Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

A Method of Calculating Countercurrent Distribution Curves of Nonideal Solutes

Robley C. Williams Jr.[a]; Lyman C. Craig[a]
[a] Rockefeller University New York, New York

To cite this Article Williams Jr., Robley C. and Craig, Lyman C.(1967) 'A Method of Calculating Countercurrent Distribution Curves of Nonideal Solutes', Separation Science and Technology, 2: 4, 487 — 499
To link to this Article: DOI: 10.1080/01496396708049716
URL: http://dx.doi.org/10.1080/01496396708049716

SEPARATION SCIENCE, 2(4), 487–499 (1967)

A Method Of Calculating Countercurrent Distribution Curves of Nonideal Solutes*

ROBLEY C. WILLIAMS, JR., and LYMAN C. CRAIG
ROCKEFELLER UNIVERSITY
NEW YORK, NEW YORK

Summary

A practical method is presented of calculating, with the aid of a digital computer, the positions and shapes of countercurrent distribution patterns of solutes which display concentration-dependent partition coefficients. The method requires knowledge of the concentration dependence of the partition coefficient in the solvent system being used. Its application can permit optimization of conditions for large-scale preparative distributions and can also yield information about purity and complicating interactions between the components of mixtures. Distributions of benzoic acid and of the three cyclic decapeptides, tyrocidines A, B, and C, are presented as examples of its use.

Many solutes examined by countercurrent distribution (CCD) have been observed to display asymmetric peaks, variations of partition coefficient across the peaks, and "traveling *K*'s" which change as the distribution proceeds (*1–4*). The binomial theoretical curves based on the assumption of a concentration-independent partition coefficient (*5,8*) are not adequate to describe distributions of such solutes. It has long been realized that these phenomena, frequently encountered in biochemistry, are a result of concentration-dependent partition coefficients of the pure solutes (*4,5*). The commonly encountered nonconstant partition coefficients are always monotonic functions of concentration. In these cases, a partition coefficient which increases with concentration produces a curve with a steep leading edge and a long trailing edge; whereas a

---

[*] This work was supported in part by a National Institutes of Health Grant AM-02493.

partition coefficient which decreases with increasing concentration produces a curve with a long leading edge and a sharp trailing edge. Often the concentration dependence of a solute can be ascribed to association behavior of that solute in one or both phases of the solvent system in which the distribution is performed (*4,5*).

One of the important advantages of separation by countercurrent distribution is that its stepwise nature permits rigorous calculation of the shapes of distribution profiles. Such calculation allows one to estimate the degree of separation of two substances which is obtained in a given number of transfers and makes possible the detailed use of the shape of the curve resulting from a distribution as a criterion of purity (*5*). Bethune and Kegeles have developed methods of predicting the positions and shapes of CCD peaks, both in the case of a solute which associates with itself (*6*) and in the case of pairs of solutes which associate with each other (*7*). Their methods are of great theoretical interest but are somewhat difficult to apply in practice; they require knowledge of the nature and equilibrium constant of each reaction and of the partition coefficient of each of the reacting species. For most solutes of biochemical interest these quantities are not known, and often even the reactions which a solute undergoes may be obscure.

It has proved helpful, therefore, to devise a simple method for predicting, with the aid of a digital computer, the position and shape of CCD curves of solutes whose partition coefficients, in the solvent system of interest, can be measured as a function of solute concentration.

METHOD OF CALCULATION

To calculate the shape and position of a CCD pattern, the partition coefficient is first measured as a function of the concentration of material in the upper phase. These measurements must span the range of concentration encountered in the distribution. The initial mass placed in each tube, the number of tubes loaded, and the volumes of upper and lower phases are specified. The validity of the calculations rests upon the assumption that partition equilibrium obtains at each step in the distribution, and that the partition coefficient of each solute species in a mixture is uninfluenced by the presence of other species. Calculation proceeds as follows. The table of experimentally measured partition coefficients, K, at upper phase concentrations C_u is used to generate a "working"

