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COUNTERCURRENT CHROMATOGRAPHY

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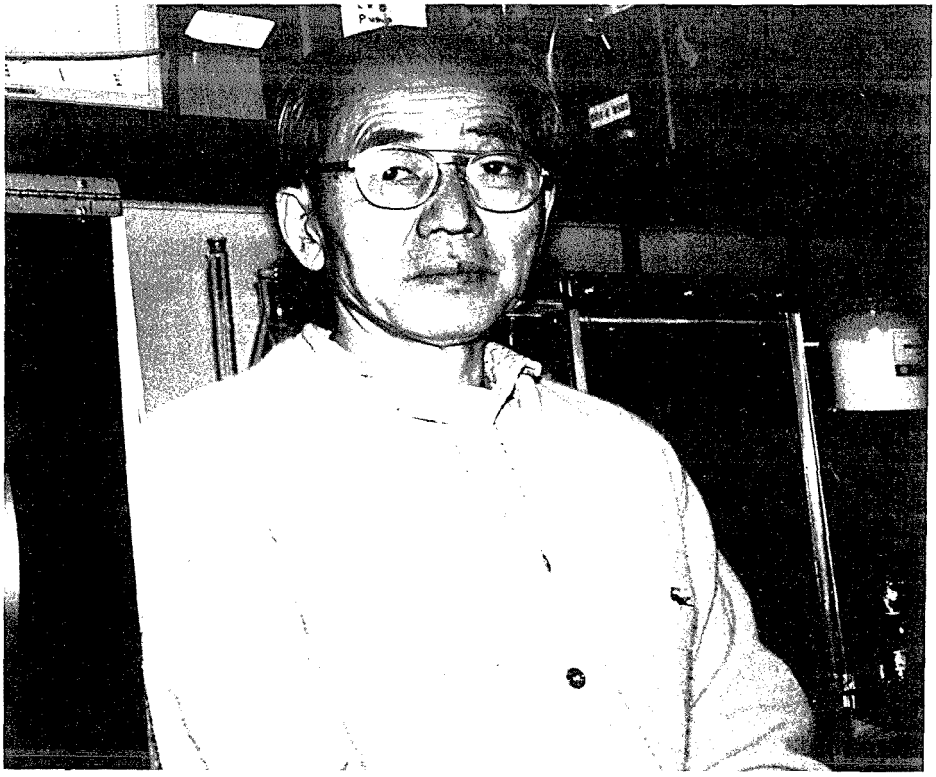

This Special Issue is Dedicated
to
Dr. Yoshiro Ito
for
Development of Countercurrent Chromatography

COUNTERCURRENT CHROMATOGRAPHY

Edited by

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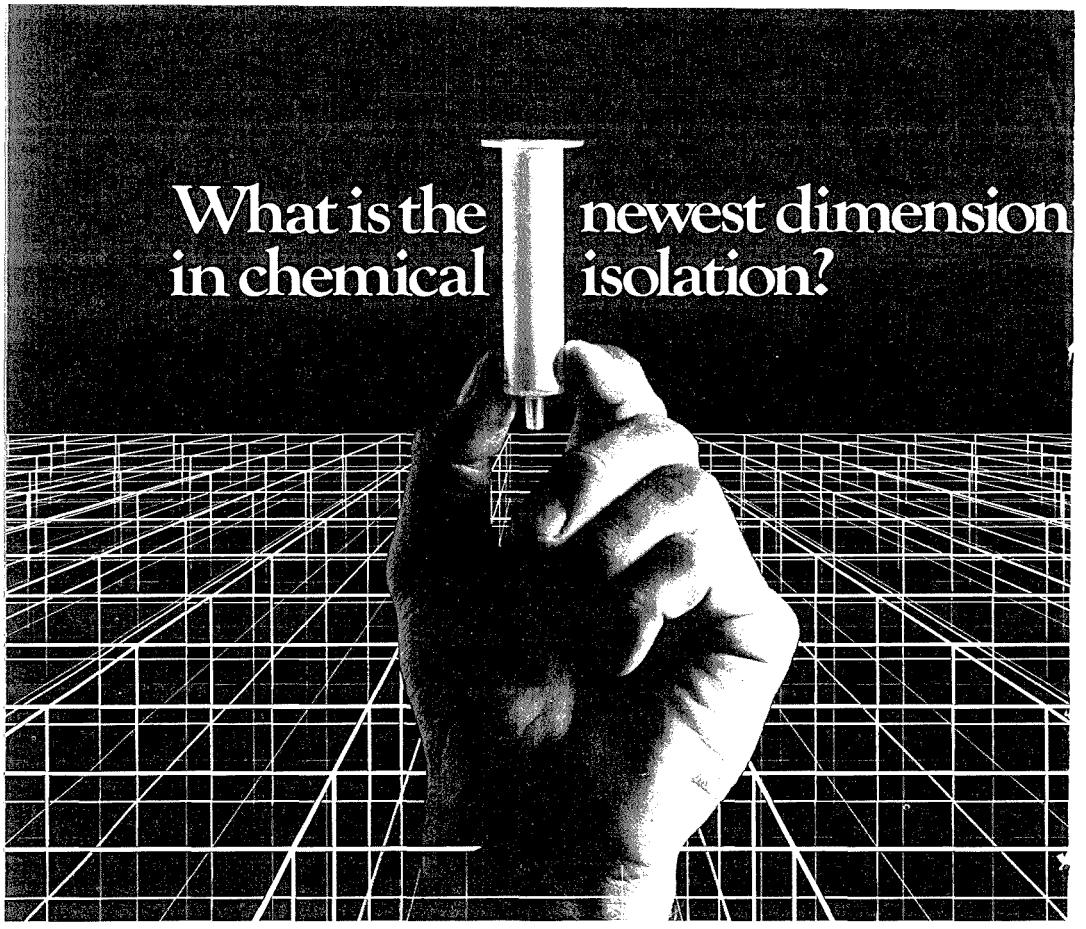

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JOURNAL OF LIQUID CHROMATOGRAPHY

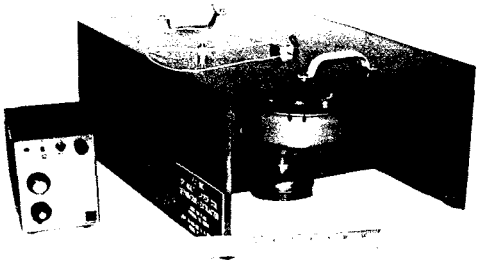
Volume 7, Number 2, 1984

Special Issue on Countercurrent Chromatography

CONTENTS

COUNTERCURRENT CHROMATOGRAPHY WITH ITO MULTI-LAYER COIL SEPARATOR-EXTRACTOR

This successful new technology is the only method which fully exploits centrifugal forces to retain one phase while another passes through it without the use of solid supports.



Solvent Systems

Most of the two phase solvent systems used for Craig CCD systems or droplet countercurrent chromatography (DCCC) can be used.

Because there are no solid supports, this technique has the following advantages:

1) No sample loss
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INTRODUCTION

The technology of countercurrent chromatography evolved from Dr. Yoichiro Ito's observation that two immiscible liquids flowing countercurrently in a helical tube, which is rotating in an acceleration field, become uniformally segmented in the coils of the helix (1). Separation of both soluble and particulate samples was demonstrated. Though tedious and limited to microgram samples, the value of the technique was immediately acknowledged by the commercial development of an instrument called the coil-plant centrifuge by Sanki Engineering, Ltd. (Japan).

In the late 1960's, as a member of the Laboratory of Technical Development at the National Institutes of Health (Bethesda, MD), directed by Dr. Robert Bowman, Dr. Ito began a systematic program to design and evaluate an extensive series of separation devices, often in collaboration with Dr. Bowman and others at NIH. With exception of droplet countercurrent chromatography, coinvented by Drs. Tanimura and Ito (2), and locular countercurrent chromatography (3), the instruments are based on a helical coil which is acted upon by either a gravitational or centrifugally induced acceleration field. Aside from their mechanical features, the devices may be classified on the basis of the cyclic variation in the direction and intensity of this field relative to the coil. The more recently designed chromatographs incorporate an ingenious flow through

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system which permits high speed rotation of the coil without kinking of the flexible influent and effluent lines, thereby avoiding the need for an often troublesome rotating fluid seal.

The process of countercurrent chromatography is essentially liquid-liquid chromatography in which the stationary liquid bed is retained in the column by an acceleration field rather than by a solid supporting matrix. Adsorption effects are thereby eliminated. The technique is particularly advantageous in the preparative (mg to g) range for polar and labile organic compounds and bio-particulate materials such as cells and cell fragments. Virtually any two-phase solvent system, either aqueous or nonaqueous, may be employed and some apparatus is particularly useful for the two-phase aqueous polymer systems developed by Albertsson (4).

The novel contributions of Dr. Ito's research to the development of chromatography include use of the helical coil as a chromatographic column, development of flow-through systems for rotated components not requiring rotating seals, and use of acceleration fields varying cyclically in both intensity and direction as a means for obtaining rapid phase separation while promoting efficient mass transfer. The reader is encouraged to consult recent reviews for detailed discussion of the features of individual countercurrent chromatographs and of the historical development of the methodology (5,6,7).

REFERENCES

1. Ito, Y., Weinstein, M., Aoki, I., Harada, R., Kimura, E. and Nunogaki, K., The Coil Planet Centrifuge, Nature, 212, 985 (1966).

2. Tanimura, T., Pisano, J.J., Ito, Y. and Bowman, R.L., Droplet Countercurrent Chromatography, Science, 169, 54 (1970).

3. Ito, Y. and Bowman, R.L., Countercurrent Chromatography: Liquid-Liquid Partition Chromatography without Solid Support, J. Chromatogr. Sci., 8, 315 (1970).

4. Albertsson, P., Partition of Cell Particles and Macromolecules, John Wiley and Sons, New York, NY, 2nd ed., 1971.

5. Ito, Y., Countercurrent Chromatography, J. Biochem. Biophys. Methods, 5, 105 (1981).

6. Mandava, N.B., Ito, Y. and Conway, W.D., Countercurrent Chromatography. Part I. Historical Development and Early Instrumentation, Amer. Lab., 14 [10], (1982).

7. Mandava, N.B., Ito, Y. and Conway, W.D., Countercurrent Chromatography. Part II. Recent Instrumentation and Applications, Amer. Lab., 14 [11], 48 (1982).

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DROPLET COUNTERCURRENT CHROMATOGRAPHY - NEW APPLICATIONS IN NATURAL PRODUCTS CHEMISTRY

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ABSTRACT

Droplet counter-current chromatography (DCCC) has found wide application for preparative separation of plant constituents and other natural products. It is particularly indicated for the isolation of polar compounds. The use of non-aqueous solvent systems allows the separation of weakly polar substances. Large-bore columns can be employed without decreasing the resolution when the flow-rate is increased. New fields of applications include essential oils, triterpenoids and steroids, plant growth regulators (gibberellins), antibiotics.

INTRODUCTION

In order to avoid complications arising from solid supports, various support-free liquid-liquid partition techniques have been recently developed (1). Among the available counter-current separation methods, droplet counter-current chromatography (DCCC) is

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becoming increasingly popular. Originally designed by Tanimura et al. (2), it has been used by Ogihara et al. (3) and Hostettmann et al. (4) for the isolation and purification of saponins and other plant glycosides. Numerous examples of separations of various classes of natural products by DCCC have been reported and recently reviewed (5, 6).

DCCC is based on the partitioning of solutes between a steady stream of droplets of mobile phase and a column of surrounding stationary phase and is carried out as follows. A pair of immiscible phases is prepared by the equilibration of a suitable mixture of solvents. Either lighter or heavier phase may be selected as mobile phase depending on the separation problem. When the lighter phase is the mobile phase (ascending mode), the instrument formed of 200-500 vertical columns interconnected in series by capillary Teflon tubes is first filled with the stationary heavier phase. The separation proceeds by delivery of the mobile phase containing the sample to the bottom of the first column. Droplets are formed which rise to the top of the column where they are delivered through the Teflon tubing to the bottom of the next column, thus generating new droplets. Under suitable conditions only the mobile phase is allowed to flow. At the end of the series of columns the mobile phase containing the components of the mixture, separated according to their partition coefficients, is collected by a fraction collector.

Although DCCC possesses limitations arising from the fact that

the efficiency of the method depends entirely upon droplet formation, it has been extensively used for separation of polar compounds during natural products isolation. Since the publication of our review on DCCC in 1980 (5), more than sixty papers dealing with the application of this method have appeared. The present paper is not an exhaustive review on all these applications. We wish to report on new interesting developements such as the use of large-bore columns and non-aqueous solvent systems which greatly increase the versatility of the technique. In addition, some selected new fields of applications in natural products chemistry will be discussed.

Use of non-aqueous solvent systems

The solvent systems generally employed in DCCC contain water as one of the components. These solvent systems allow the separation of polar compounds, but are not indicated for weakly polar or water-sensitive substances. The production of droplets possessing suitable sizes and mobilities is difficult with non-aqueous solvent systems. Recently, Becker et al. (7, 8) developed such a system formed from methanol - nitromethane - ethylacetate - n-hexane and reported successful separations in the field of essential oils. However, this system has the disadvantage of using nitromethane which is incompatible for UV detection and may react with oxidizing materials. As basic solvents for the DCCC separations, Domon et al. (9) selected methanol - n-heptane and acetonitrile - n-heptane which form two layers. But the addition of a third solvent which is miscible with both cons-

tituents is required for (i) greater selectivity by decreasing the difference in polarity between the two layers and (ii) formation of suitable droplets by decreasing the interfacial tension of both layers. Chlorinated solvents (dichloromethane or 1,2-dichloroethane) and acetone are indicated. Several ternary solvent systems could be developed. Satisfactory results were obtained with columns possessing internal diameters of 2.7 mm and 3.4 mm, respectively. The droplet formation appears to be more difficult with small-bore columns (2 mm I.D. or less).

Table 1. Non-aqueous solvent systems for DCCC

Solvent	Separated compounds	Reference
acetonitrile : dichloromethane : n-heptane (7 : 3 : 10 v/v)	triterpenoids steroids	(9)
methanol : acetone : n-heptane (4 : 1 : 5 v/v)	depsides triterpenoids steroids	(9)
methanol : 1,2-dichloroethane : n-heptane (12 : 1 : 8 v/v)	triterpenoids	(9)
methanol : nitromethane : ethyl acetate : n-hexane (3 : 2 : 2 : 9 v/v)	essential oil of chamomile	(8)
methanol : nitromethane : ethyl acetate : n-hexane (3 : 2 : 2 : 8 v/v)	valepotriates essential oil of Echinacea angustifolia	(7)

Some typical non-aqueous DCCC solvent systems are given in Table 1. Simple ternary solvent systems form droplets with suitable sizes and mobilities. However, it should be noted, that, in general, lipophilic compounds are more easily separated by classical chromatographic methods. The interest of DCCC with non-aqueous solvents lies in the separation of weakly polar substances which are unstable in the presence of water or decompose during chromatography on silica gel.

Use of large-bore columns and influence of the flow-rate on the separation.

Most of the DCCC-separations reported until now were achieved on small-diameter glass columns (2 mm I.D.) which limited the sample loading capacity. By using small-bore columns, the choice of solvent systems is restricted as the formed droplet must have a smaller size than the internal diameter of the column. Recently, columns with 2.7 mm, 3.0 mm and 3.4 mm I.D. have become commercially available. These columns allow the use of numerous solvent systems which could not be employed previously.

We have demonstrated that an increase of the internal diameter of the columns does not necessarily result in a decrease of the resolution. The following experiments were carried out. A mixture formed of three coumarins (herniarin, scopoletin, ombelliferon) and a flavanone (hesperetin) was submitted to DCCC. Separation was achieved on 2.7 mm I.D. columns (294) at different flow rates varying from

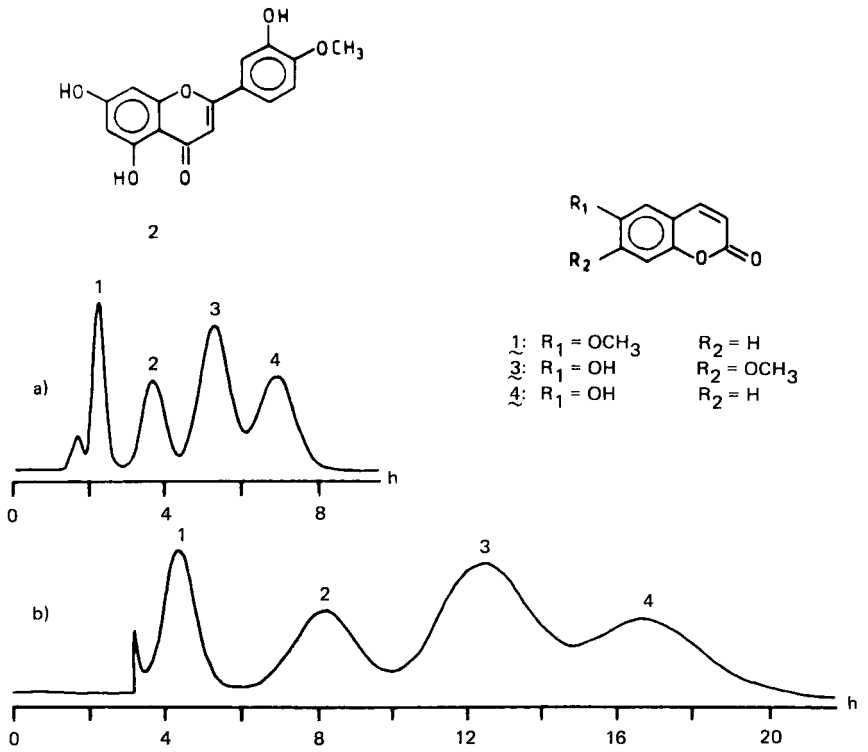
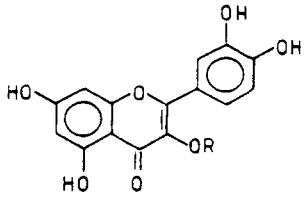


Figure 1 : Separation of hernianin 1, hesperetin 2, scopoletin 3 and ombelliferon 4 using chloroform : methanol : water (13 : 7 : 8 v/v) in the descending mode on 2.7 mm I.D. columns; eluate monitored at 254 nm.

a) at 50 ml/h b) at 18 ml/h.

15 to 80 ml/h with chloroform : methanol : water (13 : 7 : 8 v/v) in the descending mode. Figure 1 shows the results obtained at 50 ml/h and 18 ml/h.

An improvement of the separation efficiency with increasing flow rates was observed until 60 ml/h. The number of theoretical



1: R = α-L-rhamnosyl(1 6)β-D-glucosyl
2: R = β-D-galactosyl
3: R = α-L-rhamnosyl

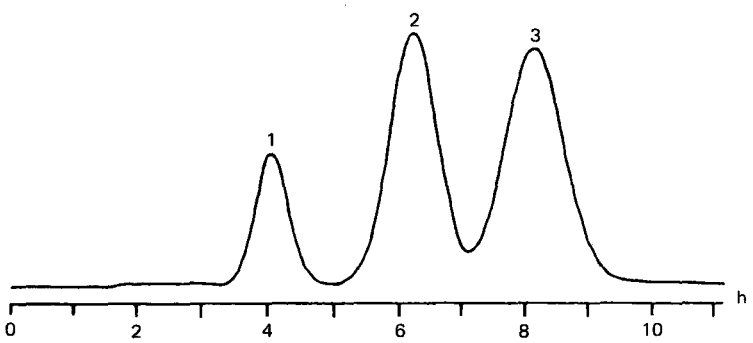


Figure 2 : Separation of rutin 1, hyperoside 2 and quercitrin 3, using chloroform : n-butanol : methanol : water (10 : 1 : 10 : 6 v/v) in the ascending mode on 2.7 mm I.D. columns at a flow rate of 48 ml/h; eluate monitored at 278 nm.

plates was improved from 120 at 18 ml/h to 240 at 60 ml/h. For higher flows no significant changes could be noted, but some stationary phase was eluted. Similar results were obtained for the separation of hesperetin and kaempferol using chloroform : methanol : water (33 : 40 : 27 v/v) in the descending mode and for the resolution of a flavonoid glycoside mixture (rutin, hyperoside, quercitrin) with chloroform : n-butanol : methanol : water (10 : 1 : 10 : 6 v/v) in the ascending mode. Figure 2 shows the glycoside separation carried out at 48 ml/h. A base-line separation is completed within 9 h.

These results can be explained by a decrease of the longitudinal diffusion and a better mass transfer between the droplets and the surrounding stationary phase when the flow rate is increased. Thus 60 ml/h seems to be an optimum flow for working with 2.7 mm I.D. columns; in such conditions the time required for a separation can be reduced to a few hours.

It should be noted that all above mentioned experiments were achieved with chlorinated solvents which have high densities and low viscosities. High flow-rate can not be obtained with very viscous solvent systems.

We have not yet studied the sample loading capacity of large-bore columns. But it appears that more than 6 g of sample can be handled in one separation process when 3.4 mm I.D. columns are employed. This has been exemplified by the isolation of pure flavonoid glycosides from a methanolic extract (6.4 g) of Lomatogonium carinthiacum (Gentianaceae) with chloroform : methanol : n-propanol : water (5 : 6 : 1 : 4 v/v) in the descending mode, using 288 columns (length 40 cm) (10).

Selected applications in natural products chemistry

Gibberellins

These diterpenes of the kaurane group are very widely used plant growth-promoting substances and are manufactured on a large scale by fermentation. Bearder and MacMillan (11) have shown that the separation of these important natural products is simpler and

more economic by DCCC than by classical chromatographic techniques. Excellent separations of various gibberellins could be achieved with dichloromethane : methanol : water (5 : 6 : 4 : v/v) or with chloroform : methanol : water (7 : 15 : 8 v/v) in the descending mode. Mixtures of gibberellin GA_3 and gibberellin GA_{13} were resolved with the solvent system chloroform : acetic acid : water (2 : 2 : 1 v/v) used in the descending mode.

Antibiotics

DCCC is of particular interest for the preparative separation of antibiotics and their metabolites. A complex mixture of various tetracyclines has been successfully analysed with chloroform : methanol : n-propanol : aq. HCl 0.01 N (9 : 12 : 1 : 8 v/v) in the ascending mode (300 columns; I.D. : 2.o mm; length 40 cm). The crude macrolide antibiotic niphithricin could be separated into niphitricin A and niphithricin B by Keller-Schierlein and coworkers (12). A sample of 628 mg of crude material afforded 37 mg of pure niphithricin B and 144 mg of pure niphithricin A.

These compounds were active against Gram-positive bacteria and fungi. The solvent system benzene : chloroform : methanol : water (15 : 15 : 23 : 7 v/v) was employed in the descending mode for the separation of the closely related antibiotics narbomycin, picromycin, methymycin and neomethymycin (13). The formula of the separated antibiotics are represented in Figure 3. A further antibiotic separation was reported recently, namely the resolution of ni-

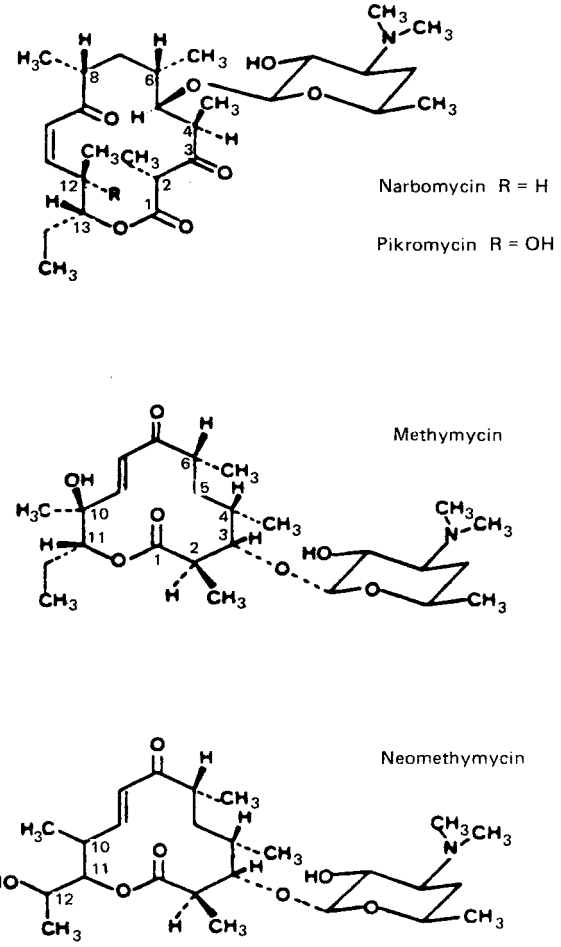


Figure 3 : Structures of macrolide antibiotics separated by DCCC with benzene : chloroform : methanol : water (15 : 15 : 23 : 7 v/v) in the descending mode (13).

phymycin I in niphymycin Iα and niphymycin Iβ with chloroform : methanol : water (35 : 65 : 40 v/v) used in the ascending mode (14).

CONCLUSION

Although DCCC possesses limitations due to the fact that its efficiency depends entirely upon droplet formation, it is increasingly applied in the field of natural products. Recent developements such as the use of large bore columns, the use of non-aqueous solvent systems and the reduction of the separation time by working at an optimum flow-rate will contribute to a wide extension of this simple counter-current method.

ACKNOWLEDGEMENTS

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REFERENCES

1. Mandava, N.B., Ito, Y. and Conway, W.D., Intern. Lab. 12 (9), 12 (1982).

2. Tanimura, T., Pisano, J.J., Ito, Y. and Bowmann, R.L., Science 169, 54 (1970).

3. Ogihara, Y., Inoue, O., Otsuka, H., Kawai, K.-I., Tanimura, T. and Shibata, S., J. Chromatogr. 128, 218 (1976).

4. Hostettmann, K., Hostettmann-Kaldas, M. and Nakanishi, K., J. Chromatogr. 170, 355 (1979).

5. Hostettmann, K., Planta med. 39, 1 (1980).

6. Hostettmann, K. in Natural Products as Medicinal Agents, Beal J.L. and Reinhard, E., ed., Hippokrates-Verlag, Stuttgart, 1981, p. 79.

7. Becker, H., Hsieh, W.Ch. and Verelis, Ch.D., Supplement Chromatographie, G.I.T., Darmstadt, 1981, p.34.

8. Becker, H., Reichling, J. and Hsieh, W.Ch., J. Chromatogr. 237, 307 (1982).

9. Domon, B., Hostettmann, M. and Hostettmann, K., J. Chromatogr. 246, 133 (1982).

10. Schaufelberger, D., Hamburger, M. and Hostettmann, K., Bull. Liais. Groupe Polyphénols (Narbonne), 11., (1983), in press.

11. Bearder, J.R. and MacMillan, J. in Gibberellins-Chemistry, Physiology and Use, Monograph 5, Lenton, J.R., ed., British Plant Growth Regulator Group, Wantage, 1980, p. 25.

12. Fiedler, H.-P., Wörner, W., Zähner, H., Kaiser, H.P., Keller-Schierlein, W. and Müller, A., J. Antibiotics 34, 1107 (1981).

13. Kaiser-Keller, H.P., PH.D. Thesis (Diss. ETH Nr. 67 74), ETH, Zürich, 1981, p. 144.

14. Bassi, L., Joss, B., Gassmann, P., Kaiser, H.P. Leuenberger, H. and Keller-Schierlein, W., Helv. Chim. Acta 66, 92 (1983).

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APPLICATIONS OF ROTATION LOCULAR COUNTERCURRENT CHROMATOGRAPHY IN NATURAL PRODUCTS ISOLATION

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ABSTRACT

Purification of natural products and the fractionation of crude plant extracts are processes which traditionally involve adsorption and/or molecular exclusion chromatography. While countercurrent chromatography avoids the irreversible adsorption and decomposition frequently encountered in adsorption chromatography, the classical countercurrent distribution technique is both time- and solvent-consuming, consequently it is of limited value today. Rotation locular countercurrent chromatography (RLCC) is one of three new countercurrent techniques, the others being droplet contercurrent chromatography (DCCC)[1] and planet coil countercurrent chromatography (PCCCC)[2], which have revived the chromatographic application of liquid-liquid partition. We have employed a commercially available RLCC apparatus in the isolation of several natural products with a broad range of polarity demonstrating the versatility of the RLCC with regard to the functionality of the compounds to be isolated.

INTRODUCTION

Rotation locular countercurrent chromatography is a technique originally proposed by Signer et al.[3] and later developed by several groups.[4-9] As a countercurrent technique, RLCC has the advantages of complete recovery of all material (i.e., no irre-

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versible adsorption) and no danger of decomposition catalyzed by a solid support or compounds adsorbed on the solid support. Furthermore, RLCC requires only a biphasic solvent system for partitioning, difficulties such as emulsion formation or an inability to form droplets are avoided with this technique.

These characteristics render RLCC suitable for the fractionation of crude plant extracts and the purification of natural products. We have applied RLCC to the facile isolation of plant and bacterial glycosides as well as flavonoid aglycones whose isolations were extremely tedious using other methods. The fractionation of a crude plant extract (Daucus carota L.) possessing plant growth regulatory activity was routinely performed with no loss of material. Finally, resolution of racemic norephedrine as its ammonium salt with a lipophilic anion, hexafluorophosphate, was also achieved employing a partitioning between an aqueous phase and a lipophilic phase containing (R,R)-di-5-nonyltartrate.[10]

APPARATUS AND MECHANISM

The RLCC apparatus (Tokyo Rikakikai Co., Tokyo, Japan) consists of sixteen glass columns (50 cm X 11 mm i.d.) mounted cylindrically about a rotational axis. Each column is divided into 37 compartments or loculi by teflon disks with a hole in the center to allow solvent flow between the compartments. The columns are connected in series with teflon tubing (1 mm i.d.). Solvent is applied via a high pressure, constant flow pump. After selecting the solvent system and determining the mode (ascending or descending, see below), the solvent serving as the stationary phase is loaded into the machine, completely expelling all air bubbles. Upon loading of the stationary phase, the columns are inclined to a 20-40° angle with the horizontal. When using the ascending mode, as in Figure 1 for the n-PrOH/n-BuOH/H_2O (1/2/3) system, the lighter mobile alcohol phase is

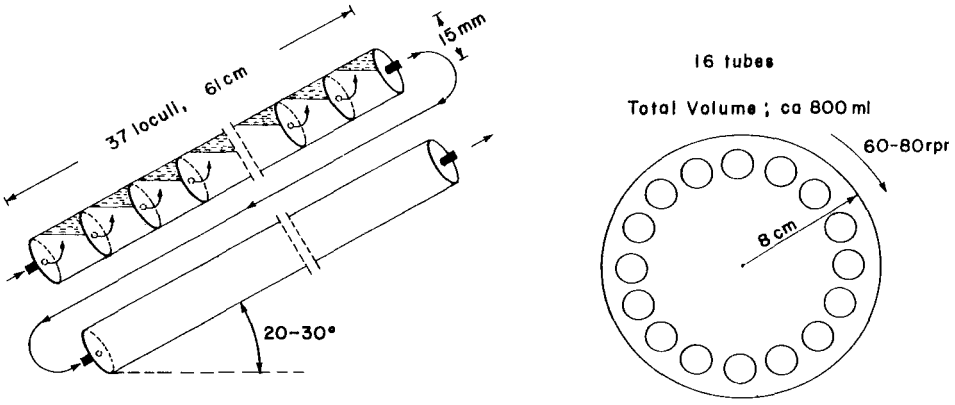


FIGURE 1

RLCC Ascending Mode. Example: n-PrOH/n-BuOH/H_2O (1/2/3); Stationary Phase: Lower Layer; Mobile Phase: Upper Layer.

applied to the bottom of the first column at a rate of 15-25 ml/hr while the columns rotate about the central axis at a speed of 60-80 rpm. Passing through the initial perforation, the alcohol phase rises to the uppermost corner of the first loculus, displacing stationary phase as its volume increases until it attains the level of the hole in the disk leading to the next loculus. The alcohol passes through this hole and enters the second loculus which is slightly elevated from the first. This process continues through all the loculi in the column until the mobile phase emerges from the uppermost loculus and is directed to the bottom of the next column in the series by the teflon tubing.

