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HIGH RESOLUTION IN COUNTERCURRENT EXTRACTION

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SUMMARY

In spite of years of experimentation and theoretical deduction a complete understanding of all the parameters contributing to high resolution in countercurrent separations is still lacking. Largely by trial and error the physical arrangements permitting high numbers of theoretical stages in a reproducible way have been established. This has stimulated extensive theoretical and mathematical interpretation but such does not insure that the complex interplay of the various parameters involved are really understood. Minor deviations from ideality ordinarily undetected with less efficient separations may furnish the real basis of the separation when high numbers of theoretical stages are brought into play. Correct interpretation therefore requires a stricter examination of the basic assumptions used in the interpretation. It is known that relatively large interfaces and rapid rates of exchange are usually required. This sets the stage for individual events very difficult to measure and promotes reliance on the measurement of overall effects.

In this paper an attempt will be made to suggest certain events and phenomena that may be important in contributing to the achievement of high resolution.

The objective of this lecture is to consider some of the important parameters of true liquid–liquid countercurrent extraction and the bearing these may have on various views concerning modern high-efficiency chromatographic processes where liquid–liquid countercurrent extraction is thought to play a predominant role. There is no argument about the gross similarity of the two processes. The basis for argument and differences of opinion arise when too close a comparison is made and exact definition of the parameters of chromatography is attempted by analogy with liquid–liquid countercurrent distribution.

One of the stumbling blocks in this comparison is the assumption that the supporting matrix in partition chromatography which immobilizes one of the phases has no adsorptive power for the solutes being separated but only sufficient interaction with the immobilized phase to hold it in place and yet not influence the solubility properties of the immobilized phase. We now know that high efficiency depends on a relatively vast expanse of surface (fine particles of supporting agent) and on an exceedingly thin layer of the immobilized phase.

Superimposed on this situation is the fact that the system is strictly continuous

and therefore by definition always in some degree of disequilibrium, where rates of exchange between the phases and the solid support can play a role. It is assumed that the diffusional distances are so short that the partition ratio is essentially satisfied at any time in flight. However, the partition ratio used for interpretation is a constant determined in bulk phases, where it is known that the concentration of solute at the interface is likely to be quite different from that in the bulk phases even without the influence of a solid surface in very close proximity.

Actually the basis of a partition ratio in two bulk phases in equilibrium can be considered as a balance of rates of solute interchange across the interface in opposite directions but this rate is influenced by the relative diffusional activities of the solute in the two bulk solutions. This reasoning is in accord with the two-film theory of mass transfer commonly used in chemical engineering. In many cases the uncertainty of diffusion in and out of highly restricted interstices must be considered.

In spite of the obvious differences between true liquid–liquid extraction and partition chromatography the considerable practical experience in designing systems for countercurrent distribution (CCD)[1–4] has apparently been of help in choosing systems for partition chromatography. The reverse, however, in our experience has been less true as have attempts at prediction on theoretical grounds*. The trial-and-error approach combined with experience and the art of choosing solvents has given the greatest success. In the more complicated systems phase diagrams have been of help[5].

The simplest countercurrent process in which we are certain that actual stages of high-efficiency numbering in the thousands have been attained is CCD[6]. It is usually a liquid–liquid process although even populations of living flies have been sorted this way[7] by substituting air for the liquid phases. Usually it is operated at complete equilibrium on each stage with the transfer being 97% perfect. This permits the mathematics of the binomial theorem and its extension to the normal curve of error to be precisely applied to distribution patterns with no assumptions required whatever. On the other hand continuous column processes such as liquid chromatography, gas chromatography, permeation chromatography, etc., can only be interpreted in terms of the theoretical plate concept which originally had its basis on the same mathematical interpretation which rigorously holds only in CCD. The rationale for this was based on analogy and includes an unknown number of assumptions, one of which is that the two processes merge for all practical purposes at high numbers of transfers for CCD or theoretical plates in the continuous process. Actual experimentation and comparison do not always support this latter assumption. In many cases where systems have been chosen so that CCD and partition chromatography are as nearly comparable as possible the differences become more pronounced as higher numbers of transfers in the case of CCD or theoretical plates in partition chromatography are reached. Experience has shown that CCD has a higher selectivity with certain mixtures while partition chromatography is superior with others. Since CCD is so directly related to the liquid–liquid partition ratio, it follows that the main difference lies in the fact that the theoretical plate of partition chromatography must depend in part on an unknown parameter or parameters and not strictly on the partition ratio. It is interesting to try to infer what certain of these parameters can be, as will be done later

* For a good review, see Morris and Morris[5].

in this paper. Inferences of interest can be derived from separation techniques other than chromatography.

