentrations as indicated in the experiments. The solvent systems were equilibrated at room temperature for at least 24 h. Columns of 1 cm diameter were packed with acid washed celite 545 (Johns-Manville) mixed with stationary phase (2:1, w/v) using 1 g of celite per 3 cm column length.

The alcoholic solutions of radioactive steroids to be chromatographed were evaporated *in vacuo*, dissolved in 0.25 ml of stationary phase, and mixed with 0.5 g of celite. Mobile phase was added to the celite and after mixing with Sudan red the slurry was applied to the top of the column. A new column was packed for every experiment.

The collection of fractions (2 or 5 ml) was started when Sudan red emerged from the column. After transferring to glass counting vials the column eluates were mixed with 10 ml scintillation solution containing 0.5 g P.P.O. and 0.03 g dimethyl-P.O.P.O.P. per 100 ml toluene.

^{3}H and ^{14}C radioactivity was measured simultaneously in a Tricarb liquid scintillation counter model 314-EX-2 on high voltage 1205 and channel settings: 10% gain (gate 3–10) and 100% gain (gate 1–10). The counting efficiencies were:

Channel	*^{14}C*	*^{3}H*
10% gain	34.8%	0.0%
100% gain	20.1%	31.2%

Computations

The scintillation counting data expressed as dpm^{3}H or dpm^{14}C were used to compute a probit analysis for each radioactive peak[7]. From this was obtained a mean retention volume M and a peak dispersion σ, as well as the standard errors of these quantities. From the isotope ratio ø for successive fractions of the mixed peak, the displacement ΔM% and its standard error were computed, using the equations derived by KLEIN, SIMBORG AND SZCZEPANIK[8], which include correction for any

differences in dispersion between the two peaks. The overall computer programs for these calculations were prepared by BARBARA KUNZE-FALKNER of the Division of Biological and Medical Research.

RESULTS

An example of the fractionation of ^{3}H labeled aldosterone from ^{14}C labeled aldosterone is illustrated in Fig. 1. The isotope ratio exhibits a remarkable increase in the mixed peak, indicative of the difference in mobility between the two isotopically labeled aldosterones.

Table I lists the pertinent data for 13 columns in which the stationary phase was successively: water, 10 %, 30 %, 50 %, and 80 % methanol and the mobile phase was toluene–ligroin (4:1). Despite the fact that within each series the columns were

TABLE I

CHROMATOGRAPHY OF ALDOSTERONE-1,2-^{3}H AND ALDOSTERONE-4-^{14}C ON CELITE PARTITION COLUMNS WITH VARIOUS STATIONARY PHASES

Stationary phase	*Column length (cm)*	*Retention volume (ml) ± S.E.*		*Dispersion (ml) ± S.E.*	
		^{14}C	^{3}H	^{14}C	^{3}H
H_2O	15	177.67 ± 0.30	178.90 ± 0.26	4.25 ± 0.27	4.15 ± 0.23
		169.56 ± 0.28	170.75 ± 0.32	4.21 ± 0.31	4.17 ± 0.31
		183.19 ± 0.11	184.45 ± 0.12	4.67 ± 0.12	4.31 ± 0.12
10 % methanol	15	106.69 ± 0.05	108.50 ± 0.14	5.75 ± 0.05	6.06 ± 0.14
		116.80 ± 0.08	118.73 ± 0.10	7.02 ± 0.08	7.18 ± 0.10
30 % methanol	30	85.33 ± 0.13	86.62 ± 0.09	6.47 ± 0.13	6.68 ± 0.10
		84.34 ± 0.20	85.77 ± 0.23	5.64 ± 0.20	5.82 ± 0.22
50 % methanol	40	104.30 ± 0.04	105.98 ± 0.01	4.46 ± 0.04	4.54 ± 0.02
		103.15 ± 0.09	104.76 ± 0.10	4.05 ± 0.08	4.06 ± 0.10
		101.26 ± 0.07	102.75 ± 0.06	4.79 ± 0.06	4.88 ± 0.04
		98.67 ± 0.06	100.13 ± 0.04	4.43 ± 0.05	4.54 ± 0.04
80 % methanol	40	116.16 ± 0.25	117.11 ± 0.24	6.20 ± 0.25	6.40 ± 0.24
		114.20 ± 0.16	115.26 ± 0.14	5.10 ± 0.17	5.21 ± 0.15

completely repacked after every run, the retention volumes and dispersions from run to run were closely reproducible. For this reason, even the small number of columns used in these studies were sufficient to obtain meaningful comparisons between the stationary phases. Table II lists the per cent displacement of the ^{3}H-aldosterone from the ^{14}C-aldosterone for each of the five series; there is a twofold increase in displacement when the stationary phase is changed from water to 10 % methanol and this displacement is maintained over most of the range, declining somewhat when the methanol concentration reaches 80 %. A plot of these displacements *versus* the methanol concentration is shown in Fig. 2, which illustrates the close agreement between the average displacements measured by probit analysis (*i.e.*, from the differences in means, Table I) and those obtained from the isotope ratio.