When the apparatus is charged with mobile phase, the sample, dissolved in the mobile phase, is loaded into a teflon sample loop (volume 4 ml) with syringe suction and subsequently transferred to the first column. Within each loculus the sample is partitioned between the mobile and stationary phases, and eluted

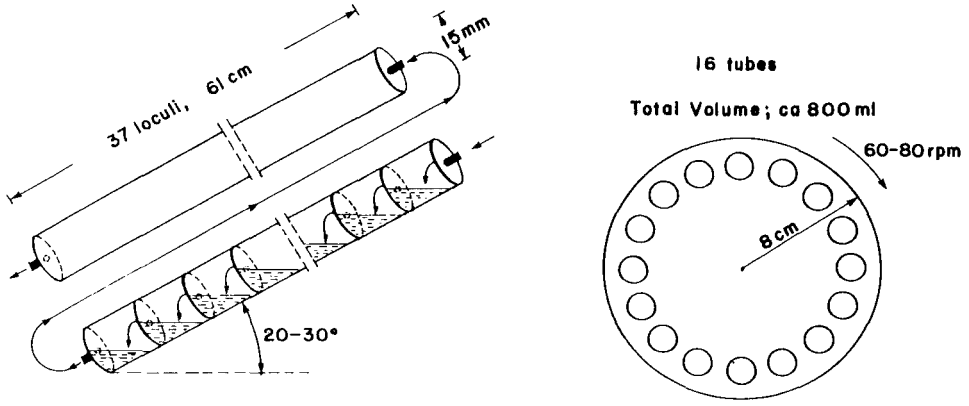


FIGURE 2

RLCC Descending Mode. Example: $CHCl_3/MeOH/H_2O$ (35/65/45); Stationary Phase: Upper Layer; Mobile Phase: Lower Layer.

through each of the columns. Any sample which remains in the stationary phase and not completely eluted from the apparatus can be displaced by flushing the columns with excess stationary phase.

The rotation of the columns serves to renew the contact surface between the two phases, therefore aiding in equilibration, though not allowing emulsions to form due to excessive agitation. The RLCC apparatus has 250-300 theoretical plates, thus the partitioning in each loculus does not represent a complete equlibration.

When the descending mode is selected for separation, as in Figure 2 for the $CHCl_3/CH_3OH/H_2O$ (35/65/45) system, the operation remains the same except that the heavier mobile phase is applied at the top of each column and descends through each loculus.

SELECTION OF THE SOLVENT SYSTEM

A preliminary screening of appropiate biphasic systems using silica gel TLC plates was employed. The TLC's of the sample

using each of the two phases were developed; those systems giving R_f values greater than 0.8 in one of the phases and between 0.2 and 0.4 in the other were potentially applicable systems.

Final selection was based on the distribution of 5 to 10 mg of sample between 5 to 10 ml of each of the the two phases. A system in which 15-25% of the sample was distributed in one of the phases is chosen. The phase which contained the higher percentage of sample functions as the stationary phase. Such systems usually enable the chromatography to be completed within 36 hrs prior to displacement of residual compounds dissolved in the stationary phase. If the equilibration gave a more equal distribution of the sample, the chromatography was completed in less time, but with less resolution. If the equilibration gave a less equal distribution (<15% in one of the phases), the time factor and hence the amount of solvent required increased significantly.

EXPERIMENTAL

All solvents were spectral grade or distilled prior to use. Biphasic solvent systems were prepared in a 2 l. separatory funnel and allowed to equlibrate overnight prior to separation of the phases. Following elution from the apparatus, the eluant was collected in ten ml fractions with an automatic fraction collector and analyzed by TLC or UV-spectrophotometry. Ultraviolet spectra were recorded on a UVIKON 810 or a JASCO UVIDEC-505 spectrometer. Circular dichroism were recorded on a Jobin-Yvon III Dichrograph.

Separation of flavonoid Aglycones

A mixture of approximately 30 mg each of hesperetin $\underset{\sim}{1}$, kaempferol $\underset{\sim}{2}$, and quercetine $\underset{\sim}{3}$ was prepared and applied to the RLCC. Solvent system: $CHCl_3/MeOH/H_2O$ (33/40/27); mobile phase: lower layer; flow rate: 48 ml/hr; detection: UV 280 nm. The results are shown in Figure 3.

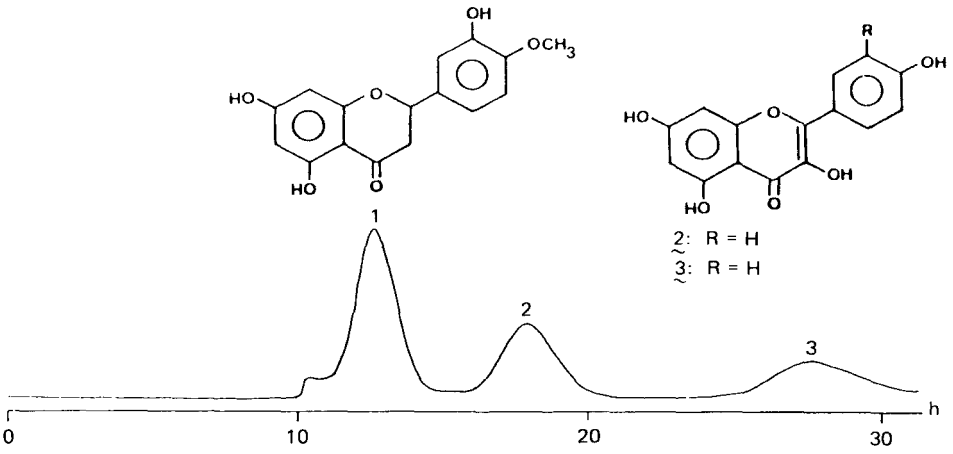


FIGURE 3

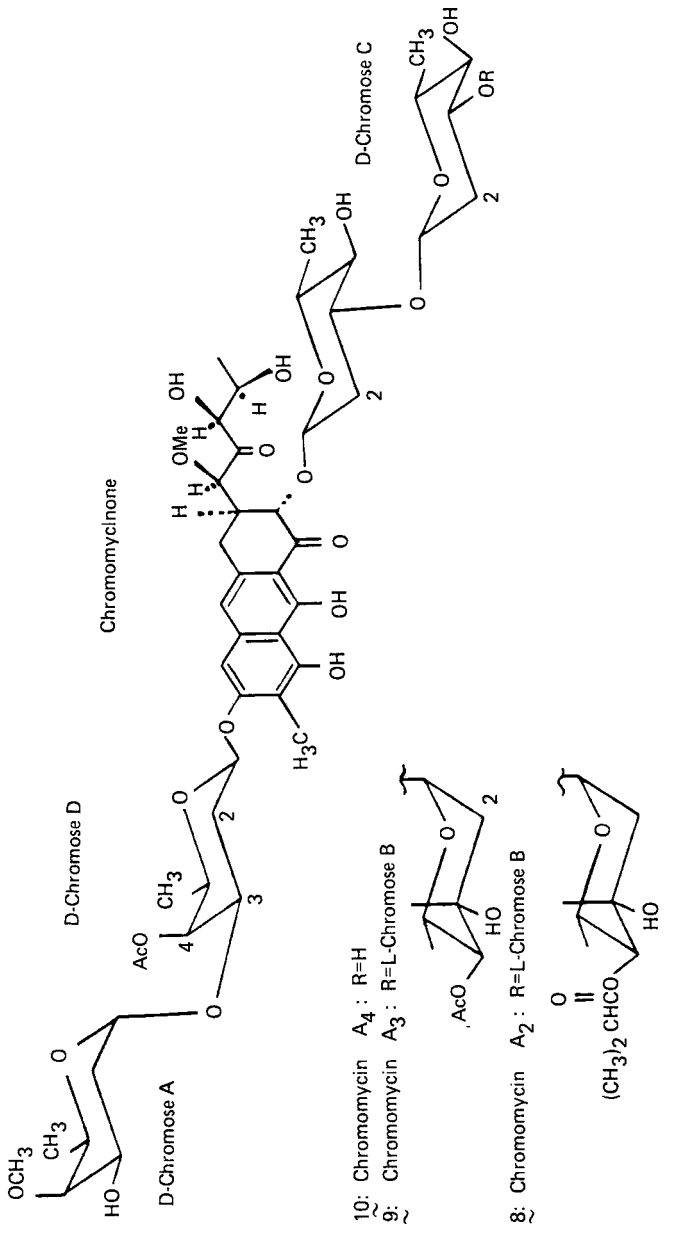
Separation of Flavonoid Aglycones. Solvent System: $CHCl_3$/MeOH/H_2O (33/40/27); Mobile Phase: Lower Layer.

Separation of Swertia perennis L. (Gentianaceae) Constituents

The crude methanol extract of the leaves and stems of Swertia perennis L. was chromatographed on a Sephadex LH-20 column (methanol).[11] The fraction (100 mg) containing three flavonoid C-glycosides 4, 5, and 6, and a xanthone 7, was applied to the RLCC. Solvent system: AcOEt/n-PrOH/H_2O (40/20/70); mobile phase: lower layer; flow rate: 48 ml/hr; detection: UV 280 nm. The results are shown in Figure 4.

Separation of Chromomycins A_2, A_3 and A_4, 8, 9 and 10, Respectively

The crude chromomycin extract[12] (700 mg) was separated using a $CHCl_3$/AcOEt/MeOH/H_2O (2/4/2/1) solvent system as shown in Figure 5. In this solvent system, 79% of the extract equilibrated in the lower phase, hence the ascending mode was employed. Mobile phase: upper phase; flow rate: 30 ml/hr; detection: TLC (silica gel, 1% oxalic acid in AcOEt). Increasing the charge to 5g of the extract gave the same results.


Chromomycinone
D-Chromose A
D-Chromose D
D-Chromose C
10: Chromomycin A4 : R=H
9: Chromomycin A3 : R=L-Chromose B
8: Chromomycin A2 : R=L-Chromose B

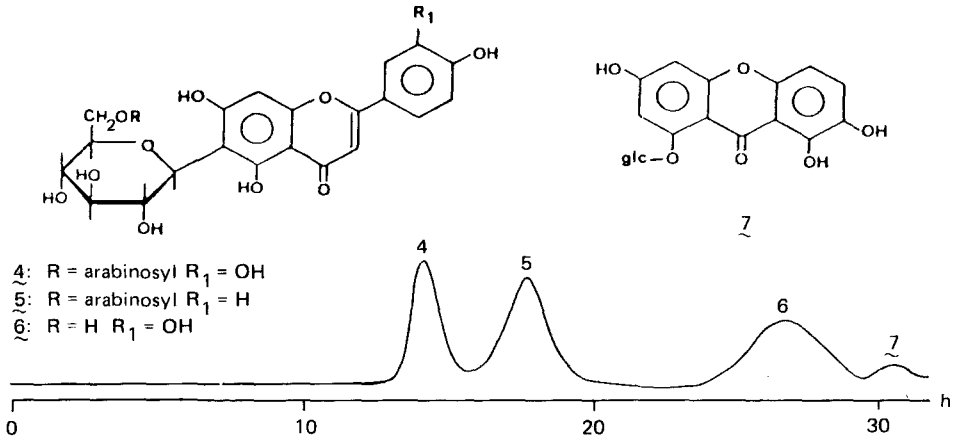


FIGURE 4

Separation of Swertia perennis L. (Gentianaceae) Constitutents. Solvent System: AcOEt/N-PrOH/H_2O (40/20/70); Mobile Phase: Lower Layer.

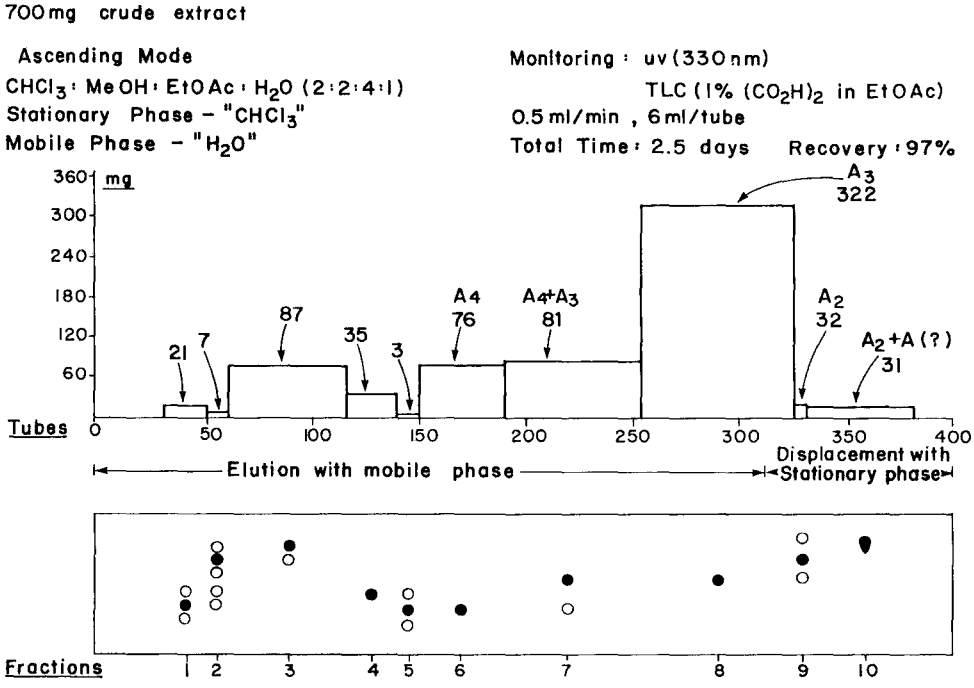


FIGURE 5

Separation of Chromomycins.

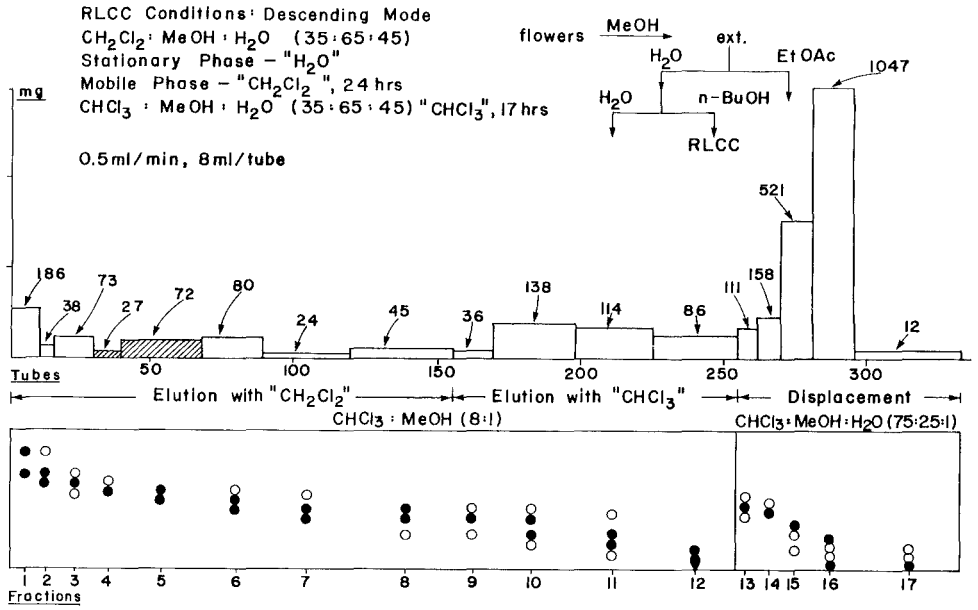


FIGURE 6

Isolation of Plant Growth Regulator from Daucus carota L., Shaded Areas are Active Fractions.

Fractionation of Extract of Daucus carota L. Flowers

The crude methanol extract of the fresh flowers of wild carrot or Queen Anne's lace, Daucus carota L., (Ammiaceae) which showed plant growth regulatory activity, was partitioned between water and ethyl acetate, and the residue from the active, aqueous layer was subsequently partitoned between water and n-butanol. The residue from the n-butanol fraction contained the biologically active compound and was applied to the RLCC as shown in Figure 6. Due to the large number of compounds in this fraction as shown by TLC, the elution of the crude fraction from the aqueous stationary phase was carried out with two separate organic phases using the descending mode. Initially a CH_2Cl_2/MeOH/H_2O

(35/65/45) was applied. In this system, the sample was only 10% partitioned into the lower layer, but TLC had shown that a major component would be quite mobile in this layer. The aqueous layer was eluted for 24 hrs with the lower layer of this system. Following this elution, the stationary phase was then eluted with the lower layer of a $CHCl_3$/MeOH/H_2O (35/65/45) system. In this second biphasic system, the sample was 20% partitioned into the lower layer. Elution with the lower phase of the chloroform containing system was continued for 17 hrs prior to displacement of residual compounds dissolved in the stationary phase using the aqueous layer of the second solvent system. These results are shown in Figure 6.

Resolution of Racemic Norephedrine

The resolution of racemic norephedrine employing the RLCC has been previously described.[10]

RESULTS AND DISCUSSION

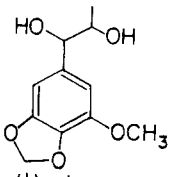
The separation of the flavonoid aglycones, Figure 3, and the Swertia perennis L. constituents, Figure 4, proved to be routine. In the case of the Swertia perennis L. constituents, the RLCC separation represents a notable improvement over a time-consuming polyamide column chromatography employing a water-methanol gradient originally used in the purification of these compounds.[11] Furthermore, the solvent system employed in this isolation could not be used on the DCCC as it would not form droplets.

The chromomycins, 8-10, are clinically used antitumor antibiotics isolated from Streptomyces griseus No. 7 culture broth; the structure of chromomycin A_1 is still undetermined.[13,14] While countercurrent chromatography appeared to be ideally suited for the purification of these compounds, the non-polar sugars of the chromomycins rendered the solvent systems employed on the DCCC ineffective. Moreover, a considerable amount of the extract

had to be separated in order to obtain the desired quantity of the chromomycins.

Using the solvent system as shown in Figure 5, the chromomycins were readily separated in gram quantities. This purification was a great simplification over the original isolation which used chromatography on silica gel employing a 1% oxalic acid solution in ethyl acetate.[12] The chromomycins were readily separated from early eluting impurities which had caused difficulties in the original isolation due to similar R_f values in adsorption chromatography. Impressively, 9 and 10 were obtained in pure states while 8, which differs from 9 only in the nature of the acid group esterified to the 4-OH group of the terminal chromose B sugar (8 is esterified with isobutyric acid while 9 is esterified with acetic acid), was obtained in a nearly pure state.

The crude methanol extract of the fresh flowers of wild carrot or Queen Anne's lace, Daucus carota L. (Ammiaceae), exhibited plant growth regulatory activity in the lettuce seed germination bioassay. This bioassay was then used as a guide for the isolation of the active compound. Final fractionation of the n-butanol fraction on the RLCC led to the isolation of two active fractions, Figure 6 fractions 4 and 5. Final purification of the active compound on C-18 reverse phase HPLC, MeOH/H_2O (25/75) lead to the phenylpropanoid 11.[15]

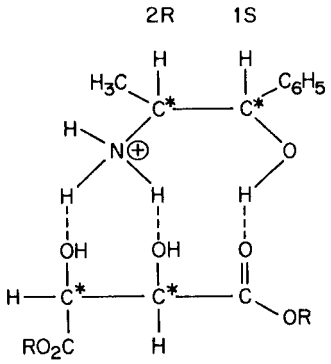


(±) - threo

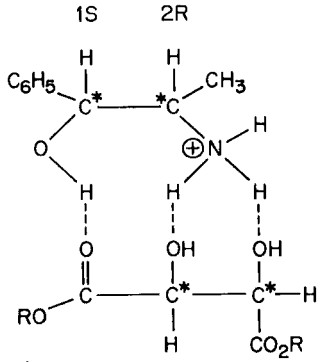
11

This application of RLCC illustrates the significant promise for the fractionation of polar components of crude natural product extracts. These compounds are traditonally troublesome to fractionate via adsorption chromatography due to irreversible adsorption, decomposition, and severe tailing. The utilization of RLCC provides a stepping stone to ultimate purifications with HPLC or another countercurrent technique such as DCCC.

The final application of RLCC described in this work, the resolution of racemic norephedrine, is based on the preliminary, elegant work of Prelog et al.[16] who resolved racemic α-amino-alcohols as their salts with lipophilic anions by partition between an aqueous phase and a lipophilic phase, 1,2-dichloro-ethane, containing (R,R)-di-5-nonyltartrate. In this work, the stationary aqueous phase was adsorbed on a Kieselguhr support. For application of RLCC, the stationary phase employed was a 0.5M sodium hexafluorophosphate solution, pH adjusted to 4 with HCl, and the mobile phase was a 0.3M solution of (R,R)-di-5-nonyl-tartrate in 1,2-dichloroethane.[10] Prelog has suggested that the racemic norephedrine forms diastereotopic complexes 12 and 13 with the (R,R)-5-nonyltartrate esters which have different partitioning properties. While baseline separation of the enantiomers



12



13

R = 5 – nonyl

was not achieved, practically pure enantiomers ($\geq$ 95%) were obtained.

CONCLUSIONS

Applications of countercurrent chromatography, especially with regard to polar extracts, can be much more suitable than column chromatography due to the high recovery of material. When elution with the mobile phase is completed, the material remaining in the stationary phase can be conveniently recovered by flushing with excess stationary phase. Moreover, countercurrent chromatography avoids the hazards of decomposition and severe tailing frequently encountered when using adsorption chromatography. Equally important is the advantage of partitioning behavior versus adsorption behavior. Thus, compounds which may not be separated via adsorption chromatography due to similar adsorption behavior may be routinely separated via partitioning, as in the case of the chromomycins.

Rotation locular countercurrent chromatography complements the recently developed DCCC and reverse phase HPLC. While RLCC has only 250-300 theoretical plates compared to approximately 1,000 for DCCC, RLCC is more practical for the fractionation of crude extracts as gram quantities can be loaded and separated with the selection of the solvent system restricted only by the requirement of being biphasic and not by the limitations of droplet formation.[17] Furthermore, application of gradient systems as well as a wider variety of non-aqueous systems are possible on RLCC.

ACKNOWLEDGMENTS

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REFERENCES

1. Hostettmann, K., Hostettmann-Kaldas, M., and Nakanishi, K. J. Chromatog., 170, 355, 1979 and references therein.

2. Ito, Y. J. Biochem. Biophys. Methods, 5, 105, 1981 and references therein.

3. Signer, R., Allemann, K., Koehli, E., Lehmann, W., Mayer, H. and Ritschard, W. Dechema Monograph., 27, 36, 1956.

4. Michaelis, W., Schindler, O. and Signer, R. Helv. Chim. Acta, 49, 42, 1966.

5. Arm, H., Grob, E. C. and Signer, R. Helv. Chim. Acta, 49, 851, 1966.

6. Signer, R. and Arm, H. Helv. Chim. Acta, 50, 46, 1967.

7. Winistorfer, P. and Kovats, E. sz. J. Gas Chromatog., 362, 1967.

8. Ito, Y. and Bowman, R. L. J. Chromatog. Sci., 8, 315, 1980.

9. Grob, E. C., Pfander, H., Leuenberger, U. and Signer, R. Chimia, 25, 322, 1971.

10. Domon, D., Hostettmann, K., Kovacevic, K. and Prelog, V. J.Chromatog., 250, 149, 1982.

11. Hostettmann, K. and Jacot-Guillarmod, A. Helv. Chim. Acta, 59, 1584, 1976.

12. Miyamoto, M., Kawamatsu, Y., Kawashima, K., Shinohara, M. and Nakanishi, K. Tetrahedron Lett., 2355, 1964; Miyamoto, M., Morita, K., Kawamatsu, Y., Kawashima, K. and Nakanishi, K. Tetrahedron, 23, 421, 1967.

13. Riccio, R. and Nakanishi, K. J. Org. Chem., 47, 4589, 1982.

14. For recent ^{1}H-NMR studies of oligosaccharide moiety of chromomycin A_3, see: Thiem, J. and Meyer, B. J., J. Chem. Soc., Perkin Trans. 2, 1331, 1979.

15. Snyder, J., manuscript in preparation

16. Prelog, V., Stojanac, Z. and Kovacevic, K. Helv. Chim. Acta, 65, 377, 1982.

17. Gram quantities may also be applied to the DCCC; with large bore columns (4.2 mm i.d.) loading capacities up to 8 g have been realized. K. Hostettmann, personal communication.

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IMPROVED SCHEME FOR PREPARATIVE COUNTERCURRENT CHROMATOGRAPHY (CCC) WITH A ROTATING COIL ASSEMBLY

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ABSTRACT

Efforts have been successfully made to improve a preparative CCC scheme utilizing a slowly rotating coil assembly by optimizing the orientation of the coiled column. By the aid of a standard set of test samples and a two-phase solvent system, the performance of the single coil was examined in both eccentric and coaxial orientations with respect to the axis of rotation. In the eccentric location 10 cm away from the rotation axis, changes in the skew angle and/or inclination of the apparatus failed to improve the separation significantly. On the other hand, the coils mounted coaxially around the rotation axis in various helical diameters all produced excellent peak resolution at critical rotational speeds due to a high level of stationary phase retention. This finding facilitated the development of a new separation column consisting of multiple layers of the coil coaxially arranged around the rotary support. The preparative capability of this multi-layer coil was demonstrated in the separations of lg-quantity samples with satisfactory results. The present scheme is amenable to be further scaled up for industrial applications.

INTRODUCTION

Countercurrent chromatography (CCC) has an advantage over liquid chromatography in that it eliminates complications arising from the use of solid supports (1). In the past preparative CCC has

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been performed with a slowly rotating coil assembly, which holds coiled columns in the eccentric positions around the horizontal axis of rotation (2,3). Recently, efforts have been made to increase the sample-loading capacity of the scheme by the use of larger-bore coils similarly arranged around the rotary shaft (4).

This paper describes the continued development of this preparative CCC scheme which involves changing the orientation of the coiled column. Experiments were performed to test both eccentric and coaxial locations of the coil on the rotary shaft. In the eccentric orientation of the coil, changing the column angle relative to the rotary shaft and/or tilting the device against the horizontal plane was found to give only slightly improved results. On the other hand, the same column mounted coaxially around the rotary shaft produced excellent peak resolution with an extremely high level of stationary phase retention. In this coaxially rotated coil, increasing the helical diameter from 3 cm to 20 cm showed little difference in peak resolution. This new finding led to the development of an efficient separation column which consists of multiple layers of coil mounted concentrically around the rotary shaft. The performance of these columns was evaluated in the separation of a standard set of DNP (dinitrophenyl) amino acid samples and a two-phase solvent system composed of chloroform, acetic acid and 0.1N hydrochloric acid at a 2:2:1 volume ratio.

PRINCIPLE

The principle of CCC with a slowly rotating coil has been described earlier (1-4). When a water-filled coil is held horizontal and slowly rotated around its own axis, any object either heavier or lighter than the water moves toward one end of the coil. This end is called the head and the other end, the tail of the coil. When such a coil contains two immiscible solvents, slow rotation soon establishes a hydrodynamic equilibrium between the two solvent phases in which the head side of the coil is occupied by nearly

equal amounts of the two phases and any excess of either phase is found at the tail end of the coil. Under this hydrodynamic equilibrium condition, the coil can be eluted with one of the phases through the head end while retaining the other phase stationary in the coil. Consequently, solutes locally introduced at the head of the coil are subjected to an efficient partition process between the mobile and stationary phases and are chromatographically separated according to their partition coefficients in the absence of solid supports. The eluate eluted through the tail end of the coil is continuously monitored for its absorbance and then fractionated as in liquid chromatography.

Peak resolution produced by this CCC scheme is greatly influenced by the volume of the stationary phase retained in the coil, i.e., the higher the retention level, the better the result. It has been observed that the retention of the stationary phase is quite sensitive to the orientation and rotational speed of the coil (5). In the coaxially rotated coil, slow rotation usually yields near 50% retention. Increasing the rotational speed of the coil radically changes the hydrodynamic equilibrium volume ratio of the two phases in the coil and, in some critical range, one of the phases almost entirely occupies the head end and the other phase, the tail end of the coil. This equilibrium condition permits a high level of stationary phase retention if the mobile phase is introduced in the proper direction (5). In the eccentric orientation of the coil, the centrifugal force field induced by the rotation tends to trap the heavier phase in the outer half and the lighter phase in the inner half of each helical turn resulting in a more or less even distribution of the two phases throughout the coil and, therefore, the retention of the stationary phase becomes rather insensitive to the rotational rate of the coil. Thus eccentrically rotated coils tend to retain the stationary phase no more than 50% of the column volume unless the axis is inclined against the horizontal plane (2,3). While hydrodynamic behavior of the solvents in the rotating coil are highly complex and difficult to predict, the optimum coil orientation is easily determined by a series of

experiments with a standard set of samples and a two-phase solvent system.

MATERIALS AND METHOD

Apparatus

Two types of rotary devices with comparable functions were employed. The first device was equipped with a rotary seal at each terminal of the rotary shaft to establish a flow-through system (2,3). In the present experiment a clamp rod was mounted on the rotary shaft to support a coiled column at an eccentric position 10 cm away from the axis of rotation. The coil was positioned at a desired skew angle while the rotary shaft was set at a desired incline against the horizontal plane (See Fig. 1, left). The second rotary device was equipped with a rotating-seal-free flow-through system similar to that reported earlier (4). In this device, the coil was coaxially mounted around the rotary shaft at various helical diameters over a spool-shaped support. In both rotary devices, rotational speed is continuously adjustable up to 400 rpm.

