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LOCAL SOLVENT STRUCTURE AS A FACTOR IN SECONDARY ISOTOPE FRACTIONATION PHENOMENA OF LABELED AMINO ACIDS ON ION-EXCHANGE COLUMNS*

PETER D. KLEIN AND PATRICIA A. SZCZEPANIK
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill. 60439 (U.S.A.)
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SUMMARY

The separation or displacement of ^{3}H-labeled amino acids from ^{14}C-labeled varieties of the same species on ion-exchange columns is influenced by the presence of unlabeled amino acid, local solvent structure and buffer composition. These factors may enhance or reduce the degree of separation observed and act by affecting the diffusion of the labeled molecules. A significant *inverse* relationship between peak disengagement and hand-broadening processes was observed.

INTRODUCTION

Radiochemically labeled amino acids are a convenient group of compounds in which to study isotope fractionation phenomena. Their simple structure, unequivocal radiopurity, diversity of labeled positions and their highly reproducible behavior on ion-exchange columns offer the opportunity to correlate isotope location with chromatographic behavior. Such studies were originally reported by PIEZ AND EAGLE[1] in 1956; subsequently, the behavior of four ^{14}C-labeled basic amino acids was studied by BELLOBONO[2,3] and, most recently, an extensive series of ^{3}H- and ^{14}C-labeled compounds were investigated by KLEIN AND SZCZEPANIK[4]. The latter study showed that in addition to the anticipated influence of isotopic substitution on chromatographic mobility, several secondary phenomena affecting isotope fractionation could be demonstrated. In particular, the fractionation of a [^{3}H]amino acid from its ^{14}C-labeled counterpart was perceptibly increased by the addition of unlabeled amino acid. This increase was proportional to the original magnitude of the fractionation and, in some instances, was as much as 50%. Additionally, the displacement of several ^{3}H-labeled amino acids from the unlabeled form was sensitive to the location of the radiocarbon label in the accompanying species of the same amino acid.

This work deals with the influence of local solvent structure upon these phenomena, the effect of structure breaking by *tert.*-butanol, and of buffer composition

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upon the separation factors of labeled amino acids. It indicates that the diffusion characteristics of labeled amino acids are limited by solvent structure and buffer composition and that their absolute displacement from each other is reduced by these factors in the eluting buffer.

EXPERIMENTAL

Radiochemicals

^{3}H- and ^{14}C-labeled amino acids were products of the New England Nuclear Corp. with a radiochemical purity of 99% or greater by paper chromatography in butanol–acetic acid–water systems. Nonradioactive amino acids were added to the chromatogram where noted, as 2.50 μmole of the L form.

Column operation

Details of column operation, aliquoting of samples, ninhydrin measurements and liquid scintillation counting have been given in the previous work[4] and were maintained throughout this study. Scintillation counting was carried out in a Beckman LS 200 instrument with automatic external standardization.

When *tert.*-butanol was added to the buffer, the desired molecule percent in the final volume was calculated and the *tert.*-butanol added before adjustment of the pH and final dilution. In changing from one concentration of butanol to another, the column resin was removed, suspended in the new buffer, repacked and buffer was pumped through the column for 24 h to establish equilibrium. At high concentrations of *tert.*-butanol (4 mol. %), omission of these steps led to fluctuations of as much as 15% in the retention of volume from run to run. No measurements were made until this variation had been reduced to 3% or less.

Computations

As previously described, established programs were used to compute the cumulative percent elution for tritium, radiocarbon and ninhydrin by fraction number.