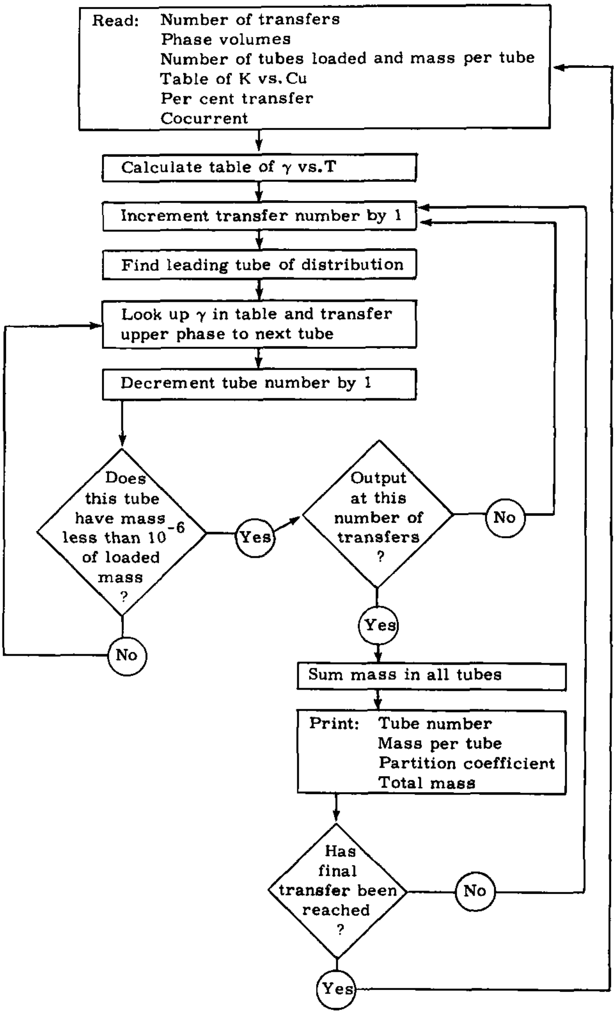

FIG. 1. Flow diagram of FORTRAN computer program used to carry out calculations of the CCD curve of a nonideal solute. Masses and phase volumes may be in any convenient units.

table of values of γ, the fraction of the total mass in a given tube which is present in the upper phase, at various values of T, the total mass in a tube. Transfers are then simulated, using the ordinary expression $T_{n,r} = \gamma_{n-1,r-1}T_{n-1,r-1} + (1 - \gamma_{n-1,r})T_{n-1,r}$, where $T_{n,r}$ is the mass of solute in the rth tube after the nth transfer, and $\gamma_{n,r}$ is the corresponding value of γ. Linear interpolation between the points of the table is used to find $\gamma_{n,r}$. In a single transfer, calculation proceeds from the leading to the trailing edge until all tubes have been calculated, whereupon a new transfer is begun.

A brief flow diagram of the FORTRAN program used to carry out these calculations is shown in Fig. 1. The program permits output at any number of transfers, provides for the simulation of a small amount of lower phase cocurrent and of the transfer of less than the full amount of upper phase, and minimizes unnecessary calculation by arbitrarily setting equal to zero the mass in all *trailing-edge* tubes which contain less than 10^{-6} of the mass initially loaded in a single tube.

EXPERIMENTAL

Materials

All solvents were distilled before use, and all were reagent grade with the exception of cyclohexane (Mathieson, Coleman, Bell Co., practical grade). Benzoic acid was primary standard grade (Mathieson, Coleman, Bell Co.).

Purified tyrocidines A, B, and C (9) were prepared from tyrocidine HCl (Wallerstein Co., lot No. ON13554) by CCD to 3000 transfers in the system chloroform/methanol/0.01 *N* HCl, 2:2:1 (*10*).