Coiled Column

Both glass and plastic coils were tested at the eccentric location. The glass coils (Kontes Scientific Glass Co., Vineland, NJ) consisted of 45 helical turns of 0.5 cm i.d., 3 cm helical diameter with a total capacity of about 90 ml. The plastic coil was prepared from a piece of 0.55 cm i.d., 420 cm long FEP (fluorinated ethylene propylene) tubing (Galtek Corp., Jonathan Ind. Ctr., Chaska, MN) by winding it on to a 2.5 cm o.d. aluminum pipe core to make about 45 helical turns with a total capacity of about 100 ml. The same plastic tubing was used to test the coaxial orientation on the second rotary device in which the tube was wound around the spool-shaped support to make a single layer coil with helical

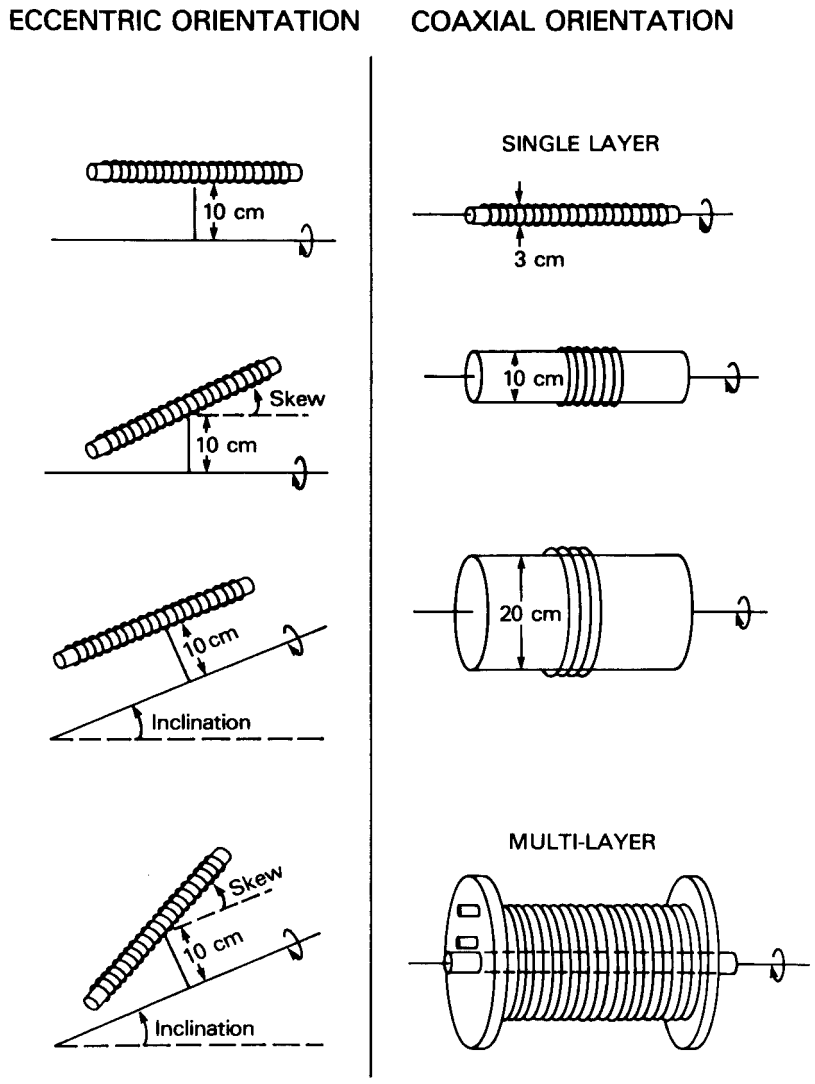

FIGURE 1. Various orientations and configurations of the coiled separation columns.

diameters of 3 cm, 10 cm and 20 cm (see Fig. 1, right top). For preparative separations, a multi-layer coil (Fig. 1, right bottom) was prepared from a 30 m long FEP tube of the same type by winding it coaxially over a 10 cm diameter, 25 cm wide spool support to make nearly 3 layers of the coil with a total capacity of about 750 ml. Each terminal of all these coiled columns was connected to 0.85 mm i.d. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ) for continuous elution.

Solvent System and Sample Solution

The two-phase solvent system used in the present study consisted of chloroform (Burdick and Jackson Laboratories, Inc., NJ), glacial acetic acid and 0.1N hydrochloric acid (Fisher Scientific Co., Fairlawn, NJ) at a volume ratio of 2:2:1. The solvent mixture was equilibrated in a separatory funnel at room temperature and separated before use.

N-2,4-DNP-D,L-glutamic acid and N-2,4-DNP-L-alanine (Sigma Chemical Co., St. Louis, MO) were selected as test samples. For comparative studies with short columns, the sample solution was prepared by dissolving the DNP amino acid mixture in the upper aqueous phase to make the concentration of each component 0.5g%, and 0.5 ml was used for each separation. For preparative-scale separations with the multi-layer coil, the sample solution was prepared by dissolving 500 mg of each DNP amino acid for a total of 1g in 30 ml of the solvent, consisting of equal amounts of the upper and lower phases.

Measurement of Phase Distribution

The distribution of the two solvent phases in the rotating coil was studied with a 0.55 cm i.d. FEP coiled tube coaxially mounted on the rotary support in helical diameters of 3 cm, 10 cm and 20 cm.

In order to facilitate observation, the lower nonaqueous phase was colored with Sudan III. The column was first filled with equal volumes of the upper and lower phases and both inlet and outlet tubes were clamped. Then the apparatus was rotated at a given rate until the two solvent phases established the hydrodynamic equilibrium, in which one of the phases predominantly occupied the head side leaving the excess volume of the other phase at the tail side of the coil. Upon stopping the rotation, the number of helical turns, n, containing the predominant phase was noted. From the total number of helical turns, N, the percentage volume occupied by the predominant phase in the equilibrated coil was calculated from the expression, 50N/n. Alternatively, for a coil with large helical diameters, the length of the segment of each phase occupying in one helical turn was directly measured to obtain percentage figures for each phase. The above procedure was repeated without renewing the column contents while changing the rotational speed of the coil to obtain a series of measurements for each helical diameter of the coil.

Separation procedure

For comparative studies with short columns, the separations of the DNP amino acids were performed as follows: The column was first filled with the stationary phase. This was followed by injection of the 0.5 ml sample solution containing 5 mg of DNP amino acid mixture through the sample port which was located on the flow line between the outlet of the pump and the inlet of the coil. Then the coil was rotated at a given rate while the mobile phase was introduced through the coil at a rate of 120 ml/h with a Milton Roy Minipump or a Chromatronix Cheminert pump. Although the elution was usually performed from the head of the coil towards the tail, the reversed elution mode (tail to head) was also applied to the coils with the coaxial orientation. The eluate through the outlet of the coil was continuously monitored for absorbance at 280 nm with an LKB Uvicord

S and LKB recorder to obtain elution profiles of the samples. After the separation was completed, the apparatus was stopped and, by connecting the inlet of the coil to a pressured N_2 line (50 psi), the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the coil. During the process of filling the column with the solvent or emptying the column contents with N_2, the coil was slowly rotated in a reversed mode (tail to head) to eliminate trapped air bubbles or remaining solvent from the coil.

Preparative-scale separations were performed with a multi-layer coil mounted coaxially on the rotary support. The column was similarly filled with the stationary phase under the reversed mode of slow rotation to eliminate trapped air bubbles from the coil. After the filling process was completed, the coil was rotated at the optimum rate of 80 rpm in the desired direction, while the sample solution, 30 ml in volume containing 1g DNP amino acid mixture as described above, was injected into the rotating coil through the sample port at a rate of 4 to 5 ml per minute. During the injection, the sample-loading syringe was kept substantially in the horizontal position so that both phases would be evenly introduced into the stream. Then the column was eluted with the mobile phase at a rate of 120 ml/h with a Milton Roy Minipump. Both the upper and the lower phases were used as the mobile phase at the identical operational conditions except that the upper phase was eluted in the normal mode (head to tail) and the lower phase in the reversed mode (tail to head). The eluates through the outlet of the coil were collected with an LKB fraction collector to obtain a 12-ml fraction in each test tube. A 20µl volume of each fraction was then mixed with 3 ml of methanol to measure the absorbance at 430 nm with a Beckman DU spectrophotometer. After the separation, the volume of the retained stationary phase was measured by emptying the column contents into a graduated cylinder by means of N_2 pressure and reversed slow rotation as described earlier.

RESULTS AND DISCUSSION

Eccentric Orientation of the Coil.

The performance of the short coils mounted 10 cm away from the axis of rotation was studied by varying both skew angle and inclination. The DNP amino acid separations obtained using glass and FEP coils are summarized in Fig. 2 where individual charts are arranged according to the applied operational conditions of coil orientation, choice of the stationary phase and the rotational speed.

In the first experimental group with the glass coil (Fig. 2A), the 0° skew, 0° inclination condition used in the previous studies (4) produced almost identical results (left column). These separations are considerably improved by changing either skew or inclination angle. The best peak resolutions are found in 0° skew, 15° inclination at 40 rpm for both nonaqueous and aqueous stationary phase groups (middle column). In the second experimental group with the FEP coiled tube (Fig. 2B), the best separations are observed at 40 rpm for the stationary nonaqueous phase and 60 rpm for the stationary aqueous phase, both under 0° skew and 0° inclination (left column). Application of either 15° skew or 15° inclination failed to give any appreciable improvement in the peak resolution. Although skew and inclination produce some significant improvement of the performance of the glass coil, the FEP coiled tube positioned at 0° skew and 0° inclination yields the best peak resolutions among all experimental groups.

Coaxial Orientation of the Coil

The FEP coiled tube mounted eccentrically for the above studies was uncoiled and rewound coaxially around the rotary shaft in three helical diameters of 3 cm, 10 cm and 20 cm to investigate the hydrodynamic behavior of the two solvent phases and the partition efficiency at various rotational speeds.

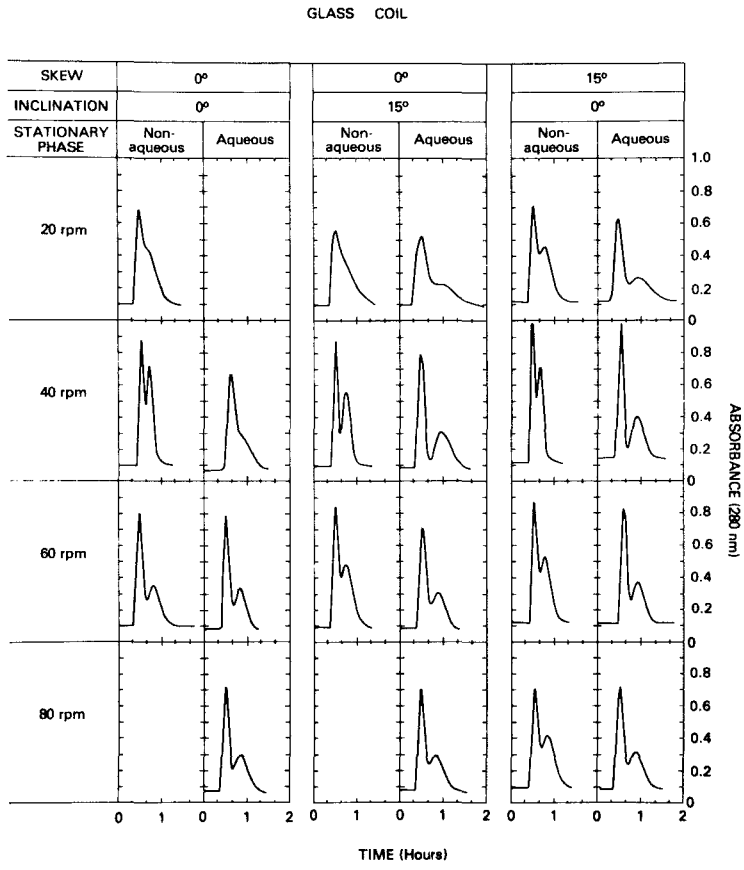

FIGURE 2. Effects of skew angle and inclination of the eccentrically rotated coil on the separations of DNP amino acids at various rotational speeds.

A. Glass Coil.

B. FEP Coil.

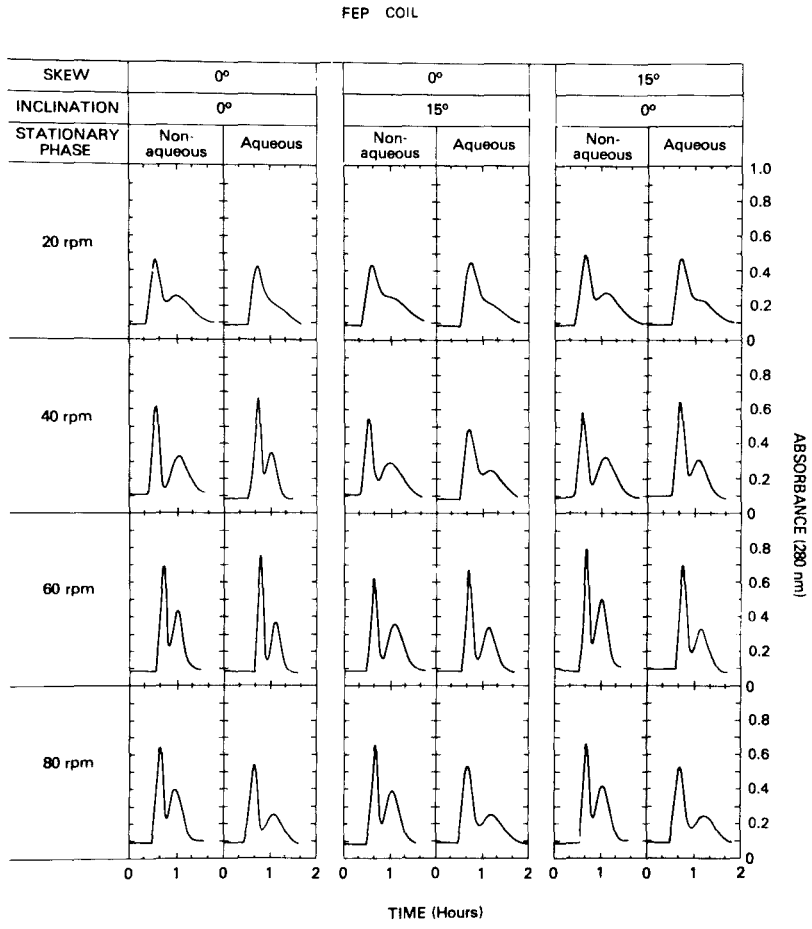

FIGURE 2B

The distribution of the two phases in the coaxially rotated coils measured at their hydrodynamic equilibrium is shown in Fig. 3. In each diagram, the volume percentage of each phase at the head side of the coil is plotted against the applied rotational speed. The three diagrams obtained from different helical diameters show common features characteristic of the coaxial orientation. In the

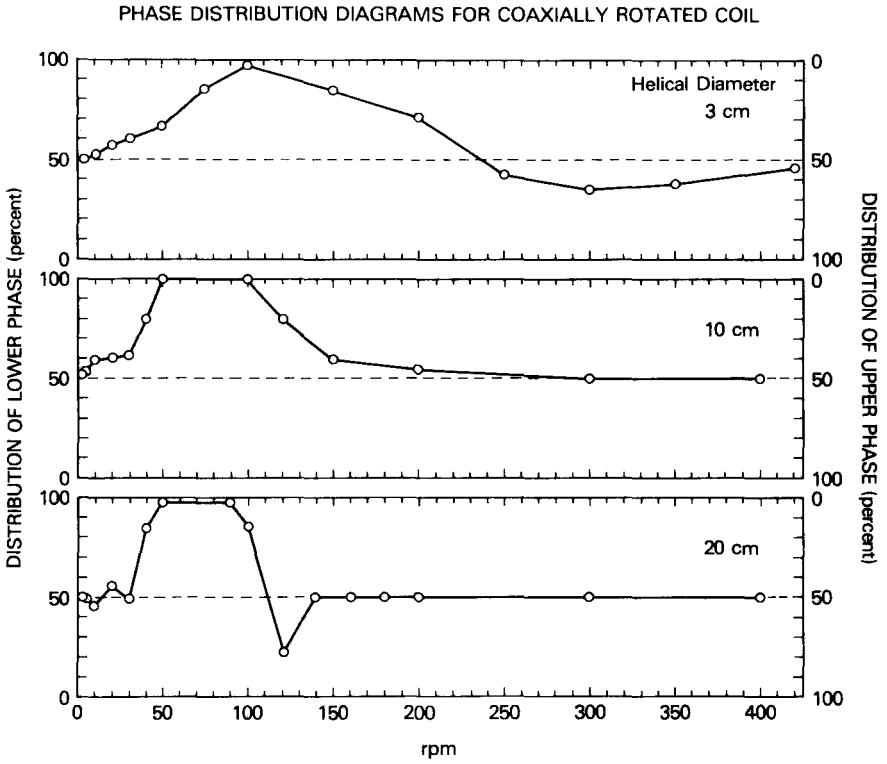

FIGURE 3. Phase distribution diagrams for coaxially rotated coils with three different helical diameters.

slow rotational speed between 0 and 30 rpm, the two phases distribute fairly evenly in the coil (stage I). When the rotational speed is increased, the lower nonaqueous phase tends to occupy more space on the head side of the coil and, at a critical speed between 60 and 100 rpm, the two phases are almost completely separated along the length of the coil, the lower phase occupying the head side and the upper phase, the tail side of the coil (stage II). After this critical range, the amount of lower phase on the head side tends to decrease rather sharply, crossing below the 50% line (stage III). A further increase in the rotational speed again yields an even

distribution of the two phases in the coil (stage IV). As the helical diameter increases all these stages tend to shift toward the lower rpm range apparently due to the enhanced centrifugal force field. For performing preparative CCC, stage II is considered to be of greater interest because the system permits retention of a large amount of the stationary phase for either phase under the proper mode of elution as described earlier (5). The uneven distribution of the two phases in the coil at the critical rpm permits an extremely high level of stationary phase retention when either the aqueous phase is eluted in the normal mode (head to tail) or the nonaqueous phase in the reversed mode (tail to head).

The partition efficiency of the coaxially rotated coils was measured by the set of DNP amino acid samples and the two-phase solvent system which were used in the studies on the eccentric orientation. The experimental results with the three different helical diameters are summarized in Fig. 4 in which individual charts are arranged according to various operational conditions. In addition to the normal elution mode (head to tail), the reversed elution mode (tail to head) was also applied for the nonaqueous mobile phase at the critical rpm range (stage II) to achieve a high level of stationary aqueous phase retention as is expected from the phase distribution diagrams shown in Fig. 3.

The overall results of these experiments with the coaxially rotated coils clearly indicate that the best peak resolutions are obtained at the critical rotational speed ranging between 60 rpm and 100 rpm, when either the aqueous phase was eluted in the normal mode (head to tail) or the nonaqueous phase in the reversed mode (tail to head). As predicted from the phase distribution diagrams in Fig. 3, the normal mode of elution with the stationary aqueous phase (middle column in each helical diameter in Fig. 4) gave unsatisfactory peak resolution due to a low level of stationary phase retention. Under the proper elution mode the resultant peak resolutions from the coaxially rotated coils are much higher than those from the eccentrically rotated coils shown in Fig. 2.

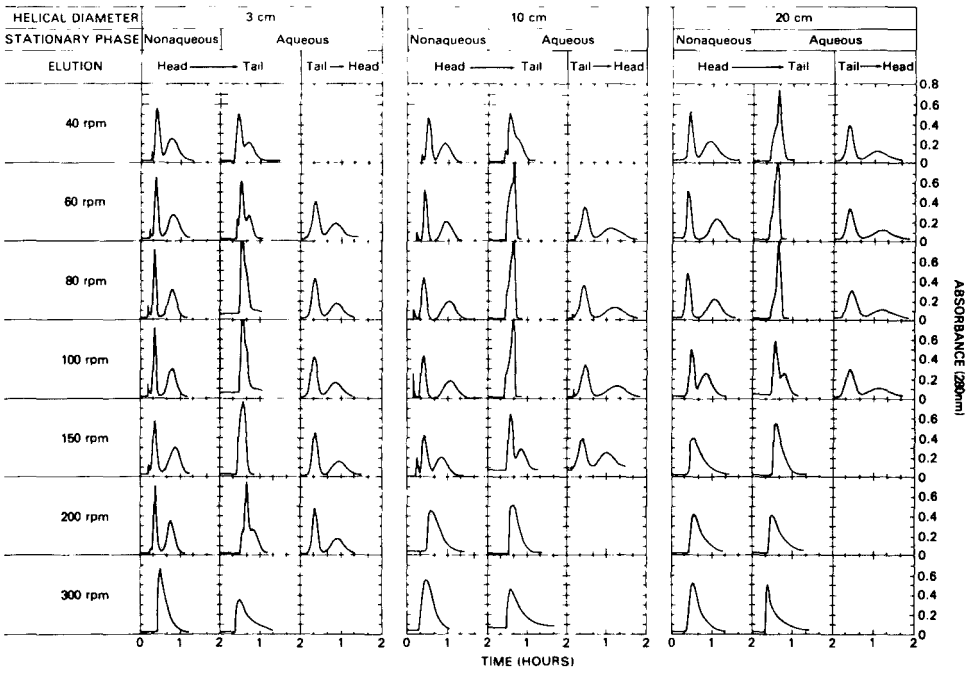

FIGURE 4. Effects of helical diameters of the coaxially rotated coil on the separations of the DNP amino acids at various rotational speeds.

Comparison of the results obtained from the three different helical diameters illustrated in Fig. 4 reveals the considerable shift of the optimal conditions toward the lower rpm range in the larger helical diameter coil, this being consistent with the finding from the phase distribution diagrams in Fig. 3. However, the satisfactory peak resolution observed in a wide range of rpm in each helical diameter suggests that the choice of a proper rotational speed such as 80 rpm would produce good separations in the coil with any helical diameter between 3 cm and 20 cm. In light of these experimental data with the coaxially rotated coils, a new column configuration called the multi-layer coil (Fig. 1, right bottom) has been made for large-scale preparative CCC.

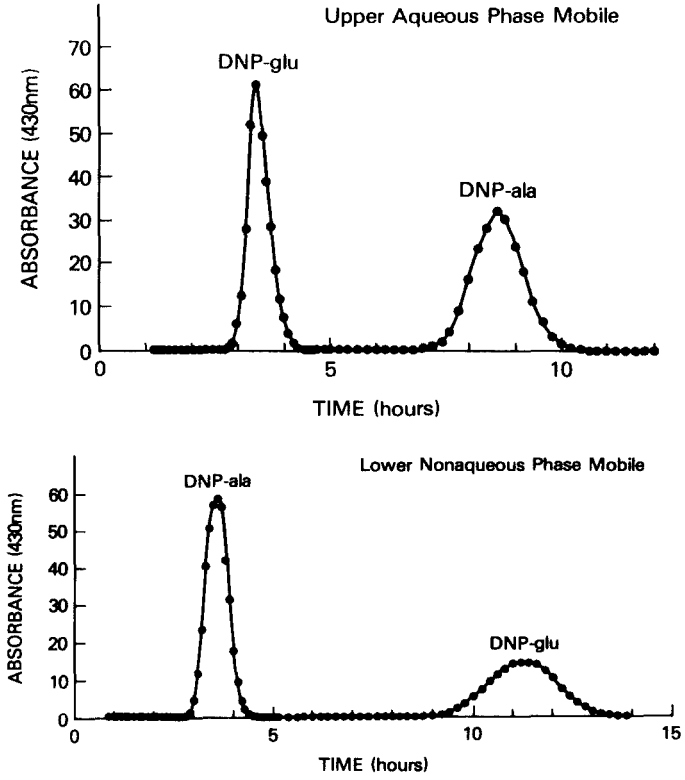

FIGURE 5. Chromatograms of DNP amino acids (1g quantity) obtained with the multi-layer coil.

A. Elution with the upper aqueous phase in the normal mode (head to tail).

B. Elution with the lower nonaqueous phase in the reversed mode (tail to head).

Preparative CCC with a Multi-Layer Coil

The preparative capability of the multi-layer coil has been demonstrated in the separations of 1g quantity of the DNP amino acid mixture under the optimum operational conditions determined by the preliminary studies on short coils as described above. Fig. 5 shows the chromatograms obtained with the multi-layer coil in which both the upper aqueous phase (A) and the lower nonaqueous phase (B) are used as the mobile phase in the suitable elution mode. The two DNP amino acids are well resolved and eluted out as symmetrical peaks. The retention of the stationary phase measured after the separation was 68% in A and 84% in B. The separations obtained with this multi-layer coil with a 750 ml capacity are much better than those from the eccentrically mounted glass coils with a similar internal diameter and having a total capacity of 900 ml (4).

The multi-layer coil has a number of advantages over the previous CCC schemes utilizing a slowly rotating coil assembly. Because of the extremely large volume of the retained stationary phase, both peak resolution and the sample-loading capacity of the column are much increased. The column is leak-free, easily prepared and relatively inexpensive. The compactness of the column reduces the size of the apparatus saving cost and space in the research laboratory. The present scheme is amenable to be scaled up for large-scale industrial applications.

ACKNOWLEDGEMENT

We are deeply indebted to Mr. Waldo Groves for fabrication of the instrument.

REFERENCES

1. Ito, Y., Countercurrent Chromatography, J. Biochem. Biophys. Met. 5, 105 (1981).

2. Ito, Y. and Bowman, R. L., Preparative Countercurrent Chromatography with a Slowly Rotating Helical Tube, Anal. Biochem. 78, 506 (1977).

3. Ito, Y. and Bowman, R. L., Preparative Countercurrent Chromatography with a Slowly Rotating Helical Tube, J. Chromatogr. 136, 189 (1977).

4. Ito, Y. and Bhatnagar, R., Preparative Counter-Current Chromatography with a Rotating Coil Assembly, J. Chromatogr. 207, 171 (1981).

5. Ito, Y., New Continuous Extraction Method with a Coil Planet Centrifuge, J. Chromatogr. 207, 161 (1981).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(2), 275-289 (1984)

SOLVENT SELECTION FOR COUNTERCURRENT CHROMATOGRAPHY BY RAPID ESTIMATION OF PARTITION COEFFICIENTS AND APPLICATION TO POLAR CONJUGATES OF p-NITROPHENOL

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ABSTRACT

A rapid and moderately precise technique to measure partition coefficients of UV-absorbing solutes in solvent systems for countercurrent chromatography is described and applied to p-nitrophenol and its conjugates with glucose, sulfuric acid and glucuronic acid. It involves equilibration of one ml of each phase with solute in a narrow test tube, removal of the entire lower phase, dilution with methanol and calculation of the partition coefficient as the ratio of the absorbance values of each dilution at any suitable wavelength. The polar conjugates of p-nitrophenol can be separated by countercurrent chromatography using ethyl acetate as mobile phase and aqueous KH_2PO_4 as stationary phase.

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INTRODUCTION (1,2)

Separation of organic compounds by solvent-solvent partition has been facilitated by the recent design of numerous instruments to carry out the process of countercurrent chromatography (3,4). One of the most versatile of these instruments, the horizontal flow-through coil planet centrifuge (5,6), in which the stationary phase is retained in a helical coil by centrifugal force, is employed in the present investigation. Countercurrent chromatography is complementary to other forms of chromatography and is particularly suited for preparative separations of polar compounds and compounds which are stable over a limited range of pH, characteristics found in many natural products. The use of PTFE tubing for the chromatographic column and the absence of any solid supporting matrix for the stationary phase precludes sample loss by adsorption or surface catalysis. These features also facilitate accurate prediction of chromatographic retention based simply on a knowledge of the partition coefficient in the solvent system employed.

The attribute of the centrifugal apparatus to use as the mobile phase either phase of virtually any immiscible liquid-liquid pair allows the chromatographer to exploit a much wider range of solvent polarity and selectivity than was heretofore possible. To exploit this opportunity and to optimize solvent selection for a particular separation requires extensive appraisal of the partitioning characteristics of analytes in the available phase systems.

The methodology reported here was developed to provide a rapid technique, capable of moderate precision, for the estimation of partition coefficients of UV-absorbing compounds in the interval of 0.1 to 10, which is the range desired to achieve separation within a reasonable time. In essence, the method consists of distributing about 200 μg of compound between equal

(1 - 2 ml) volumes of each phase, separating the layers, equally diluting each layer with 5 ml of methanol, measuring the absorbance of each layer using any wavelength which provides readings in a usable range (0 - 1A), not necessarily corresponding to an absorbance maximum, and calculating the partition coefficient as the ratio of the two absorbance readings. The unique features of the procedure are the narrow diameter of the culture tube employed for equilibration and the transfer of the lower layer, which is experimentally simpler and more precise than transfer of the upper layer.

The procedure is illustrated using p-nitrophenol (PNP) and its conjugates with glucose (PNP-GS), sulfuric acid (PNP-S) and glucuronic acid (PNP-GN) in several solvent systems with 1 M phosphate buffers as the aqueous phase.

EXPERIMENTAL

Reagents (2)

p-Nitrophenol (PNP), p-nitrophenyl-β-D-glucopyranoside (PNP-GS), potassium p-nitrophenyl sulfate (PNP-S) and p-nitrophenyl-β-D-glucuronide (PNP-GN) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade. Buffers were prepared by mixing equimolar solutions of H_3PO_4, KH_2PO_4 and K_2HPO_4 to obtain the desired pH as measured using a pH meter.

Apparatus

Countercurrent chromatography was done with a horizontal flow-through coil planet centrifuge (5,6) using the planet gear drive at 400 rpm, β 0.25 and a column consisting of 5 m of 2.6 mm ID PTFE tubing wound (98 turns) on a 12.5 mm rod. Column volume was 25 ml. Solvent was delivered with a Milroyal model

HDB-1-30R pump (Laboratory Data Control, Riviera Beach, FL). Column effluent was monitored at 280 nm using either an LKB Uvicord S monitor (LKB Instruments, Inc., Rockville, MD) with a 1.8 mm cell and an LKB model 6520 recorder or a Glenco model 5480 UV monitor fitted with a 3 mm cell and a Houston-OmniScribe recorder.

Spectrophotometric measurements for partition coefficient determination were made using either a Beckman model DU or a Varian model 634 spectrophotometer with 1-cm cells.

Culture tubes were Kimble no. 45066-A, 13 x 100 mm with teflon-lined caps. Disposable Pasteur pipets should have tip diameters of approximately 1 mm and should be free of chips. A Universal Repipet® dispenser model 3010-A-U (Labindustries, Berkeley, CA) was used to dispense methanol for dilution.

Methods

Partition coefficients. Approximately 0.2 ml of a methanol solution containing approximately 1 mg/ml (7) of test compound was evaporated with an air stream in a 13 mm diam. culture tube. Exactly 1 ml (2 ml where indicated) of each of two mutually saturated solvents was added by pipet (8). The tube was closed with a teflon-lined cap and shaken gently for 10 min (9), then centrifuged briefly. A disposable Pasteur pipet attached to a 2-ml syringe-type filling device (10) was inserted with the tip touching the bottom of the culture tube and the lower layer was precisely removed and transferred to a second tube containing 5 ml of methanol or other appropriate diluent (11) previously added using a Repipet®. The pipet used for the transfer was then rinsed by inspiring and expelling a portion of the solution. The same volume of diluent was added to the upper phase. The absorbance of each solution was determined at any convenient wavelength, usually on the longer wavelength side of the absorbance maximum, chosen to provide a reading not greater than

1.0A for either solution. Minor corrections for solvent absorbance were made by subtracting absorbances of similarly diluted aliquots of mutually saturated blank solvents. The partition coefficient was calculated as the ratio of the net absorbance values.

Countercurrent chromatography. A solvent system was prepared by shaking 300 ml of ethyl acetate and 120 ml of 1 M KH_2PO_4 (pH 4.5) in a separatory funnel. After filling the column by pumping in the organic phase at a rate of 1.34 ml/min the apparatus was rotated at 400 rpm and exactly 15 ml of aqueous phase introduced by means of a loop injector. The stationary phase volume was calculated by subtracting the unretained portion of aqueous phase as measured by collecting the column effluent in a 25-ml graduated cylinder. The difference between the stationary phase volume, V_S, and the total column volume (25 ml) is the volume of mobile phase (V_m). A sample mixture was prepared by evaporating 0.5-ml aliquots of individual methanol solutions of the test compounds in a test tube and dissolving the residue in stationary aqueous phase to provide a solution containing PNP (0.25 mg), PNP-GS (0.5 mg), PNP-S (0.5 mg) and PNP-GN (0.5 mg) in 0.5 ml. An 0.5-ml aliquot was injected using a sample loop of 18 gauge (1.02 mm ID) PTFE tubing. Effluent was monitored at 280 nm with an LKB monitor using a 1.8 mm cell.

In studies on the effect of KH_2PO_4 concentration on the resolution of the glucoside and sulfuric acid ester, solvent systems were prepared by equilibrating 750 ml of ethyl acetate, presaturated with water, with 150 ml of 1 M, 0.75 M or 0.5 M KH_2PO_4. Instrument settings were the same as indicated heretofore for the mixture of four compounds. An 0.5-ml sample contained PNP (0.25 mg), PNP-GS (0.5 mg) and PNP-S (0.6 mg). Effluent was monitored at 280 nm with a Glenco monitor using a 3 mm cell.