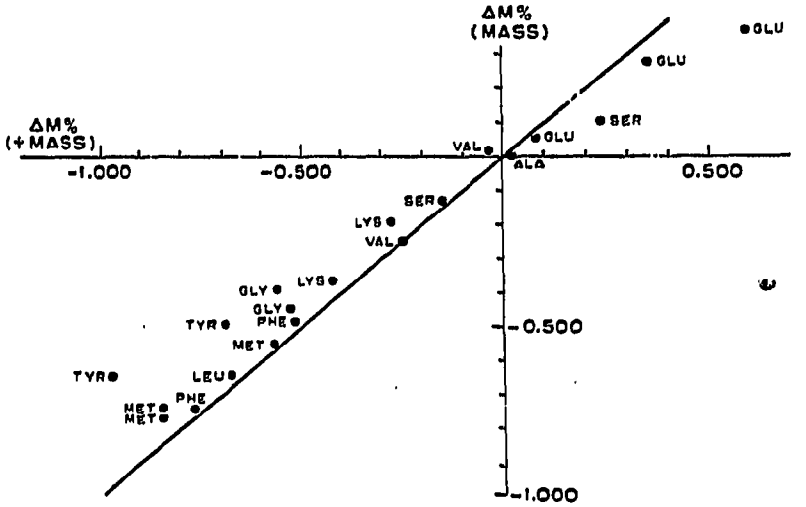

Fig. 1. Correlation between displacements ($^{3}H/^{14}C$) in the absence and presence of 2.5 μmoles of unlabeled amino acid. The line indicates normal expectation of 1 : 1.

From these values, a probit analysis yields the midpoint M of the peak and its dispersion σ. Displacements were calculated from differences in M, or from the isotope ratio (and specific activities), using the equations of KLEIN *et al.*[5].

RESULTS

The departure point for the present work is shown by the data in Fig. 1, in which the $^{3}H/^{14}C$ displacement obtained between specific pairs of tritium and radiocarbon forms of the same amino acid in the absence of added amino acids is plotted against the same displacement obtained when 2.5 μmoles of unlabeled amino acid is added to the chromatogram. As previously noted, if random fluctuations were responsible for the differences between two values, the overall collection would be expected to show both increases and decreases, and a slope for all values equal to 1.00. Instead, a slope of 1.211 $\pm$ 0.062 was obtained. Although the separation factor between two compounds is invariant with sample size under ideal conditions, the usual departure from ideality is a *decrease* with increasing sample size, not the increase encountered here. This suggested that the unlabeled amino acid was modifying the environment of the radioactive species during migration, perhaps by altering the local solvent structure in the eluting buffer. Two categories of evidence were found for this belief: that obtained from *tert.*-butanol, and that from the use of ammonium formate buffers.

TABLE I

ISOTOPIC DISPLACEMENTS AND THE EFFECT OF *tert.*-BUTANOL IN ELUTING BUFFER

Amino acid	^{3}H *position*	^{3}H/*mass* (% × 10^{3})	^{14}C *position*	^{14}C/*mass* (% × 10^{3})	$^{3}H/^{14}C$ (−*mass*)	$^{3}H/^{14}C$ (+*mass*)
Glutamic acid	3	443 ± 23	1	282 ± 19	170 ± 15	187 ± 10
	3	440 ± 36	3,4	10 ± 32	429 ± 24	399 ± 15
Glycine	2	− 98 ± 8	1	278 ± 11	−380 ± 17	−345 ± 20
Methionine	Methyl	−493 ± 29	1	100 ± 24	−662 ± 11	−623 ± 10
Phenylalanine	gl[a]	−359 ± 12	1	213 ± 10	−560 ± 7	−599 ± 5
	gl	−334 ± 33	3	39 ± 41	−380 ± 5	−395 ± 10
Serine	3	165 ± 10	1	207 ± 15	− 34 ± 16	− 37 ± 15
Tyrosine	3′,5′	−325 ± 12	1	287 ± 18	−637 ± 15	−568 ± 15

[a] Generally labeled.

Of the compounds known to disrupt local solvent structure and hydrogen bonding, *tert.*-butanol was selected because of its miscibility in all proportions with water, its compatibility with ninhydrin analyses, and its absence of quenching in the liquid scintillation counting solvent. In the first series of experiments, *tert.*-butanol was incorporated at a level of 2.5 mol. % into sodium citrate buffers of pH 3.28 and 4.25. Eight pairs of ^{3}H- and ^{14}C-labeled amino acids were chromatographed in the absence and presence of unlabeled amino acid, and the displacements shown in Table I were obtained. Each value is the result of three to five separate chromatograms. Inspection of these values shows that 2.5 mol. % *tert.*-butanol abolishes the effect of added mass on the displacement between ^{3}H and ^{14}C forms, *i.e.*, no further increase takes place. These data, plotted in the manner used in Fig. 1, yielded a slope

of 0.982 ± 0.036 in a linear regression by the method of least squares. Moreover, the individual displacements for [3-^{3}H]glutamic acid and for uniformly ^{3}H-labeled phenylalanine from their respective unlabeled forms are unchanged by ^{14}C-labeled forms of the same amino acid labeled in different locations.