In trying to gain information bearing on these parameters it is instructive to consider certain anomalous CCD patterns obtained with biochemical solutes which show a strong solute–solute interaction behavior. Fig. 1 gives a pattern obtained with serum albumin[8] in a system made with water and 2-butanol containing a low concentration of trichloroacetic acid (TCA). The striking thing about this pattern is that the protein band is much too narrow for the numbers of transfers applied. If the theoretical plate count based on the width of the band for this run is calculated it would differ from the actual plates applied by a factor of 7 or more. At higher concentration levels the band can be even more narrow, due to the reversible binding between the protein and the TCA. The partition ratio actually was concentration dependent in spite of the symmetrical nature of the band. A common interpretation found in chromatography is to use symmetry of a band to indicate ideality.

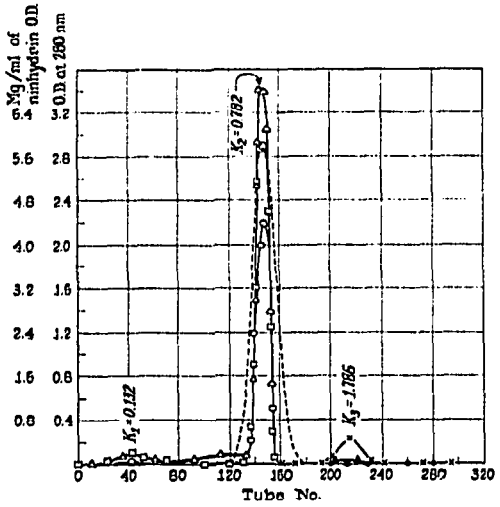

Fig. 1. An example of too narrow a band in countercurrent distribution. ○—○, Ninhydrin lower phase; ●—●, ninhydrin upper phase; □—□, 280 nm lower phase; ×—×, 280 nm upper phase; △—△, mg/ml lower phase; ▲—▲, mg/ml upper phase; ....., theoretical. K_1, K_2 and K_3 are partition ratios.

Irrespective of theory narrow bands are the result desired in countercurrent processes if they are reproducible. Such narrow bands and high plate counts, however, do not contribute much to a real understanding of the separation parameters involved. In the case of CCD the anomalous behavior shown in Fig. 1 was immediately apparent and its basis could be determined easily because of the nature of the process.

Interesting patterns in this connection were obtained with tRNA mixtures. The naturally occurring mixture contains somewhere in excess of fifty individual nucleotides of poor stability, all of molecular weight approximating 30,000. CCD gave the first individual tRNA[9] from this complicated mixture. The interesting patterns shown in Fig. 2 were obtained by Goldstein *et al.*[10]. The upper patterns (a) show a