RESULTS AND DISCUSSION

Efficacy of lower phase transfer

To test the efficacy of lower layer transfer, the partition coefficient of PNP was measured in two solvent systems in which partitioning into the upper layer was less than 0.02. In this case, virtually all of the PNP will be in the lower layer and any increase in the partition coefficient over the expected value will represent incomplete transfer of the lower layer. The expected value was determined in a separate experiment using larger phase volumes in which an aliquot was removed by pipet from each phase so as to eliminate the possibility of error by contamination of one phase with traces of the other.

With an aqueous lower phase, Table 1, contamination of upper phase by residual lower phase results in an absorbance reading for the upper phase no greater than 0.020 units, which represents a contamination of the upper phase with less than 2.1% of lower phase using either 1-ml or 2-ml phase volumes.

Transfer of a lower phase organic layer, Table 2, is even more precise, resulting in an excess absorbance reading of 0.002, which represents a contamination of upper phase by lower phase of well under 1%.

Reproducibility

The reproducibility of the method is demonstrated by the data in Table 3 summarizing replicate measurements of the partition coefficient of PNP between 0.2 M HOAc and EDC with relative standard deviations of 1.4% and 0.9% using 1-ml and 2-ml phase volumes respectively.

Application to polar conjugates of PNP

Partition coefficients determined for a variety of solvent systems for possible use in separation of polar conjugates of

TABLE 1

Efficacy of Lower Aqueous Phase Transfer

Sample: 100 μg p-nitrophenol
Upper Phase: heptane
Lower Phase: 50% methanol in water
Diluent and Analytical Wavelength: methanol, 312 nm

PHASE VOLUMES		ABSORBANCE OF DILUTED PHASES		$K_{U/L} = \frac{\text{UPPER-EXPECTED}}{\text{LOWER-BLANK}}$
		UPPER	LOWER	
1 ML		0.031	1.088	0.019
		0.036	1.089	0.024
		0.029	1.076	0.018
		0.034	1.145	0.021
		0.032	1.127	0.020
	EXPECTED	0.010	---	
	BLANK	0.003	0.005	
	MEAN ± SD			0.020 ± 0.002
2 ML		0.030	0.898	0.024
		0.022	0.874	0.015
		0.021	0.918	0.013
		0.023	0.863	0.016
		0.027	0.917	0.020
	EXPECTED	0.009	---	
	BLANK	0.005	0.007	
	MEAN ± SD			0.019 ± 0.004

TABLE 2

Efficacy of Lower Organic Phase Transfer

Sample: 100 μg p-nitrophenol
Upper Phase: 0.2 M HOAc
Lower Phase: 10% pentanol in ethylene dichloride
Diluent and Analytical Wavelength: methanol, 312 nm

PHASE VOLUMES		ABSORBANCE OF DILUTED PHASES		$K_{U/L} = \frac{\text{UPPER-EXPECTED}}{\text{LOWER-BLANK}}$
		UPPER	LOWER	
1 ML		0.017	1.162	-0.003
		0.017	1.108	-0.003
		0.018	1.114	-0.002
		0.022	1.122	0.002
		0.022	1.125	0.002
	EXPECTED	0.020	---	
	BLANK	0.000	0.004	
	MEAN ± SD			-0.002 ± 0.001
2 ML		0.014	1.001	-0.003
		0.015	0.987	-0.002
		0.018	1.010	0.001
		0.015	0.975	-0.002
		0.014	0.992	-0.003
	EXPECTED	0.017	---	
	BLANK	0.002	0.007	
	MEAN ± SD			-0.002 ± 0.001

TABLE 3

Reproducibility of K Determination

Sample: 100 µg p-nitrophenol
Upper Phase: 0.2 M HOAc
Lower Phase: ethylene dichloride
Diluent and Analytical Wavelength: methanol, 312 nm

$K_{UPPER/LOWER}$

	1 ML PHASES	2 ML PHASES
	0.222	0.217
	0.220	0.215
	0.226	0.214
	0.221	0.213
	0.219	0.219
MEAN ± SD	0.222 ± 0.003	0.216 ± 0.002

PNP are summarized in Table 4. Most represent single determinations using 1 ml of each phase. Solvents range in polarity from heptane to neat isopropanol. All provided 2-phase systems with 1 M phosphate buffers. Partition coefficients greater than 20 or less than 0.05 were not considered to be sufficiently accurate for intercomparison and, in any case, are beyond the desirable range for countercurrent chromatography.

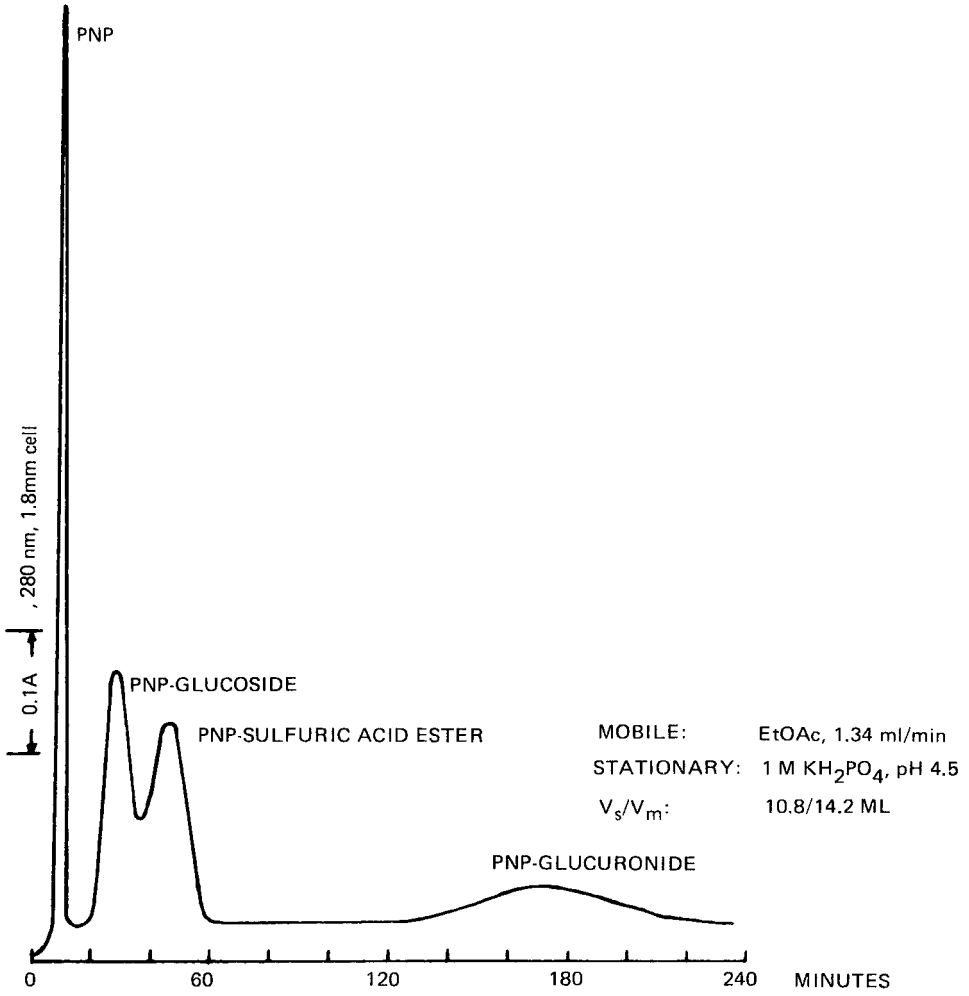
It is apparent that the four compounds could be separated using 1 M KH_2PO_4 as the stationary phase and either stepwise elution with a series of solvents or with a gradient of EtOAc or an alcohol in heptane. A separation using the isocratic system 1 M KH_2PO_4-EtOAc is shown in Fig. 1. Note that the partition coefficients, $K_{s/m}$, calculated from the chromatographic capacity factors, k',

$$K_{s/m} = K_{A/O} = k' \frac{V_m}{V_s}$$

TABLE 4

Partition Coefficients for Polar Conjugates of p-Nitrophenol

AQUEOUS PHASE, 1 MOLAR PHOSPHATE	ORGANIC PHASE	$K_{AQUEOUS/ORGANIC}$			
		PNP	PNP-GS	PNP-S	PNP-GN
pH 4.00	EtOAc			4.4	4.5
	HEPTANE	>20			
	HEPTANE + 5% EtOAc	1.3			
	HEPTANE + 10% EtOAc	0.4			
	HEPTANE + 50% EtOAc	<0.05			
	HEPTANE + 95% EtOAc		5.6	5.9	
	HEPTANE + 20% n-BuOH		>20	>20	
	ETHER	<0.05	>20	>20	
	MTBE		20	10	
KH_2PO_4	MTBE + 10% i-PrOH		3	4.3	
	MTBE + 10% n-BuOH		3	7.7	
pH 4.5	EtOAc	<0.05	2.5	5.0	14
	EtOAc + 10% i-PrOH		1.0	2.0	6.2
	EtOAc + 15% i-PrOH	0.01	0.7	1.4	4.7
	EtOAc + 20% EtOH			0.8	4.6
	EtOAc + 20% n-BuOH			0.7	3.5
	EtOAc + 20% i-PrOH			0.6	3.5
pH 5.50	n-BuOH			0.2	2.7
	HEPTANE				
	HEPTANE + 5% EtOAc	1.9			
	HEPTANE + 10% EtOAc	0.6			
	EtOAc	<0.05	2.2	3.7	
	EtOAc + 10% i-PrOH		0.8	1.1	>20
pH 7.00	EtOAc + 15% i-PrOH		0.6	0.6	>20
	EtOAc + 25% i-PrOH		0.3	0.2	12
	EtOAc + 35% i-PrOH		0.22	0.1	3.2
	EtOAc + 50% i-PrOH		0.17	<0.05	0.9
	n-BuOH		0.4	0.1	3.8
	i-PrOH			<0.05	0.2



	PNP	PNP-GS	PNP-S	PNP-GN
k' (ccc)	0	1.7	3.2	15
k (ccc)	0	2.3	4.2	20

FIGURE 1. SEPARATION OF p-NITROPHENOL AND ITS CONJUGATES WITH THE COIL PLANET CENTRIFUGE; PLANET GEAR DRIVE, 400 RPM, β 0.23; COLUMN 5 m OF 2.6 mm ID PTFE, 98 TURNS ON 12.5 mm CORE, VOL. 25 ml; SAMPLE 0.5 ml.

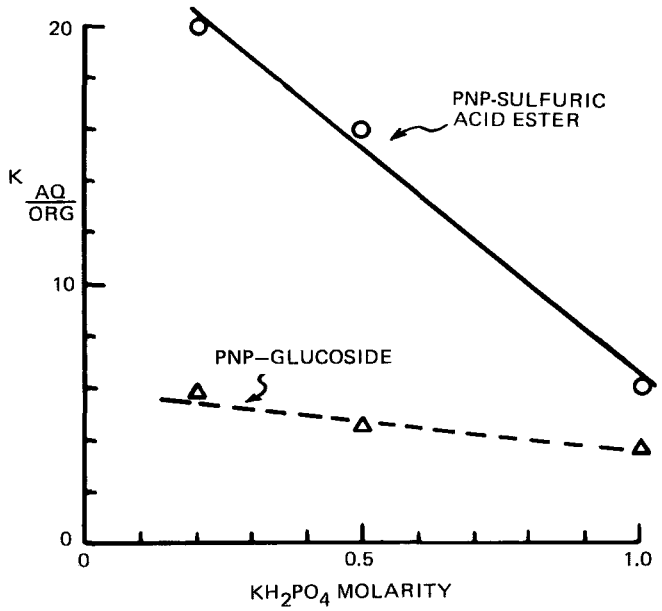


FIGURE 2. EFFECT OF BUFFER MOLARITY ON PARTITION COEFFICIENT WITH ETHYL ACETATE.

are in good agreement with those presented in Table 4. The glucoside and sulfuric acid ester are incompletely resolved.

Effect of buffer concentration

Measurement of the partition coefficients of PNP-GS and PNP-S between EtOAc and KH_2PO_4 solutions, Fig. 2, showed that $K_{A/O}$ for the ionized sulfuric acid ester is inversely related to the salt concentration, while $K_{A/O}$ for the neutral glucoside is relatively unchanged. As seen in Fig. 3, the compounds are completely resolved by countercurrent chromatography using a buffer concentration of 0.50 M.

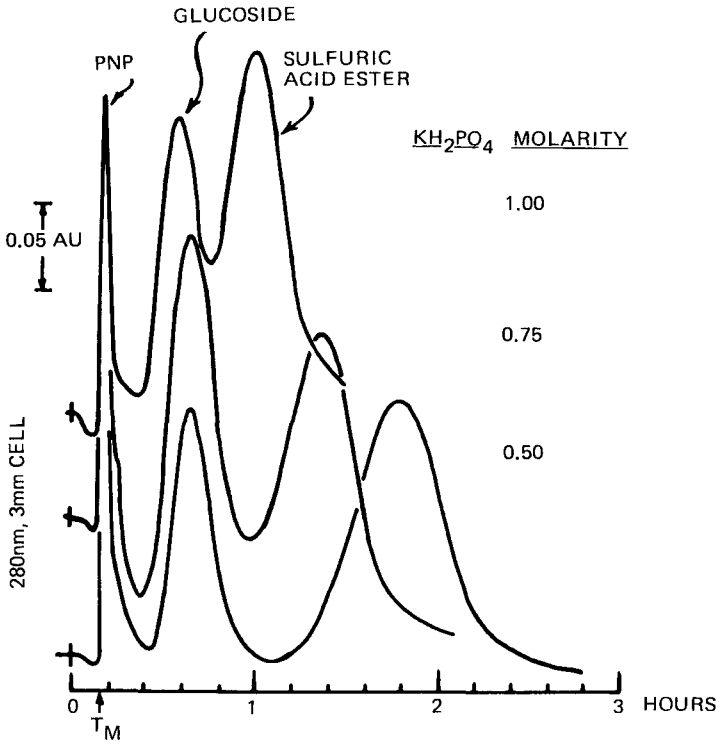


FIGURE 3. EFFECT OF KH_2PO_4 CONCENTRATION ON RESOLUTION OF PNP, PNP-GS AND PNP-S USING ETHYL ACETATE AS THE MOBILE PHASE. OTHER CONDITIONS IDENTICAL TO THOSE IN FIGURE 1.

Conclusion

The micromethod described for measuring partition coefficients is rapid, and sufficiently precise to be useful for screening solvent systems for use in countercurrent chromatography. A rapid HPLC procedure for analytical determination of PNP, PNP-S and PNP-GN in biological fluids has been reported (12). Though the countercurrent chromatographic separation reported here is slower, the capacity of the countercurrent chromatograph is greater, and the wide choice of solvents per-

mits greater flexibility in choosing conditions for separation. These features are often advantageous in isolation or preparative purification of polar substances such as drug conjugates as well as other biochemicals or natural products.

REFERENCES

1. Presented in part as paper no. 611, Pittsburgh Conference, Atlantic City, N.J., March 8-13, 1982.

2. Abbreviations used: PNP, p-nitrophenol; PNP-GS, p-nitrophenyl-β-D-glucopyranoside; PNP-S, potassium p-nitrophenyl sulfate; PNP-GN, p-nitrophenyl-β-D-glucuronide; PTFE, polytetrafluoroethylene; HOAc, acetic acid; EDC, 1,2-dichloroethane; EtOAc, ethyl acetate; MTBE, methyl t-butyl ether; i-PrOH, isopropanol; n-BuOH, n-butanol; $K_{U/L}$, $K_{S/m}$, $K_{A/O}$; partition coefficient expressed as concentrations in upper/lower, stationary/mobile and aqueous/organic phases respectively.

3. Ito, Y., Countercurrent Chromatography, J. Biochem. Biophys. Methods, 5, 105, 1981.

4. Mandava, N.B., Ito, Y. and Conway, W.D., Countercurrent Chromatography: I, Historical development of differential extraction and description of early countercurrent chromatographic instrumentation, Amer. Lab., 14 [10], 62 (1982), II, Newer instrumentation for analytical and preparative use and for particle separation and applications of countercurrent chromatographic methodology, Amer. Lab., 14 [11], 48, 1982.

5. Ito, Y., A new horizontal flow-through coil planet centrifuge for countercurrent chromatography: I. Principle of design and analysis of acceleration, J. Chromatogr., 188, 33, 1980.

6. Ito, Y., A new horizontal flow-through coil planet centrifuge for countercurrent chromatography: II. The apparatus and its partition capabilities, J. Chromatogr., 188, 43, 1980.

7. A concentration of 1 mg/ml is convenient and generally appropriate, but with compounds with very high absorptivity

such as p-nitrophenol a lower concentration or smaller aliquot may be used to avoid the need to employ an analytical wavelength far removed from the absorption maximum.

8. It is convenient to use either a 1-ml tuberculin syringe fitted with a blunt needle or a positive displacement plunger pipet.

9. Vigorous hand shaking leads to emulsion formation. A wrist-action shaker or simply repeated inversion is preferable.

10. This is conveniently made from a 2-ml glass syringe having the barrel lightly coated with petroleum jelly and with cut-off 1-ml latex dropper bulb forced over the delivery end to attach the Pasteur pipet.

11. While methanol is a generally useful diluent, it precipitates salts. Although these are separated by centrifugation, an indeterminate error, not serious for screening purposes, may arise because of a volume change affecting the aqueous phase. Aqueous methanol (50%) largely avoids salt precipitation and is miscible with most organic solvents. In instances where spectral shifts may result from pH differences in dilutions of upper and lower layers it is necessary to incorporate a suitable acid, base or buffer into the diluent.

12. Diamond, G. and Quebbemann, A.J., Rapid Separation of Conjugates by Reversed-Phase High-Performance Liquid Chromatography, J. Chromatogr., 177, 368, 1979.

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EVALUATION OF NONAQUEOUS SOLVENT SYSTEMS FOR COUNTERCURRENT CHROMATOGRAPHY USING AN HPLC ASSAY TO DETERMINE PARTITION COEFFICIENTS OF A MIXTURE OF COMPOUNDS

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ABSTRACT

A reversed phase HPLC assay was used to measure partition coefficients for salicylic acid, salicylamide and salicyluric acid in two-phase systems employing neat or aqueous ethylene glycol, formamide, or methanol and the organic solvents: ethylene dichloride, ethyl acetate, diethyl ether and methyl isobutyl ketone. These solvent systems permit preparative separation by countercurrent chromatography of compounds with limited aqueous solubility. Salicylic acid, salicylamide and salicyluric acid are readily separated in the system employing ethylene glycol as the stationary phase and diethyl ether as the mobile phase.

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INTRODUCTION (1,2)

Selection of solvent systems for the separation of compounds by countercurrent chromatography is expedited by a knowledge of the partition coefficients of the solutes in the various solvent systems. Partition paper chromatography (3) and TLC (4,5) have been suggested as means for surveying possible solvents for separation of mixtures by liquid-liquid partition; but errors may arise in these methods as a result of unpredictable adsorption to the supporting matrix. The approach described here involves partition of a mixture of solutes between small volumes of immiscible liquid phases and subsequent measurement of the concentration of solutes in each phase using reversed phase liquid chromatography.

Because of their strong solubilizing properties, the polar solvents ethylene glycol (EG) and formamide (F) as well as 80% EG in water and 80% methanol (M) in water are examined for possible use as stationary phases for countercurrent chromatographic separation of compounds having relatively low aqueous solubility. A mixture of salicylic acid (SA), salicylamide (SAM) and salicyluric acid (SU) is employed as a test mixture. The solubilities of SA and SAM and the partition coefficients of these and SU are determined in two-phase systems consisting of the above solvents and methylene dichloride (MDC), ethylene dichloride (EDC), ethyl acetate (ETAC), diethyl ether (ET) and methyl isobutyl ketone (MIK).

EXPERIMENTAL

Reagents

Methanol was HPLC grade. All other chemicals were reagent grade.

Apparatus

Countercurrent chromatography was done with a horizontal flow-through coil planet centrifuge (6,7) using the planet gear drive at 400 rpm, β 0.25 and a column consisting of 5 m of 2.6 mm ID PTFE tubing wound (98 turns) on a 12.5 mm rod. Column volume was 25 ml. Solvent was delivered with a Beckman Accuflow pump. Column effluent was monitored at 254 nm using an LKB Uvicord S monitor (LKB Instruments Inc., Rockville, MD) with a 1.8 mm cell and an LKB model 6520 recorder.

HPLC was done using a modular system consisting of a Glenco HPLPS-1 pump, Glenco SV-3 injection valve with 50 µl loop, Glenco 5480 monitor (254 nm) and an Alltech C-18 column (10 µ, 4.6 x 250 mm). Samples (25 µl) were injected using a Valco 50-µl syringe.

Methods

HPLC. Chromatographic separation of SA, SAM and SU was examined using mobile phases composed of 0.05 M Na formate buffer:MeOH; 70:30 (v/v) with buffers of pH 3.00, 3.25, 3.50 and 4.00. Routine chromatography employed the pH 3.25 buffer. Flow-rate was 1 ml/min and monitoring was at 254 nm. Calibration plots were prepared by assay of 20-, 50-, 100-, and 200-fold dilutions in mobile phase of stock solutions containing 4, 8 and 2 mg/ml of SA, SAM and SU respectively.

Partition coefficients. Two ml of a stock solution in ethylene glycol (or formamide or aqueous methanol) containing 4, 8 and 2 mg/ml of SA, SAM and SU respectively, was gently mixed with 2 to 5 ml of a second, immiscible solvent for 30 min at room temperature in a 15-ml graduated centrifuge tube closed with a teflon-lined cap. After centrifugation, 10- to 50-fold dilutions of each layer were prepared in HPLC mobile phase using volumetric flasks and 25- to 250-µl Kirk transfer pipets. Aliquots of solvents (EDC, etc.) not miscible with the mobile phase were evaporated in an air stream and the residue diluted with

mobile phase. Aliquots (25 µl) of diluted solutions were assayed by HPLC using the pH 3.25 mobile phase. Retention times for SU, SAM and SA were 10, 11.2 and 15.2 min respectively. Partition coefficients were calculated as

$$K_{u/l} = \frac{h_u \, d_l}{h_l \, d_u}$$

where h_u, h_l, d_u amd d_l represent the peak heights and dilutions of the upper and lower layers respectively. To minimize error, dilutions were chosen to provide approximately equal values of h_u and h_l.

Solubilities. Saturated solutions of SA and SAM in several solvents were prepared by allowing an excess of compound in 1 ml of solvent in a capped vial to remain for several days at room temperature with intermittent mixing. Aliquots of the supernatant liquid were measured with Kirk microliter pipets, diluted with HPLC mobile phase (pH 3.25) and assayed by HPLC.

Countercurrent Chromatography. Retention of ethylene glycol when using ethyl acetate and diethyl ether as mobile phases was measured for various flow rates at β 0.25 and various rates of revolution by observation, under stroboscopic illumination, of the number of coils occupied by 5.0 ml of ethylene glycol (ethyl acetate or ether-saturated) dyed with acid fuchsin (8). The fraction of column volume, F_s, occupied by stationary phase is then given by

$$F_s = \frac{v_s}{n \, v_c}$$

where v_s is the volume of dyed stationary phase introduced, v_c is the volume of a single coil of the column and n is the number of coils containing dyed stationary phase under the particular conditions of flow-rate, rate of rotation and β.

Solvent systems for separation by countercurrent chromatography were prepared by mutually saturating ethylene glycol and ethyl acetate or diethyl ether in a separatory funnel. After filling the column by pumping in the upper phase at a rate

of 1.35 ml/min, the apparatus was rotated at 400 rpm and exactly 15 ml of solvent-saturated ethylene glycol introduced by means of a loop injector. The stationary phase volume, V_S, was calculated by subtracting the unretained portion of the stationary phase as measured by collecting the column effluent in a 25-ml graduated cylinder. The difference between the stationary phase volume and the total column volume (25 ml) is the mobile phase volume (V_m). An 0.5-ml aliquot of an ethylene glycol solution containing 1.1, 3.0 and 2.5 mg/0.5 ml of SU, SAM and SA respectively, was injected using a sample loop of 18 gauge (1.02 mm ID) PTFE tubing. Effluent was monitored at 280 nm with an LKB monitor using a 1.8 mm cell.

The number of theoretical plates, N, for each solute was calculated using the conventional formula

$$N = 16\left(\frac{t_R}{w}\right)^2$$

where t_R is the retention time and w the peak-width at the base.

The partition coefficient, $K_{S/m}$, was calculated from the capacity factor, k', by multiplying by the phase volume ratio

$$K_{S/m} = k'\left(\frac{V_m}{V_S}\right) = \frac{t_R - t_m}{t_m}\left(\frac{V_m}{V_S}\right)$$

where V_m and V_S are the volumes of mobile and stationary phases respectively and t_R and t_m are the retention times and holdup times respectively.

RESULTS AND DISCUSSION

HPLC

Evaluation of k' as a function of mobile phase pH, Fig. 1, indicated that a mobile phase composition of 0.05 M Na formate buffer, pH 3.25:MeOH; 70:30 (v/v) provided satisfactory resolution of SU, SAM and SA as shown in Fig. 2. Peak heights were linearly related to injected concentrations over the concentration ranges

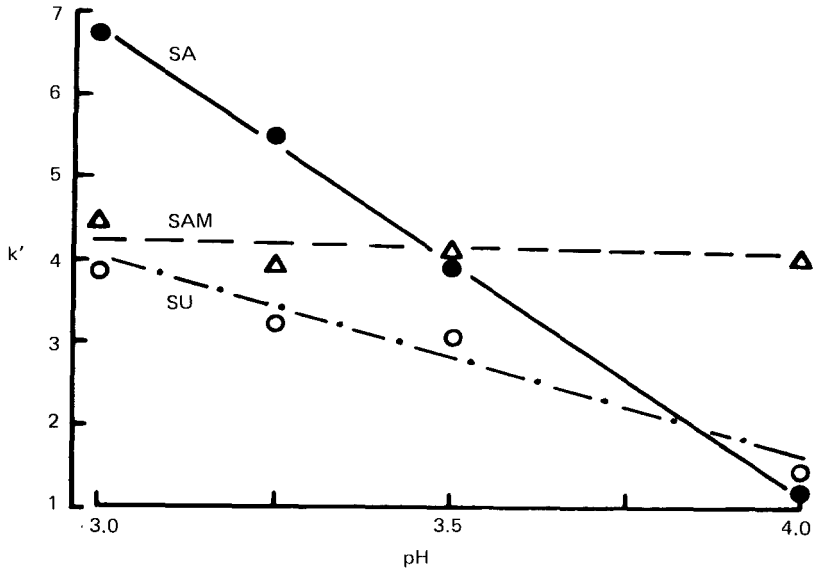

FIGURE 1. HPLC Capacity factor, k´, as a function of pH of mobile phase consisting of 30% methanol in 0.05 M sodium formate buffer.

employed: 10-100 µg/ml for SU, 40-400 µg/ml for SAM and 20-200 µg/ml for SA with correlation coefficients (3 injections at each of 4 concentrations) of 0.9995, 0.994 and 0.998, respectively.

Solubilities

Solubilities of SA and SAM in several solvents and solvent mixtures, most of which were nonaqueous, are summarized in Table 1. The solubilities are appreciable in most of the solvents. While the solubility of SU was not measured, preparation of several saturated solutions showed that it is more soluble than either SA or SAM in most of these solvents. It should also be noted that mutual saturation of two immiscible solvents, such as ETAC/EG, results in a significant increase in the solubilities of SA and SAM compared with their solubilities in the neat solvents.

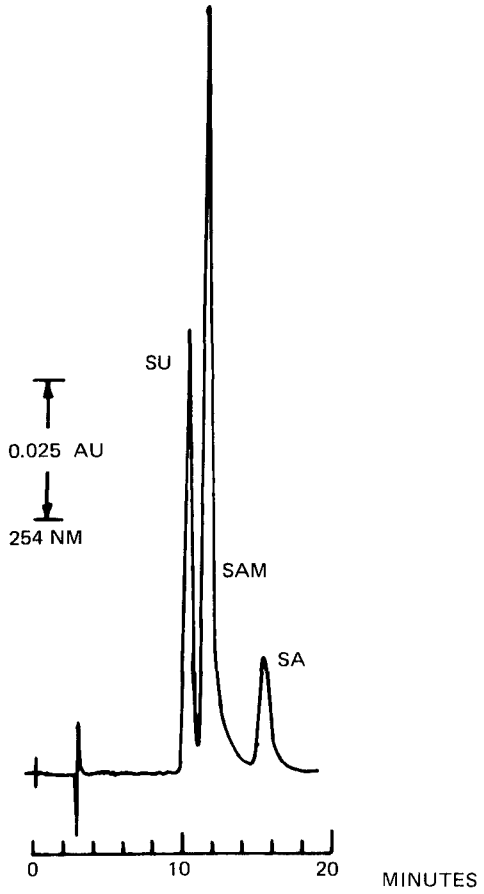

FIGURE 2. HPLC separation of salicyluric acid (SU), salicylamide (SAM) and salicylic acid (SA) on a C-18 column with mobile phase composed of 0.05 M sodium formate buffer: methanol; 70:30 (v/v), pH 3.25 at a flow rate 1 ml/min. Sample is 25-µl of an ethylene glycol stock solution containing 2, 8 and 4 mg of SU, SAM and SU, respectively, which was diluted 1/20 with mobile phase.

TABLE 1

Solubilities of Salicylic Acid and Salicylamide at Room Temperature

Solvent	Solubility, mg/ml	
	Salicylic Acid	Salicylamide
Ethyl Acetate	164	111
80% Ethyl Acetate in Ether	192	100
50% Ethyl Acetate in Ether	208	77
Diethyl Ether	283	32
Methyl iso-Butyl Ketone	186	103
80% Methanol	206	79
Chloroform	38	8
Ethylene Dichloride	21	22
Ethylene Glycol	120	105
80% Ethylene Glycol in Water	60	53
Formamide	230	118
1% Acetic Acid in Formamide	188	140
1% Acetic Acid in 60% Formamide in Water	296	40
Ethyl Acetate/Ethylene Glycol		
upper phase	270	166
lower phase	283	150

Partition Coefficients

The partition coefficients of SA, SAM and SU in several solvent systems are summarized in Table 2. The relative solvent volumes used in the determination are indicated. For systems containing three or more solvents, the partition coefficient may be expected to vary with the relative solvent volumes employed, since the solvents were not mutually saturated prior to equilibration. This potential variation must be taken into account when preparing large volumes of solvent for use in countercurrent chromatography.

Countercurrent Chromatography

The systems ETAC/EG and ET/EG were examined for use in countercurrent chromatography with EG as the stationary phase.

TABLE 2

Partition Coefficients Determined by HPLC

Solvents (2)		Vol. B[2] / Vol. A	Partition Coefficient C_A/C_B		
A	B		SA	SAM	SU
83% EG[1]	MDC	2.1	5.2	5.4	>20
EG	MDC	1.5	4.7	4.2	>20
EG	EDC	2.5	12	12	>20
80% EG[1]	ETAC	1	0.19	0.50	1.19
EG	ETAC	2.5	0.59	0.92	2.48
EG	ET	2.5	0.65	4.36	13.5
EG	50% ETAC in ET	1	0.47	1.69	4.17
EG	80% ETAC in ET	1	0.61	1.30	2.85
EG	MIK	2.5	0.47	0.89	2.53
F	ET	2.5	>20	4.35	>20
1% HOAc in F	ET	1	>20	3.57	>20
1% HOAc in 60% F[1]	ET	1.3	3.70	1.31	16.7
1% HOAc in 60% F[1]	ETAC	1.3	4.00	0.39	5.0
F	EDC	2.5	>20	5.00	>20
80% M[1]	EDC	2.5	0.75	1.45	2.7

[1] in water
[2] relative volume ratio before equilibration

As summarized in Table 3, the amount of EG retained varies from 28 to 44% and 38 to 49% of column volume, with ETAC and ET respectively as mobile phase over a wide range of flow rate and rate of revolution of the column holder.