Measurement of Partition Coefficients

Partition coefficients were measured at 30 to 40 concentrations over the range spanned by the distributions. Concentrations were assayed spectrophotometrically, care being taken to correct for possible differences of the extinction coefficient from one phase to the other. Measurements on benzoic acid were carried out in single tubes, but because of difficulties caused by evaporation, the CCD machine was used as a dilution and equilibration device for the tyrocidines.

Countercurrent Distributions

Benzoic acid was distributed to 200 transfers in the system cyclohexane/0.1 *N* HCl. Concentrations were measured spectrophotometrically at 273 mμ. Forty tips were applied at each equilibration stage to assure complete equilibration.

All distributions of the tyrocidines were carried out in the same system (see above). Previous to the 3000 transfer distribution described below, the peptides were dialyzed against a large volume of upper phase to prevent a shift in the relative volumes of the phases (*10*). A small volume (approx. 0.1 ml) of cocurrent was supplied to make up for a slight evaporation of the lower phase. The amount of each of the peptides used in calculating the distribution was estimated by appropriate summation of the observed pattern at 3000 transfers. Because separation is always incomplete in the distribution, this procedure is necessarily somewhat lacking in precision. The *qualitative* conclusions presented below are, however, not appreciably affected by even fairly large variations in the choice of input masses.

Computer Program

The accuracy of the computer program was checked with a linear dependence of K on C_u, by hand calculation at 10 transfers and, with a constant K, by comparison with a machine-computed Gaussian curve at 500, 1000, and 2000 transfers. As a further check, the mass in all tubes at the end of each distribution was summed. Deviations of no more than 1 part in 10^4 were found in single tubes. Departures of the total mass at the end of the distribution from the input mass were typically of the order of 10^{-4} of the total mass at 3000 transfers when calculations were done on an IBM 7044 computer but were found to become as high as 2.5×10^{-2} of the input mass when carried out on a CDC 160G, probably as a result of the shorter word length used by the second computer.

Benzoic Acid

The results of a distribution of benzoic acid to 200 transfers are shown in Fig. 2, together with the applicable plot of K vs. C_u. The calculated curve was prepared using as input 60 points read from the line indicated in the K vs. C_u plot. The small discrepancy between the calculated and observed curves near the peak of this distribution is probably due to the presence in the sample of a very small amount of impurity of high K. The ordinary binomial theoretical curve, with a width at half-height of 16 tubes, would give a strikingly poor fit to the observed curve; and the similar binomial curve based on the high K observed at the starting con-

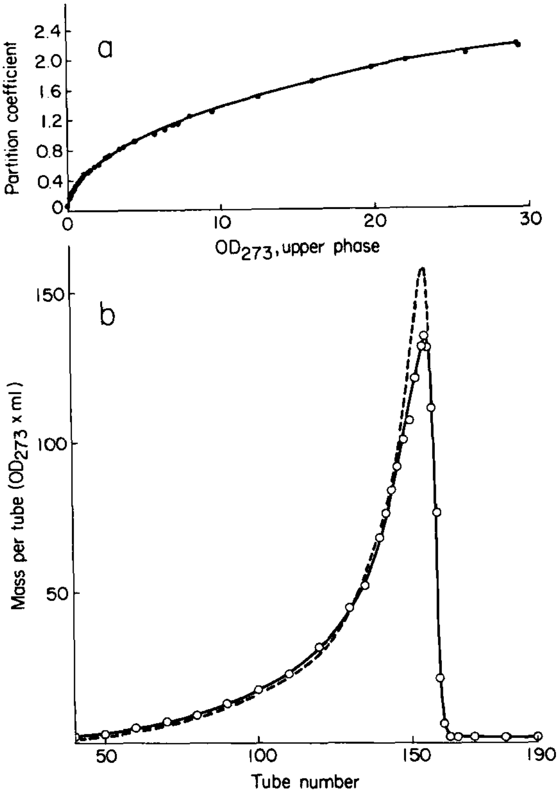

FIG. 2. (a) Partition coefficient of benzoic acid as a function of its concentration in the upper phase of the system cyclohexane/0.1 *N* HCl. (b) Countercurrent distribution of benzoic acid to 200 transfers in the same system. The points and solid line are experimental; the dashed line is predicted by the present method. Conditions: 9.8 ml upper phase, 5.0 ml lower phase, 0.468 g loaded in first 10 tubes, 94% of upper phase transferred at each step.

centration would, of course, provide an even poorer representation of the observed peak.