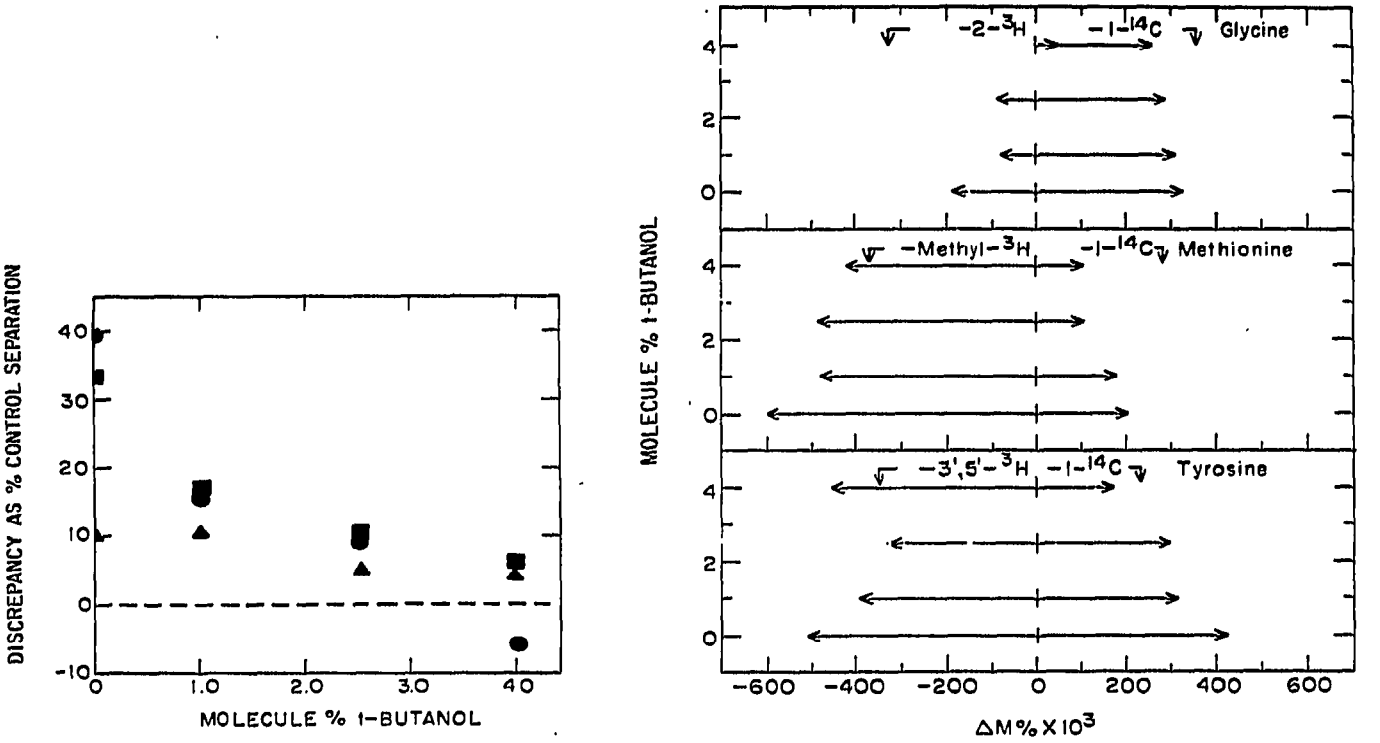

Fig. 2. The influence of *tert.*-butanol on the difference in displacement between ^{3}H and ^{14}C species in the absence and presence of 2.5 μmoles of unlabeled amino acid. Circles: glycine; squares: tyrosine; triangles: methionine.