As expected from the partition coefficients (Table 2), countercurrent chromatography, using ETAC/EG on a column consisting of a 5 m length of 2.6 mm i.d. PTFE tubing wound as 98 turns on a 12.5 mm core, did not resolve SA and SAM, although both were reasonably separated from SU.

The three compounds were fully resolved by countercurrent chromatography in the ET/EG system as shown in Fig. 3. The the-

TABLE 3

Retention of Ethylene Glycol as Stationary Phase Using Either Ethyl Acetate or Diethyl Ether as Mobile Phase with Planet Gear Drive and β 0.25

Mobile Phase Flow Rate, ml/min	Revolutional Rate, RPM	Stationary Phase Retention, F_S ETAC	ET
0.62	300	0.43	0.49
	400	0.44	0.48
	600	0.43	0.48
1.30	300	0.38	0.43
	400	0.40	0.44
	600	0.40	0.44
1.95	300	0.33	0.40
	400	0.36	0.42
	600	0.36	0.43
2.74	300	0.28	0.38
	400	0.32	0.40
	600	0.34	0.41

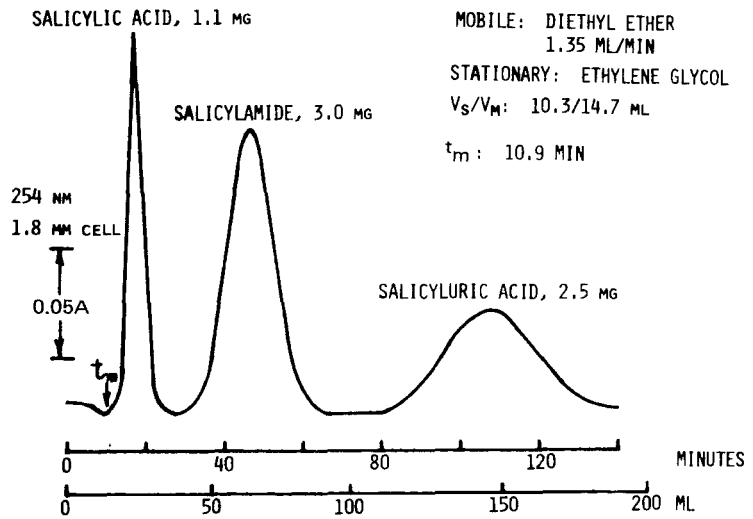

FIGURE 3. Countercurrent chromatographic separation of SA, SAM and SU.

oretical plate numbers, N, were 32, 48 and 79 for SA, SAM and SU respectively. The partition coefficients, 0.74, 4.34 and 12.7, calculated from the capacity factors, k', 0.52, 3.04 and 8.91 for SA, SAM and SU respectively, are in good agreement with the values measured by HPLC (Table 2).

The high boiling point of EG mitigates against its use as a mobile phase in countercurrent chromatography; however, its solubilizing properties recommend its use as a stationary phase. In preparative applications solutes recovered by evaporation of the mobile phase will be contaminated with ethylene glycol. This may often be removed simply by washing with water. In instances where water is undesirable, azeotropic distillation with toluene or heptane may be employed to remove ethylene glycol at a moderate temperature (9).

Conclusion

HPLC is a useful means for measuring partition coefficients for evaluation of solvent systems for countercurrent chromatography, particularly in those instances where UV-absorbing solvents are to be considered, and where only a mixture, such as a natural product extract, is available for evaluation. Where pure solutes are available, and the search is confined to UV-transparent solvents, development of an HPLC assay may be unduly time consuming, and use of a previously described simple partitioning system will usually be faster (10).

Because of their good solubilizing properties and immiscibility with a number of moderately polar solvents, ethylene glycol and formamide may be advantageously employed as stationary phases in countercurrent chromatography.

REFERENCES

1. Presented in part as paper no. 495, Pittsburgh Conference, Atlantic City, N.J., March 7-12, 1983.

2. Abbreviations used: TLC, thin-layer chromatography; EG, ethylene glycol; F, formamide; M, methanol; SA, salicylic acid; SAM, salicylamide; SU, salicyluric acid; MDC, methylene dichloride; EDC, ethylene dichloride; ETAC, ethyl acetate; ET, diethyl ether; MIK, methyl isobutyl ketone; PTFE, polytetrafluoroethylene; HPLC, high pressure liquid chromatography; $K_{u/l}$, $K_{s/m}$, partition coefficient expressed the ratio of concentrations in upper and lower layers or stationary and mobile phases respectively; k′, capacity factor.

3. Socewinski, E., Waksmundzki, A. and Maceijewicz, W., Determination of Optimum Solvent Systems for Countercurrent Distribution from Paper Chromatographic Data, Anal. Chem., 36, 1903, 1964.

4. Hostettmann, K., Hostettmann-Kaldas, M. and Nakanishi, K., Droplet-Countercurrent Chromatography for the Preparative Isolation of Various Glycosides, J. Chromatogr. 170, 355, 1979.

5. Hostettmann, K., Hostettmann-Kaldas, M. and Sticher, O., Preparative Scale Separation of Xanthones and Iridoid Glycosides by Droplet-Countercurrent Chromatography, Helv. Chim. Acta, 62, 2079, 1979.

6. Ito, Y., A new horizontal flow-through coil planet centrifuge for countercurrent chromatography: I. Principle of design and analysis of acceleration, J. Chromatogr., 188, 33, 1980.

7. Ito, Y., A new horizontal flow-through coil planet centrifuge for countercurrent chromatography: II. The apparatus and its partition capabilities, J. Chromatogr., 188, 43, 1980.

8. Ito, Y. and Bowman, R.L., Preparative Countercurrent Chromatography With a Slowly Rotating Helical Tube, J. Chromatogr., 136, 189, 1977.

9. Horsley, L.H., Azeotropic Data, American Chemical Society, Washington, D.C., 1952.

10. Conway, W.D. and Ito, Y., Solvent Selection for Countercurrent Chromatography by Rapid Estimation of Partition Coefficients and Application to Polar Conjugates of p-Nitrophenol, J. Liq. Chromatogr., in press, 1983.

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PLANT HORMONE ANALYSIS BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Countercurrent chromatography (CCC) has been successfully applied for the separation of plant hormones; namely, indole auxins, gibberellins, cytokinins and abscisic acid. In our present study three different types of CCC devices were evaluated for their performance in separation of plant hormones with a special emphasis on analysis and purification of abscisic acid (ABA). A large-scale preparative CCC apparatus consisting of a slowly rotating coil assembly was used for preliminary separations of ABA from a large volume of crude plant extracts. The toroidal coil planet centrifuge (CPC) for analytical-scale separations was subsequently applied for purification of ABA, the final confirmation being obtained by HPLC and combined gas chromatographic-mass spectrometric method. This two-step procedure utilizing preparative CCC and toroidal CPC was successfully applied for determination of ABA content in several plant tissues. A recently introduced high-speed CCC apparatus was tested for semipreparative separation of ABA and indole-3-acetic acid. The method yielded high peak resolution within 2 hours.

*Part of the experimental work described in this paper was performed at the Plant Hormone Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705, where the author was previously employed.

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INTRODUCTION

Several new innovations in the analytical methods, especially in chromatography, have greatly improved our separation capabilities recently that have helped to detect trace quantities in the parts-per-million (ppm) to parts-per-billion (ppb) range of several organic compounds. This is especially true for the separation and analysis of pesticides and plant growth substances. The chromatographic methods that received official and legal acceptance as the test methods include gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC) and a combined gas chromatography-mass spectrometry (GC-MS) which are capable of detecting trace (ppb) amounts in plant and animal tissues, foods and pharmaceuticals, besides pollutants from water and air. Furthermore, advances in the analytical methods for separation work are continuously sought for accurate and rapid determination in efficient and reliable manner either to complement or to supersede the existing techniques. A few such advances as coupling the liquid chromatography to mass spectrometer (LC-MS), one mass spectrometer to another mass spectrometer (MS-MS) and the GC and LC instruments to Fourier transform infrared spectrometers (GC-IR and LC-IR) which are further coupled to computers for total automation appear to be in the-developmental stages as a future generation of analytical instruments.

One recent development based on the principles of liquid chromatography and countercurrent distribution is known as the countercurrent chromatography (CCC) which has been introduced to separation science by Ito and his coworkers (1). They have been continuously refining the method for efficient separation of not only simple organic molecules but also high molecular weight polymers and cell particles. Several prototype instruments

were devised by Ito, and some of them are now commercially available (1,2). We have evaluated three instruments for their separation and analytical capabilities in the present work.

Several plant hormones and other growth substances appear to regulate the growth, development and reproduction of all higher plants (3). They are present at ppm to ppb levels in the plant tissue. They are required to study several metabolic changes including the stress-related phenomenon and the photosynthetic process, and also other biochemical and physiological implications during the plant growth and developmental process. We are especially interested in the analysis of ABA because of its involvement in the seed dormancy, stomatal closure and environmental stress besides its primary inhibitory function during the plant growth. Several analytical methods such as HPLC, GLC and GC-MS are now used for separation of plant hormones, and particularly for the analysis of ABA from plant tissues (3). As a continuation of our ongoing program for refinement and development of analytical methods for plant hormone analysis, we have examined the countercurrent chromatography for its potential applications in this field (4) and now report here our assessment of three CCC instruments for possible routine laboratory use.

MATERIALS AND METHODS

Apparatus

In the present study the following three types of CCC devices were evaluated for their performance. The general operating conditions of these instruments are summarized in TABLE 1.

TABLE 1.

General Operating Conditions for CCC Instruments in Plant Hormone Analysis

CCC Instrument	Flow rate (ml/hr)	Revolutional speed (rpm)
1. Toroidal coil planet centrifuge	2.5-4.0	450-500
2. Preparative CCC with rotating coil assembly	180-190	40-50
3. High-speed preparative CCC: small column	75-80	800-850
medium column	240	800

1) Toroidal Coil Planet Centrifuge (Toroidal CPC):

Figure 1 shows a simple table top model of the toroidal coil planet centrifuge (5,6) which is used for analytical-scale separations ranging from a few micrograms to milligram quantities. It has a rotatory frame driven by a motor around the stationary pipe mounted on the central axis of the centrifuge and holds a pair of symmetrically spaced cylindrical holders (10 cm from the central axis), one of which (15 cm O.D.) has a coiled column while the other holder (10 cm O.D.) carries a counterweight to balance the centrifuge system. Each holder is equipped with a plastic gear (Winfred N. Berg, Inc.) which is coupled to an identical sun gear mounted around the central stationary pipe. The gear arrangement produces the desired planetary motion of the holders, i.e., revolution around the central axis of the apparatus and rotation about its own axis at the same angular velocity in the same direction. For mechanical stability of the centrifuge, the free end (right side) of the rotatory frame is coaxially connected to a short coupling pipe, which is supported by a stationary wall member of the centrifuge through a ball bearing. The instrument employs a long column, typically 50 m of 0.55 mm I.D. PTFE tubing (Zeus Industrial Products, Raritan, N. J.) wound (8500 turns) on a 1.5 mm flexible core (13-m long)

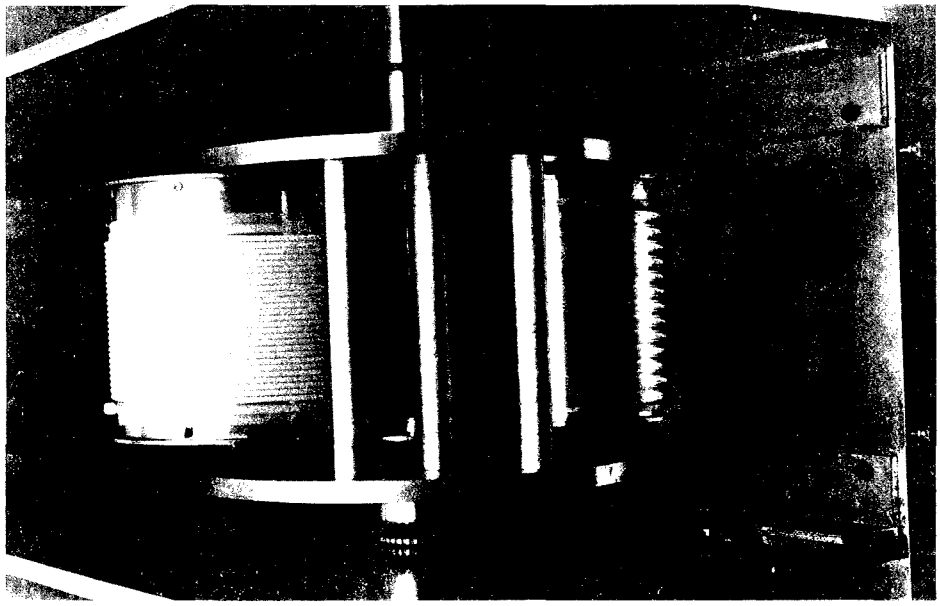

Figure 1. Toroidal coil planet centrifuge.

which is, in turn, coiled onto a (15 cm diameter) column holder. The gear drive provides a centrifugal force field that varies in intensity and direction as the column holder revolves around the central axis. The speed is adjustable up to 1000 rpm providing a maximum centrifugal force of about 450 xg. with a motomatic speed control unit (Electro-Craft).

The coiled column (total capacity: 18 ml) is first filled with a stationary phase of the pre-equilibrated two-phase solvent with the aid of either a Chromatronix Cheminert metering pump or a Milton-Roy pump. The test sample solution (50 µl) is typically introduced through a sample injection port and the mobile phase is pumped (pressure: 400-500 psi) through the column (2-4 ml/hr)

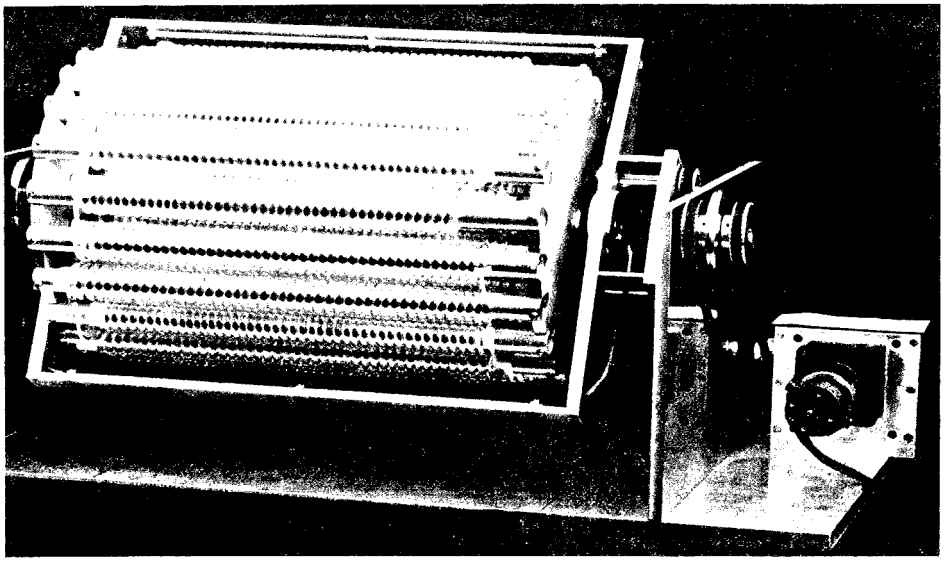

Figure 2. Preparative Countercurrent Chromatograph

while the apparatus is run at a desired revolutional speed (400-500 rpm). The column provides from 2000 to 6000 theoretical plates. The eluate is continuously monitored with either an LKB Uvicord III or an LKB Uvicord S at 206, 260 or 280 nm, depending on the test samples, and then collected with a fraction collector.

2) Preparative Countercurrent Chromatograph with Rotating Coil Assembly. A bench-top model of preparative CCC (FIG. 2) that performs efficient separations of gram-quantity samples was used to handle crude extracts of plant samples. The instrument design (2) consists of a coiled tube assembly that slowly rotates around its horizontal axis in a gravitational field. The stationary phase is retained by gravity in a large-diameter coil typically made of 50 turns of glass tubing with 0.5 cm

I.D., 2.5 cm helical diameter, and 90 ml capacity. A maximum of 30 columns can be mounted on the holder, 10 on the inner ring and 20 on the outer ring. The desired number of columns can be connected in series tail-to-head with PTFE tubing. Although the rotational speed of the column assembly can be regulated up to 300 rpm, a maximum rate of 50-100 rpm is ideal for the glass column assembly. A large column (500 helical turns) consisting of 10 coils on a 2.5-cm diameter core connected in series has a capacity of approximately 900 ml. When 1 g sample (in 30 ml solvent) is introduced into the column, it is separated with a theoretical plate efficiency ranging from 800 to 1000 when the mobile phase is pumped at 120 ml/hr (revolution speed: 40-60 rpm depending on the stationary phase used).

3) High-speed Preparative Countercurrent Chromatograph. This newly introduced CCC centrifuge performs fast and efficient separations in both analytical and preparative scales. It belongs to a member of the coil planet centrifuge which produces a synchonous planetary motion identical to that in the toroidal CPC. The design of the apparatus (FIG. 3) is also identical to that in the toroidal CPC except that the separation column consists of multiple layers of coiled PTFE tubing coaxially wound around a spool-shaped holder of 10 cm in diameter. In order to facilitate the preparation of this multi-layer coil column, the holder is made removable from the rotatory frame simply by loosening a pair of screws.

The unique feature of this CCC scheme is derived from an intriguing hydrodynamic motion of the two immiscible solvents in the multi-layer coil. Under the synchronous planetary motion the two solvent phases are subjected to a rapid countercurrent flow along the length of the coil, the upper phase traveling

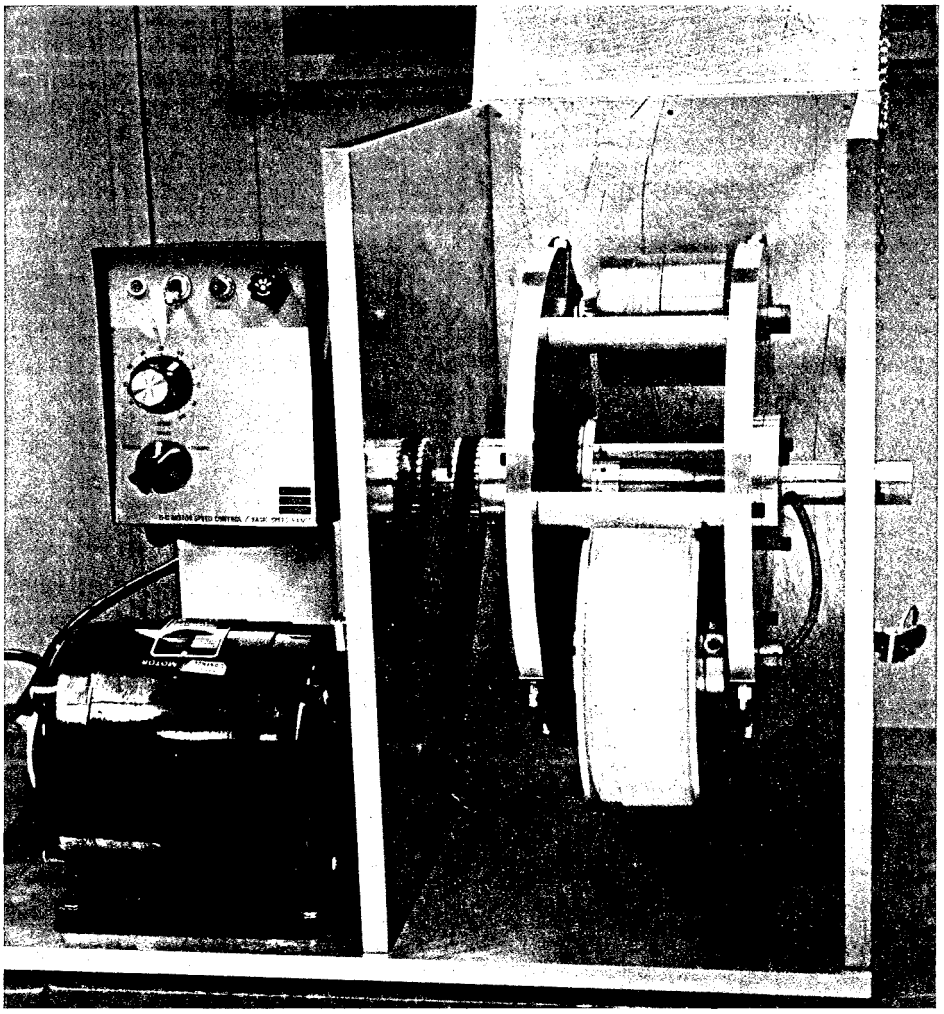

Figure 3. High-speed countercurrent chromatograph

toward the internal head-end and the lower phase, the external tail-end of the coil. This hydrodynamic motion establishes highly efficient partitioning of solutes with an excellent retention of the stationary phase under an unusually high flow rate of the mobile phase. Therefore, the method yields high peak resolution in a few hours of elution.

In the present study, separations are performed with two different I.D. columns - the medium size column with 130 m long. 1.6 mm I.D. and 285 ml capacity and the small size column with 170 m long, 1.0 mm I.D. and 140 ml capacity. The coil is first filled with the stationary phase followed by sample injection through the sample port. Then, the mobile phase is pumped into the column while the apparatus is run at 800 rpm. Both the sample solution and the mobile lower phase are introduced through the internal head-end of the coil. (If the mobile phase is the upper phase, they should be introduced through the external tail-end of the coil.) The flow rate applied to the medium column is 240 ml/hr and that to the small column, 80 ml/hr. The eluate is continuously monitored with an LKB Uvicord S at 260 nm and fractionated with an LKB fraction collector.

Sources of Hormone Samples

All of the test compounds were obtained from several commercial sources and used without further purification. Scarification of Zoysia grass seeds with alkali followed by extraction with diethyl ether-ethyl acetate (1:1) gave an extract which was acidified ($p_H 2$) with hydrochloric acid. The crude acidic extract was purified by HPLC on a reversed-phase μ Bondapak C_{18} column (Waters Associates) using methanol water (80:20) and the fraction corresponding to the retention volume of standard ABA sample was

collected and analyzed by CCC. Soybean seeds (400 g) were homogenized to a powder suspension with Polytron for 15 min with 700 ml of a mixture of methanol-water (80:20) and the homogenate was stored for 16 hr. After removing the insoluble material, the filtrate was concentrated to remove methanol. The solution was adjusted to p_H 2 and extracted with methylene chloride which was evaporated to give the acidic plant extract. The extract was then analyzed by CCC. Avocado fruit, after maceration, was extracted with 80% aqueous methanol (20:80) and kept at 4°C overnight. After removing the insoluble material, and the filtrate was concentrated. The aqueous extract was adjusted to p_H 2 with hydrochloric acid and extracted with diethyl ether (P.H. Terry unpublished procedure). Evaporation of solvent yielded an acidic extract which was subjected to CCC separation. Final confirmation for ABA was obtained by GC-MS analysis of the corresponding methyl ester.

RESULTS AND DISCUSSION

1) Toroidal CPC. Previously, we have reported the partition coefficients for indoles, gibberellins, cytokinins and ABA in different two-phase solvent systems. These systems were used for countercurrent chromatographic separation of plant hormones utilzing a toroidal coil planet centrifuge, referred here as the analytical CCC (4). We found that the naturally occurring indoles in the plant tissue can be separated in two-phase solvent systems, namely hexnane-ethyl acetate-methanol-water (0.6:1.4:1:1) and chloroform-acetic acid-water (2:2:1) because of their differences in partition coefficients ranging from 0.1-2.0. However, in the present study, we have chosen the latter solvent system, in

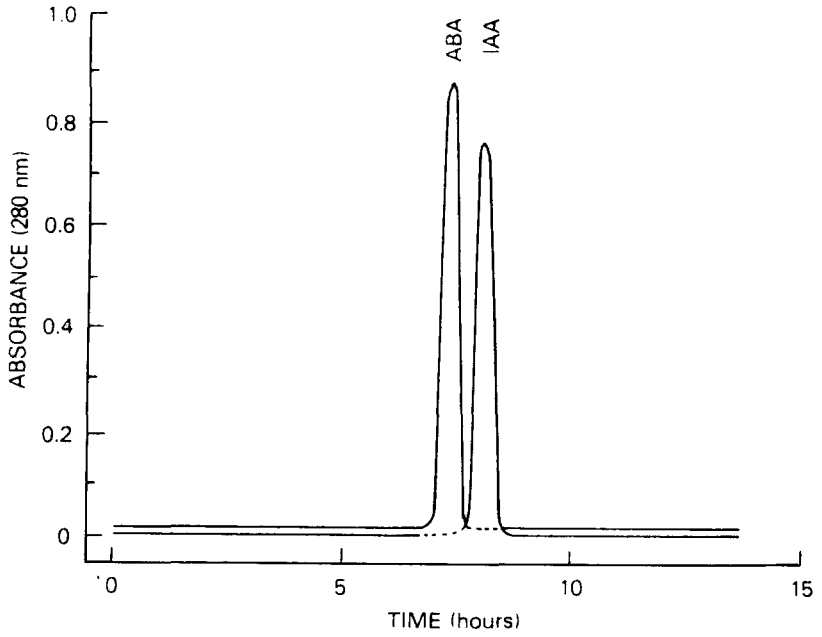

Figure 4. Separation of ABA and IAA by toroidal coil planet centrifuge

which ABA is readily separated from the indoles. Our previous paper (4) also reported the effective separation of gibberellins (GA_3,GA_4 and GA_7) by CCC in ethyl acetate-methanol 0.5M phosphate buffer, p_H 5.9 (3:1:2). Further, we have demonstrated that, for separation of cytokinins (zeatin and 6-isopentenyl adenine and their ribosides), either phase of the solvent system containing ethyl acetate-methanol-0.5M phosphate buffer, p_H 7 (3:1:3) could be used as the stationary phase which is analogous to normal and reversed phases in liquid chromatography.

In the present work, we have first standardized the procedure for separation of ABA with a mixture of ABA and indole-3-

acetic acid (IAA) in chloroform-acetic acid-water (2:2:1) keeping the organic phase stationary (FIG. 4). For ABA analysis from plant tissue, an acidic extract from Zoysia grass seed was subjected to separation by HPLC on a reversed-phase μ Bondapak C_{18} column using methanol-water (80:20) and a fraction corresponding to the retention volume of the standard ABA sample was collected. This fraction (100 μg) in 100 μl of methanol was introduced, via sample port, on to CCC column which contained the stationary organic phase and eluted with aqueous phase. The peak corresponding to the retention time of standard ABA was collected and the solvent evaporated to give a sample residue (22 ng) which was confirmed as ABA by HPLC under the conditions mentioned above (FIG. 5). A portion of the sample was converted into the methyl ester with diazomethane and subjected to GC-MS analysis which showed that the sample from Zoysia grass seed extract is indeed ABA methyl ester (FIG. 6).