Tyrocidines A, B, and C

The partition coefficients of tyrocidines A, B, and C as functions of their concentrations are shown in Fig. 3. Thev are similar in shape and give rise to similar skewed distribution curves. It will be seen that at high concentrations the partition coefficients of the three peptides are quite different from one another and change little with concentration but that at lower concentrations they decline rapidly and approach each other. This behavior is expected to produce, for each peptide, a CCD curve having a sharp leading edge and a long trailing edge; better separation of mixtures of the peptides would be expected at higher loading concentrations.

In Fig. 4 is shown a distribution of a single one of the peptides, tyrocidine B, at relatively low loading concentration. The extreme skewing of the peak is evident, as is the rather good fit of the predicted curve to the experimentally observed curve. Figure 5 gives the results at four stages of a distribution carried out for the purpose of preparing pure tyrocidines A, B, and C, together with the appropriate calculated curves. The effect of the concentration dependence of the partition coefficients is clearly seen. Near the peaks the curves are fairly symmetrical, reflecting the relative constancy of the partition coefficients at high concentration. The trailing edges, however, are very much elongated as a result of the

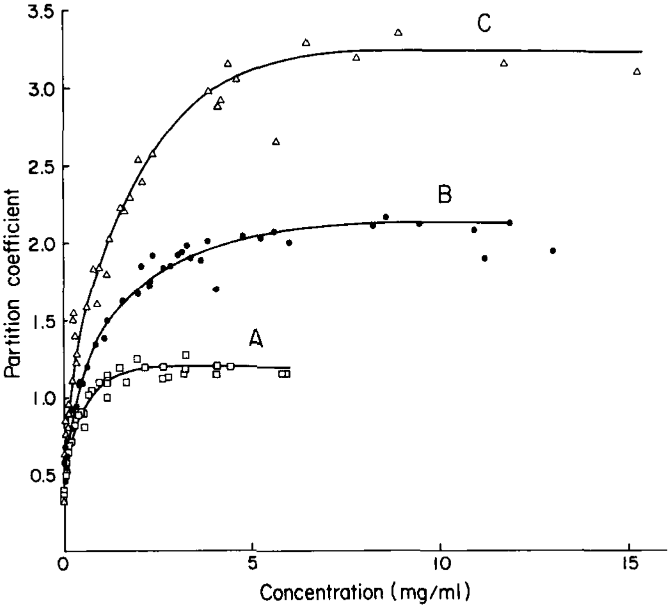

FIG. 3. Partition coefficients of tyrocidines A, B, and C as a function of their concentration in the upper phase of the system chloroform/methanol/0.01 *N* HCl, 2:2:1. Tyrocidine A, □; tyrocidine B ●; tyrocidine C, △.