Fig. 3. Individual isotope/mass displacements for tritium and radiocarbon varieties of glycine, methionine and tyrosine as a function of *tert.*-butanol concentration.

Selected pairs of [3',5'-^{3}H,1-^{14}C]tyrosine, [2-^{3}H,1-^{14}C]glycine and [methyl-^{3}H, 1-^{14}C]methionine were then chromatographed in sodium citrate buffers with 0, 1, 2.5 and 4 mol. % *tert.*-butanol, with and without added mass. As seen in Fig. 2, the discrepancy between the two types of chromatograms decreased rapidly with increasing *tert.*-butanol concentration.

In the same series, it appeared worthwhile to determine the individual ^{3}H/mass and ^{14}C/mass displacements to see if the action of *tert.*-butanol could be attributed to a general effect on the solvent or to a specific form of interaction between the labeled and unlabeled forms of an amino acid. Fig. 3 illustrates the individual displacements from mass as a percent of the unlabeled amino acid mobility for each of the three amino acids: glycine, methionine and tyrosine at each of the *tert.*-butanol concentrations tested. In general, the ^{14}C/mass displacements were reduced by increasing *tert.*-butanol concentrations, although the extent varied with each amino acid and was not linearly related to *tert.*-butanol concentration. ^{3}H/mass displacements were less consistent in the direction and extent of change. There was, therefore, no evidence

TABLE II

$^{3}H/^{14}C$ SEPARATION FACTORS FOR [4,5-^{3}H]- AND [1-^{14}C]PROLINE ON ION-EXCHANGE COLUMNS WITH AMMONIUM FORMATE BUFFER, pH 3.28

No added mass					
M (ml)	137.50	138.04	137.96	137.10	
σ	0.938	0.922	0.938	0.927	
$\Delta M\%$	−0.428	−0.321	−0.410	−0.375	Average: −0.384 ± 0.024
2.5 μmole added mass					
M (ml)	139.95	138.20	137.90		
σ	1.094	0.940	0.914		
$\Delta M\%$	−0.353	−0.358	−0.377		Average: −0.363 ± 0.007

TABLE III

$^{3}H/^{14}C$ SEPARATION FACTORS OF [4,5-^{3}H]- AND [1-^{14}C]PROLINE ON ION-EXCHANGE COLUMNS WITH SODIUM CITRATE BUFFERS, pH 3.28

No added mass					
M (ml)	187.84	187.37	186.90		
σ	1.499	1.526	1.428		
$\Delta M\%$	−0.530	−0.532	−0.509		Average: −0.524 ± 0.006
2.5 μmole added mass					
M (ml)	189.88	189.38	189.55	189.65	
σ	2.093	2.102	2.078	2.354	
$\Delta M\%$	−0.722	−0.699	−0.780	−0.703	Average: −0.726 ± 0.018

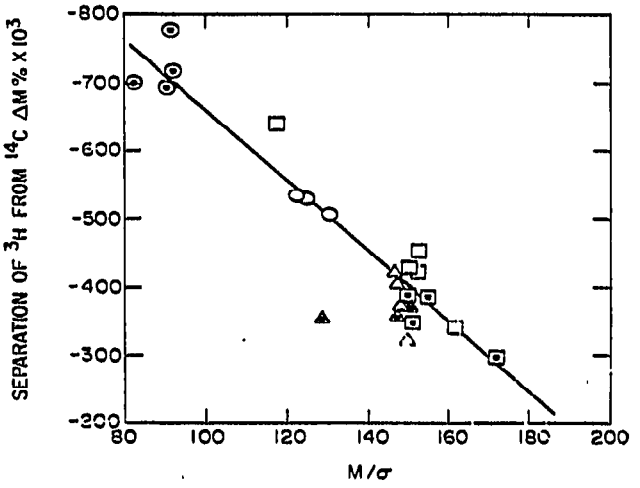

Fig. 4. Relationship of displacement between [4,5-^{3}H]proline and [1-^{14}C]proline to column efficiency. Circles: sodium citrate, pH 3.28; triangles: ammonium formate, pH 3.28; squares: ammonium formate, pH 3.10. Open symbols: without added mass; filled symbols: 2.5 μmoles unlabeled amino acid added.

of a specific effect by *tert.*-butanol on either ^{3}H or ^{14}C/mass fractionation in the separation process.