2) Preparative CCC. To simplify the clean-up procedure of the crude plant extracts prior to CCC analysis of ABA, we have used preparative CCC which is capable of handling gram quantities. The chromatographic profile obtained from preparative CCC run for a standard mixture of ABA (100 mg) and IAA (100 mg) utilized a solvent system composed of 1600 ml chloroform, 1600 ml acetic acid and 800 ml water. The chromatograph was operated at 45 rpm and the column was filled with aqueous (upper) phase and eluted with organic (lower) phase maintaining the flow rate at 3.1 ml/min (UV detection at 260 nm). After standardizing the chromatographic operating conditions (retention times at a given flow rate for eluting solvent and revolutional speed) for standard ABA and IAA mixture (FIG. 7), the system was applied for plant sample analysis. The sample from plant extract (see Materials and Methods) (up to 1 g sample in 1 ml methanol) was introduced via sample port (a Rheodyne injector was attached to the instrument for sample introduction). By applying the above flow rate

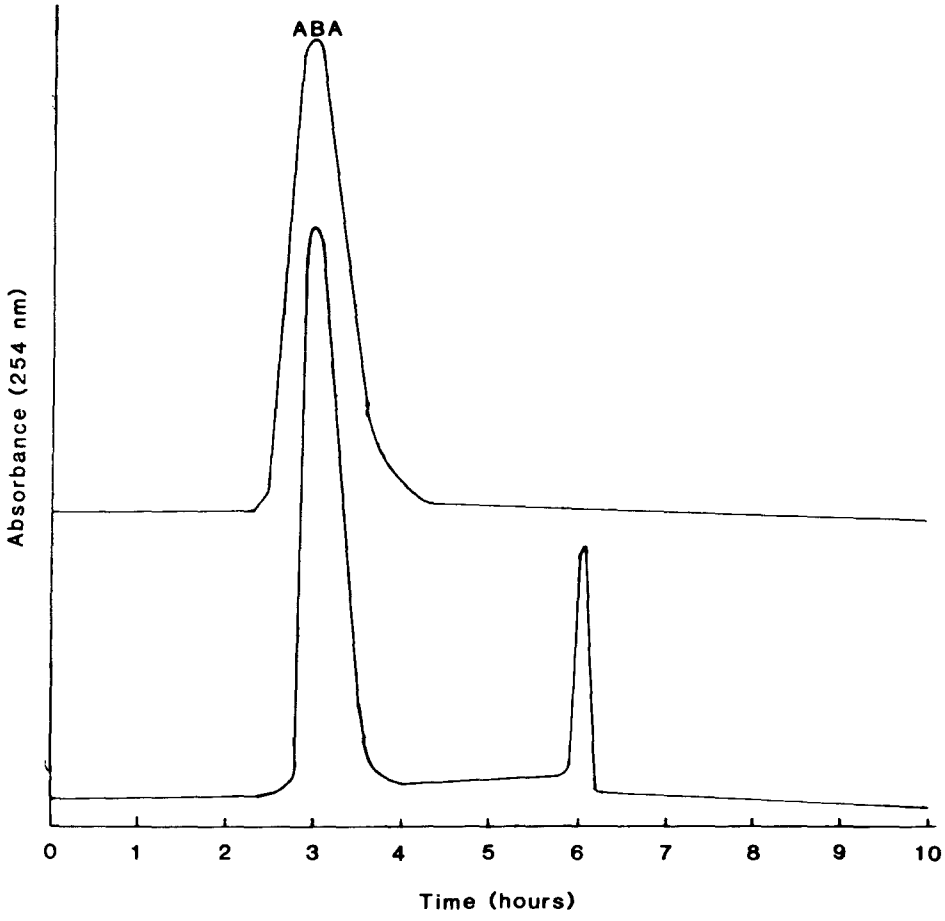

Figure 5. HPLC profile of ABA from Zoysia grass seed after CCC separation

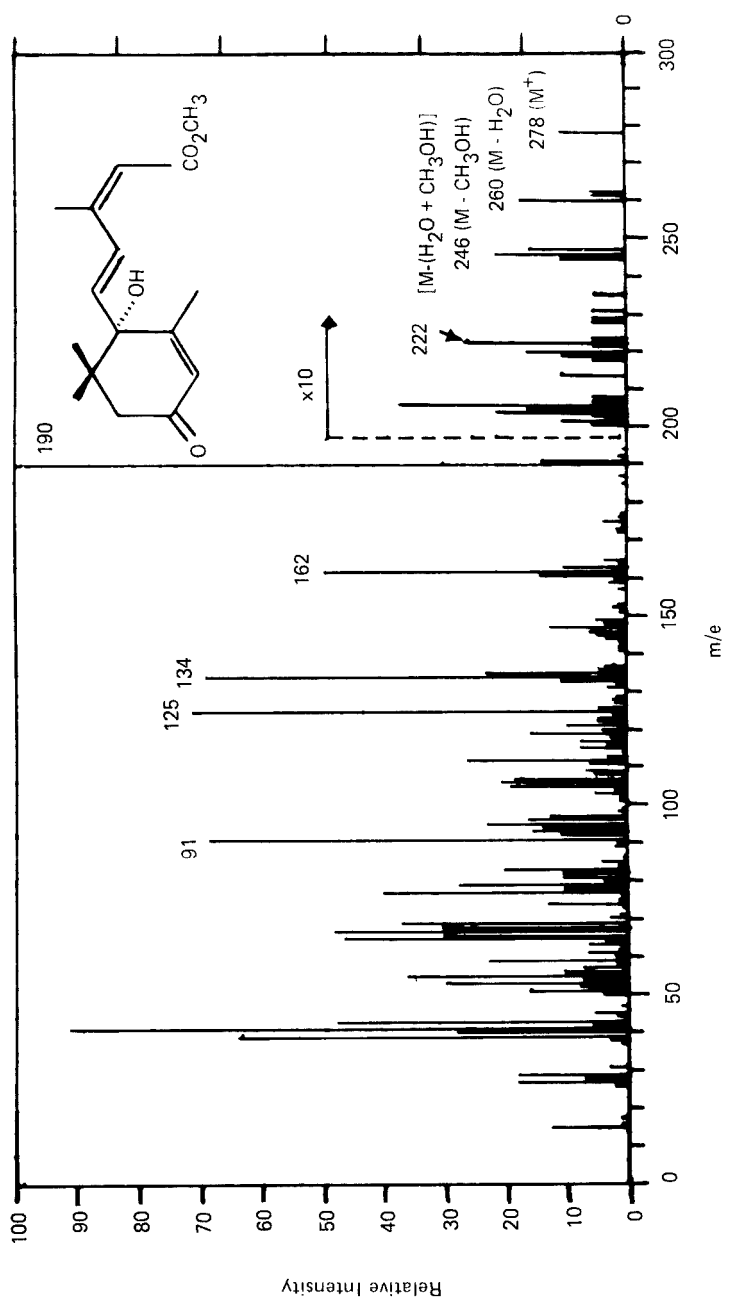

Figure 6. MS of ABA-methyl ester obtained from CCC separation of ABA

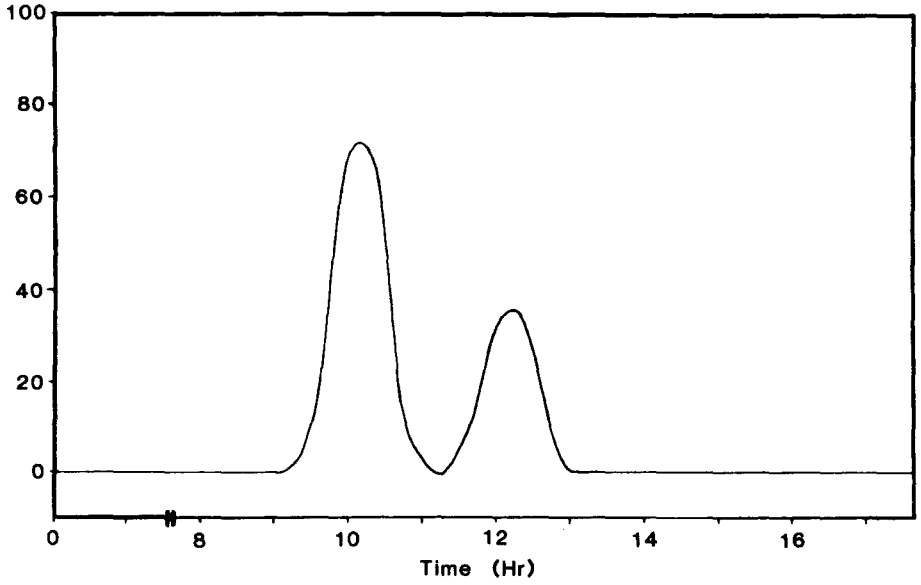

Figure 7. Separation of ABA and IAA by preparative CCC

under the optimum revolutional speed of 50 rpm, the eluate was monitored at 260 nm with a UV detector and the fraction corresponding to the retention time of standard ABA was collected, thus separating it from other undesired compounds. To insure that this fraction contained ABA, it was qualitatively checked for spectrum by an ultraviolet spectrophotometer. This fraction after evaporation of the solvent was subjected to toroidal CPC which separated ABA from other contaminants. Final confirmation for ABA from the sample (purified in this manner) was obtained by GC-MS analysis of the corresponding methyl-ester. The two-step procedure (preparative CCC followed by analytical CCC) eliminated the need for time consuming precolumn clean-up work of crude plant extracts.

TABLE 2.

Abscisic Acid Analysis by Countercurrent Chromatography

	Plant sample	ABA content* (ng/g plant tissue)
1.	Zoysia grass seed	22
2.	Soybean seed	26
3.	Avocado fruit	15

* The values reported here are only approximate (estimated error, σ = 4.86) and based on the average of three experiments.

Although HPLC is generally considered to be faster and more efficient method than CCC, we noticed the following disadvantages in the hormone analysis:

1) As for the sample size limitations, even on preparative HPLC column, the sample loading capacity cannot exceed 200-500 mg of the crude plant extracts.

2) Deterioration of expensive HPLC columns during clean-up with strong solvents sometimes causes the need for replacement of columns and this incurs additional expenses.

Utilizing the 2-step CCC procedure, we have analyzed representative classes of plant tissues (TABLE 2) to demonstrate its potential use for ABA analysis.

3) High-Speed Preparative CCC. While our aforementioned work using analytical and preparative CCC instruments was in progress, another CCC prototype for semi-preparative analysis was reported (7). The main advantage of using this CCC instrument is that it has shorter time duration for analysis as compared to other in-

struments (described above) without sacrificing resolution during separation. It can also be used for both analytical and preparative works because the coiled column assemblies can be changed depending on the size of the sample. In other words, a set of coiled column assemblies contains different sizes of the columns which can handle from micrograms (small column akin to analytical CCC column) to gram quantities (large column). A design similar to this CCC instrument is now commercially available (P.C. Inc., Potomac, MD). To determine its potential for plant hormone analysis, milligram amounts of a mixture of indole compounds were tested for the efficiency in the resolution of peaks during separation into individual components (FIG. 8A). The resolution of this mixture by the toroidal CPC (FIG. 8B) had already been reported. It is clear from Figure 8 that this instrument is far more superior than the other CCC instruments because of the differences in compound separation times and loading capacity of the sample. To further demonstrate its utility for ABA analysis, we have separated a mixture containing 50 mg ABA and 50 mg IAA. The separation was completed within 2 hours and the solvent required for CCC run was about 200 ml (FIG. 9). This is comparable to a typical HPLC run which takes about 2 hr and requires about 200 ml solvent. The potential use for this instrument appears to be great because of the shorter times (thereby quick runs) and lesser amount of solvent as compared to the preparative CCC.

This work suggests the possibility for an alternative analytical method in the separation science, particularly for trace analysis such as hormone analysis from plant tissue. At the present stage of the development, the CCC method supplements (complements) HPLC. The main advantage in CCC is that the column has no solid support thereby eliminating such problems as sample adsorption, deterioration (loss) of sensitive/ labile samples, besides the expenses involved for column purchases, which are

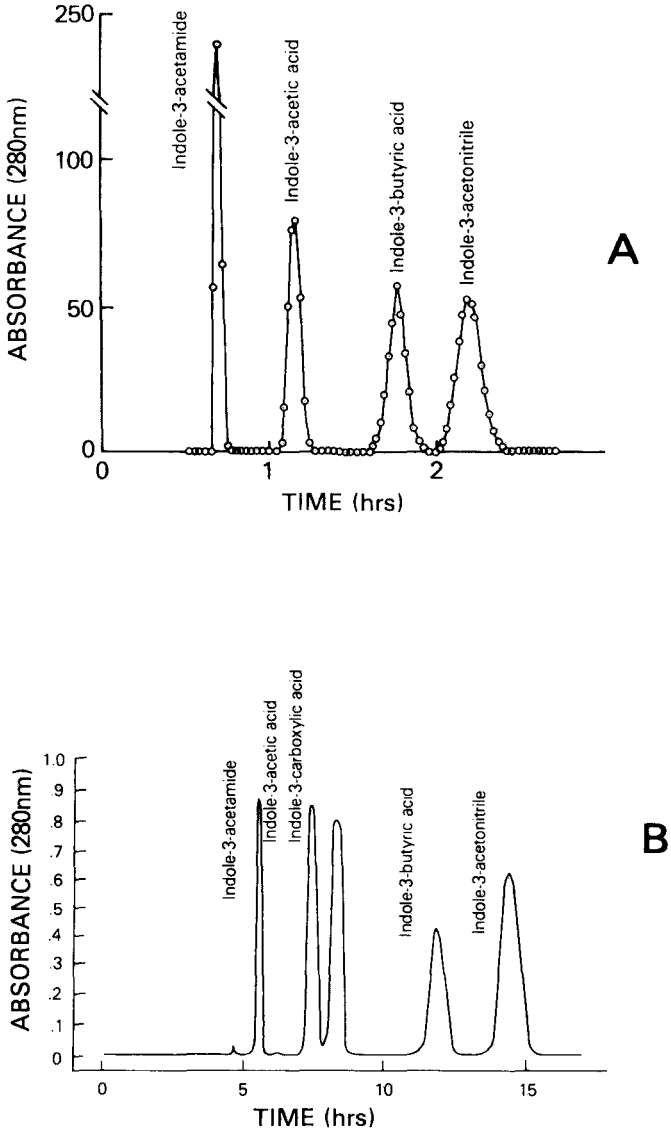

Figure 8. Separations of indole hormones by semi-preparative (A) and analytical (B) CCC. A: Chromatogram obtained by high-speed CCC; B: Chromatogram obtained by Toroidal CPC

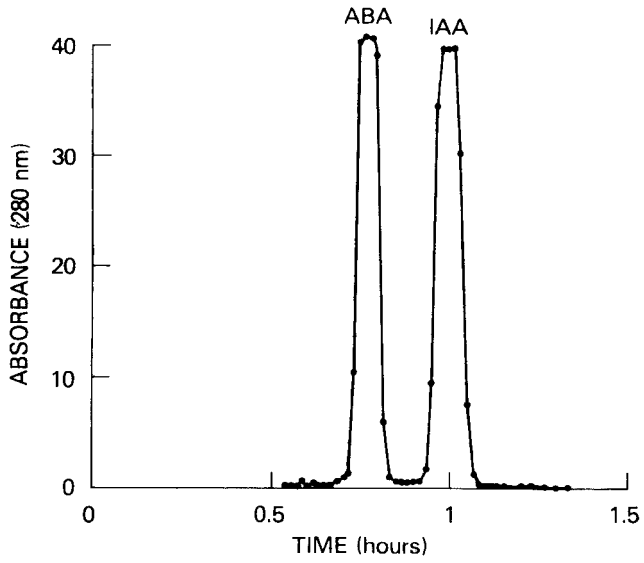

Figure 9. Separation of ABA and IAA by high-speed semi-preparative CCC

sometimes encountered in liquid chromatography. Also, there is no need to get different types of normal and reversedphase columns (as in HPLC) because the mobile and the stationary phase solvents can be interchanged and the column clean-up as well as the sample recovery are easily accomplished. For many researchers working with limited funds, cost for the purchase of the instrument is of a great concern and the CCC instruments offer alternative source because they are available under $10,000. In other words, this type of separation method appears to be an obvious choice when one considers the price and advantage of not using solid support in the column as long as the CCC and HPLC separations are comparable in the separation and analysis of organic compounds.

DISCLAIMER

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by any Agency of the United States Government and does not imply its approval to the exclusion of other products that may also be suitable.

REFERENCES

1. Mandava, N.B., Ito, Y. and Conway, W.D., Countercurrent Chromatography, Part I. Historical Development and Early Instrumentation, Amer. Lab., 14 [10], 62 (1982).

2. Mandava, N.B., Ito, Y. and Conway, W.D., Countercurrent Chromatography, Part II. Recent Instrumentation and Applications, Amer. Lab., 14 [11], 48 (1982).

3. Mandava, N.B., Plant Growth Substances, ACS Symposium Series, Volume 111, American Chemical Society, Washington, D.C., 1979, 310 p.

4. Mandava, N.B. and Ito, Y., Separation of Plant Hormones by Counter-Current Chromatography, J. Chromatogr., 247, 315 (1982).

5. Ito, Y., The Toroidal Coil Planet Centrifuge without Rotating Seals Applied to Countercurrent Chromatography, Anal. Biochem., 102, 150 (1980).

6. Ito, Y., Toroidal Coil Planet Centrifuge for Counter-Current Chromatography, J. Chromatogr., 192, 75 (1980).

7. Ito, Y., Sandlin, J. and Bowers, W.G., High-Speed Preparative Counter-Current Chromatography with a Coil Planet Centrifuge, J. Chromatogr., 244, 247 (1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(2), 323-340 (1984)

GRAM QUANTITY SEPARATION OF DNP (DINITROPHENYL) AMINO ACIDS WITH MULTI-LAYER COIL COUNTERCURRENT CHROMATOGRAPHY (CCC)

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ABSTRACT

The efforts have been successfully made to extend the preparative capability of the high-speed CCC scheme with a multi-layer large capacity coiled column. The apparatus is a table top model of a horizontal flow-through coil planet centrifuge which produces a synchronous planetary motion of the column holder. The separation column was prepared from a single piece of 70 m long, 2.6 mm i.d., PTFE tubing coiled around the spool-shaped holder to form multiple layers of the coil with a total capacity of about 400 ml. The performance of the apparatus was assessed with a standard set of DNP amino acid samples and a two-phase solvent system composed of chloroform, acetic acid and 0.1N HCl (2:2:1). Preparative capability of the method was evaluated in terms of the retention level of the stationary phase and peak resolution for various sample size ranging from 0.05g to 2g. The effects of sample volume, sample concentration and the choice of the sample diluent on the separation were studied. The results indicated that both the retention level and the peak resolution tend to decrease with the increase of the sample volume applied at a given concentration. For separation of 1 gram quantity, best results were obtained by applying the sample dissolved in a small volume (10 ml) consisting of equal amounts of the two phases. Overall results indicate that the present scheme is capable of efficient separation for gram quantity of samples in a short period of time. The preparative capability may be further increased by the use of a larger-diameter and/or longer coil.

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INTRODUCTION

Countercurrent chromatography (CCC) has a great potential in performing preparative-scale separations (1). The method eliminates all complications arising from the use of solid supports and retains a large volume of the stationary phase in the free space in the column to provide a large sample-loading capacity. The preparative CCC schemes developed in the past, however, requires relatively long separation times ranging from an overnight to a few days for completing a sizable separation (2-5). Recently, we have developed a new CCC scheme which produces highly efficient separations in short periods of time (6,7). The scheme uses a separation column consisting of multiple layers of the coil around a spool-shaped holder which is subjected to a synchronous planetary motion by the use of a coil planet centrifuge. With the use of 1.6 mm i.d. tubing, the scheme has produced separation of various biological samples in the order of 100 mg quantities within 3 hours. Efforts have been successfully made to scale up the sample loading capacity of the present scheme by using larger-diameter tubing. This paper describes the results of 1-g quantity separations of dinitrophenyl (DNP) amino acid mixture with a 2.6 mm i.d. multi-layer coil. Effects of sample dose, sample volume and sample diluents on the retention of the stationary phase and the partition efficiency are studied to optimize the operational conditions.

PRINCIPLE

As described earlier (1), the mechanism of countercurrent chromatography (CCC) is based on the complex hydrodynamic motion of two immiscible solvent phases in a coiled tube. When a water-filled coil is held horizontal and rotated around its own axis, anything that is heavier than water (glass bead) or lighter than water (air bubble) will tend to move towards the head end of

the coil. This is in accordance with the Archimedean screw principle. When two immiscible solvents are introduced into the coil, a hydrodynamic equilibrium is established whereby a certain volume of each phase occupies each helical turn (equilibrium volume ratio) and any excess of either phase remains at the tail end of the coil. This hydrodynamic equilibrium behavior of the two solvent phases can be efficiently used for solute partitioning. The column is first filled entirely with the stationary phase and the mobile phase is then pumped through the head of the coil. As the mobile phase occupies the first helical turn, hydrodynamic equilibrium (above) is established, thus producing a large retention of the stationary phase in the first helical turn. This process repeats until the mobile phase reaches the tail end of the coil after which only the mobile phase is displaced.

The amount of stationary phase retained in the coil is dependent upon two factors. The first is the initial equilibrium volume ratio before the elution is started. This ratio determines the maximum attainable retention level of the stationary phase. When the mobile phase is introduced into the inlet of the coil some of the stationary phase is displaced thus altering the equilibrium ratio. The other factor that determines retention of the stationary phase is the returning rate of the stationary phase. The higher the rate of the returning stationary phase, the higher the percent of retention, but always within the maximal attainable equilibrium volume ratio. In order to achieve a satisfactory retention level of the stationary phase, a large initial equilibrium volume ratio must be obtained in addition to a high rate of flow of the returning stationary phase. In the above simple scheme described, the initial equilibrium volume ratio meets the criteria to obtain a high level of retention; however, the returning rate of the stationary phase is insufficient if a high elution rate is to be applied.

Recently modifications have been made to the above scheme so that both requirements are satisfied (8). In Figure 1 cylindrical holder coaxially mounted with a planetary gear is coupled to an

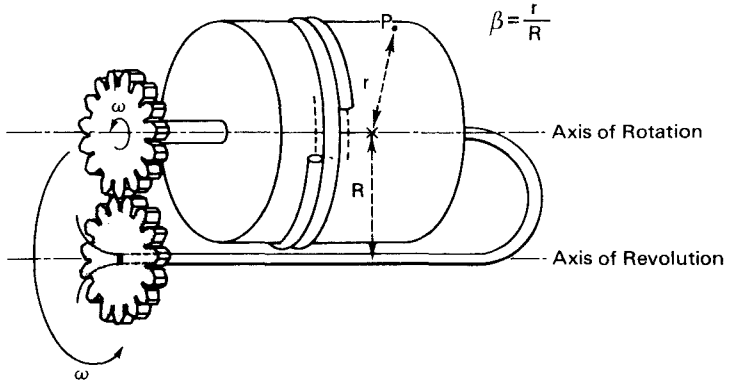

Figure 1. Synchronous planetary motion of the coil holder.

identical stationary sun gear (shaded) that is mounted along the central axis of the apparatus. This type of arrangement produces a beneficial planetary motion of the coil holder. The holder revolves around the central axis of the apparatus and simultaneously rotates about its own axis at the same angular velocity in the same direction. The holder always maintains its axis parallel to and at a distance R from the central axis of the apparatus.

This synchronous planetary motion produces a type of centrifugal force field that establishes a favorable hydrodynamic equilibrium that produces higher retention of the stationary phase. Experimental observations revealed that the magnitude and acting pattern of this centrifugal force field favor the heavier phase to remain in the peripheral layers of the coil (tail) and the lighter phase to remain in the internal layers of the coil (head). The centrifugal force field produced by this planetary motion is highly dependent upon the location of point P on the holder. Location of point P, expressed as β, is the ratio between the radii of rotation and revolution (r/R). When β is greater than 0.25, the centrifugal force vector is always directed outwardly from the coil (9). In addition to the beneficial hydrodynamic situation produced by this planetary motion, physical factors pertaining to the solvent system

also play a critical role in the retention of the stationary phase. These physical parameters include relative density, viscosity, and tube wall affinity.

When the upper phase is much lighter, has a greater tube wall affinity and is less viscous than the lower phase, then complete separation of the two phases is achieved along the entire length of the coil. The upper phase completely occupies the head of the coil while the lower phase occupies the tail side. In this situation retention of the stationary phase, be it the lower phase (tail to head elution) or the upper phase (head to tail elution), is maximized. However, if the upper phase is more viscous or has less tube-wall affinity than the lower phase, the above said separation of phases may not take place. Instead, a hydrodynamic equilibrium is established whereby the upper phase dominates the head side of the coil. In this situation the choice of which phase is to be the mobile phase is limited. Due to the hydrodynamic equilibrium a small portion of the stationary phase will remain in the head end of the coil. Therefore, if the upper phase is used as the mobile phase (tail to head elution) there would be a continuous carryover of the stationary phase (lower phase) from the head end since a small portion of the stationary phase always remains in the head side due to the equilibrium state. With continual loss of the stationary phase, solute resolution would drastically diminish. In this situation one is limited to the lower phase as the mobile phase since the tail end is completely occupied by the lower phase.

To solve the dilemma of limited mobile phase availability a recent modification has produced a situation where either phase can be used as the mobile phase with relatively the same percentage of stationary phase remaining in the column. The improved coil is made by winding a single piece of PTFE tubing onto a spool-shaped holder with a pair of large flanges (6,7). The tubing is wound around the holder in a continuous fashion in which multiple layers are formed. This multi-layer configuration produces a centrifugal force field gradient created from the internal layer of the coil (head end) toward the external layer of the coil (tail end). This gradient

forces the upper phase to move toward the head and the lower phase toward the tail. Due to this gradient the hydrodynamic equilibrium is altered in such a way that there is more complete separation of the two phases along the entire length of the coil. When there is complete separation of the phases, either phase can be used as the mobile phase without carryover of the stationary phase.

EXPERIMENTAL

Apparatus

The apparatus is a table top model of a horizontal flow-through coil planet centrifuge with a multi-layer coil as previously described (7). Figure 2 illustrates a cross-sectional view of our prototype. The motor drives a rotary frame about a stationary central shaft by means of a pair of toothed pulleys and a toothed belt. The rotary frame holds a freely rotating multi-layer coil column and a counterbalance equidistant from the central stationary shaft. The coil holder is coupled to the stationary shaft by means of a stationary sun gear, attached to the central shaft, and a planetary gear attached to the column holder. This arrangement produces the desired synchronous planetary motion, one rotation per one revolution in the same direction. Both holder and counterbalance are removable from the rotary frame by means of loosening a pair of screws. This easily facilitates changing coils with tubing of different i.d. The coil itself is comprised of a spool equipped with two large flanges, around which a single piece of 70 m long, 2.6 mm i.d. PTFE tubing is wound, thus producing a multi-layer arrangement. Each terminal of the coil is attached to an appropriate diameter flow tube. These flow tubes are passed through a side hole leading to the lumen of the central shaft of the column holder and emerge at the most proximal end of this shaft. The flow tubes are then passed through a side hole of the short coupling pipe that leads to the lumen of the central stationary shaft. The flow tubes are protected from metal contact

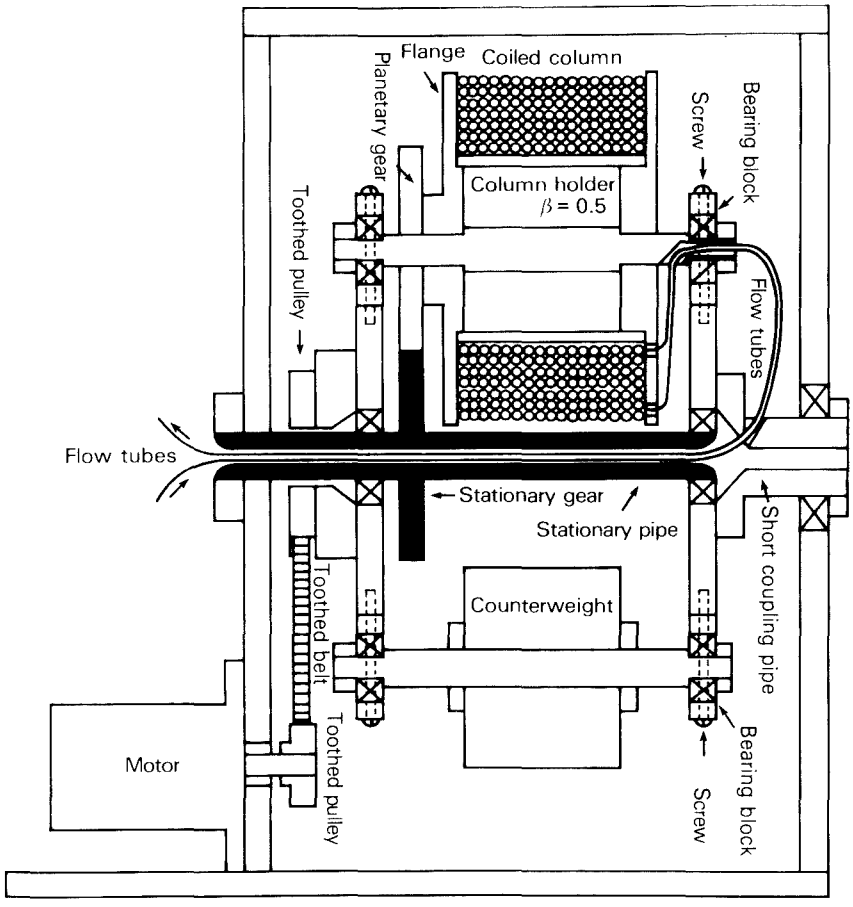

Figure 2. Cross-sectional view through the central axis of the apparatus.

by a piece of lubricated Tygon tubing along the entire length of the flow tubes to the point where they emerge from the lumen of the central stationary shaft. Revolutional speed (0-1000 rpm) is controlled with high accuracy and stability by the use of a speed control unit (Electro Craft or Bodine Electric Co.). The solvent is pumped through the column by means of a Beckman Accu Flow Pump or Milton Roy Mini Pump, while the effluent is monitored with an LKB Uvicord S at 280 nm and fractionated into test tubes with an LKB fraction collector for further analysis.

The multi-layer coil is prepared from a single piece of PTFE tubing, 2.6 mm i.d. and 70 m long. The tubing is tightly wound onto a spool equipped with two large flanges, thus providing boundaries for which the multi-layer configuration can be obtained. To prevent movement of the multi-layer coil column with respect to the column holder, each layer of coil is attached to the flanges with a piece of fiberglass enforced tape across the width of the coil. To further enhance greater stability at higher revolutional speeds, the same fiberglass tape is wrapped (one continuous layer) around the circumference of the coil. Around this layer of tape is wound a single piece of wire in which the ends were anchored to each flange. Around the wire another single layer of tape is wrapped in effect, sandwiching the wire between the two layers of tape. This arrangement firmly anchors the multi-layer coil to the holder.

Reagents

The DNP amino acid samples used in this study include N-2,4-DNP-L-valine, N-2,4-DNP-L-alanine, N,N-di(2,4-DNP)-L-cystine, N-2,4-DNP-DL-glutamic acid, and N-2,4-DNP-L-aspartic acid and they were obtained from Sigma Chemical Co., Saint Louis, MO. Organic solvents used in the two-phase solvent system were mostly of chromatographic grade. Chloroform was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ and Burdick and Jackson Laboratories, Inc., Muskegon, MI, glacial acetic acid from MCN

Manufacturing Chemists, Inc., Cincinnati, Ohio, and Fisher Scientific Co., Fairlawn, NJ, and hydrochloric acid from Fisher Scientific Co., Fairlawn, NJ.

Preparation of Solvent System and Sample Solution

The two-phase solvent system was prepared by mixing chloroform, acetic acid and 0.1N hydrochloric acid at a 2:2:1 volume ratio. The mixture was equilibrated in a separatory funnel at room temperature and separated before use.

The sample solutions were prepared by dissolving a mixture of the 5 DNP amino acids in the upper and/or lower phase and stored in the dark at 4°C. For the first set of experiments where the sample concentration remained constant at 5g% (Figures 3 and 4), the sample solution was prepared by dissolving 1g each of DNP-valine, DNP-alanine, DNP-glutamic acid, DNP-aspartic acid, and 200 mg of diDNP-cystine (because of lower solubility) in 84 ml of the stationary phase. For the second set of experiments where the sample dose was fixed at about 1g for each run (Figure 5), the sample solution was prepared by dissolving 250 mg each of DNP-valine, DNP-alanine, DNP-glutamic acid, DNP-aspartic acid, and 50 mg of diDNP-cystine in 10 ml, 20 ml or 40 ml of the mobile and/or the stationary phase.

Separation Procedure

The column was first filled with the stationary phase followed by the introduction of the sample solution through the injection port. The mobile phase was then pumped through the column while the apparatus was run at a revolutional speed of 800 rpm. The eluate through the outlet of the column was continuously monitored with an LKB Uvicord S at 280 nm and fractionated into test tubes with an LKB fraction collector. Introduction of the sample solution and solvent phases is dependent upon which phase is chosen to be the mobile phase. When the mobile phase is the lower phase,

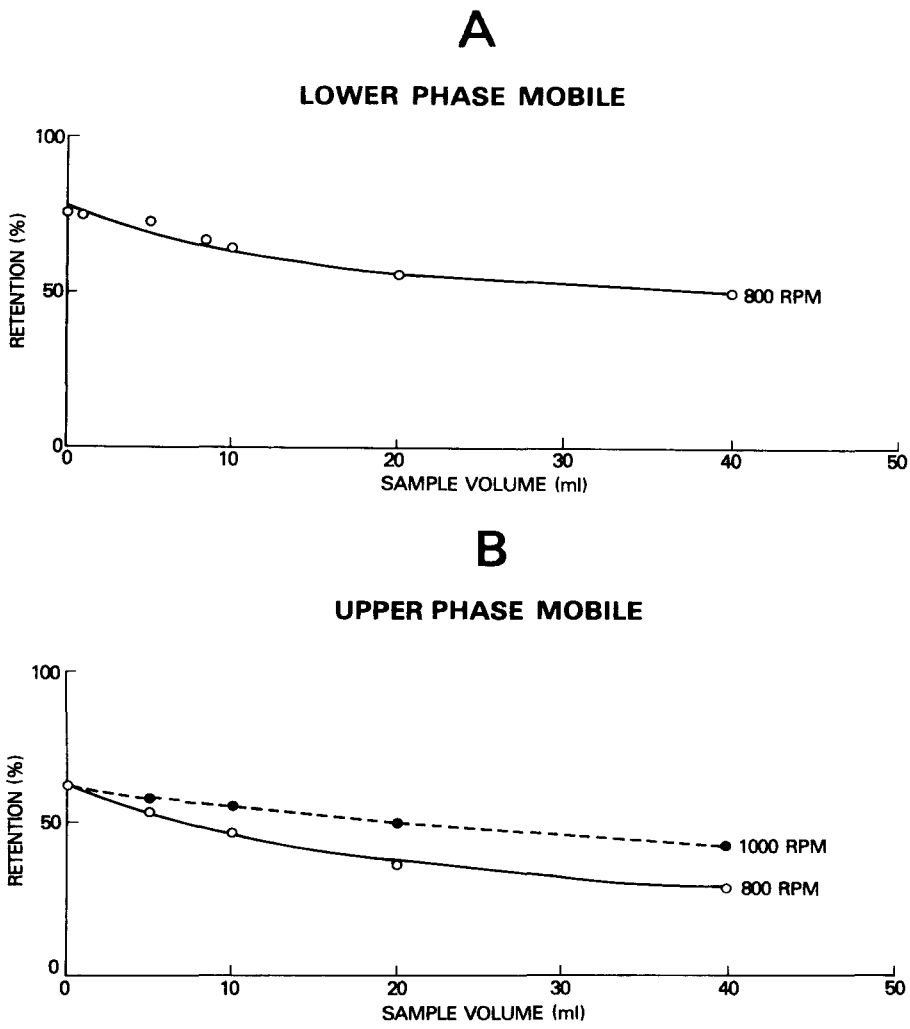

Figure 3. Effects of sample volume on retention level of the stationary phase.
(A) Lower Phase Mobile
(B) Upper Phase Mobile.