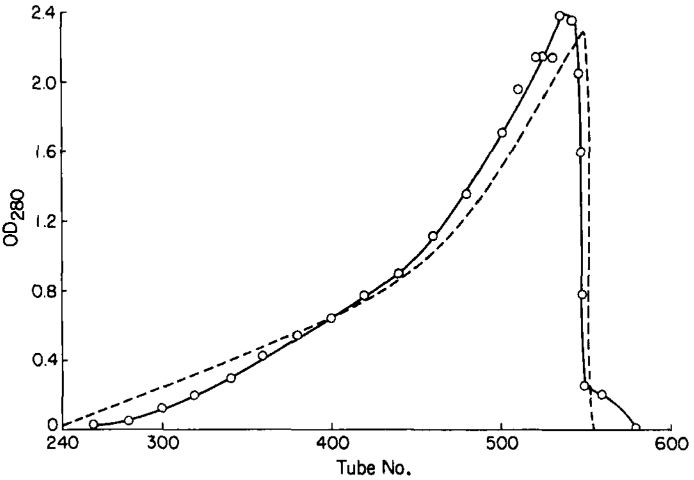

FIG. 4. Countercurrent distribution of tyrocidine B at low concentration at 1008 transfers in the system chloroform/methanol/0.01 *N* HCl, 2:2:1. The points with the solid line are experimental; the dashed line is predicted. Conditions: 5.0 ml upper phase, 3.0 ml lower phase, 250 mg loaded in first 50 tubes.

rapid decrease of the partition coefficients at low concentrations. The degree of impurity to be expected from tailing of one band into another may be estimated from the calculated curves. Thus, in its peak tube, the band of tyrocidine B would be estimated to contain about 3% of tyrocidine C; and that of tyrocidine A would probably contain about the same amount of each of tyrocidines B and C. These impurities have not been detected chemically because the differences between the peptides involve the replacement of trytophan by phenylalanine, a difference difficult to detect by amino acid analysis. A low-molecular-weight species of high extinction coefficient, tentatively identified as tyrocidine C has, however, been seen in ultracentrifuge experiments with tyrocidine B (*12*).

It is, therefore, clear (1) that the ordinary binomial approximation is inadequate to describe the separation of these peptides, (2) that the present method describes the skewed shape of the peak of a single one of these peptides rather well, (3) that this skewing will cause tailing of the leading peaks (tyrocidines B and C) into the ones behind, and (4) that the relative amount of impurity caused by this tailing becomes greater as the concentration decreases.

