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ISOTOPE SEPARATION FACTORS OF ^{14}C-AMINO ACIDS IN ION-EXCHANGE ON RESINS WITH CARBOXYLIC GROUPS

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SUMMARY

The overall isotope effect in ion exchange for ^{12}C–^{14}C amino acids with isoionic points > 7 was measured by an automated procedure. Isotope separation factors were then calculated by use of breakthrough curves at 25° and the height equivalent to a theoretical plate for the chromatographic column employed. They were found to be chiefly correlated with the pK_1 of the basic groups which are concerned in exchange interactions.

The use of continuous flow radioactivity detectors in the study of ion-exchange processes involving labelled molecules is well-known in the literature[1]. More recently this technique has gained widespread acceptance, mainly because it does not interfere in any way with subsequent manipulation of the effluent, as in amino acid colorimetric analysis. A comprehensive study on this technique was published by PIEZ[2], who was also able to verify the isotope effect in amino acid analysis during the exchange on cationic resins with sulphonic groups.

In previous papers[3] by the present author, the exchange of labelled amino acids, with an isoionic point between 7.5 and 10.5, was studied on a buffered cationic resin with carboxylic groups. The elementary separation factor, *i.e.* the equilibrium constant K for the isotope exchange reaction

$$(A^+R^-)_{res} + (^*A^+)_{sol} \rightleftarrows (^*A^+R^-)_{res} + (A^+)_{sol} \quad (1)$$

where R^- is the anion of the resin macromolecule, A^+ and $^*A^+$ are the ^{12}C and ^{14}C amino acid cations, respectively (the subscripts refer to resin and solution phases), was calculated for 1-^{14}C-DL-lysine from the overall isotope effect using the breakthrough curve and the height equivalent to a theoretical plate for the chromatographic column employed. In the present report the procedure was automated, and a continuous flow radioactivity detector connected in series with a recording ultraviolet spectrophotometer, so that the analyzer could run unattended and breakthrough curves started at any time.

A schematic drawing of the equipment is given in Fig. 1. The apparatus is basically an automatic amino acid analyzer[4], with some modifications, in order to record breakthrough curves, instead of elution chromatograms. The cationic exchange

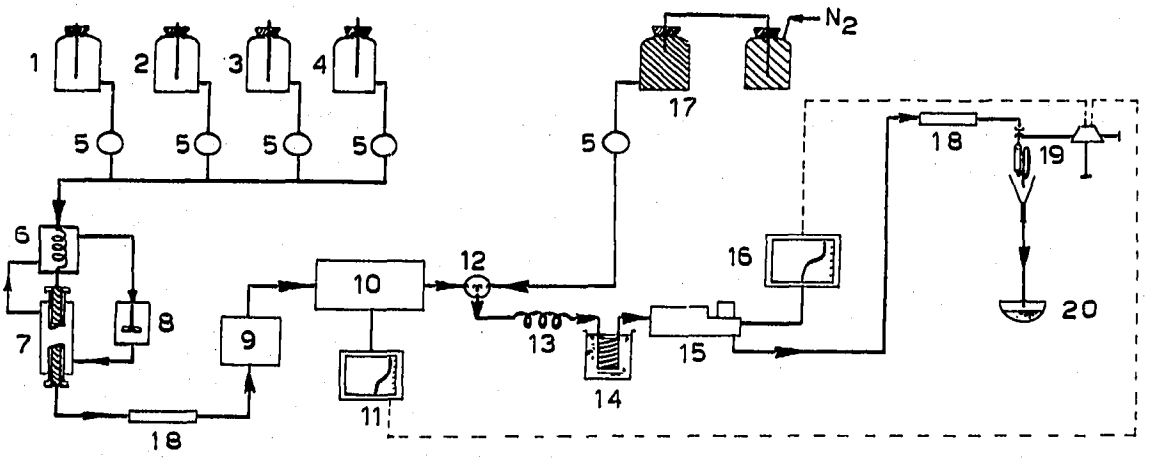

Fig. 1. 1 = Buffer solution reservoir; 2 = amino acid solution reservoir; 3 = water reservoir; 4 = acid (regenerating solution) reservoir; 5 = micro pump; 6 = preheater; 7 = 150-cm chromatographic column; 8 = thermostatic unit; 9 = pH-meter and recorder; 10 = radioactivity flow detector; 11 = radioactivity recorder; 12 = T-fitting; 13 = mixing coil; 14 = colorimetric reaction coils; 15 = spectrophotometer; 16 = absorbance recorder; 17 = ninhydrin solution reservoir; 18 = flow meters; 19 = siphon stand; 20 = waste.