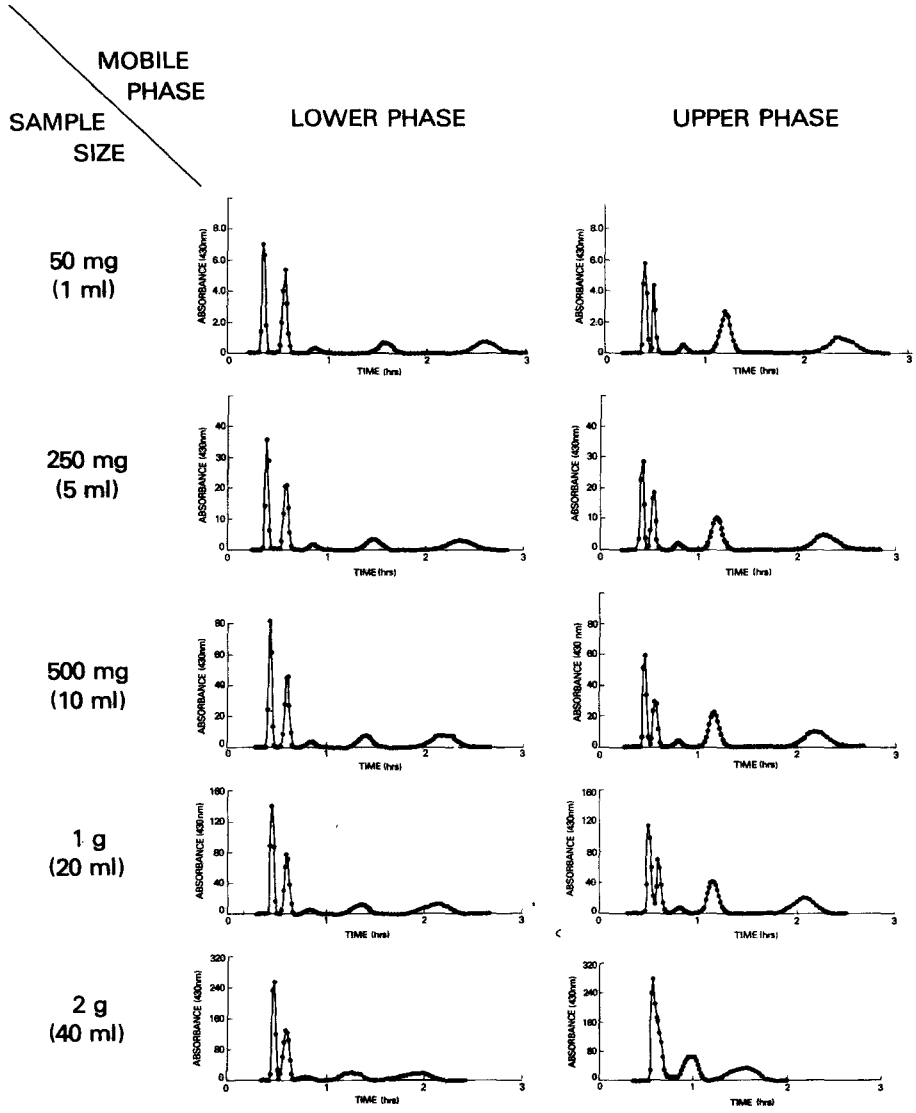

Figure 4. Effects of sample size on separation of a set of DNP amino acids.
Order of Elution:
Lower Phase Mobile (left column): DNP-valine, DNP-alanine, diDNP-cystine, DNP-glutamic acid, DNP-aspartic acid.
Upper Phase Mobile (right column): DNP-aspartic acid, DNP-glutamic acid, diDNP-cystine, DNP-alanine, DNP-valine.

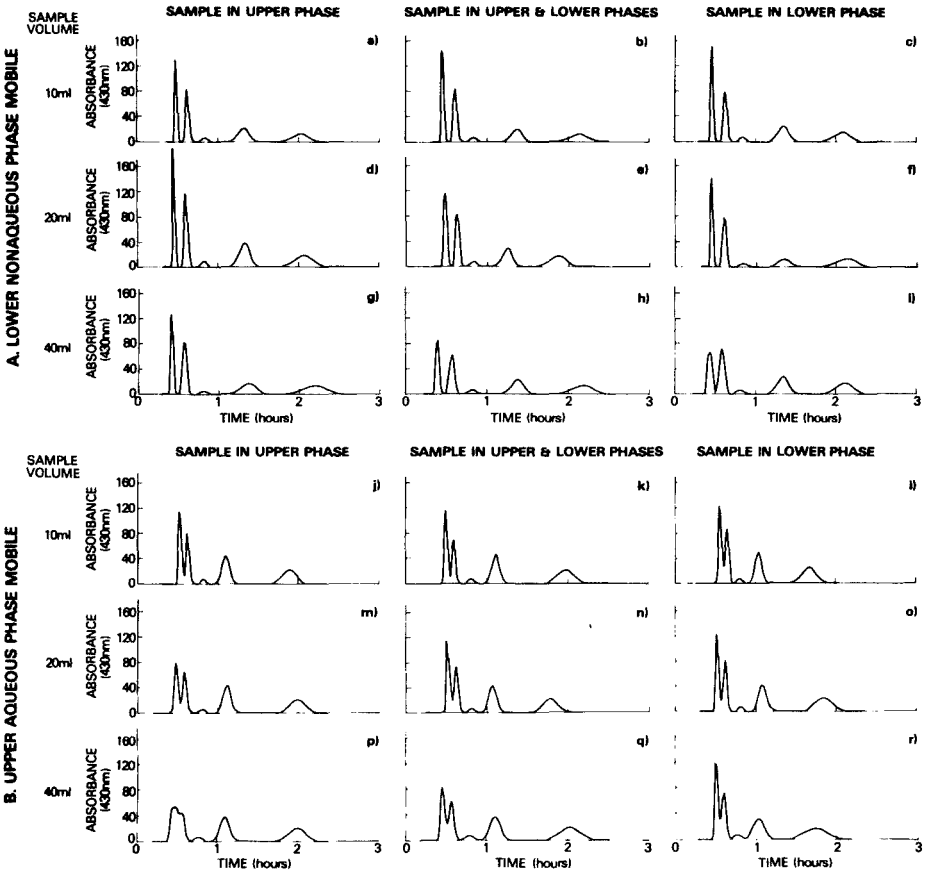

Figure 5. Effects of sample volume and sample diluent on separation of a set of DNP amino acids. Order of elution see Figure 4. The retention levels of the stationary phase for these separations are listed in TABLE II.

both sample solution and mobile phase are introduced at the head end of the coil (internal terminal). When the mobile phase is the upper phase, they are introduced through the tail terminal of the coil (external terminal). Upon completion of the separation, nitrogen gas at 100 psi was applied to the inlet of the column. Column contents were collected in a graduated cylinder and percent retention of the stationary phase remaining in the column was calculated. Aliquots of each fraction collected during the separation were mixed with 3 ml of methanol and analyzed the absorbance at 430 nm with a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

Effects of the sample size on the retention of the stationary phase and partition efficiency have been investigated on separation of 5 DNP amino acids with a two-phase solvent system composed of chloroform, acetic acid, and 0.1N hydrochloric acid at a 2:2:1 volume ratio. Both lower nonaqueous and upper aqueous phases were used as the mobile phase at 500 ml/h under 800 rpm unless otherwise indicated. The sample was dissolved in the stationary phase at 5 g%.

Figure 3 shows effects of the sample dose on the retention of the stationary phase where the retention volume expressed in percentages relative to the total column capacity is plotted against the applied sample volume. The results clearly indicates a general trend that the retention of the stationary phase decreases with the increased sample size for both mobile phase groups. The results also show that the retention level of the nonaqueous phase (B) is substantially lower than that of the aqueous phase (A). However, this low retention level of the nonaqueous phase is improved by applying a higher revolutional speed at 1000 rpm as indicated by the dotted line (B).

Chromatograms obtained from these experiments are illustrated in Figure 4 where individual charts are arranged according to the sample dose and the mobile phase. Although the peak resolution gradually decreases with the increased sample dose, the integrity

TABLE I

Comparison of Stationary Phase Retention with Increased Sample Volume (Sample Concentration Constant)

Sample Volume (ml)	Sample Wt (mg)	UP (%)	LP (%)
1.0	50.0	73.3	57.9
5.0	250.0	70.3	53.8
10.0	500.0	60.8	46.6
20.0	1000.0	56.4	39.5
40.0	2000.0	51.3	28.1

TABLE II

Comparison of Stationary Phase Retention with Sample Dissolved in Increasing Volumes of UP, LP, ULP (Sample Dose Constant 1g)

A. Lower Nonaqueous Phase Mobile

Sample Solvent	UP	ULP	LP
10 ml	50.6	54.4	48.8
20 ml	53.8	48.7	56.4
40 ml	64.1	60.8	57.3

B. Upper Aqueous Phase Mobile

Sample Solvent	UP	ULP	LP
10 ml	39.2	44.2	33.2
20 ml	45.6	39.5	41.0
40 ml	46.4	46.8	40.0

UP = Upper Phase, LP = Lower Phase, and ULP = Upper and Lower Phases

of the individual peaks is well preserved in all charts except for the 2 g run with the mobile upper phase. This lowest peak resolution coincides with the lowest retention level of the stationary phase at 28% (See Figure 3B and TABLE I).

The above results strongly suggest that the retention level of the stationary phase plays a critical role in partition efficiency in the present method. Among a number of factors involved in the retention of the stationary phase, we consider the density difference between the two phases most important. The greater is the density difference, the higher retention level is expected. In the solvent system utilized in the present study, the specific gravity for the upper aqueous and the lower nonaqueous phases measured 1.11 and 1.34, respectively. Introduction of the sample mixture into the solvent system reduces not only the original density of each phase but also the density difference between the two phases. This explains the fact that the application of the large sample dose tends to lower the retention level especially when the sample is dissolved in the nonaqueous stationary phase. Alteration of other factors such as interfacial tension, viscosity, etc. may also contribute to the loss of the stationary phase. For example, lowered interfacial tension tends to produce emulsification of the phases which hinders countercurrent movement of the two phases and results in carryover of the stationary phase.

Effects of the sample volume and the choice of the sample diluents on the peak resolution were studied on separations of 1g quantity of the DNP amino acid mixture. The results are shown in Figure 5 and TABLE II where both the lower nonaqueous phase (A) and the upper aqueous phase (B) are used as the mobile phase.

These chromatograms clearly show that the choice of the sample diluent makes little difference in resolution for both early and late appearing peaks until the sample volume is increased to 40 ml. With a large sample volume a significant decrease in peak resolution is observed in early appearing peaks especially when the sample is dissolved entirely in the mobile phase (Figure 5, i and

p). The loss of peak resolution becomes minimized if the sample is dissolved in the stationary phase (Figure 5, g and r). Further observation reveals that chromatograms i and p (Figure 5) with the lowest resolution in the early peaks show the best resolved late peaks among all 40 ml sample groups. This peculiar elution profile of the early and late peaks can not be attributed to the loss of the stationary phase since all these separations yielded satisfactory retention levels as shown in TABLE II. Instead, this phenomenon may be clearly understood on the basis of the two-phase interaction within the sample compartment at the beginning of the partition process.

When the sample mixture is introduced with the stationary phase, the eluting mobile phase will pick up each individual component at a different rate according to the partition coefficient. The components which favor the partition in the mobile phase, hence producing the early appearing peaks, are quickly depleted from the sample compartment of the stationary phase and concentrated in a small volume of the mobile phase, resulting in a sharp sample band. Therefore, a large volume of sample can be injected into the column without causing significant peak broadening of early appearing peaks. Although the components favoring the partition in the stationary phase are not concentrated by the mobile phase, they are subjected to the partition process in the column for longer periods of time to produce broader but better resolved peaks. Therefore, the initial band width for these components becomes less significant for separation. The above effects become reversed when the sample mixture is introduced with a large volume of the mobile phase. In this case the components favoring the partition in the stationary phase, thus producing the late appearing peaks, are concentrated in the stationary phase at the beginning of the column, while other components tend to produce broader peaks affected by the sample volume. Because these early appearing peaks are eluted close together, the loss of resolution among those peaks becomes serious compared with the late eluted peaks. By dissolving the sample in equal volumes of the upper and

lower phases, resolution can be relatively maintained for both early and late appearing peaks. There is a decrease in resolution when the sample is dissolved in a large volume of the upper and lower phase mixture but not to the degree seen when the sample is dissolved in a large quantity of the mobile phase.

As discussed above, in large-scale separations the best results are usually attained by dissolving the sample mixture entirely in the stationary phase. However, in some instances the sample mixture contains a component or components having low solubility in the stationary phase and, therefore, an enormously large volume of the stationary phase is required to dissolve the sample. In this situation the addition of the mobile phase to the sample solution will give beneficial effects in reducing the sample volume and at the same time securing the formation of two phases in the sample compartment upon introduction into the column.

The overall results of the experiments described above indicate that the present scheme produces efficient separations for gram quantity of samples in a short period of time. Sample-loading capacity of the scheme may be increased by the use of a larger-diameter and/or longer coil. We believe that the present countercurrent chromatographic scheme will be useful in separation and purification of natural products and synthetic drugs.

ACKNOWLEDGEMENT

The authors give special thanks to William G. Bowers for fabrication of the apparatus.

REFERENCES

1. Ito, Y., Countercurrent Chromatography, J. Biochem. Biophys. Methods, 5, 105 (1981).

2. Tanimura, T., Pisano, J. J., Ito, Y., and Bowman, R. L., Droplet Countercurrent Chromatography, Science, 169, 54 (1970).

3. Ito, Y., and Bowman, R. L., Countercurrent Chromatography: Liquid-Liquid Partition Chromatography Without Solid Support, J. Chromatogr. Sci. 8: 315 (1970).

4. Ito, Y., and Bowman, R. L., Preparative Countercurrent Chromatography with a Slowly Rotating Helical Tube, J. Chromatogr. 136, 189 (1977).

5. Ito, Y., and Bhatnagar, R., Preparative Counter-Current Chromatography with a Rotating Coil Assembly, J. Chromatogr. 207, 171 (1981).

6. Ito, Y., Efficient Preparative Counter-Current Chromatography with a Coil Planet Centrifuge, J. Chromatogr. 214, 122 (1981).

7. Ito, Y., Sandlin, J., and Bowers, W. G., High-Speed Preparative Counter-Current Chromatography with a Coil Planet Centrifuge, J. Chromatogr. 244, 247 (1982).

8. Ito, Y., New Continuous Extraction Method with a Coil Planet Centrifuge, J. Chromatogr. 207, 161 (1981).

9. Ito, Y., Toroidal Coil Planet Centrifuge for Counter-Current Chromatography, J. Chromatogr. 192, 75 (1980).

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SYNERGISTIC USE OF COUNTERCURRENT CHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE PURIFICATION OF SYNTHETIC PEPTIDES

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ABSTRACT

Examples are given demonstrating how countercurrent chromatography (CCC) and high performance liquid chromatography (HPLC) can be used together to purify synthetic peptides. In one example, CCC provided a preliminary purification of Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys by enabling separation of ultraviolet absorbing, ninhydrin-negative material from the desired peptide. Final purification was achieved with HPLC without risk of harming the HPLC column. In a second example Tyr-Ala-Ala-Nle-Ala-Ala was completely purified by CCC with the CCC separation rapidly and conveniently monitored by HPLC. CCC appears to be a very useful technique for the peptide chemist.

INTRODUCTION (2)

In previous reports we described the solid phase synthesis (3) and purification by countercurrent chromatography (CCC)

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(3,4) of a peptide having the sequence: Tyr-Ala-Ala-Nle-Ala-Ala-Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys. This purified peptide was subsequently coupled to bovine serum albumin and the conjugate injected into rabbits generated antibody to the peptide (5). In order to estimate which portion of the peptide contains antibody combining sites, the following peptides were prepared by solid phase synthesis: Tyr-Ala-Ala-Nle-Ala-Ala and Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys. This report describes the purification of these synthetic peptides and illustrates how CCC can be used in conjunction with high performance liquid chromatography (HPLC) to facilitate the frequently arduous task of peptide purification.

EXPERIMENTAL

Reagents

Water used for CCC and HPLC was obtained by passing our standard laboratory grade water through a Critical Applications Adsorption Column (Hydro Services and Supplies, Inc., Durham, NC) which contains highly purified, activated charcoal and has a 0.2 μm polycarbonate filter (Nucleopore Corp, CA) attached to its outlet. Butanol and acetonitrile were obtained from Burdick and Jackson Laboratories (Muskegon, MI). Trifluoroacetic acid (TFA) Sequanal Grade was purchased from Pierce Chemical Co. (Rockford, IL). The enzymes D-amino acid oxidase and leucine aminopeptidase (LAP) were purchased from Sigma Chemical Co. (St. Louis, MO) and carboxypeptidase A (CPA) was obtained from Boehringer Mannheim (Indianapolis, IN). Other chemicals and solvents were reagent grade or better.

Peptides were prepared by a modification of the Merrifield solid-phase method (6) using a Vega (Vega Biochemicals, Tucson, AZ) Model 96 automated synthesizer. The benzyl group was used to protect Ser, Asp and Glu; Arg, Tyr and Lys were protected by p-toluenesulfonyl, o-bromobenzyloxycarbonyl and o-chlorobenzyl-oxycarbonyl groups, respectively. Carboxyl terminal residues were coupled to the resin as their cesium salts (7). The average load obtained was 0.4 mmoles amino acid/g resin. Peptides were custom-cleaved with HF by Peninsula Labs. (Belmont CA).

Apparatus

CCC was performed with the new horizontal flow-through coil planet centrifuge whose design and function has been described in

detail elsewhere (8). In brief, the rotary frame of the centrifuge contains a pair of column holders; each is subjected to a specific mode of synchronous planetary motion provided by a set of gears and pulleys. In this work a column mounted on the gear-side holder was used. This gear-side holder (coupled with gears) rotates about its own axis and simultaneously revolves around the central axis of the apparatus at the same angular velocity and in the same direction. The column is made of PTFE tubing (2.6 mm inner diameter) and has a total capacity of 260 ml.

HPLC was performed with an Altex Model 312 MP (Altex Instruments, Berkeley, CA) system which is a microprocessor controlled, gradient system described in detail elsewhere (9). Separations were performed with 0.39x30 cm μBondapak C_{18} columns (Waters Associates, Milford, MA). The particle size of the column packings was 10 microns. Runs were monitored at 206 nm with a Model 970A variable wavelength detector from Tracor Instruments (Austin, TX). To maintain a stable baseline a back pressure was placed on the outlet tubing as previously described (9).

Amino acid analyses were performed with a Durrum (Dionex Corp, Sunnyvale, CA) D-500 amino acid analyzer equipped with a fluorescence detector. Amino acids were made fluorescent by reaction with o-phthalaldehyde.

Procedures

For CCC of both synthetic peptides the solvent system butanol:acetic acid:water (4:1:5 by volume) was used with the aqueous, lower phase serving as the mobile phase. This two-phase solvent system was thoroughly equilibrated in a separatory funnel at room temperature and separated before use. The column was filled with stationary phase as previously described (4) and the samples (100 mg) dissolved in mobile phase (10 ml) were injected through the sample port into the column which was rotated at 400 or 500 rpm. Following sample addition, the mobile phase was pumped through the rotating column at the desired flow rate using a Milton Roy miniPump (American Scientific Products, Baltimore, MD). Column eluates were continuously monitored with an LKB (LKB Instruments, Gaithersburg, MD) Uvicord S detector at 280 nm.

For HPLC work a stock solution obtained by mixing 10 ml of TFA with 100 ml of purified water was used to prepare the chromatographic solvents. Solvent A was prepared by diluting 5

ml of the stock solution to 1 liter with water and solvent B was prepared by diluting 4.25 ml of the stock solution to 1 liter with acetonitrile.

CPA digestions were performed in bicarbonate solution and LAP digestions were performed in 0.1 M TRIS buffer, pH 8.6-.005 M $MgCl_2$. Oxidations with D-amino acid oxidase were performed using a modification of a published procedure (10).

RESULTS AND DISCUSSION

The profile obtained from CCC for the synthetic nonapeptide is shown in Figure 1. Thin-layer chromatography (TLC) of aliquots from the CCC fractions using the solvent system butanol: pyridine:acetic acid:water (90:60:18:72 by volume) indicated which tubes should be pooled and that fractions eluting prior to and later than the pooled fraction contained little ninhydrin-positive material, even though their ultraviolet absorption was higher. The stationary phase also contained ninhydrin-negative, ultraviolet absorbing material. HPLC of the pooled fraction (Figure 2) indicated that this fraction was still heterogeneous. However, comparison of Figure 2 with Figure 3, which is an HPLC run of the synthetic starting material, indicates that CCC has provided a most useful preliminary purification of the peptide. Final purification was achieved with HPLC by running isocratically at 15% B with a flow rate of 0.5 ml/min. The purified synthetic peptide was obtained in 50% yield and was subsequently shown to contain an antigenic determinant recognized by antibody raised to Tyr-Ala-Ala-Nle-Ala-Ala-Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys (5).

CCC was used to completely purify Tyr-Ala-Ala-Nle-Ala-Ala. The chromatogram showed two large, partially separated peaks. Aliquots were removed from tubes comprising each peak but located far from the region of overlap, and these aliquots were evaporated to dryness. TLC using the solvent system butanol:acetic acid: water (4:1:5 by volume) indicated that each tube contained a single ninhydrin-positive peptide and that the peptide that eluted later in CCC also appeared to have a slightly larger R_f. Each fraction had the same amino acid composition, i.e. the expected composition. Aliquots of these purified peptides were

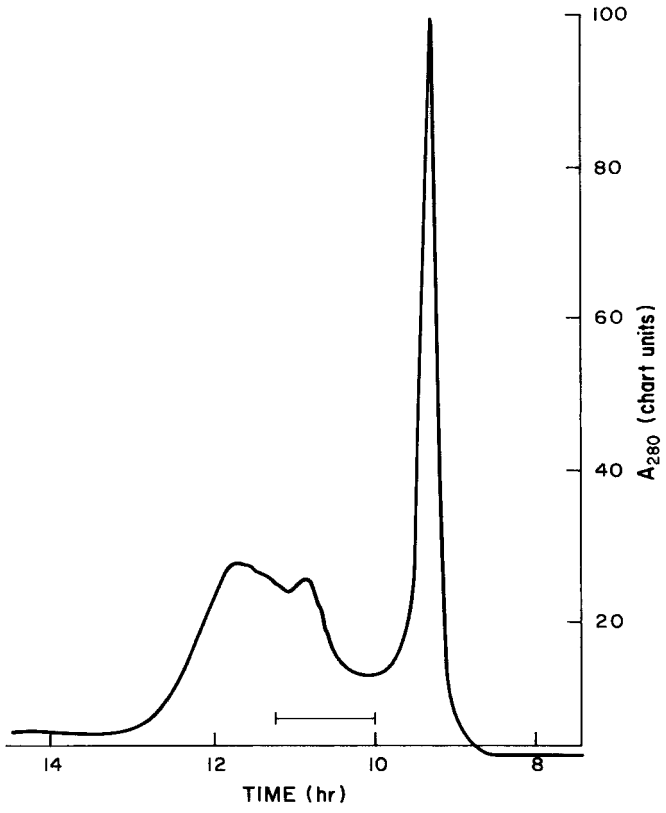

FIGURE 1: Countercurrent chromatogram of Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys. revolution:500 rpm; flow rate: 15ml/hour; fraction volume: 3 ml. The region underlined in the figure was pooled and purified by HPLC.

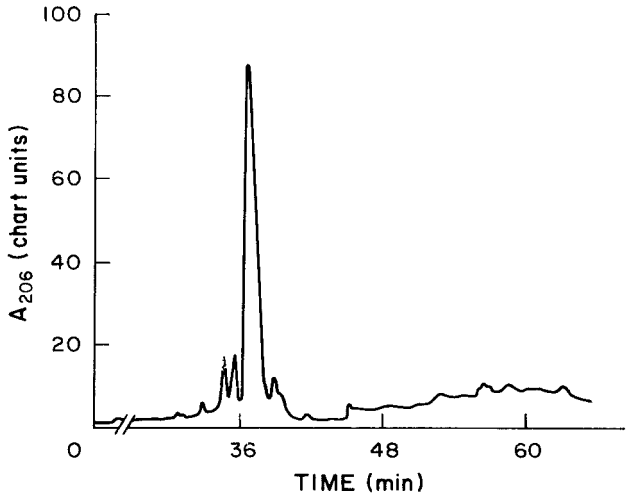

FIGURE 2: HPLC of an aliquot from the pooled fraction shown in Figure 1. The sample was run isocratically at 0% B for 5 min and then a linear gradient from 0% B to 60% B in 60 min was employed.

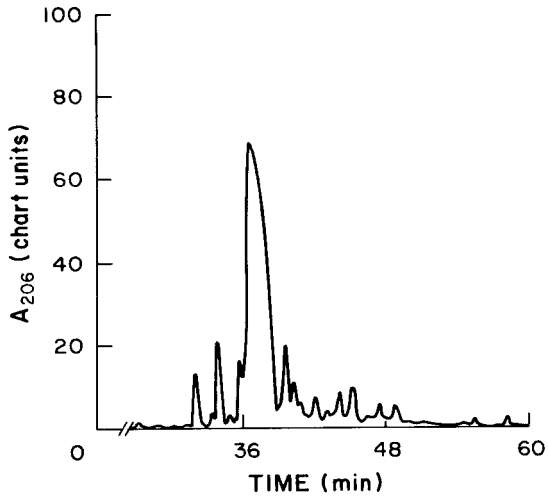

FIGURE 3: HPLC of Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys (500 μg) before CCC. Conditions for HPLC are the same as in the legend to Figure 2.

then used to develop an HPLC technique for monitoring the CCC run. By running isocratically at 10% B at a flow rate of 1 ml/min, both peaks could be readily separated in 18 minutes. Aliquots from appropriate tubes comprising the CCC run were then analyzed by HPLC. The results (Figure 4) clearly indicate which tubes contained pure peptide and which tubes contained mixtures of the two peptides. The fraction which eluted later in CCC when the organic phase was used as the stationary phase, also appeared to be more hydrophobic on HPLC. Based on the data given in Figure 4, tubes 101-107 and 115-150 were pooled to give Fraction I and Fraction II respectively.

Physical and biochemical techniques were used to characterize the two fractions and determine which was the desired peptide. Mass spectra showed some differences, but it could not be concluded which was the desired peptide. Mass spectra did not show the presence of bromine suggesting that the heterogeneity was not due to incomplete deblocking of the o-bromobenzyloxycarbonyl group used to protect the tyrosyl residue during peptide synthesis. Exopeptidase digestions indicated differences between the two fractions. CPA digestion of Fraction I liberated both alanine and nor-leucine while CPA digestion of Fraction II only liberated alanine. LAP digestion of Fraction I liberated tyrosine, alanine and nor-leucine in a molar ratio of 1:4:1 while LAP digestion of Fraction II liberated tyrosine and alanine in equal molar ratios but nor-leucine was absent. Treating lyophilized acid hydrolysates of each peptide with D-amino acid oxidase gave tyrosine, alanine and nor-leucine in a molar ratio of 1:4:1 for Fraction I while a tyrosine to alanine molar ratio of 1:4 was obtained for Fraction II. Thus, the difference between Fraction I and Fraction II was that Fraction I contained L-Nle and Fraction II contained D-Nle. (A comparison of CPK space-filling models of the two hexapeptide isomers shows that with Fraction II, the butyl side-chain (of Nle) is quite exposed while with Fraction I the butyl side chain is better able to fold back on other groups in the same peptide. Thus, model work indicates that Fraction II would appear more hydrophobic on CCC and HPLC.) Since D-amino acid oxidase treatment of an acid hydrolysate of Tyr-Ala-Ala-Nle-Ala-Ala-Met- Arg-Asp-Val-Val-Leu-Phe-Glu-Lys, which had been prepared earlier using the same synthetic reactions, showed that the peptide contained no D-Nle, it must be concluded that Fractions I and II were obtained this time because our synthetic intermediate (t-butyloxycarbonyl-nor-leucine) was

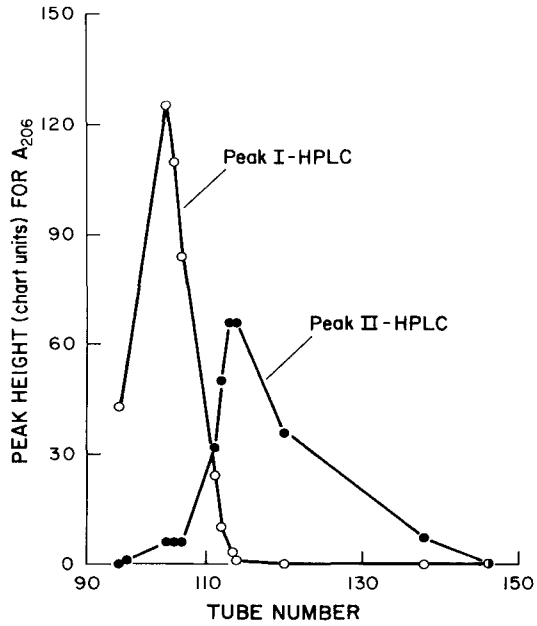

FIGURE 4: Countercurrent chromatogram of Tyr-Ala-Ala-Nle-Ala-Ala as monitored by HPLC. Conditions for CCC were: revolution: 400 rpm; flow rate: 10 ml/hour; fraction volume: 2 ml.

a racemic mixture. Fraction I contained no antigenic determinants recognized by antibody raised to this pentadecapeptide (5).

In a previous paper (3,4) it was noted that CCC appears to be ideally suited to the purification of synthetic peptides, since it gives peptide products free of salts, resin by-products, products of deblocking, and blocked peptides. This paper illustrates further that CCC can be used either for a preliminary purification with a final purification by HPLC or that CCC can be used for complete purification with the run conveniently monitored by HPLC. Thus far, only a few solvent systems have been used for CCC of peptides (3,4,11,12). Hopefully, other laboratories will employ CCC and provide additional solvent systems for the peptide chemist.

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REFERENCES

1. Correspondence should be sent to Dr. G.J. Putterman, Dept. of Ophthalmology and Visual Sciences, Yale University School of Medicine, New Haven, CT; Ms. Spear's present address: ISI, Philadelphia, PA; Dr. Perini's present address: Dept. of Pharmacology, University of Michigan School of Medicine, Ann Arbor, MI.

2. Abbreviations: CCC = countercurrent chromatography; HPLC = high performance liquid chromatography; TFA = trifluoroacetic acid; LAP = leucine aminopeptidase; CPA = carboxypeptidase A; TLC = thin-layer chromatography.

3. Putterman, G.J, Perini, F., White, E.L. and Ito, Y. Purification of Synthetic Peptides By Means of Countercurrent Chromatography With the Horizontal Flow-Through Coil Planet Centrifuge. Peptides: Structure and Biological Function, Proc. Sixth American Peptide Symposium, Gross, E. and Meienhofer, J., eds., Pierce Chemical Co., Rockford, IL, 1979, p.113.

4. Ito, Y. and Putterman, G.J. New Horizontal Flow-Through Coil Planet Centrifuge For Countercurrent Chromatography III. Separation and Purification of Dinitrophenyl Amino Acids and Peptides. J. Chromatogr. 193,37,1980.

5. Aldenderfer, P.H., Meade-Cobun, K.S. and Putterman, G.J. Unpublished results.

6. Merrifield, R.B. Solid Phase Peptide Synthesis I. The Synthesis of a Tetrapeptide. J. Amer. Chem. Soc., 85,2149,1963.

7. Gisin, B.F. The Preparation of Merrifield-Resins Through Total Esterification With Cesium Salts. Helvet. Chim. Acta 56,1476,1973.