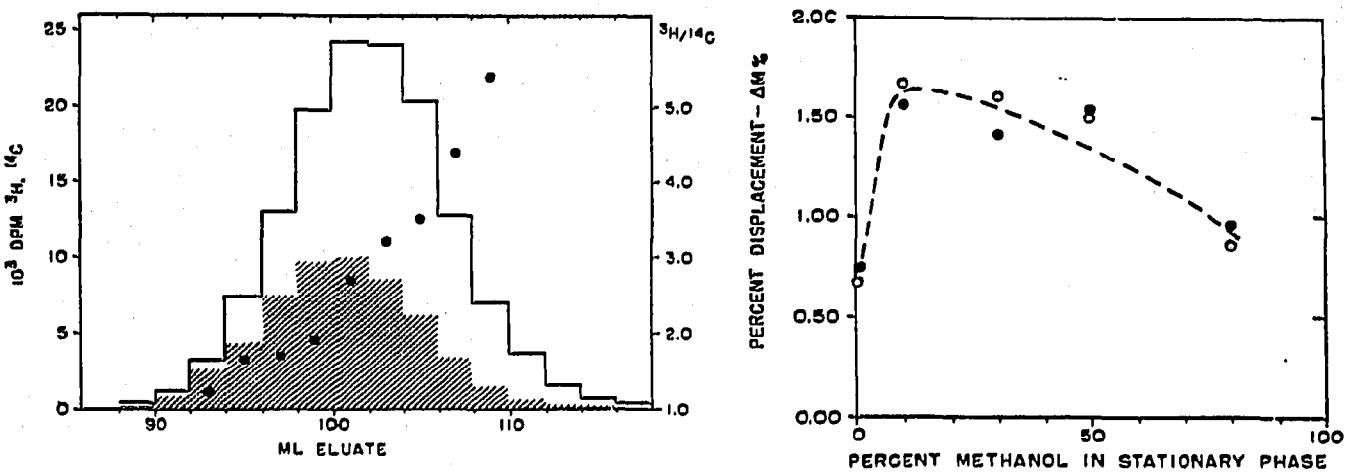


Fig. 1. The fractionation of aldosterone-1,2-^{3}H from aldosterone-4-^{14}C during partition chromatography on 40 cm celite column. Cross hatched area: ^{14}C; outline: ^{3}H; dots: ^{3}H/^{14}C. (Solvent system toluene–ligroin (4:1)/50 % methanol.)

Fig. 2. The effect of methanol concentration on the isotopic fractionation of ^{3}H-aldosterone from ^{14}C-aldosterone. Open circles: as determined by probit analysis; solid circles: from isotope ratio measurement.

