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Interpretation of enantioselective activity of albumin used as the chiral selector in liquid chromatography and electrophoresis

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Abstract

The possibility of constructing a hypothesis on albumin enantioselectivity allowing consistent interpretation of retention and selectivity data for D,L-monocarboxylic acids, D,L-dicarboxylic acids and D,L-amino acids, measured by liquid chromatography and capillary zone electrophoresis, is demonstrated. An explanation of effects caused by high temperature, mildly alkaline aqueous solutions and methanol, consistent with the explanation of the retention and selectivity data, is also possible. The hypothesis considers the conformational variability of albumin.

1. Introduction

Recently, we published a study of albumin as a chiral selector for liquid chromatographic [1–3] and electrophoretic [4] chiral separations. Aqueous buffers served as the albumin wetting medium in both separation techniques. The chromatographic sorbent was prepared by chemical bonding on albumin on a hydroxyethylmethacrylate (HEMA) polymeric matrix possessing a high hydrolytic stability [5,6]. This allowed us to widen the range in which the influence of pH on the separation selectivity was investigated from the acidic and neutral regions, applicable with silica gel-based sorbents [7], to the whole pH range of the albumin titration curve. Racemates of monocarboxylic acids, dicarboxylic acids and amino acids served as solutes in the study.

Fast and slow changes in albumin enantioselectivity, influenced by the pH value, were observed. The well known fast, virtually immediate and reversible changes, effective over the whole pH range of the albumin titration curve, are commonly utilized as the most effective tool in the control of both solute retentions and its separation selectivity [7,8]. The shape of the pH dependence of retention data was the same for compounds of a similar structure, e.g., for amino acids. However, characteristic differences in the shape of the dependence were found for monocarboxylic acids, dicarboxylic acids and amino acids. The same is valid for the influence of pH on the separation selectivity changes [3].

Slow changes in the albumin enantioselectivity and in its capability to bind solutes of various

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types occur in weakly alkaline mobile phases in the pH range 7–10. The changes, irreversible in aqueous medium and qualitatively identical for solutes of a particular type, e.g., amino acids, may be qualitatively different for solutes of different structural types, e.g., amino acids and dicarboxylic acids. Treatment with methanol and subsequent washing with an aqueous buffer of the appropriate pH restores the albumin enantioselectivity altered by the slow process [3]. Qualitatively, the same irreversible changes in both retention and separation selectivity are observed for tryptophan as a solute with the sorbent heated to 90°C in a buffer of pH 5 and than washed with methanol and mobile phase [2].

None of the published models, ideas or hypotheses on the enantioselectivity of albumin, based on liquid chromatographic experiments, allows a satisfactory interpretation of our results. The hypothesis described below, allowing such an interpretation, stems from our experiments with bovine serum albumin (BSA) chemically bonded to the porous matrix HEMA [1–3] and from the successful transfer of these results to capillary zone electrophoresis [4], from common constituents of hitherto published chromatography-based ideas and hypotheses on albumin enantioselectivity and from a knowledge of albumin research.

2. Hypothesis

Albumin enantioselectivity is conceived as the general capability of albumin to discriminate differences in the steric arrangements of the members of enantiomeric pairs. The chromatographic properties of a sorbent with chemically bonded albumin are conferred by both the matrix and albumin. The capability of an albumin macromolecule, chemically bonded to a solid matrix at a few points only, to interact with solutes and discriminate the members of enantiomeric pairs may be approximated by those of dissolved albumin.

The requirement for three interaction points, necessary for chiral discrimination [9], does not necessitate three qualitatively different interactions. Cooperating interactions may be either attractive or repulsive; a preponderance of attractive interactions results in the solute retention. A change or a difference in the sum of the mutual solute–albumin interactions is reflected in a corresponding retention change or in a retention difference, respectively. The influence of a solute–albumin interaction on the retention of the solute depends on the intensity of the interaction. As a result, coulombic interactions are the most important.

Coulombic interactions come into effect by means of electrostatic fields formed by electrical charges. An electrostatic field interacting with a charge may be created by one charge, and also by a set of charges of identical or opposite polarities. As a result, a space point in which the resulting field, created by contributions from two or more albumin charges, interacts most strongly with the field of a solute charge acts as the coulombic interaction point of albumin, dependent on albumin conformation. A coulombic interaction point independent of albumin conformation is created by one charged albumin group only. Albumin charges forming inner ionic pairs (interacting charges) [10] do not participate in albumin–solute interactions.