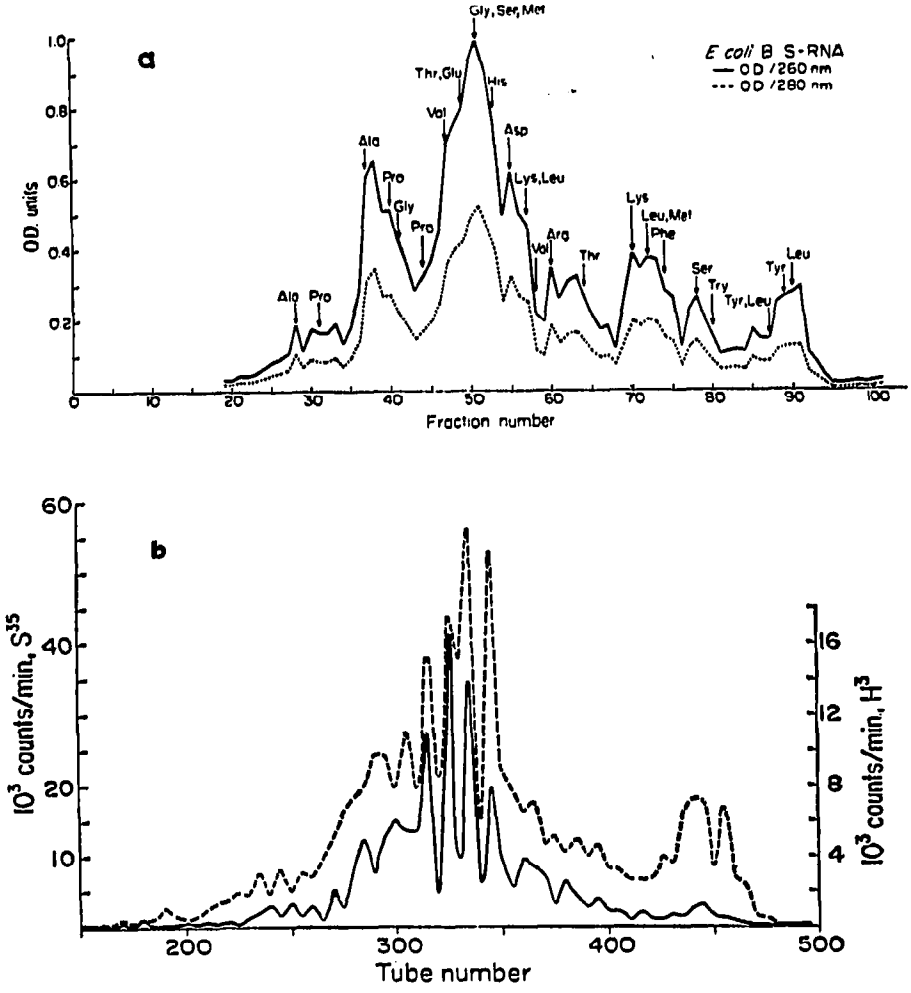

Fig. 2. CCD patterns obtained with tRNA mixtures.

distribution of 1000 transfers monitored at 260 and 280 nm. The location of individual nucleotide bands was determined by amino acid incorporating assay. Some of the bands were broader than theory based on the number of transfers but others were much too narrow. It is very likely that the selectivity is due to the change in conformation of the nucleotides on going from one phase to the other.

In another run with material doubly labelled by growing the bacteria in isotopic media the lower patterns (b) were obtained at 476 transfers. The profiles here were obtained by radioactive counting. The striking feature of the central peaks is their sharpness. If one did not know that 476 transfers had been applied one could calculate 3000 theoretical stages by the mathematics used in chromatography.

Strong solute–solute interactions are often found with polypeptides, nucleotides, fatty acids, etc. Indeed, this property is often essential for the role they play in living cells and probably contributes greatly to the selectivity found in tissues. It can be taken advantage of in countercurrent processes. For instance, the naturally oc-

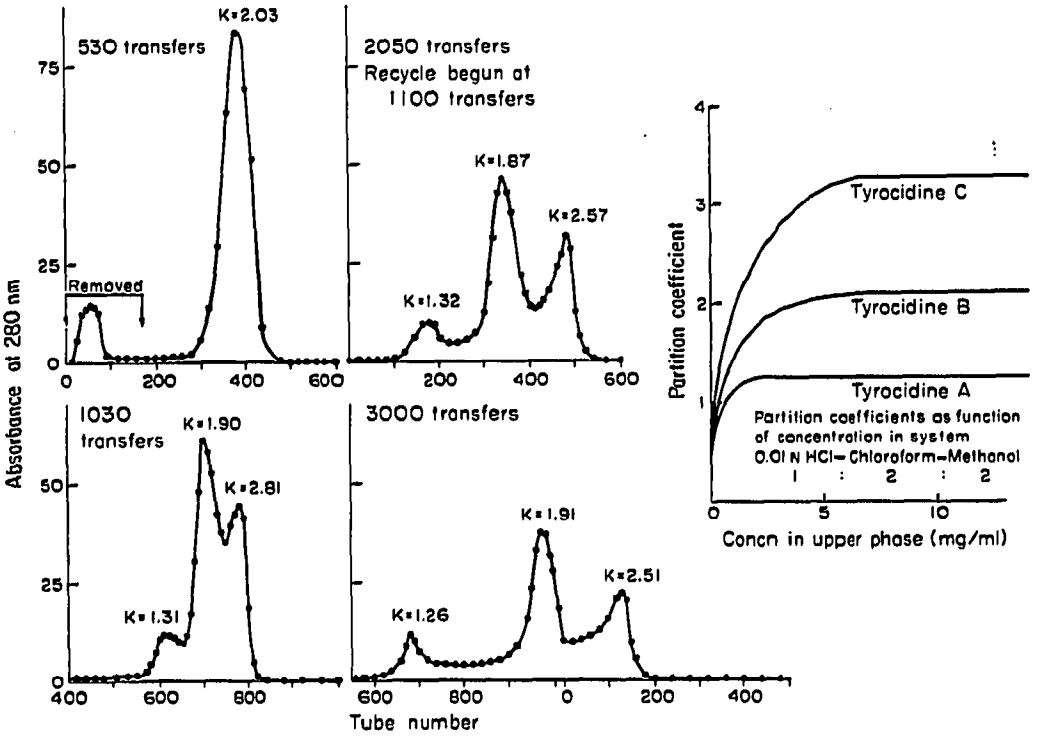

Fig. 3. CCD patterns obtained with associating solutes. K = partition ratio.