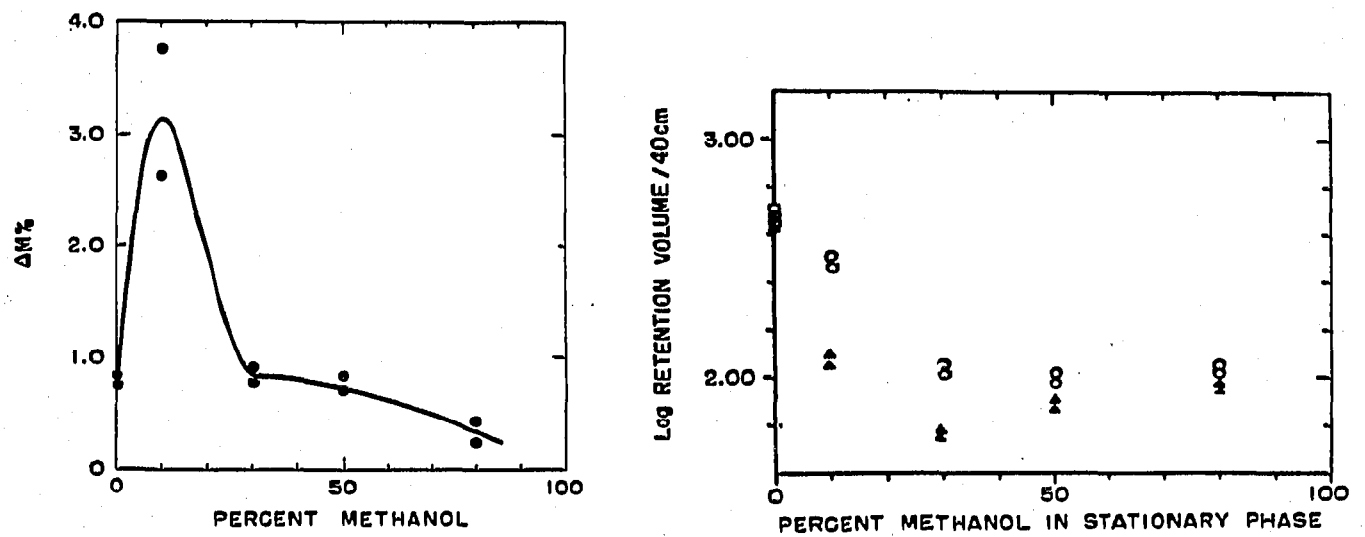


Fig. 3. The effect of methanol concentration in stationary phase on the isotopic fractionation of ^{3}H-cortisone from ^{14}C-cortisone.

Fig. 4. The effect of methanol concentration in the stationary phase on the log retention volumes of aldosterone (circles) and cortisone (triangles).

TABLE II

ISOTOPE FRACTIONATION FACTORS DETERMINED FROM ISOTOPE RATIO MEASUREMENT FOR ALDOSTERONE-4-^{14}C AND ALDOSTERONE-1,2-^{3}H ON VARIOUS STATIONARY PHASES

Run	H_2O	*10 % Methanol*	*30 % Methanol*	*50 % Methanol*	*80 % Methanol*
	ΔM % ± S.E.	*ΔM % ± S.E.*	*ΔM % ± S.E.*	*ΔM % ± S.E.*	*ΔM % ± S.E.*
1	0.73 ± 0.07	1.50 ± 0.14	1.32 ± 0.11	1.53 ± 0.07	0.88 ± 0.10
2	0.68 ± 0.11	1.63 ± 0.10	1.53 ± 0.17	1.73 ± 0.15	1.05 ± 0.10
3	0.85 ± 0.04			1.43 ± 0.06	
4				1.47 ± 0.06	
Average	0.75 ± 0.06	1.56 ± 0.06	1.41 ± 0.09	1.54 ± 0.09	0.96 ± 0.08

A similar series of columns was packed and run to determine the effect of the stationary phase composition of the separation of ^{3}H-cortisone from ^{14}C-cortisone. The column data for these runs are shown in Table III and the displacements obtained are shown in Fig. 3. An even more striking effect of the methanol concentration can be seen in the region of 10 % methanol, where the fractionation effect attains a value of more than 3 % and then declines to less than 0.3 % at 80 % methanol.

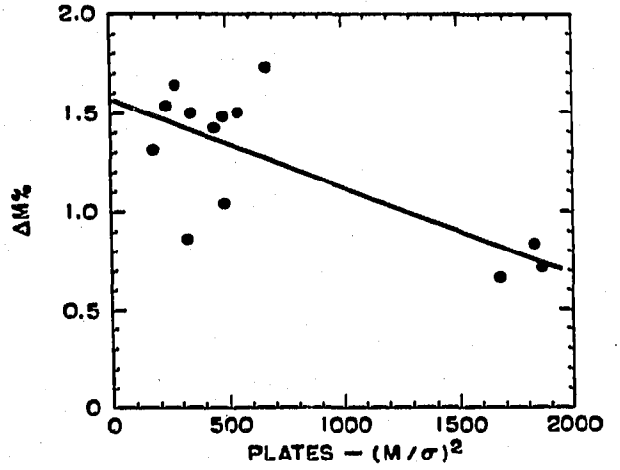
The effect of the stationary phase composition on the log retention volume of aldosterone and cortisone is shown for a standardized column length of 40 cm in Fig. 4. Instead of the anticipated straight line, there are two components to the relationship, one decreasing the retention volume, the other maintaining or increasing it. These intersect somewhere in the region of 30 % methanol for both steroids. Furthermore, cortisone, which is less polar than aldosterone and should be expected to have a lower retention volume at all methanol concentrations, displays an anomalously high retention volume on those columns in which water is the stationary phase. There is, however, no anomalous behavior in either series ascribable to, or related to, the changes in isotope fractionation.