The ionization state of a solute follows from its distribution diagram. The ionization state of albumin is described by the albumin titration curve (Fig. 1) [11]. The change in the albumin ionization state is connected with changes in albumin conformation in general; the former may also be attended by a change in the number of functional groups capable of forming hydrogen bonds.

The types, number and accessibility of binding sites capable of interacting with a given solute depend on the albumin conformation. The mobile phase composition, pH, temperature and other experimental variables affect the solute retention and the separation selectivity via changes in albumin charge, in albumin conformation and in solute ionization state.

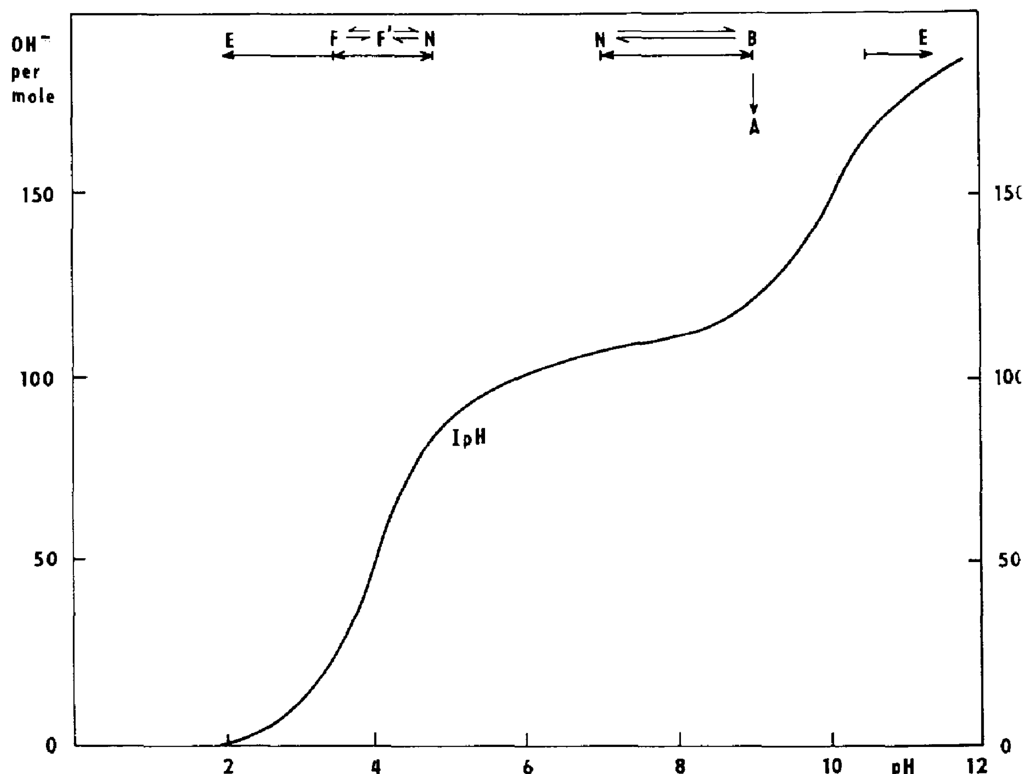

Fig. 1. Titration curve of human serum albumin according to Peters [11], with a diagram of albumin conformations according to Foster [10].

3. Interpretation of pH dependences

3.1. Monocarboxylic acids

Different retentions of D- and L-enantiomers at any pH < 11 and a monotonic decrease in their retention with increasing pH are characteristic of monocarboxylic acids (Fig. 2). The steep pH dependence of the retention of monocarboxylic acids may be understood as an indication that a strong and pronouncedly pH-dependent interaction is involved in their interaction with albumin. The marked influence of 1-propanol on the retention of both enantiomers [3] reveals the contribution of hydrophobic interaction to retention. Supposing that D,L-indolelactic acid is bound at the binding site of indolyl compounds [12], the hydrophobic interaction of the indolyl group and the coulombic interaction of the ionized carboxylic group with albumin should also be the participating interactions in the retention of this acid. One can assume that the third interaction is due to the hydrogen bond formed by the hydroxyl group attached to the asymmetric carbon atom and that it is responsible for chiral discrimination.