curring mixture of the tyrocidine antibiotics proved difficult to separate by any technique except CCD. They are mixtures of closely related cyclic decapeptides which associate strongly in aqueous solution. The way the individual bands emerge from each other in a 1000-tube train is shown in Fig. 3 by analysis at several different numbers of transfers[11]. Examination of the patterns will show that little resolution occurs at 530 transfers, but by 1030 transfers partial separation has occurred which is improved further at 2050 transfers, but at the highest number of transfers, 3000, the resolution deteriorates. Apparently the association equilibrium slightly favors a homoassociation as compared to heteroassociation within a given concentration range. Other systems which repressed association did not give as good a resolution.

One of the most fundamental differences between CCD and chromatographic processes is that one is an equilibrium process and the other is at least in part a rate process. Most theoretical treatments of chromatography assume this difference to be minimal or essentially non-existent. Otherwise a reasonable theory becomes difficult. Since a flowing phase is required in chromatography it is obvious that rates of exchange between the stationary and flowing phases must be involved to some degree. Rates of diffusion across interfaces must therefore be considered.

In thinking about this question some years ago we decided to study in depth some separation system in which the separation parameters would depend mainly or exclusively on rates of diffusion. This led us to the development of the technique we have called "Thin-Film Dialysis"[12]. It involved an extensive study of the rates of dif-

fusional transfer across semipermeable membranes. In order to accomplish our objective it was necessary to find a membrane or to modify the properties of the membrane so that it would behave toward a given test solute exclusively as a mechanical sieve. That is to say there would be no adsorption or solution type of absorption, ion exchange or other solute interaction with the membrane. Cellophane proved to be the most suitable membrane and we learned how to modify it so that all fixed charges were removed[13] and to alter the porosity at will so that a given solute could be either passed or retained. By comparison of rates with those of rigid model solutes of known size and shape we could interpret diffusion rates through the membrane so as to estimate Stokes radius to $\pm$ 3%. The approach offers a versatile tool for studying solute–solute interactions, binding phenomena, self-association, concentration effects, conformation, temperature effects, etc. All these are obviously the same parameters which must be considered in achieving high selectivity in CCD and in chromatography. The amazing individuality and conformational sensitivity to slight changes in the solvent demonstrated for polypeptides, for instance, is of particular interest for their separation.