In previous work, the relationship between displacement and added mass had not been examined in the case of tritium and radiocarbon pairs of proline because of the insensitivity of ninhydrin measurements. To circumvent this difficulty, the gas chromatographic method of GEHRKE AND STALLING[6] was adapted to the column samples. In the formation of the *n*-butyltrifluoroacetyl derivative, however, a consistent interfering contaminant, traceable to the sodium citrate was encountered. In order to avoid this contaminant, the column buffer was changed to ammonium formate, pH 3.28. This also permitted the buffer salts to be removed by sublimation under reduced pressure. Table II shows, however, that the separation of [4,5-^{3}H]-proline from [1-^{14}C]proline, chromatographed on an ammonium formate column is unaffected by the addition of unlabeled mass. This is a function of the eluting buffer, not the amino acid itself, according to the results shown in Table III, in which sodium citrate and ammonium formate columns of a similar retention volume are compared. The sodium citrate buffer series exhibited a 36% increase in displacement when 2.5 μmoles of unlabeled proline were added, while the same conditions on the ammonium formate column produced a small, but insignificant decrease.

TABLE IV

$^{3}H/^{14}C$ SEPARATION FACTORS FOR [4,5-^{3}H]- AND [1-^{14}C]PROLINE ON ION-EXCHANGE COLUMNS WITH AMMONIUM FORMATE BUFFERS, pH 3.10

No added mass						
M (ml)	177.32	176.32	174.92	182.59	181.73	
σ	1.511	1.154	1.161	1.208	1.130	
ΔM%	−0.645	−0.458	−0.432	−0.428	−0.347	Average: −0.462 ± 0.49
2.5 *μmole added mass*						
M (ml)	175.59	173.23	182.62	182.59		
σ	1.177	1.120	1.067	1.210		
ΔM%	−0.395	−0.398	−0.307	−0.345	Average: −0.360 ± 0.022	

Examination of the results in Tables II and III reveals that the formate columns result in a smaller absolute displacement between [4,5-^{3}H]proline and [1-^{14}C]proline and that the values are accompanied by proportionately smaller values for the peak dispersion. Correspondingly, the increase in separations seen upon the addition of mass to the chromatograms on sodium citrate columns is accompanied by an increase in peak dispersion. Accordingly, the displacement values in Tables II and III were plotted against the respective values for M/σ, as shown in Fig. 4. The values for the ammonium formate columns and sodium citrate columns were analysed independently by linear regression analysis to obtain the values shown in Table IV. Both columns showed similar values for the slope and intercept, permitting the data from both columns to be combined in a single regression analysis. This analysis establishes that the separation between ^{3}H- and ^{14}C-labeled proline is inversely related to the column efficiency.