The decrease in retention with increasing pH may be explained easily as the consequence of a decline in the total positive albumin charge. Weakening of the coulombic interaction and alkaline unfolding of albumin with pH increase enhance the relative contribution of non-coulombic interactions; this results in a small transient increase in separation selectivity. The decrease in enantioselectivity at pH > 9 results from the strengthening of the repulsion of the solute anion

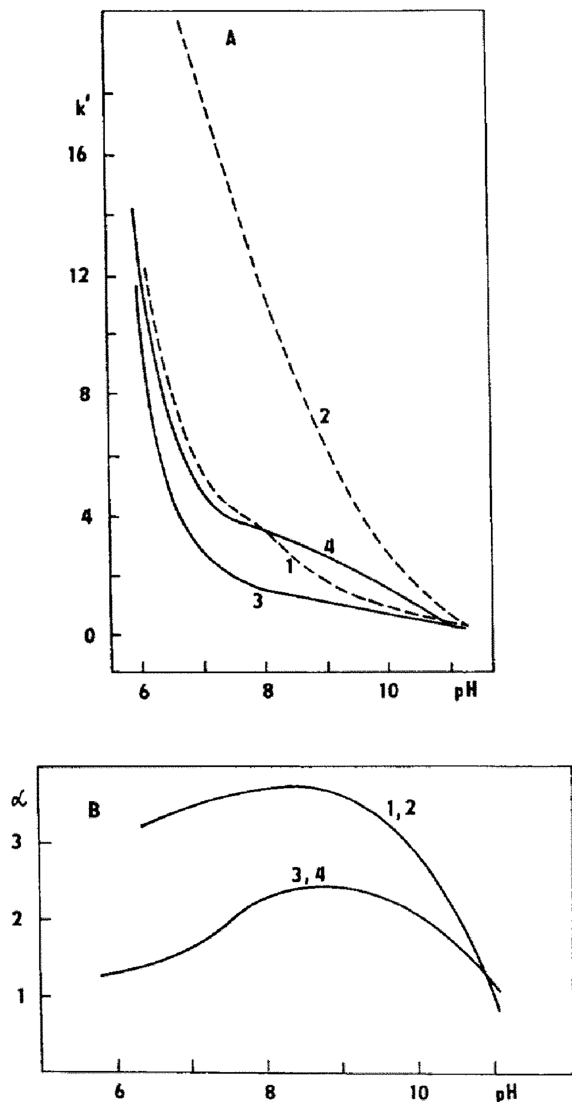

Fig. 2. pH dependences of (A) retention and (B) separation selectivity of D,L-indolelactic acid (1,2) and of N-benzoyl-D,L-phenylalanine (3,4) on the HEMA-BSA sorbent [3].

by the growing negative charge of albumin. At pH 11, the attractive interactions are totally eliminated by anionic repulsion.

The pH dependences of the retentions of N-benzoyl-D,L-phenylalanine may be explained analogicously, assuming that the difference in the interaction of the substituted amine group in the D- or L-enantiomer with albumin is critical for chiral discrimination. The group may interact with albumin hydrophobically (benzene ring) or by the hydrogen bond (carbonyl or secondary amine group).

3.2. Dicarboxylic acids

The retention and selectivity dependences of all three dicarboxylic acids studied on pH [3] have the form illustrated in the Fig. 3. The