resin was Amberlite CG 50 (BDH; Rohm and Haas) minus 400 mesh. The finest particles were isolated and fractionated by repeated sedimentation in water to obtain beads in a narrow range of size distribution (10–14 μm). The particle size was determined microscopically with the resins swollen in water, and the fractions carefully inspected before use. The 150 cm chromatographic column was made of precision bore borosilicate tubing, had an internal diameter of 0.35 cm and was flanged at the top and bottom so that the teflon fittings described by JONSSON AND SAMUELSON[5] could be attached. Buffers and solutions[3,6] were stored in Mariotte flasks and fed by means of micro pumps (Beckman, type 74608, Fullerton, Calif., U.S.A.) to the chromatographic column, after preheating in a jacketed coil 0.5 m long, made of teflon tube, with an inner diameter of 2 mm. The temperature was maintained constant at 25 $\pm$ 0.1° by the circulation of water from a thermostatic unit through the jackets of the preheater and the column. The bed height was 120 cm, when the resin was buffered at pH 6.7. All solutions were prepared with CO_2-free, triply distilled water. Each reservoir bottle was connected to a de-aerator in order to prevent the formation of gas bubbles. The resin was buffered at the pH of maximum capacity (pH_{max}) for each amino acid[3,6] and washed with CO_2-free water. A 10^{-3}–10^{-4} M amino acid solution (containing 10–30 μ C/l of labelled compound), the pH of which was carefully adjusted to pH_{max} by means of a small addition of $NaHCO_3$ or HCl solution, depending on the case, was then passed through the column. Change-over of operations was performed with the aid of solenoid-operated valves, which were actuated by a robot-timer (Industri AB Reflex, Stockholm, Sweden), not indicated in the figure. Pre-set times were obtained empirically during preliminary runs. The effluent from the column was continuously monitored for pH and subsequently passed into a flow detector (Packard Tri-Carb, Model 320, Lagrange, Ill., U.S.A.) for the radioassay of labelled amino acids[7]; the apparatus was equipped with a strip chart recorder (Packard, Model 380). The column effluent was then mixed in a commercial T-fitting with the ninhydrin reagent stored at a slight overpressure[8] under nitrogen in a dark Mariotte flask and delivered at a constant rate by a micro pump.

The solution passed through teflon capillary tubing (30 cm long) to achieve mixing. The colour was then developed in a teflon reaction coil about 20 m long and with an inner diameter of 1 mm, kept in a thermostated bath at 100°. Absorbance was read at 570 nm in a spectrophotometer (Spectronic, type 600E, Bausch and Lomb, Rochester, N.Y., U.S.A.), the output of which was recorded (Speedomax type G potentiometer recorder, Leeds and Northrup, Philadelphia, Pa., U.S.A.). In order to check the flow rate (0.150 cm^3/min per gram of dry resin) at regular intervals, flow meters were inserted in the line. To record the flow rate of the effluent, event markers, as described by LINDQVIST[9] and LARSEN[8], were connected to the absorbance and radioactivity recorders. The event markers were actuated by a mercury switch in the syphon stand of a fraction collector (Komby Rak, Terzano, Milan, Italy).

The equilibrium constant K of reaction (1) at 25° for labelled amino acids (I)–(IV) (see Table I) was calculated from the overall isotope effect φ for the breakthrough curve by means of the following equations[3]:

$$K = 1 + [(\varphi - 1)H_{EZ}/H_T N_{EZ}] \quad (2)$$

where H_{EZ} and H_T are the height of the exchange zone and the total height of the chromatographic column, respectively, and N_{EZ} is the number of effective plates of the exchange zone;

$$\varphi = (1/NA_{s_0}) \int_0^N n\mathrm{d}A_s \quad (3)$$

where N is the overall exchange capacity (gmoles) of the buffered resin in the column towards the amino acid at the experimental pH value, A_{s_0} the specific activity of the ^{14}C amino acid solution at the exhaustion point, A_s the specific activity, and n the overall gmoles of the amino acid at any point of the breakthrough curve. The integral of eqn. (3), which was evaluated graphically, is an obvious extension of the previously employed relation[3] for the evaluation of φ.

Results are summarized in Table I. ^{14}C-amino acids (I), (II) and (IV) were commercially available compounds (Radiochemical Centre, Amersham, Bucks, Great Britain), while (III) was synthesized[3]. ^{12}C-Amino acids were all analytically pure reagents (BDH, Poole, Great Britain), critically examined for purity[10].

TABLE I

ISOTOPE SEPARATION FACTORS IN ION EXCHANGE OF ^{12}C–^{14}C AMINO ACIDS

No.	*Amino acid*	pH_{max}	$(K-1) \times 10^{5}$ [a] *at 25° (eqn. 2)*	pK_1' [b]
I	L-(+)-histidine 2-^{14}C-imidazole ring	4.5	4.5 ± 0.5 (4) [c]	5.97 (pK_2')
II	1-^{14}C-DL-lysine	6.7	7.7 ± 0.6 (5)	10.28 (pK_3')
III	6-^{14}C-DL-lysine	6.7	5.9 ± 0.5 (6)	8.90 (pK_2')
IV	L-(+)-arginine, guanidine, ^{14}C	7.7	8.6 ± 0.7 (6)	13.2 (pK_3')

[a] Average value, with standard deviation, measured on 4–6 replicate chromatograms.
[b] K_1' is the acid dissociation constant.
[c] The figure in parentheses is the number of determinations.