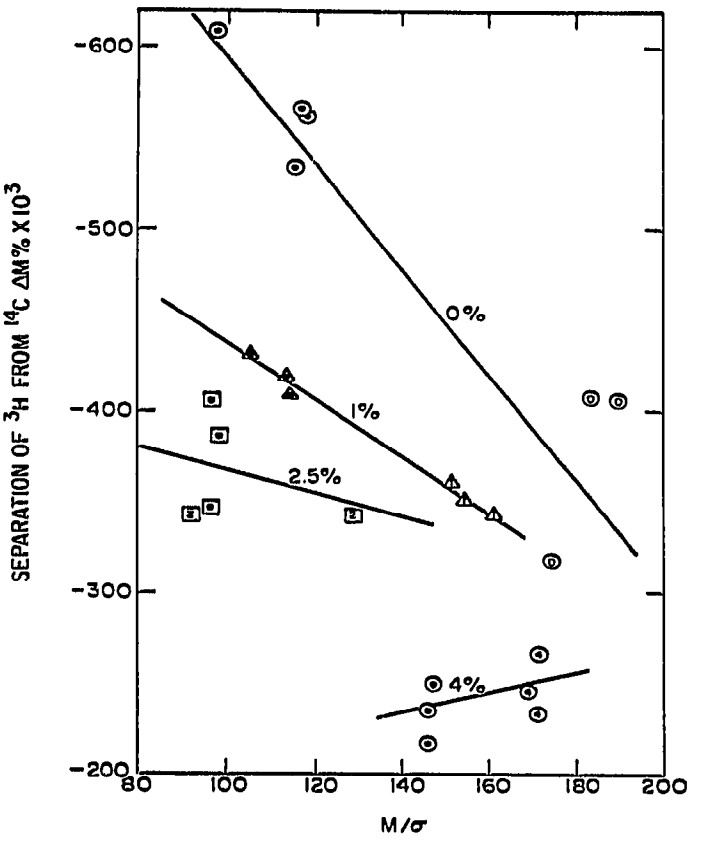

Fig. 5. Displacement between ^{3}H- and ^{14}C-labeled glycine *vs.* column efficiency in sodium citrate buffers containing various levels of *tert.*-butanol. Open symbols: no added mass; filled symbols: mass added.

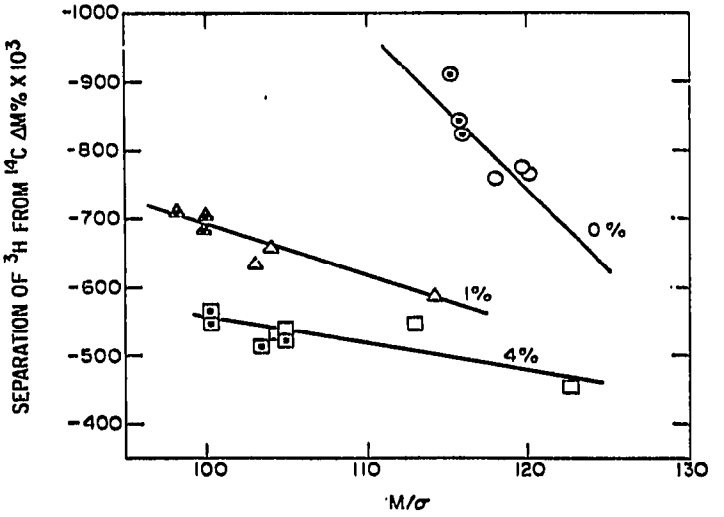

Fig. 6. Displacement between ^{3}H- and ^{14}C-labeled methionine *vs.* column efficiency in sodium citrate buffers containing various levels of *tert.*-butanol. Open symbols: no added mass; filled symbols: mass added.

The $^{3}H/^{14}C$ displacements obtained with the three amino acids in the *tert.*-butanol series were then plotted in a similar manner. Figs. 5 and 6 illustrate these plots for glycine and methionine. Within each series of concentrations, there was a significant inverse dependence of displacement on column efficiency. The value of this negative slope decreased as the concentration of *tert.*-butanol increased and, in the case of glycine, the highest concentration of *tert.*-butanol resulted in a positive slope in the displacement: efficiency relationship.

TABLE V

REGRESSION ANALYSES OF DISPLACEMENT *versus* COLUMN EFFICIENCY

	Buffer system		
	Sodium citrate	*Ammonium formate*	*Combined data*
Intercept ($\Delta M\%$)	1.1614 ± 0.0895	1.0416 ± 0.1923	1.1747 ± 0.0626
Slope ($\Delta M\%/(M/\sigma) \times 10^3$)	−4.99 ± 0.84	−4.32 ± 1.29	−5.20 ± 0.46

DISCUSSION

Studies and reports of isotope fractionation in molecules of biological interest until recently have met scepticism and incredulity in the acceptance of their existence. The gradual accumulation of systematic information, demonstrable reproducibility and significant correlations has authenticated the ability of conventional separation processes to disengage isotopic species from one another. This, in turn, has opened the way to recognition of secondary phenomena present in the separation process that limit, or extend, the resolution of labeled species. SIMON AND HEUBACH[7], for example, traced an inordinately large enrichment of ^{3}H-labeled formaldehyde during fractional distillation to the greater stability of the hydrated formaldehyde species when tritium was present, a factor unrelated to the differences in vapor pressure of the pure formaldehyde species themselves. CEJKA *et al.*[8] were able to alter the displacement between ^{3}H- and ^{14}C-labeled steroids by as much as ten fold by changes in the composition of the stationary phase of the partition column used in the separation process.