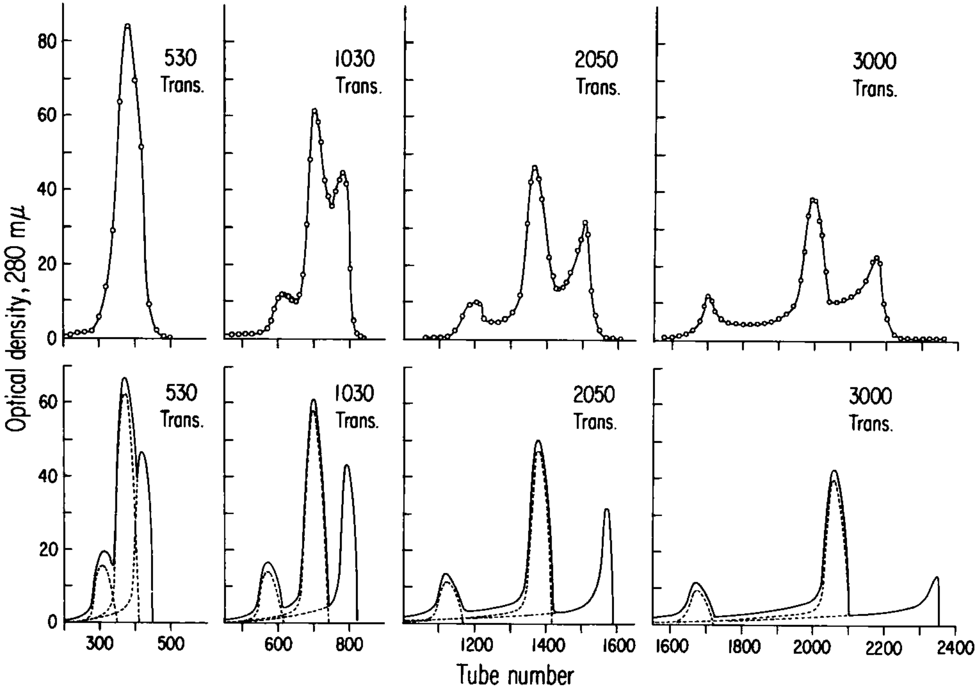

FIG. 5. Countercurrent distribution of the naturally occurring mixture of tyrodicines A, B, and C in the system chloroform/methanol/0.01 *N* HCl, 2:2:1. Experimental (upper) and predicted (lower) curves are shown at four stages in the distribution. The leading peak is tyrocidine C; the trailing peak is tyrocidine A. The dashed lines in the predicted curves represent the concentrations of the individual peptides and the solid line their sum. Conditions: 3.0 ml upper phase, 3.0 ml lower phase, 0.1 ml cocurrent, 97% of upper phase transferred at each step. Load: 10 g of crude tyrocidine HCl in first 50 tubes, of which about 2 g is gramicidins, removed at 500 transfers. Assumed for calculation: 1.75 g of tyrocidine A, 3.90 g of tyrocidine B, 2.15 g of tyrocidine C.

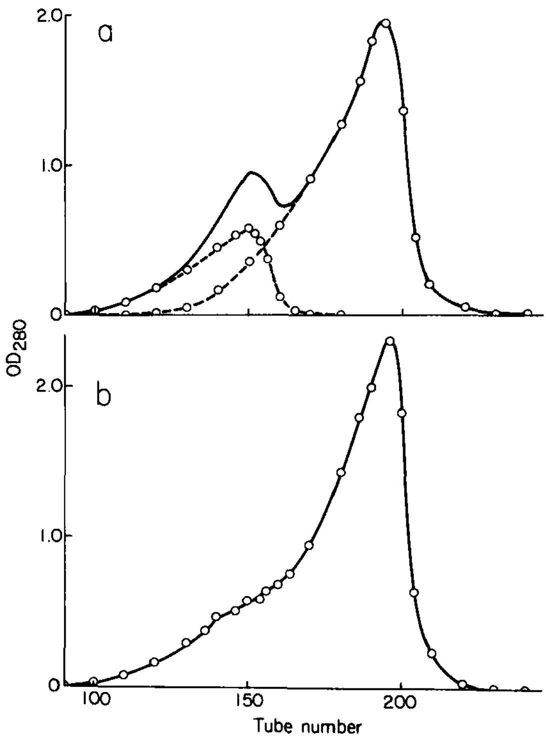

FIG. 6. Countercurrent distributions of tyrocidines B and C to 315 transfers in the system chloroform/methanol/0.01 *N* HCl, 2:2:1. (a) 55 mg of C (leading peak) and 24 mg of B (trailing peak) distributed separately. The solid line represents the sum of the two peaks. (b) The same quantities of B and C distributed together. Conditions: 5 ml upper phase, 3 ml lower phase; materials loaded in first five tubes.

It will also be seen in Fig. 5, especially at 530, 1030, and 2050 transfers, that the separation is further complicated by other factors. The concentration of material between the peaks is higher, and the separation of the peaks not as great, as would be expected on the basis of the calculations performed on the assumption that the partition coefficient of each solute is independent of the presence of other solutes. From previous work, both experimental (*1*) and theoretical (*7*), one is led to expect that this lack of resolution is brought about by reversible association reactions between different peptides of the type $X + Y \leftrightarrows X{\cdot}Y$, in addition to reactions of the type $nX \leftrightarrows X_n$, which the present method is capable of describing.

Another pair of experiments verified this expectation. Tyrocidine C (55 mg) and tyrocidine B (24 mg) were each distributed *separately* to 315 transfers (Fig. 6a). A *mixture* of the same amounts of each peptide was then distributed to the same number of transfers (Fig. 6b). It may readily be seen by comparison of the two patterns that these two solutes do not distribute independently of each other. As expected (7), the resulting effect is seen to be a decrease in separation. In all probability a reversible B·C complex is formed which has a partition coefficient intermediate between that of tyrocidine B and tyrocidine C. Referring again to Fig. 5, it becomes clear that the formation of this sort of reversible complex can account for the discrepancies between the predicted and observed curves. The predicted curves presented do not, therefore, represent a completely accurate picture of the concentration distribution of the three peptides but represent a considerably better approximation than the binomial curves, which would be calculated on the assumption of concentration-independent partition coefficients. In addition, the ability to account for the effect of complexes of the $nX \leftrightarrows X_n$ type enables us to detect and evaluate more clearly the effect of complexes of the $X + Y \leftrightarrows X{\cdot}Y$ type.