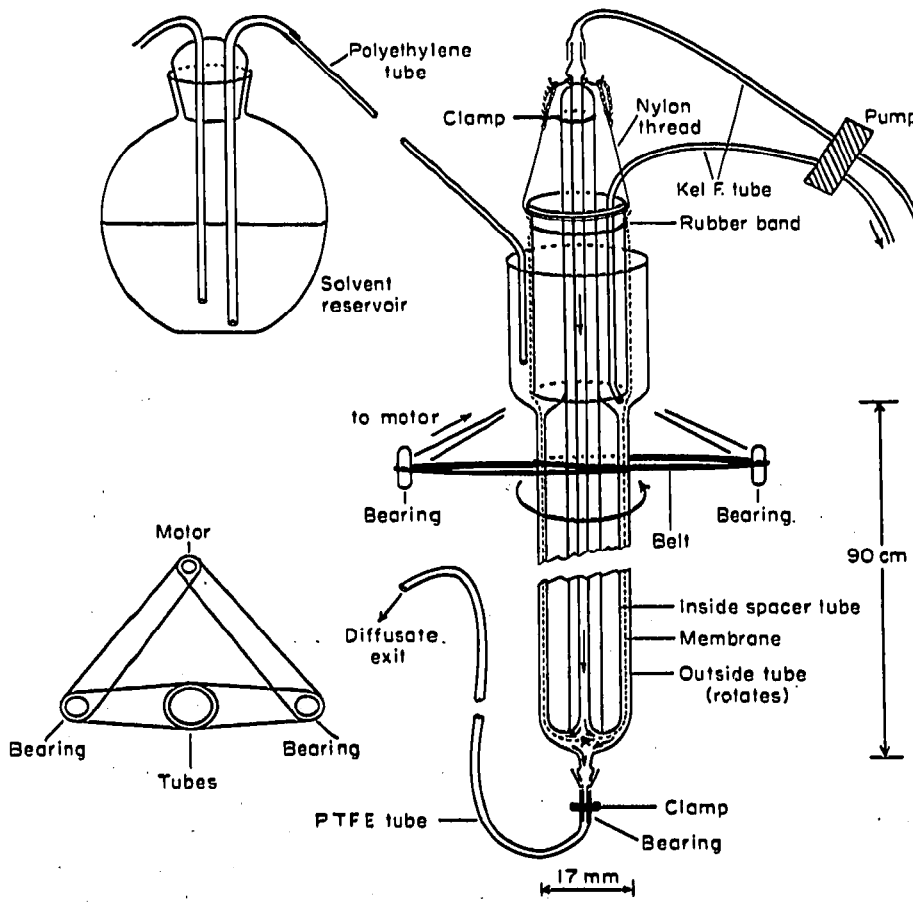

Fig. 4. Schematic drawing of a thin-film countercurrent dialyzer.

A rather surprising finding emerging from the thin-film dialysis study was that with the most inert membranes of high resistance the rate of transfer across the membrane apparently is controlled by a two-dimensional barrier at the surface of the membrane where the solute enters on the high-concentration side[14]. This is to say the rate of diffusional transfer was directly proportional to the probability of a molecule finding a pore large enough to enter and thus begin its passage through the matrix of the membrane. Its diffusion through the membrane and out on the other side was found to be much faster than its entrance. There are many implications of this finding but I shall try to develop only one for its implication in chromatography.

For practical reasons in biochemistry we developed a continuous thin-film countercurrent dialyzer[15], shown in Fig. 4, in which two thin films of solution are made to flow either countercurrently or cocurrently with a treated cellophane membrane separating them. The rates of flow are accurately controlled by precision pumps. Channeling is prevented by spinning the outer tube and even if the rate of flow of the outer film is stopped entirely longitudinal convection is prevented by the spinning outer glass tube. If now a pulse of a solution of a test solute is introduced into the retentate inner flowing stream a situation analogous to permeation chromatography is established in which the emergence of a solute from this stream in the column is retarded due to its penetration through the membrane into the stationary phase and back again.

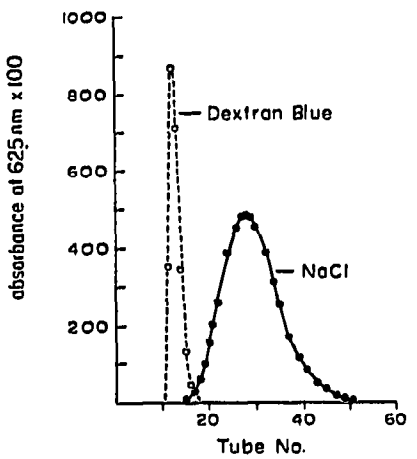

Fig. 5. Diffusate patterns when the diffusate stream of the countercurrent dialyzer is stationary.

It was found that a solute too large to penetrate the membrane would emerge as a discrete band as shown in Fig. 5 and that a small solute such as sodium chloride would emerge later as a symmetrical broader band completely separated from the large solute. But what about two solutes differing in size but both able to penetrate the membrane? In answer to this question it was found that solutes of size larger than sodium chloride did not emerge with the peak concentration much displaced from that of sodium chloride but instead only as a wider band that extended before the front of the sodium chloride band as well as behind it. Lengthening the dialysis column by recycling only made the resolution worse. This is due to the fact that while

there is a highly selective influence due to size on diffusion from the moving retentate stream to the immobilized stream there is a corresponding retardation when the larger solute diffuses back from the immobilized stream to the moving stream. The slower penetration of the larger molecule from the moving stream causes it to begin to emerge sooner than the smaller thus controlling the front of the band but the same slower rate from the stationary solution in the reverse direction causes it to emerge later and a broader band results. This behavior, Fig. 6, was found to be exactly as expected, as shown with bacitracin A, molecular weight 1420, and sodium chloride, and is analogous to operating a CCD train at disequilibrium or a chromatographic column at much too fast a rate of flow.