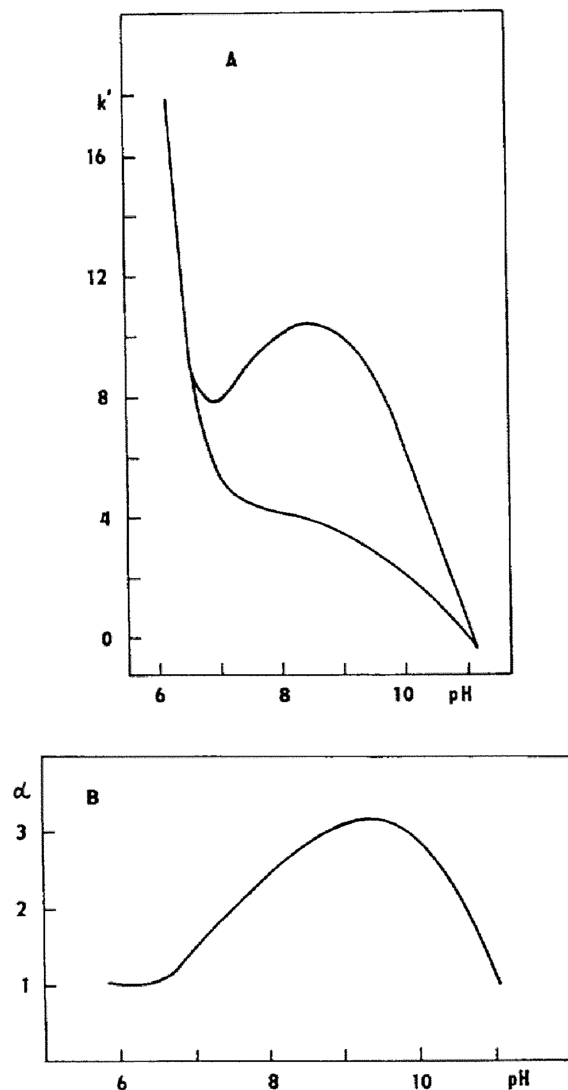

Fig. 3. pH dependences of (A) retention and (B) separation selectivity of N-2,4-dinitrophenyl-D,L-glutamic acid as the representative of dicarboxylic acids, separated on the HEMA-BSA sorbent [3].

necessity to utilize a mobile phase containing an alcohol, the strong decrease in retention with increasing pH and the unmeasurable difference in the retentions of the D- and L-enantiomers at pH < 7 indicate that chiral discrimination is probably caused by a relatively weak non-ionic interaction. The nature of the interaction is determined by one of the two non-ionized functional groups bonded to the asymmetric carbon atom.

Strong attractive interactions of negatively charged groups of the solute with positive albumin domains [10] prevail in the vicinity of the albumin isoelectric point. The anionic interaction points become weaker with increase in pH. As a result, the retentions of both enantiomers of a dicarboxylic solute decrease. Near pH 7, the contribution of the non-ionic interaction, causing the differentiation of the retentions of D- and L-enantiomers, becomes effective. Basic unfolding of the albumin macromolecule [10] enhances the differentiating non-coulombic interaction more strongly. As a result, the decrease in retentions is interrupted and the separation selectivity increases transiently. The increases in both the retention and selectivity are stopped by continuing deprotonation of the basic albumin functional groups near pH 9. At higher pH, the positive albumin charge vanishes totally; anionic repulsion outweighs the non-ionic attractive interaction at first and then even precludes the diffusion of solutes to negatively charged pores of the sorbent. As a result, the electrostatically excluded solutes are eluted from the column in a retention volume lower than that of uncharged non-retained solutes near pH 11.

3.3. *Amino acids*

In contrast to mono- and dicarboxylic acids, the pH dependence of the retention data of amino acids is characterized by low retentions in the acidic region, independent of both pH and the steric configuration of the considered amino acid (Fig. 4); for D-amino acids, less retained at pH > 6, this independence reaches the alkaline