TABLE III

CHROMATOGRAPHY OF CORTISONE-1,2-^{3}H AND CORTISONE-4-^{14}C ON CELITE PARTITION COLUMNS WITH VARIOUS STATIONARY PHASES

Stationary phase	*Column length (cm)*	*Retention volume (ml) ± S.E.*		*Dispersion (ml) ± S.E.*	
		^{14}C	^{3}H	^{14}C	^{3}H
H_2O	15	241.54 ± 0.20	243.40 ± 0.17	12.70 ± 0.20	13.20 ± 0.18
		249.66 ± 0.14	251.35 ± 0.22	13.56 ± 0.16	13.53 ± 0.23
10 % methanol	15	52.14 ± 0.07	53.38 ± 0.13	5.24 ± 0.08	4.93 ± 0.14
		52.56 ± 0.04	53.73 ± 0.22	4.36 ± 0.05	4.42 ± 0.28
30 % methanol	40	58.35 ± 0.05	58.92 ± 0.05	3.18 ± 0.05	3.23 ± 0.05
		60.39 ± 0.02	60.86 ± 0.02	3.52 ± 0.02	3.54 ± 0.02
50 % methanol	40	82.10 ± 0.12	82.47 ± 0.11	4.63 ± 0.12	4.42 ± 0.11
		78.46 ± 0.08	78.90 ± 0.04	4.02 ± 0.07	3.87 ± 0.03
80 % methanol	60	166.48 ± 0.03	167.04 ± 0.05	7.32 ± 0.03	7.18 ± 0.05
		149.19 ± 0.10	149.74 ± 0.04	7.02 ± 0.10	6.67 ± 0.04

When the numbers of plates generated in the columns (computed as $(M/\sigma)^2$) were plotted against the per cent displacement obtained, a significant relationship was observed and is shown for aldosterone in Fig. 5 and for cortisone in Fig. 6. Both figures indicate that the greater the number of plates in the column, the smaller the per cent displacement which occurs between the two labeled forms of the steroid. In the case of aldosterone, a linear regression of the points yielded a slope of $-4.272 \cdot 10^{-5}$ with a standard error of $1.212 \cdot 10^{-5}$; the probability of this being a zero slope is 0.002. In the cortisone data, the curvature of the relationship precluded a linear regression analysis, but the inverse correlation of displacement with plate number is easily seen.



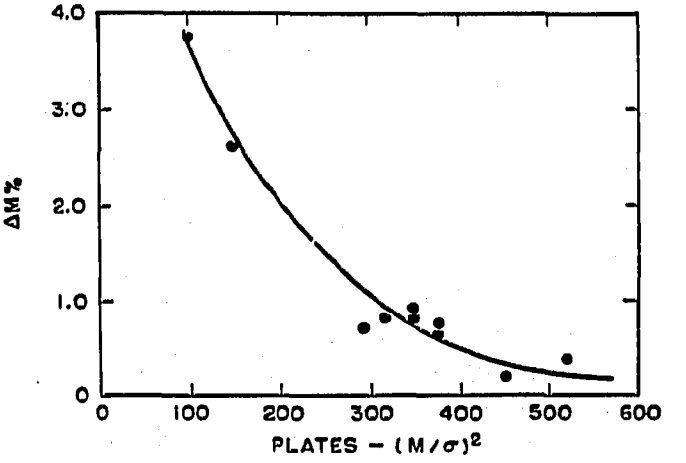


Fig. 5. The relationship between per cent displacement of ^{3}H-aldosterone from ^{14}C-aldosterone and the numbers of plates generated in the column.

Fig. 6. The relationship between per cent displacement of ^{3}H-cortisone from ^{14}C-cortisone and the numbers of plates generated in the column.

The unexpected *inverse* relationship shown in Figs. 5 and 6 suggested that at least a portion of the fractionation was due to an isotope effect exerted through some associative process present in the early stages of the chromatography. If present, such associative interaction would decay as the number of plates (and hence the number of transfer from stationary to mobile phase and back) increased; that is to say, it should be maximal with a short column and diminish as the column length increases. To investigate this possibility, two columns were packed with celite in which the stationary phase was water and the mobile phase was toluene. The column lengths were 8.5 and 28.5 cm and the fractionation of dual-labeled cortisone was determined on each column. The results are shown in Table IV. They indicate that on the short column, the per cent displacement of the two labeled forms of cortisone was more than threefold larger than on the long column, or conversely, extending the length of the column reduced the difference in migration rates by nearly three-quarters.