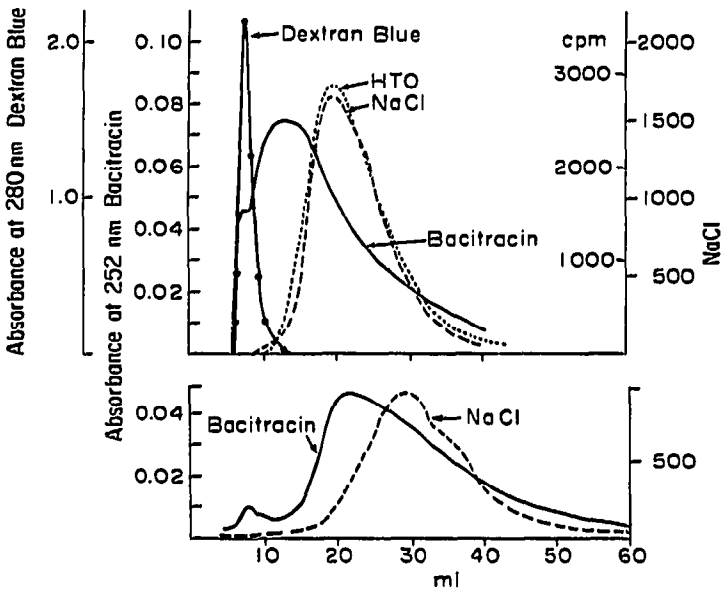

Fig. 6. Diffusate patterns, when the diffusate stream is stationary, of two solutes differing in size but both dialyzable. Upper patterns = one pass. Lower patterns = two passes. HTO = tritiated water.

How then does the highly reproducible and selective band formation come about in permeation chromatography? It is considered a partition process whose main parameter is either partial exclusion or relative rates of permeation into the gel matrix from the moving solvent and out again[16–18].

Perhaps insight into this problem can be gained from the high-size selectivity demonstrated by thin-film dialysis[12]. If the limiting rate of the overall process is the probability of a molecule finding a "pore" or solvent-filled crevice in the matrix a plausible explanation of the overall effect can be given. Once the molecule has entered a solvent-filled crevice the probability of its moving out again by diffusional activation, barring adsorption, would be high and very rapid. This part of the process would not be expected to be size selective. However, it would eliminate the cause of spreading of the band for the larger solute by eliminating the trailing edge. Such a mechanism is in complete accord with the restricted diffusion theory of Ackers[19]. If

true, it means that permeation chromatography is strictly a rate process even if for purposes of calculating theoretical plates it is interpreted as a partition process.

It was suggested many years ago[20] in liquid–liquid extraction that the rate of transition of the solute from one phase to the other might be different from that in the reverse direction and that this could be the basis of selectivity in a particular system. If the two phases should differ markedly in polarity, this difference in rate would then be expected to be greatest because of the greater energy difference in crossing the interface. In any case it was found with antimalarial drugs that very high selectivity was achieved when the system was a buffer of high ionic strength with diethyl ether or a hydrocarbon[21] as the organic phase. The nature of the buffer was important. In the penicillin field a 2 *M* phosphate–ether combination was most selective[22]. Much later Agpar *et al.*[23] found a concentrated phosphate solution equilibrated with a mixture of isopropanol and formamide to be the system of choice for the CCD separation of the tRNA mixture.

In the light of modern-day views of the nature of the forces holding long-chain polymers in specific shapes or conformations, it seems highly probable that the conformation of a long-chain polynucleotide with no covalent cross-links would be quite different in one of the phases of the system than in the other. In support of this view was the finding that the tRNAs were dialyzable in salt solution in porous membranes but not in aqueous solutions of low ionic strength[24]. A specific conformation for the tRNA molecule has been proposed for its active form, which depends largely on hydrogen bonding of base pairs[25]. This conformation, however, should be easily deformable, depending on the solution environment, and the rate of transition from the conformation in one phase of the CCD system to that in the other could be the single parameter which permits the surprising separation of such a complicated mixture of very similar solutes.

Irrespective of the correctness of the suggestions in this paper concerning the parameters which contribute to selectivity, many of the ideas originally developed in the liquid–liquid CCD process have been or can be exploited in chromatography. These include demonstration of the importance of buffers[21], ionic strength, ion pairing or complexing agents[1], the use of volatile buffers, procedures for correcting non-ideal systems and various other modifications. The recycling procedure was first developed for CCD[1–4] and the CCD trains were specifically designed to make recycling convenient.

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