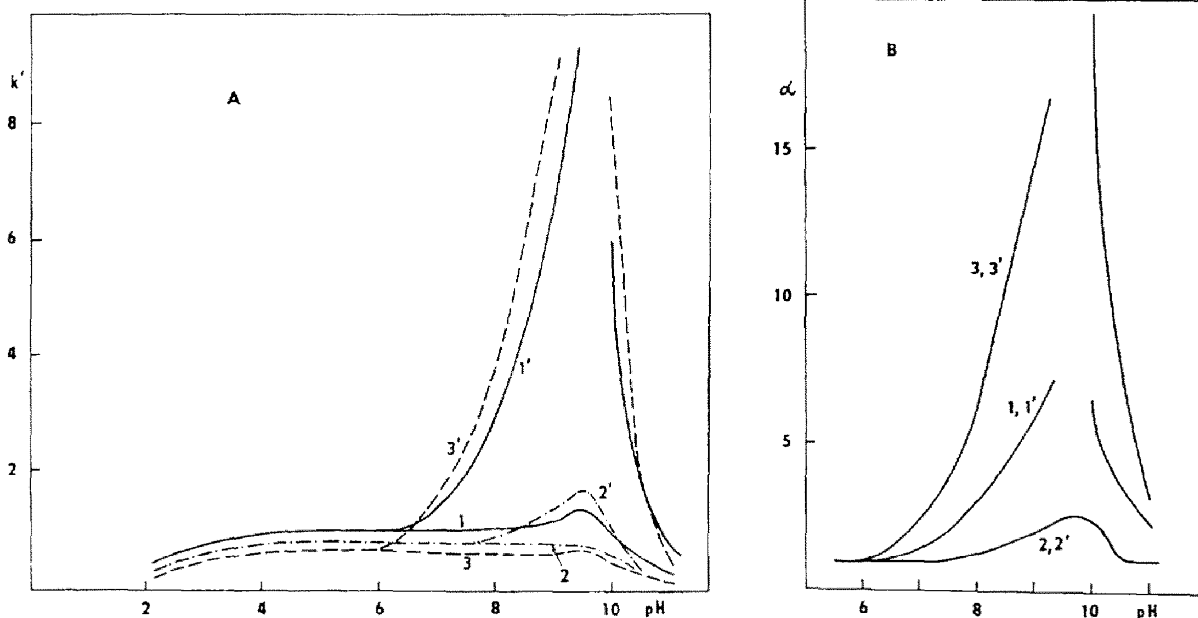

Fig. 4. pH dependences of (A) retentions and (B) separation selectivity of D,L-tryptophan, 5-hydroxy-D,L-tryptophan and D,L-kynurenine on the HEMA-BSA sorbent [3]. (A) 1 = D-tryptophan; 1' = L-tryptophan; 2 = 5-hydroxy-D-tryptophan; 2' = 5-hydroxy-L-tryptophan; 3 = D-kynurenine; 3' = L-kynurenine. (B) 1, 1' = D,L-tryptophan; 2, 2' = 5-hydroxy-D,L-tryptohan; 3, 3' = D,L-kynurenine.

region. The magnitude of retentions in this pH range is comparable to those on HEMA matrix without bonded albumin [3], free of charged groups. As a result, a strong coulombic interaction of the carboxylic group of amino acids with albumin is either absent or compensated for by a coulombic repulsion interaction in the pH region of constant retentions of amino acids.

Let us suppose that the change for the enantioselectively non-specific bonding of amino acids by albumin to enantioselectively specific bonding is not attended by migration of solutes. Then, an attractive non-coulombic interaction between amino acids and albumin must be active at least in one of the interaction points of the binding site.

A binding site allowing for contributions from coulombic interactions of both charged groups of an amino acid in the acid pH range does not make it possible to explain experimentally established data. The idea of the formation of coulombic interaction points, starting from approximately pH 6, allows a formal interpretation of dependences in Fig. 4 if the binding site given in Fig. 5A is considered. However, the formation of positively charged interaction points above the albumin isoelectric point is not probable, taking into consideration the structure of the tryptophan binding site [12].

A simple explanation of the dependences in Fig. 4 is possible by presuming the absence of coulombic interaction of the carboxylic group of amino acids with albumin and considering the formation of a hydrogen bond between the carboxylic group and albumin (Fig. 5B). Applying such an approach to D,L-tryptophan, it is advantageous to assume that the albumin interaction point, forming the hydrogen bond, is identical with the hydroxyl group of Tyr 30 having $pK_a = 10.07$ [10].

Starting from the structure of the binding site given in Fig. 5B, a pH-independent retention of amino acids may result from non-ionic interactions of the carboxylic anion and the aryl group with albumin. The increase in the retention of the D-enantiomer may be explained as the consequence of alkaline unfolding of albumin. The formation of a negatively charged interaction point of albumin, causing a steeper increase in the L-enantiomer interaction, may result from splitting off of the inner ionic pairs with increasing pH due to either deprotonation of the histidine amino group ($pK_a = 6.5$) or from alkaline unfolding of albumin at pH > 7. The stopping of the retention increase in the proximity of pH 10 and the subsequent retention decrease may result from three processes: dissociation of the protonated amine group of the amino acid, disappearance of the hydrogen bond caused by dissociation of an albumin hydroxylic group and repulsion of the amino acid anion by the growing excess of the negative albumin charge.