DISCUSSION

The method described above permits calculation of countercurrent distribution curves of solutes which display partition coefficients dependent on concentration. As such, it can be useful (1) in estimating levels of purity when separation is incomplete, (2) in deciding how many transfers to apply to a given mixture to obtain a desired degree of separation of its components, and (3) in selecting optimum concentrations and solvent systems in which to carry out large-scale preparative distributions. In addition, it is seen to be useful for detecting cases in which the partition coefficient of one component of a mixture is affected by the the presence of other components of the mixture. Its chief limitations lie in the amount of labor involved in the measurement of $K(C_u)$ and in the fact that the components of a mixture must be available in pure form before their partition coefficients can be measured. In practice it seems feasible to approximate the latter condition by using components of moderate purity.

The skewing of the CCD peaks described here is caused by concentration dependence of the activities of the solutes, which may be described as association: probably a simple monomer-dimer equilibrium in the case of benzoic acid (*13*), but a considerably more complex set of equilibria in the case of the tyrocidines, which display a high degree of reversible association in many solvents (*11,12*). It is worthwhile to note that, with the closely related solutes usually separated by CCD, if each of two solutes associates with itself to form reversible homopolymers, the chances are good that they will associate with each other to form reversible heteropolymers.

The complex nature of this type of interaction is clearly shown in Fig. 5. Here at the highest concentration, up to 530 transfers, no visible separation results. One must conclude that the formation of heteropolymers is favored at least as much as the formation of homopolymers. By 1030 transfers this has ceased to be true. In this concentration range the formation of homopolymers is favored and separation is observed, as predicted from the partition isotherms of Fig. 3. This holds past 2050 transfers but by 3000 transfers the unavoidable dilution of the long distribution brings the concentration level to the point where the lower end of the partition isotherm becomes much more important and poor separation results.

The separation of Fig. 5 is particularly interesting because it clearly shows a selective type of interaction with concentration dependence. Indeed, this is the basis of the separation. It can well be profitable in attempting the more difficult separation of large, naturally occurring biopolymers to try to set up conditions so that this type of interplay can be recognized and fully exploited.

Acknowledgment

The authors are grateful to Dr. D. A. Yphantis for the gift of computer time and for much helpful discussion during the course of this work.

REFERENCES

1. Y. Sato, G. T. Barry, and L. C. Craig, *J. Biol. Chem.*, **170**, 501 (1947).
2. G. T. Barry, Y. Sato, and L. C. Craig, *J. Biol. Chem.*, **188**, 299 (1951).
3. E. H. Ahrens, Jr., and L. C. Craig, *J. Biol. Chem.*, **195**, 299 (1955).
4. P. von Tavel and R. Signer, *Advan. Protein Chem.*, **9**, 237 (1956).

5. L. C. Craig and D. Craig, *Technique of Organic Chemistry*, Vol. III (A. Weissberger, ed.), Wiley (Interscience), New York, N.Y., 1950.
6. J. L. Bethune and G. Kegeles, *J. Phys. Chem.*, **65**, 433 (1961).
7. J. L. Bethune and G. Kegeles, *J. Phys. Chem.*, **65**, 1755 (1961).
8. J. D. Gregory and L. C. Craig, *Ann. N.Y. Acad. Sci.*, **53**, 1015 (1951).
9. M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, **4**, 11 (1965).
10. M. A. Ruttenberg, thesis, Rockefeller University, New York, 1965.
11. M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, **5**, 2857 (1966).
12. Unpublished observations of the authors, 1966.
13. A. K. M. Shamsul Huq and S. A. K. Lodhi, *J. Phys. Chem.*, **70**, 1354 (1966).

Received by editor May 2, 1967
Submitted for publication May 15, 1967