As was previously stated[3], the isotope effect was only significant if the labelled C-atom was adjacent to the basic function or if it constituted the carboxylic group itself. The present results are in agreement with preliminary measurements[3], substantiating a correlation between isotope separation factors and pK_1' of basic groups which are concerned in exchange interactions, as is shown in Table I (K_1' = acid dissociation constants): the isotope effect increases with increasing basicity of these groups. The secondary isotope effects under study and their variation with radiocarbon position are probably caused to a large extent by an activation energy effect on the rate of chromatographic displacement, the frequency factor effect being either small or constant. Changes in non-bonding interactions and tunnelling effects must be unimportant, since no bond rupture is involved, except in interactions which concern quaternary basic groups and active sites of the resin molecule[6]. The only effects remaining which might influence the activation energy are anharmonicity, inductive interactions and hyperconjugative interactions. It is difficult to assess the relative importance of these factors, because the quantitative use of small secondary isotope effects is not a reliable tool for ascertaining mechanisms. From the correlation between isotope separation factors and pK_1' of the basic functions, which are adjacent to the centre of isotopic substitution, a marked dependence on the slightly different dissociation constants of these functions in the labelled and unlabelled amino acids studied could be argued. The source of this difference may arise from considerations of zero-point vibration for the anharmonic system, as well as from inductive effect or hyperconjugative release of electrons from the isotopically substituted C–C or C–H bonds. Inductive and hyperconjugative influence on secondary deuterium isotope effects has been thoroughly discussed[11] but C–C hyperconjugation may also and probably does have a significant role and should not be neglected[12]. In the labelled molecules of Table I, particularly, the net effect is an "increased" basicity of the nitrogen atom, which enhances affinity towards the resin macromolecule. Similar conclusions were recently reached in the examination of the primary isotope effects shown by amines on cation exchange resins, the main factor affecting constants of isotope exchange reactions being the ratio of the ionization constants for the two pure isotopic forms of the amines. These factors, rather than the somewhat greater electronegativity of ^{14}C, as was postulated by PIEZ AND EAGLE[14], can adequately account for all the isotope effects observed and for the increase in retention volume of radiocarbon-labelled amino acids, during ion-exchange chromatography[3,15], which is usually maximal when the carboxyl group is labelled. Vanishing secondary isotope effects, which were observed in some instances[15], could be considered from this point of view as a result of a fortuitous cancellation between opposing and apparently counterbalanced effects.

REFERENCES

1 E. SCHRAM AND R. LOMBAERT, *Biochem. J.*, 66 (1957) 20 P; *Anal. Chim. Acta*, 17 (1957) 417; *Arch. Intern. Physiol. Biochim.*, 68 (1960) 845; *Anal. Biochem.*, 3 (1962) 68; E. SCHRAM AND R. CROKAERT, *Biochem. J.*, 66 (1959) 1253; B. L. FUNT AND A. HETHERINGTON, *Science*, 129 (1959) 1429; H. W. SCHARPENSEEL AND K. H. MENKE, *Tritium in the Physical and Biological Sciences*, Vol. 1, I AEA, Vienna, 1962, p. 281.
2 K. A. PIEZ, *Anal. Biochem.*, 4 (1962) 444.
3 I. R. BELLOBONO, *Intern. J. Appl. Radiation Isotopes*, 17 (1966) 257; *Rend. Ist. Lombardo Sci. Lettere*, A, 99 (1965) 336.

4 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190; K. A. PIEZ AND L. MORRIS, *Anal. Biochem.*, 1 (1960) 187.
5 P. JONSSON AND O. SAMUELSON, *Sci. Tools*, 13 (1966) 17.
6 I. R. BELLOBONO, *Ann. Chim. (Rome)*, 51 (1961) 1210.
7 E. RAPKIN AND L. E. PACKARD, *Proc. Univ. New Mexico Conf. Organic Scintillation Detectors*, AEC Document TID 7612, 1961.
8 I. LARSEN, *Sci. Tools*, 12 (1965) 24.
9 B. LINDQVIST, *Tek. Vetenskaplig Forskning*, 31 (1960) 125.
10 P. B. HAMILTON AND R. A. ANDERSON, *Anal. Chem.*, 31 (1959) 1504.
11 E. S. LEWIS, *Tetrahedron*, 5 (1959) 143; V. J. SHINER, JR., *Tetrahedron*, 5 (1959) 243; A. STREITWIESER, JR., R. H. JAGOW, R. C. FAHEY AND S. SUZUKI, *J. Am. Chem. Soc.*, 80 (1958) 2326.
12 P. B. D. DE LA MARE, *Tetrahedron*, 5 (1959) 107.
13 D. NOWLIN AND J. E. POWELL, *U.S. At. Energy Comm. Report IS-1059*, 1964.
14 K. A. PIEZ AND A. EAGLE, *Science*, 122 (1955) 968; *J. Am. Chem. Soc.*, 78 (1956) 5284.
15 P. D. KLEIN AND P. A. SZCZEPANIK, *Anal. Chem.*, 39 (1967) 1276.