In the previous work[4], the highly visible and reproducible effect of added mass on $^{3}H/^{14}C$ separations suggested that these secondary phenomena were related to specific forces operating in ion-exchange chromatography. In particular, these appeared to involve hydrogen bonding or local solvent structure which could be disrupted by small changes in the concentration of co-migrating species. This appears to be substantiated by the influence of *tert.*-butanol in abolishing these phenomena. The known capability of *tert.*-butanol to disrupt both hydrogen bonds and local solvent structure, and the consequences of its incorporation, points directly to the influence of those solvent molecules immediately surrounding the migrating species on the differential migration which occurs. It may also be expected that the *tert.*-butanol affects the ionization of the isotopic species, and it might be argued that this influence has not been differentiated from any effect on the solvent. Two factors rule out this argument: first, the comparison was made with and without mass and the assay for the presence of the phenomenon is thus normalized for overall changes

in ionization and/or retention volumes. Under these conditions, the discrepancy between the two test chromatograms was clearly related to the presence of mass and the concentration of alcohol employed. Secondly, the measurements of individual isotope/mass displacements demonstrate the absence of major changes in these quantities and point to the solvent as the site of action.

The solvent is also implicated by the effect of the ammonium formate buffers in which the hydrogen bonding is increased to such an extent that the addition of 2.5 μmoles of mass is insufficient to alter the separation between ^{3}H- and ^{14}C-labeled proline. This enhanced bonding in solvent structure is differentiated from the reduced bonding achieved by *tert.*-butanol by the smaller absolute displacement between the isotopic species, and by the narrower peak dispersions.

Both the *tert.*-butanol series and the ammonium formate/sodium citrate series show a significant inverse dependence of isotopic separation on column efficiency; that is, an enhanced separation of the isotopic species which is accompanied by broader peak dispersions. Such a relationship has been noted previously be CEJKA and co-workers with radiochemically labeled pairs of cortisone and of aldosterone[8]. They attributed this to associative behavior between the like members of the ^{3}H and ^{14}C species of each steroid, a process which has been evoked in the explanation of amino acid solubilities and specific interactions by SU AND SHAFER[9].

CEJKA and his collaborators predicted that the existence of such an associative process would be much more prominent on shorter columns than on long ones. They were able to produce a separation that was threefold larger than originally observed by reducing the column length by two thirds.

At first glance, it is paradoxical to find an increased distance between peak centers accompanied by an increased peak dispersion. SAHA AND SWEELEY[10] have discussed the role of these two processes in the resolution of isotopic species and regarded them as opposing one another in the peak disengagement process. It is of interest therefore to determine whether or not a true increase in resolution was achieved in these experiments. GLUECKAUF gives the formula for the fractional impurity η as:

$$\eta = \frac{M_a M_b}{M_a^2 + M_b^2}\left(0.5 - \int_0^{f(n,\alpha)} e^{-1/2t^2}\,dt\right),$$

where M_a and M_b are the mole fraction of each component, and

$$f(n,\alpha) = (n)^{1/2}(\alpha^{1/4} - \alpha^{-1/4}),$$

where n and α are the number of plates and the separation factor, respectively[11]. For the same pair of compounds, maintaining a constant fractional impurity, the change in plate number (or increase in dispersion) possible when α_1 is increased to α_2 is given by the equality:

$$\frac{(n_1)^{1/2}}{(n_2)^{1/2}} = \frac{M_1\sigma_2}{M_2\sigma_1} = \frac{(\alpha_2^{1/4} - \alpha_2^{-1/4})}{(\alpha_1^{1/4} - \alpha_1^{-1/4})}.$$

If the values for α_1 and α_2 are taken from the sodium citrate series for proline, an increase in α from 1.00525 to 1.00726 could be accompanied by an increase in the

ratio σ_2/σ_1 of 1.00647 without altering the resolution. Clearly, the performance of the column is degraded under these circumstances, since the increase in dispersion is almost three orders of magnitude greater.

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