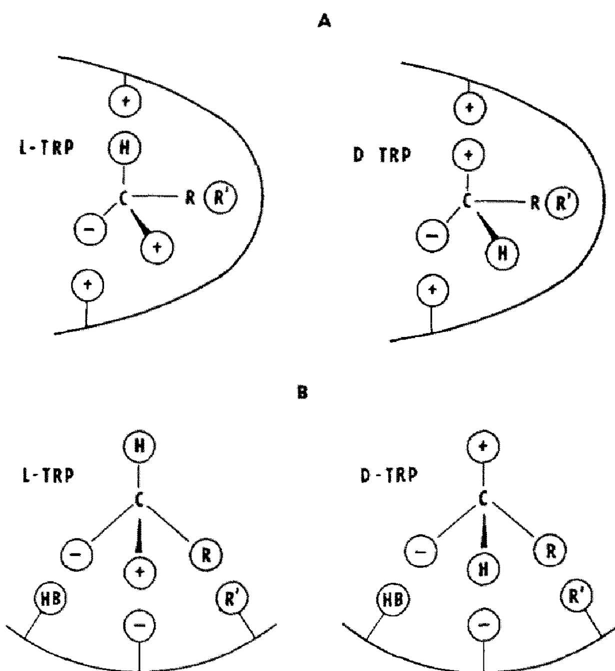

Fig. 5. Structure of hypothetical binding sites for interpretation of dependences from Fig. 4. (A) binding site with coulombic interaction of the ionized carboxylic group; (B) binding site with the hydrogen bond formed by the ionized carboxylic group.

4. Other results

The versatility of the hypothesis allowing a consistent interpretation of the pH dependences of retention and selectivity determined for three

types of compounds in the range of 10 pH units is mainly due to the incorporation of the conformational variability. The link between albumin conformation and its ability to bind enantioselectively solutes of various types enables one to understand alterations in albumin enantioselectivity as a result of changed pH, temperature and methanol treatment [2–4].

The slow decrease in D- and L-tryptophan retentions and in the separation selectivity of D,L-tryptophan, evoked by mobile phases of pH 7–10 [3], and the loss of enantioselectivity of dissolved albumin for amino acids in the same pH region, observed in capillary zone electrophoresis [4], suggest that the binding sites capable of discriminating D- and L-amino acids are linked with an albumin conformation that is unstable in that pH region. The same holds for binding sites capable of discriminating monocarboxylic acid enantiomers [4]. It follows from the albumin conformation equilibria in Fig. 1 that the B conformation is relevant. The increase in separation selectivity of dicarboxylic acids may then be ascribed to the link with the increase in the content of the A form, arising spontaneously in that pH range. The A form is identified as a covalently stabilized conformation with equivalent carboxylic groups, incapable of interacting with albumin positive charges [10]. Evidently, an analogous situation must hold for positive charges of albumin.

Organic solvents are stronger solvation agents for albumin than water [11]. The restitution of albumin enantioselectivity for amino acids and monocarboxylic acids (damaged by prolonged action of pH > 7) by methanol can be interpreted as follows. Methanol, being a sufficiently strong solvation agent, breaks the bonds causing stability of the A form in aqueous medium. During subsequent flushing of the column with aqueous buffer, methanol is replaced by water, present in great excess. The rehydrated chain of the albumin macromolecule is liable to conformational changes determined by the buffer pH.

The N–B–A transition of albumin in mildly alkaline medium is catalysed by sulfhydryl residues of the mercaptalbumin, created to a greater extent at elevated temperature [10]. Therefore, the short (10 min) mild (up to 60°C) heating of the electrophoretic background electrolyte, containing dissolved albumin [4], speeds up the disappearance of the electrolyte enantioselectivity for amino acids and monocarboxylic acids and also the stabilization of the electrolyte enantioselectivity for dicarboxylic acids. The markedly higher rate of the process in the free solution may be ascribed to the absence of kinetic deceleration of the process, effective in the matrix pores.

The loss of ability of chemically bonded albumin to separate D,L-tryptophan, observed after heating the chromatographic column at 96°C in a stream of mobile phase of pH 5.6, and restoration of the column enantioselectivity by methanol treatment [2] may be a consequence of above-mentioned processes or processes analogous to them.

Although the hypothesis presented on the enantioselectivity of albumin makes it possible to treat all our experimental observations consistently [1–4], it can be hardly considered as anything more than a contribution to the likely explanation of the enantioselectivity of albumin acting as a chiral selector. It needs further thorough verification. However, knowledge gained from additional separation experiments only cannot lead to a pronounced improvement in the ideas presented.

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