DISCUSSION

There have been few systematic attempts to influence the degree of isotope fractionation in heteroatomic molecules. LIBERTI, CARTONI AND BRUNER[9] have shown

TABLE IV

VARIATION OF ISOTOPE DISPLACEMENT WITH COLUMN LENGTH DURING THE PARTITION CHROMATOGRAPHY OF CORTISONE-1,2-^{3}H AND CORTISONE-4-^{14}C

Column length (cm)	*Retention volume (ml) ± S.E.*		*Dispersion (ml) ± S.E.*		*ΔM %*	*Plates generated in column*
	^{14}C	^{3}H	^{14}C	^{3}H		
8.5	65.80 ± 0.27	66.72 ± 0.32	9.51 ± 0.29	9.36 ± 0.35	2.33 ± 0.60	48
28.5	253.84 ± 0.23	255.56 ± 0.20	12.70 ± 0.24	12.93 ± 0.21	0.64 ± 0.04	400

that the stationary phase of a gas–liquid chromatographic column affects the degree to which perdeuterobenzene is resolved from benzene. They determined the separation factors on squalene and on silicone oil at various temperatures and have computed the heats of solution in each phase as well as the entropy change. DAVIDSON, MANN AND SHELINE[10] have measured the influence of resin cross-linking in the isotopic fractionation of $H^{14}COOH$ from $H^{12}COOH$ by ion exchange chromatography. They found an increase in the separation factor from 1.0028 at 2 % cross linking to 1.0044 at 8 % and a maximum effect of 1.0059 (equivalent ΔM %: 0.59) at 10 %. KLEIN, SIMBORG AND SZCZEPANIK[8] have shown that the spatial separation of ^{3}H and ^{14}C in the cholesterol acetate molecule influences the degree of fractionation found during absorption chromatography: the closer the ^{3}H to the ^{14}C, the greater the displacement which was observed.

The results which have been obtained with doubly labeled aldosterone and cortisone indicate that parameters in the chromatographic system itself can influence the degree of separation. These are perhaps the most interesting of the possibilities to date, since they are much more amenable to experimental variation and study. The ability to enhance the fractionation to the degree where the difference in mobility is more than 3 % and then to reduce this by more than tenfold by changing the stationary phase composition offers opportunities for study not heretofore available. From the standpoint of the primary objective, that of reducing the degree of fractionation, these experiments offer encouragement that solvent systems in which fractionation is minimal can be found, though whether or not fractionation can be eliminated is still problematic.

It is difficult to conceive of these responses to stationary phase composition as being those of primary isotope effects in the partition process. Although such effects do occur, and may indeed be present in these chromatograms, the existence of maxima in the effect of methanol concentrations and the fractionation response to changes in column length are much more characteristic of associative processes such as micelle formation. Since the degree of displacement (*i.e.*, the effective separation factor) *decreases* under conditions presumed to disrupt such associative forms, the quite surprising inference appears to be that the formation of such aggregates may itself be the isotope-sensitive process. A somewhat comparable interpretation has been offered for the unusually large isotope effects observed during the distillation of aqueous ^{3}H-formaldehyde by SIMON AND HEUBACH[11]. Inasmuch as the distillation of HOH from HTO resulted in far less enrichment than for the formaldehydes, the fractionation could not be due to a primary effect of ^{3}H on vapor pressures. They attributed

the fractionation to a difference in the hydration equilibrium of formaldehyde in which the formation of the hydrate was favored for the labeled formaldehyde over the unlabeled formaldehyde. In confirmation of this mechanism, they were able to show that as the pressure at which the distillation was carried out was reduced, the fractionation effect became correspondingly smaller. Further experiments in the present series to determine the proportion of fractionation effects which are derived from each source are being carried out. These will establish the irreducible minimum component due to isotope effects in the partition process itself.

SUMMARY

The isotope fractionation occurring during the partition chromatography of aldosterone-1,2-^{3}H and aldosterone-4-^{14}C and of cortisone-1,2-^{3}H and cortisone-4-^{14}C has been shown to be influenced by the composition of the stationary phase used to prepare the column. Displacements corresponding to separation factors as high as 1.030 and as low as 1.003 have been observed for the two labeled varieties of cortisone.

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