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Note

Direct chiral resolution in an aqueous two-phase system using the counter-current distribution principle

BJÖRN EKBERG and BÖRJE SELLERGREN
Department of Pure and Applied Biochemistry, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
and
PER-ÅKE ALBERTSSON*
Department of Biochemistry, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
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The use of proteins for direct chiral resolution has recently progressed considerably[1]. Following on the findings of McMenamy and Oncley[2], Stewart and Doherty[3] wer able to separate D- and L-tryptophan on immobilized bovine serum albumin. This technique has been further developed by Allenmark *et al.*[4] to a high-performance liquid chromatographic (HPLC) procedure, whereby a variety of racemic compounds have been resolved. However, the low capacity of these chromatographic systems make them useful mainly for analytical purposes.

This paper reports the application of partition in an aqueous polymer two-phase system for direct chiral resolution. The phase system consists of two immiscible aqueous phases and is obtained by mixing two polymers with water[5–7]. Such phase systems can dissolve proteins and other biomolecules under mild conditions. The partition coefficient (K = concentration in upper phase divided by concentration in the lower phase) of a protein depends, among several other factors, on the type and concentration of the polymers of the phase system and on the ionic composition. Under suitable conditions, a protein can be confined almost completely to one of the phases while low-molecular-weight compounds partition more equally between the phases. Hence, if the protein can bind selectively to one of the enantiomeric forms of a racemic mixture, such a phase system can be used for chiral resolution. We have used bovine serum albumin confined to the lower phase of a dextran–poly(ethylene glycol) phase system to separate D- and L-tryptophan.

MATERIALS AND METHODS

Chemicals and phase system

Dextran 40 was obtained from Pharmacia (Uppsala, Sweden) and poly(ethylene glycol), PEG 6000 (now renamed PEG 8000) from Union Carbide (New York, U.S.A.). The phase system was 10% (w/w) Dextran 40, 7% (w/w) PEG 6000, 0.1 M sodium chloride, and 50 mM sodium bicarbonate buffer (pH 9.2). Bovine serum albumin (6.5 g) (Sigma, No A-3912, Fraction V) was added per 100 g of the phase system. The albumin-containing phase system was shaken and allowed to settle in

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the cold room (4°C). Tryptophan (D and L) was from Sigma and L-[side-chain-2,3-^{3}H]-tryptophan, specific activity 50 μCi per nmol, was obtained from Amersham International (U.K.).

Counter-current distribution

An automatic thin-layer counter-current distribution apparatus[5,6] with 60 cavities was used. To each cavity numbered 1–59 was added 0.79 ml of each phase. To cavity number 0 was added 0.79 ml of bottom phase and 0.79 ml of top phase, the latter containintg 9 μmol of each enantiomer or 18 μmol of the racemate of tryptophan. The shaking time was 40 sec and the settling time 12 min. After 60 transfers (4°C) the contents of the cavities were collected in a fraction collector. The upper phase of each third phase was diluted to give a suitable absorbance at 280 nm. The radioactivity both in the upper phase and in the total system was measured on a beta-scintillation counter. Lumagel (Lumac, 6372 AD Schaesberg, The Netherlands) was used as scintillation fluid.

Measurements of binding between L-tryptophan and serum albumin

In tubes 0–5 the radioactivity of the upper phase and the radioactivity of the total phase system were determined. By difference, the radioactivity of the lower phase was obtained. From the partition coefficient of the L-tryptophan the amount of free L-tryptophan in the lower phase was calculated. Since the total radioactivity of the lower phase was known, the L-tryptophan bound to serum albumin could be calculated. (See Fig. 2 in ref. 7 for further details.)

RESULTS AND DISCUSSION

The partition of serum albumin depends, among other factors, on the molecular weight of the polymers and on the ionic composition. For the purpose of this work we wanted a phase system in which serum albumin had a low partition coefficient. This can be accomplished by choosing a dextran with a relatively low molecular weight[8] and sodium chloride as the dominating salt. In the system employed here, 95% of the serum albumin was in the lower phase and the free tryptophan partitioned more equally between the phases (partition coefficient, 1.2).

When the enantiomers were applied separately to counter-current distribution the profiles shown in Fig. 1 were obtained. The enantioselectivity is apparent; the L-enantiomer was more retained in the lower phase (G = 0.13) than the D-enantiomer (G = 0.39). The apparent separation factor (G_D/G_L) was 3.1. It should therefore be possible to obtain several optically pure fractions.

A similar result was obtained when the racemic mixture was applied (Fig. 2). No resolution of the racemic was observed when the mixture was run under identical conditions but without serum albumin. A small amount of tritium-labelled L-tryptophan was added and most of the tritium label gave a peak at the same position as the unlabelled L-form in Fig. 1. Part of the tritium label was found in tubes 20–40, however. This is probably due to labelled impurities (degradation products) of the tritium-labelled L-tryptophan used, which was two years old.

By determining the partition of the tritium-labelled L-tryptophan in tubes 0–5 we were able to calculate the concentration of free and bound L-tryptophan in the lower phase, containing serum albumin. A Scatchard plot of these data is shown in

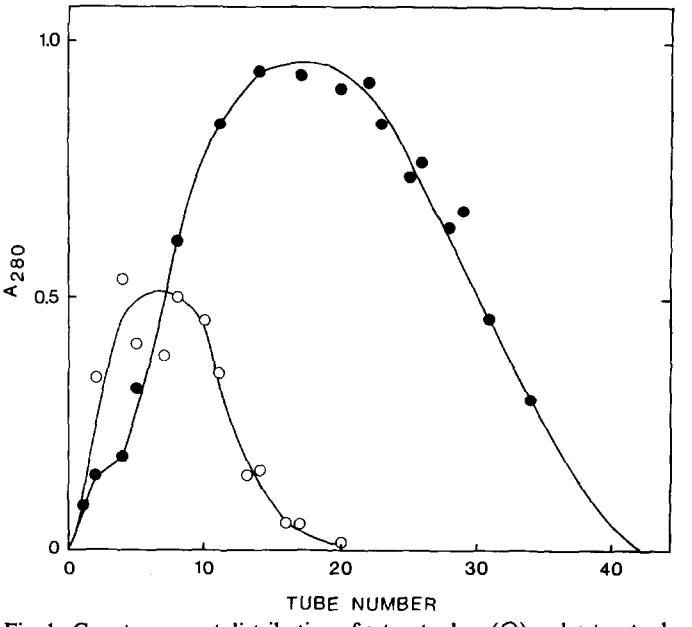

Fig. 1. Counter-current distribution of L-tryptophan (○) and D-tryptophan (●) run separately (9 μmol). The absorbance in the upper phase of each third tube was measured at 280 nm.

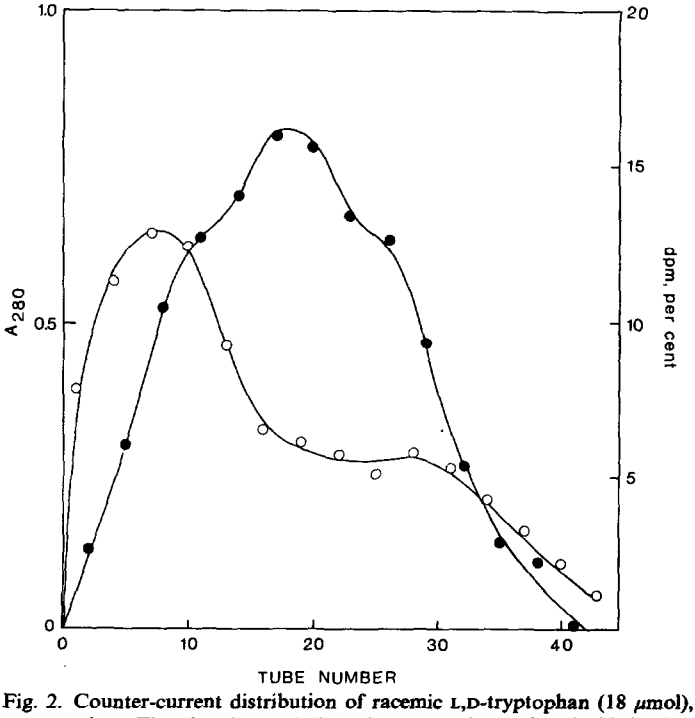

Fig. 2. Counter-current distribution of racemic L,D-tryptophan (18 μmol), with trace amounts of [^{3}H]-L-tryptophan. The absorbance (●) in the upper phase of each third tube was measured at 280 nm. To another set of each third tube 1.0 ml of distilled water was added, to give a one-phase system from which aliquots were taken for estimation of [^{3}H]-L-tryptophan content (○). Radioactivities are given as a percentage of the total amount of radioactivity added.

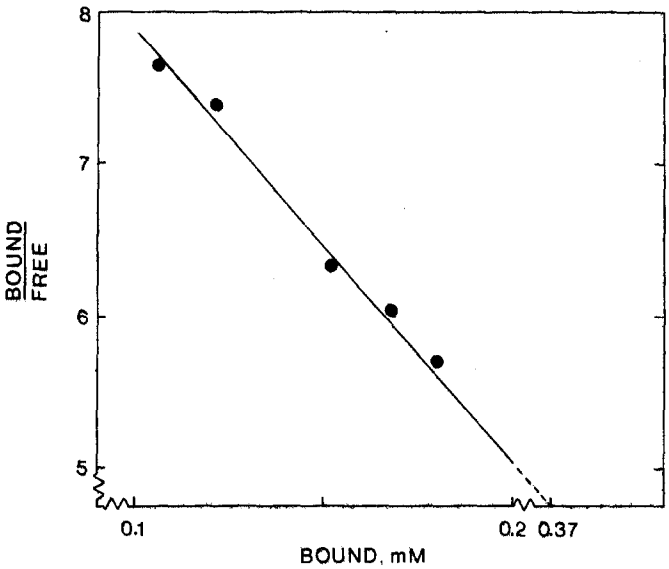

Fig. 3. Scatchard plot of the binding between L-tryptophan and bovine serum albumin, calculated with values from tubes 0–5.

Fig. 3. An association constants of $2.9 \cdot 10^4\ M^{-1}$ was obtained, which agrees well with published values obtained by other methods[2,9–12]. The number of binding sites was 0.4 which is less than 1, which might be expected from other studies[2,10–12]. The lower number of binding sites in some studies may be due to fatty acids present in the bovine serum albumin preparation. Since the serum albumin used in this study was essentially free from fatty acids a more likely explanation may be the inhibitory action of the phase polymers. Poly(ethylene glycol) may mimic decanol, which has been found to reduce the binding of tryptophan to bovine serum albumin[2].

In conclusion, our results show that on aqueous polymer two-phase system can be used for the direct chiral resolution of a racemic mixture if an enantioselective binding protein is included in the phase system. These systems may be used for both analytical and preparative purposes. It is reasonable to assume that the variety of racemic compounds resolved by Allenmark *et al.*[4] could be successfully resolved by the phase system used in this study. Since liquid–liquid partition is easy to scale up, the aqueous polymer phase systems should be of interest for large-scale separation of enantiomers.

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