8. Ito, Y. New Horizontal Flow-Through Coil Planet Centrifuge For Countercurrent Chromatography II. The Apparatus and Its Partition Capabilities. J. Chromatogr. 188,43,1980.

9. Putterman, G.J., Spear, M.B., Meade-Cobun, K.S., Widra, M. and Hixson, C.V. A Rapid Isolation of Human Chorionic Gonadotropin and Its Subunits By Reversed-Phase High Performance Liquid Chromatography. J. Liq. Chromatogr. 5,715,1982.

10. Wellner, D. and Lichtenberg, L.A., Assay of Amino Acid Oxidase, Methods in Enzymol. 17B, 593, 1971.

11. Knight, M. Ito, Y., and Chase, T.N. Preparative Purification of the Peptide Des-Enkephalin-γ-Endorphin. Comparison of High-Performance Liquid Chromatography and Countercurrent Chromatography. J. Chromatogr. 212,356,1981.

12. Knight, M., Tamminga, C.A., and Chase, T.N. Purification of Synthetic Peptides by Countercurrent Chromatography on the Coil Planet Centrifuge. Abstr. Pittsburgh Conf. Anal. Chem. and Applied Spectros. Atlantic City, 1982, p. 614.

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PURIFICATION OF SOLID-PHASE SYNTHESIZED PEPTIDES ON THE COIL PLANET CENTRIFUGE

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ABSTRACT

The analytical flow-through coil planet centrifuge, an instrument for countercurrent chromatography, performs the preparative purification of synthetic peptides. Various two-phase solvent systems have been tried with either phase mobile to purify many synthesized peptides. A series of N-terminal fragment peptides of cholecystokinin octapeptide (CCK 26-33) were synthesized by solid-phase techniques and purified on the coil planet centrifuge. The peptides were sulfated and chromatographed again. For hydrophobic peptides, purification is effected in solvent systems with a mobile aqueous phase. The n-butanol, acetic acid and water system (4:1:5 by volume) with the lower phase mobile was utilized. For sulfated peptides, the neutral system, 0.2 M ammonium acetate and n-butanol was generally applied.

INTRODUCTION

The horizontal flow-through coil planet centrifuge (2) has been used extensively for the preparative purification of peptides prepared for neuropharmacological experiments. Countercurrent chromatography (CCC) has been a useful method for separating truncated or deletion sequences and possible side products bearing

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protecting groups or products due to the oxidation of Trp and Met residues. Opioid peptides, cholecystokinin fragments and analogues have been synthesized by solid-phase techniques and purified in various two-phase solvent systems. The elution of the peptides was modified by changes in the composition of the solvent system to afford better separations. The equipment and methodology used in these experiments including the development of the solvent systems will be described. With this experience hopefully the behavior of peptides in the chromatography will become more predictable. One solvent system useful for purifying any synthetic product or adequate as an initial and routine purification step is a goal of our developmental work.

EXPERIMENTAL

Synthesis Of Peptides

Peptides were synthesized by standard solid-phase procedures whereby N-t-butyloxycarbonyl-L-amino acids including β-Benzyl-L-Asp and 2Br carbobenzoxy-L-Tyr (Bachem, Torrance, CA; Peninsula, Belmont, CA; and Chemalog, So. Plainfield, NJ) were coupled in the presence of dicyclohexylcarbodiimide to an amino acyl resin (copolymer of polystyrene and divinylbenzene (BioRad, Richmond, CA) (3). The amino-protecting group was removed by 25% trifluoroacetic acid in methylene chloride and neutralized by 10% triethylamine in methylene chloride and the next amino acid was coupled in sequence. After deprotection, removal from the solid support was effected by reaction in anhydrous hydrogen fluoride in the presence of ethyl methyl sulfide and anisole. The syntheses were conducted automatically in the Model 990 B synthesizer (Beckman, Palo Alto, CA). After synthesis the peptides were submitted to countercurrent chromatography as described below. After purification the peptides were analyzed for purity by TLC, HPLC and amino acid analysis of a mercaptoethanesulfonic acid hydrolysis at 110°C, for 22 hr. Peptides of the cholecystokinin fragment series were sulfated by reaction with the pyridine · sulfur trioxide complex. After removal of solvent, the reaction was treated with 1 M $NaHCO_3$ and

lyophilized and purified by countercurrent chromatography. The products were analyzed for homogeneity and for the presence of the sulfate group by spectroscopic techniques.

Apparatus

The countercurrent chromatography was carried out in the analytical flow-through coil planet centrifuge. Two instruments were available for our use. The Kontes prototype model (Vineland, NJ) mounted with coiled columns of either 1.5 mm i.d. fluorinated ethylene propylene (FEP) coiled tubing with a total volume of 150 ml or 2.6 mm i.d. FEP tubing with a volume of 260 ml. The other instrument was built by the Laboratory of Technical Development (National Heart, Lung, and Blood Institute, Bethesda, MD) and mounted with PTFE tubing with a volume of 260 ml. The coil is mounted on the gear side. Further details of the design of the instrument are described elsewhere (4). The Kontes prototype instrument with accessory equipment is shown (Figure 1). The coil is driven by an EC motomatic drive (Model E-652-M) (Electro-Craft Corp.). The solvent pump is a Milton Roy sapphire piston minipump, 16/160 ml/hr capacity, (Riviera Beach, FL). The sample loading device, an MTS 4-way slide valve, 0.8 mm bore (Pierce, Rockland, IL) is connected between the pump and coil. The rest of the components are Instrumentation Specialties Co. I.S.C.O. (Lincoln, NE) equipment; Model 328 fraction collector, Type 6 optical unit and Model UA-5 absorbance monitor and recorder.

Methods

Crude peptide in amounts ranging from 200 to 600 mg which were typical synthetic yields but usually not more than 300 mg were chromatographed. The coil was filled with stationary phase, either upper or lower phase of an equilibrated two-phase solvent system. All solvent systems included n-butanol as the upper phase. Solvents were reagent grade from Baker (Phillipsburg, NJ) or Fisher Scientific Co. (Fair Lawn, NJ). Water was deionized, Hydro Service and Supplies (Durham, NC) and distilled or charcoal filtered. The

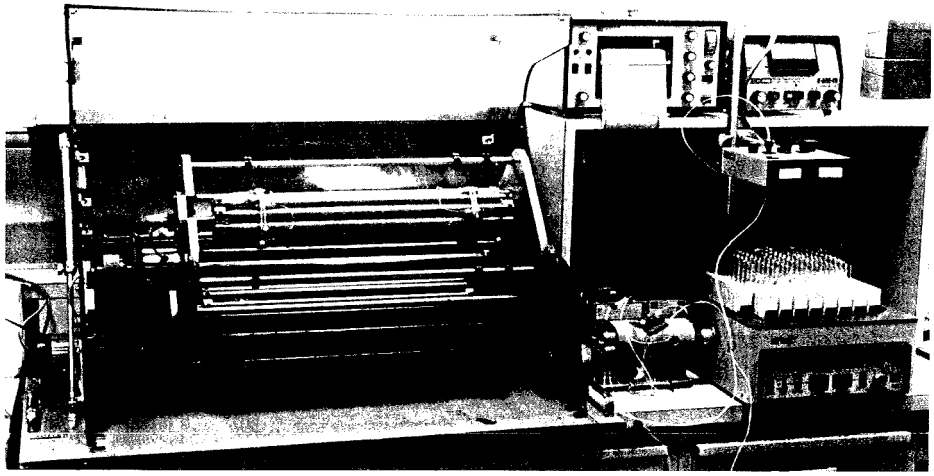

FIGURE 1

The analytical flow-through coil planet centrifuge, Kontes prototype instrument (serial no. 2) and accessory equipment described in the text. On the gear side (upper coil) is mounted the coil for preparative chromatography. On the pulley side (lower coil) is the 0.55 mm i.d. coil for analytical scale chromatography.

sample was dissolved in 3 to 5 ml of the solvent system and charged via the 4-way slide valve. The mobile phase was pumped at a flow rate of 24 ml/hr while the coil was rotated at 400 rpm in the counterclockwise direction. The effluent was monitored at 280 nm through a 40 microliter flow cell in the optical unit. The flow was down through the flow cell when the upper phase was used as the mobile phase and upward for an aqueous mobile phase. Fractions of 6 ml were collected. Samples were usually loaded late in the day and left to proceed overnight. The chromatography of an unknown sample was allowed to proceed for 3 to 4 column volumes. If necessary, the column contents were pumped out while fractions were collected and the coil rotated at 20 rpm in the clockwise direction as recommended by Y. Ito (4). Fractions containing peptide were pooled and concentrated by rotary evaporation, lyophilized and analyzed by TLC, HPLC

and amino acid analysis. The coil was washed with acetone and water between runs.

RESULTS

The peptides purified to date by the coil planet centrifuge method are listed in Tables 1 and 2 with the solvent systems utilized and resulting partition coefficients. The solvent systems are described in Table 1. Most of the peptides are cholecystokinin octapeptide (CCK 26-33) related peptides which were synthesized and purified in the conditions listed in Table 2. The peptides were sulfated and repurified in the systems shown in the table. Other peptides prepared in our laboratory are presented in Table 1.

The volume of elution of the substance permits the calculation of the partition coefficient (K) of the sample. The partition coefficient defined as (solute concentration in the mobile phase)/(solute concentration in the stationary phase) is computed as the gas chromatographic K:

V_m = excluded volume = solvent front tube x volume of fractions

V_c = total capacity of coil (260 ml or 150 ml)

V_s = $V_c - V_m$; retained stationary phase volume

K (Peak fraction x fraction volume - V_m) = V_s

For a sample eluting at peak tube 85 and solvent front at tube 26 in the 260 ml coil and fractions of 6 ml:

$$V_m = 26 \text{ X } 6 \text{ ml} = 156 \text{ ml}$$
$$V_s = 260 \text{ ml} - 156 \text{ ml} = 104 \text{ ml}$$
$$V_{85} = 510 \text{ ml} - 156 \text{ ml} = 354 \text{ ml}$$
$$K_{85} \ (354 \text{ ml}) = 104 \text{ ml}$$
$$K_{85} = .29$$

TABLE 1

Synthesized Peptides

Name and Sequence	Solvent System	Mobile Phase	K
CCK Asp Tyr (SO_3H) Met Gly Trp Met Asp Phe NH_2	NH_4OAc*	Upper	.27
($D\text{-}Ala^4$) CCK8 Asp Tyr (SO_3H) Met D-Ala Trp Met Asp Phe NH_2	EBHAW*	Upper	1.22
(Lys^{31}) CCK 26-31 Ac Asp Tyr (SO_3H) Met Gly Trp Lys	BAW*	Upper	.91
(Tfa Lys^{31}) CCK 26-31 Ac Asp Tyr Met Gly Trp Tfa Lys	NH_4OAc	Upper	1.22
βLPH 61-69 Tyr Gly Gly Phe Met Thr Ser Glu Lys	.1%TFA*	Lower	1.26
βLPH 66-77 Thr Ser Glu Lys Ser Gln Thr Pro Leu Val Thr Leu	.1%TFA	Upper	.19
(Tyr^{11}) Head activator peptide PCA Pro Pro Gly Gly Ser Lys Val Ile Leu Tyr	BAW	Lower	1.51

*NH_4OAc = 0.2 to 0.5M ammonium acetate, n-butanol (1:1 by volume)
EBHAW = ethanol, n-butanol, hexane, acetic acid, water (1:3:2:1:5)
BAW = n-butanol, acetic acid, water (4:1:5)
.1% TFA = n-butanol, 0.1% trifluoroacetic acid (1:1)

The purification of two peptides are shown in Figures 2 and 3. The chromatography of Ac Asp Tyr Met Gly Trp NH_2 (unsulfated Ac CCK 26-30 amide) in the BAW system is presented in Figure 2. The peptide which had an R_f of .58 in silica gel TLC with the BAW system (4:1:1) was purified in the BAW system with the lower phase mobile. The results are shown in Figure 2. The main peak of material emerged at fraction 149, K of .14 and contained 102 mg of pure peptide from a 0.5 m mole synthesis.

The isolation of a sulfated peptide Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2 (Ac CCK 26-31 NH_2) from the sulfation reaction and side

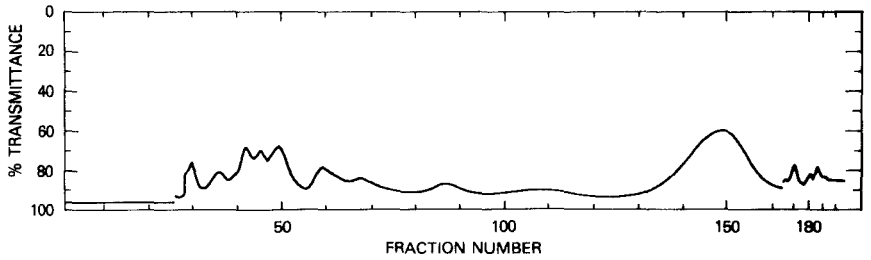

FIGURE 2

Purification of Ac Asp Tyr Met Gly Trp NH_2 in the BAW system with the lower phase as mobile phase. The 260 ml coil planet centrifuge with LKB (Bromma, Sweden) Uvicord S monitor and 6 channel recorder was used for this separation. The solvent front emerged at tube 26. Peptide, 102 mg, was collected from tubes 130 to 161 and contents of coil were pumped out beginning at fraction 163.

TABLE 2

Cholecystokinin Fragment Peptides

Sequence	Solvent System	Mobile Phase	K
Asp Tyr Met Gly Trp Met Asp NH_2	BAW	Lower	2.40
Asp Tyr (SO_3H) Met Gly Trp Met Asp NH_2	BAW	Upper	.27
Ac Asp Tyr Met Gly Trp Met Asp NH_2	BAW	Lower	.03
Ac Asp Tyr (SO_3H) Met Gly Trp Met Asp NH_2	NH_4OAc	Upper	.03
Ac Asp Tyr Met Gly Trp Met Asp	BAW	Lower	.49
Ac Asp Tyr (SO_3H) Met Gly Trp Met Asp	BAW	Upper	.88
Ac Asp Tyr Met Gly Trp Met NH_2	NH_4OAc	Upper	8.80
Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2	NH_4OAc	Upper	.90
Ac Asp Tyr Met Gly Trp Met	BAW	Lower	.17
		Upper	5.08
Ac Asp Tyr (SO_3H) Met Gly Trp Met	NH_4OAc	Upper	.03
Ac Asp Tyr Met Gly Trp NH_2	BAW	Lower	.14
Ac Asp Tyr (SO_3H) Met Gly Trp NH_2	NH_4OAc	Upper	.16
Ac Asp Tyr Met Gly	BAW	Upper	.48
Ac Asp Tyr (SO_3H) Met Gly	BAW	Upper	.12

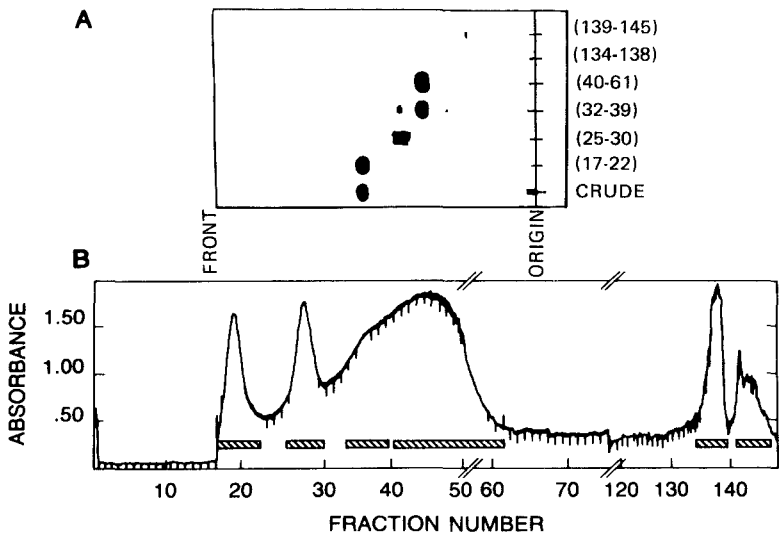

FIGURE 3

Panel A is the TLC analysis of the CCC fractionation (panel B) of the sulfation reaction of Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2. The pooled fractions indicated by diagonal lines were concentrated and lyophilized and 5 µg analyzed by TLC on silica gel in BAW (4:1:1) and developed by the Ehrlich spray (3). Unsulfated peptide, 206 mg, had been reacted with 660 mg of pyridine·sulfur trioxide complex and later treated with 15 ml of 1 M $NaHCO_3$ and neutralized with HCL. The dried mixture was chromatographed in the Kontes instrument in the 0.4 M NH_4OAc system. The solvent front emerged at fraction 17 and at fraction 118, the contents were pumped out. The first peak, 17-22, contained 102 mg of unsulfated peptide, fractions 25-30 contained 10 mg, 32-39 had 24 mg of the sulfated peptide and 40-61, 42 mg of pure sulfated peptide. Fractions 134-138 and 139-145 contained large amounts of salt.

products is shown in Figure 3. The TLC analysis of the products is included in the figure.

DISCUSSION

The partition coefficients indicate the relative elution volume and is determined by the hydrophobicity of the peptides. The majority of peptides in this group have many aromatic amino acids in their sequence and thus are highly hydrophobic. The R_f of these peptides in silica gel TLC are 0.5 or higher in the BAW system. Most of the peptides when chromatographed on the coil planet centrifuge eluted close to the solvent front in the BAW solvent system with the upper phase mobile. Better separation of impurities was achieved when the lower phase was used as the mobile phase. The peptide emerged at a later time and was distributed in a wider peak. If in TLC the R_f was high, it was better to elute in the lower K mode and if the peptide was hydrophilic, as indicated by a low R_f on TLC and there were more hydrophobic contaminants, it was useful to elute with the upper phase mobile. However if the peptide was not eluted after three column volumes then pumping out contents of the coil was warranted. A satisfactory fractionation was achieved in many cases.

Ac Asp Tyr Met Gly Trp Met as seen in Table 2 was an example of the direct determination of K's in both phases used as the mobile phase. In the BAW system with lower phase mobile, the K was 0.17 with the peak at fraction 128; and with the upper phase used as the mobile phase, the K was 5.08 (peak at tube 27). The experimental results agree with the theoretical expectation of the K's being the inverse of each other.

A means of decreasing the elution of peptides in the upper phase was to adjust the composition of the solvent system such that the sample was less soluble in the upper phase. This could be done by substituting ethyl acetate for n-butanol in the BAW system or using the system ethanol, n-butanol, hexane, acetic acid and water (EBHAW, 1:3:2:1:5). The peptide D-Ala^4 CCK 26-33 was eluted with

the solvent front in the system ethyl acetate, n-butanol, acetic acid and water (1:3:1:5) and in EBHAW it was eluted later with a K of 1.22.

The peptides were sulfated and repurified in either the BAW or NH_4OAc system with the upper phase mobile. The sulfation reaction mixture contained large quantities of sodium bicarbonate, NaCl and pyridine · sulfur trioxide decomposition products which remained in the lower phase as the peptides were extracted during the chromatography. The NH_4OAc system was preferred if adequate separation was achieved because the tyrosine sulfate ester function remained stable in the neutral pH of this system. The sulfation reaction mixture of Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2 was chromatographed in 0.4 M NH_4OAc and after two peaks (one of which was unsulfated peptide) a wide peak emerged (Figure 3). The earlier fractions in this peak were heterogeneous so the fractions were pooled separately from the rest of the peak. In the figure the elution of the chromatography and the TLC analysis of the peak contents are presented. The order of elution correlated with the R_f of the pooled fraction. The sulfated peptide was the third peak, fractions 40-61 (containing 42 mg) and on TLC had an R_f of .35. Thus the fractionating potential of CCC can be assessed by TLC. In order to separate the impurities better, product from a resynthesis plus the pool of fractions 32-39 from the synthesis in Figure 3 were combined and chromatographed in 0.2 M NH_4OAc. The sulfated peptide was eluted as a symmetrical peak at fraction 77 (not shown). The product was very pure by TLC. The lower ionic strength of the NH_4OAc decreased partitioning in the upper phase and thus resulted in better fractionation in this case. The peptide Ac Asp Tyr Met Gly Trp Tfa Lys was also better fractionated with 0.2 M than with 0.4 M NH_4OAc. In some cases the sulfated peptide had a low K and separation from the salt required another step, usually HPLC. This was the case for Ac CCK 26-32 amide, CCK 26-31, Ac CCK 26-29 and Ac CCK 26-30.

Generally the solvent systems selected for CCC application have been those described for countercurrent distribution (5).

These solvents have negligible absorbance in the ultraviolet range so the effluent can be monitored. The system 0.1% trifluoroacetic acid, n-butanol (1:1) has been used for β Lipotropin 61-69 (6) and for des enkephalin γ endorphin (β LPH 66-77) (7). Pyridine acetate (0.1% acetic acid, n-butanol and pyridine 11:5:3) which absorbs in the UV has been utilized for relatively hydrophilic peptides with upper phase mobile, but a dual flow cell would be necessary if monitoring is desired. All the solvent systems used in this work are volatile hence desalting steps are unnecessary. The examples where these other solvent systems were used are listed in Table 2.

CONCLUSION

From this experience we have found countercurrent chromatography on the coil planet centrifuge a convenient alternative to CCD because the apparatus is small in comparison to the glass manifold and resolution is better. As in CCD total recovery of material is always possible. If a run is to be repeated in another system, the sample is easily recovered. Furthermore, the capacity of the coil is enough for the yields of average solid-phase syntheses. The time required for purification may be longer than that for HPLC, but the sample can be loaded and left unattended usually overnight thus requiring less personal involvement than HPLC. The coil planet centrifuge has established itself well as a "work horse" instrument in our peptide chemistry laboratory.

ACKNOWLEDGEMENTS

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REFERENCES

1. Abbreviations used: CCC = countercurrent chromatography; CCD = countercurrent distribution; TLC = thin layer chromatog-

raphy; HPLC = high performance liquid chromatography; K = partition coefficient; R_f = mobility relative to front; UV = ultraviolet; Tfa = trifluoroacetyl; NH_4OAc = ammonium acetate; B = n-butanol; H = hexane; A = acetic acid; W = water; E = ethanol; CCK = cholecystokinin; TFA = trifluoroacetic acid; Ac = acetyl; LPH = lipotropin.

2. Ito, Y. and Bowman, R.L., J. Chrom. 147, 221 (1978).

3. Stewart, J.M. and Young, J.D., Solid-Phase Peptide Synthesis, W.H. Freeman Press, San Francisco, 1969.

4. Ito, Y., Meth. Enzymol., Vol. 91, Academic Press, New York, 1983, p. 335.

5. Reference 3, p. 49.

6. Knight, M. and Klee, W.A., J. Biol. Chem. 254, 10426 (1979).

7. Knight, M., Ito, Y. and Chase, T.N., J. Chrom., 212, 356 (1981).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(2), 363-384 (1984)

TOROIDAL COIL COUNTERCURRENT CHROMATOGRAPHY: A FAST SIMPLE ALTERNATIVE TO COUNTERCURRENT DISTRIBUTION USING AQUEOUS TWO PHASE PARTITION

Principles, Theory and Apparatus

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ABSTRACT

The principles, theoretical basis and equipment for continuous two phase toroidal coil chromatography are described. Rat liver homogenates were subjected to analytical subcellular fractionation by toroidal coil chromatography in a phase mixture of 3.3% (w/w) dextran T500, 5.4% (w/w) poly(ethylene glycol) 6000, 10 mM sodium phosphate-phosphoric acid buffer, pH 7.4, in 0.26 M sucrose containing 0.05 mM Na_2EDTA and 1 mM ethanol. The distribution of organelles, as reflected by their marker enzymes, was compared to that obtained by discrete counter-current partition in a 17 transfer apparatus. Toroidal coil chromatography showed enhanced resolution of certain organelles. In particular, almost complete separation of plasma membrane from endoplasmic reticulum was achieved and some resolution of plasma membrane from lysosomes was obtained. It is concluded that toroidal coil chromatography offers a potentially useful alternative approach to organelle separation techniques.

INTRODUCTION

The toroidal coil centrifuge is conceptually a simple piece of apparatus, comprising a helically wound tube mounted

circumferentially round a horizontal rotating disc. While it can be classified as a counter current chromatography process (1), its operation is similar to a continuous flow zonal rotor and its separation principles are those of a standard liquid-liquid chromatography column. Whereas centrifugation techniques separate on size, shape and density, the toroidal coil rotor offers the potential of separation based on partition between two immiscible liquids.

Separation of sample components with markedly different partition coefficients can be achieved in one or two test-tube partition steps involving mixing, settling and subsequent transfer of one of the phases to another test tube. However, samples with similar partition coefficients can only be resolved by using multiple discrete transfer or extraction techniques, such as counter-current distribution (2) or flow-through methods, such as liquid-liquid chromatography (3). The prime factor that makes toroidal coil chromatography different from the above techniques is that it uses centrifugation to hold one of the immiscible liquids stationary in the outer segments of the helical coil while the other phase is eluted through it.

Toroidal coil chromatography therefore can be considered as a continuous form of counter current distribution or a form of liquid-liquid chromatography without the solid support. It maintains the high retention volume of a phase partition process without an upper limit on the number of transfers. Adsorption problems and column contamination are minimised as the column requires no solid support to retain the stationary phase.

While applications using aqueous/organic phase systems have been described (4), the major application of the technique will be using polymer phase systems. The prime advantage of these polymer phases is that they are aqueous and the interfacial tension between the phases is extremely low - between 3 or 4 orders of magnitude less than aqueous/organic systems. However the very properties that make the phase systems an ideal

partitioning medium for cells and macromolecules create problems when it comes to the implementation of the phase systems as a separation technique. For example a partition step in a test tube with a typical aqueous/organic phase system would take less than a minute, while with a polymer phase system could take anywhere from twenty minutes to an hour, because of the prolonged settling time of these phase systems.

The major advantage of the toroidal coil centrifuge over conventional phase partition techniques is its simplicity of setting up and operation, the reduced operating time and its potential for far greater resolution. Cell separations are possible with apparatus of this kind, provided the coils slowly rotate relative to the centrifugal acceleration field to avoid sample sedimentation. The principle of cell separation using such an apparatus has already been demonstrated (5) using a non-synchronous flow-through coil planet centrifuge. However, this apparatus is much more complicated to construct and the principle of phase mixing more complex.

This publication describes the construction and operation of the toroidal coil centrifuge, examines the theory and demonstrates its reproducibility and resolving power by showing a clear fractionation of endoplasmic reticulum from plasma membrane in rat liver homogenate. The distribution of organelles is compared to a similar fractionation performed on a 17 transfer discrete counter current distribution apparatus, where the settling stage is enhanced using centrifugation (6). Detailed analysis of the phase system, operating and machine parameters will be described elsewhere (7).

DESCRIPTION OF APPARATUS

The Toroidal Coil (Figure 1)

The toroidal coil consists of 18 gauge PTFE tubing (i.d. 1.07 mm, o.d. 1.63 mm) wound onto a flexible 4.85 mm diameter

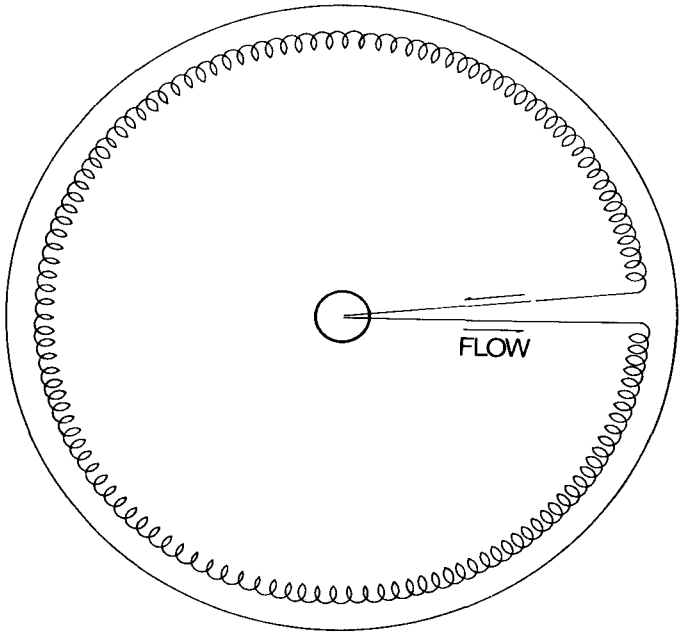

FIGURE 1

Plan view of toroidal coil, showing right handed helix with clockwise flow and rotation.

former. The helical coil so formed is mounted circumferentially on a disc at a radius of 21 cm. The disc has a shoulder to support the toroidal coil radially. There are, in addition, six clamps locating it. The coil can be continuous with the inlet and outlet leads. Alternatively, 24 gauge tubing (i.d. 0.72 mm, o.d. 1.07 mm) can be used. This can be sleeve joined without connectors and considerably reduces the dead volume of the inlet/outlet system.

The leads supplying liquid to and from the rotor have to be connected to the outside. Rotating seals could be used, but are prone to leakage. Methods eliminating the use of seals are simple to use and offer a number of advantages including reduced leakage and wear, and better sterility. One disadvantage is that

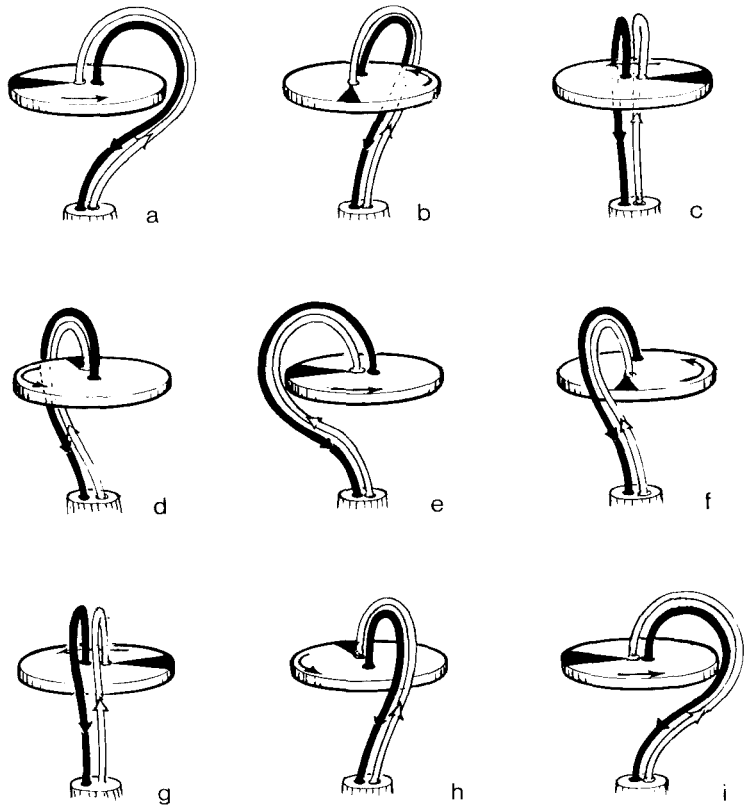

FIGURE 2

Sequencial diagrams showing the principle of seal-less connections to a rotating disc.

the rotor is more complex. The principle of connecting tubes to and from a rotating disc (8) is a simple one (figure 2). The leads enter the centrifuge along the centre line of rotation above the disc, pass round the outside and enter it from underneath. If the disc is then rotated at, say, 1000 rev/min and the leads are constrained to rotate about the same axis at 500 rev/min around the outside of the disc, then the leads will not twist.

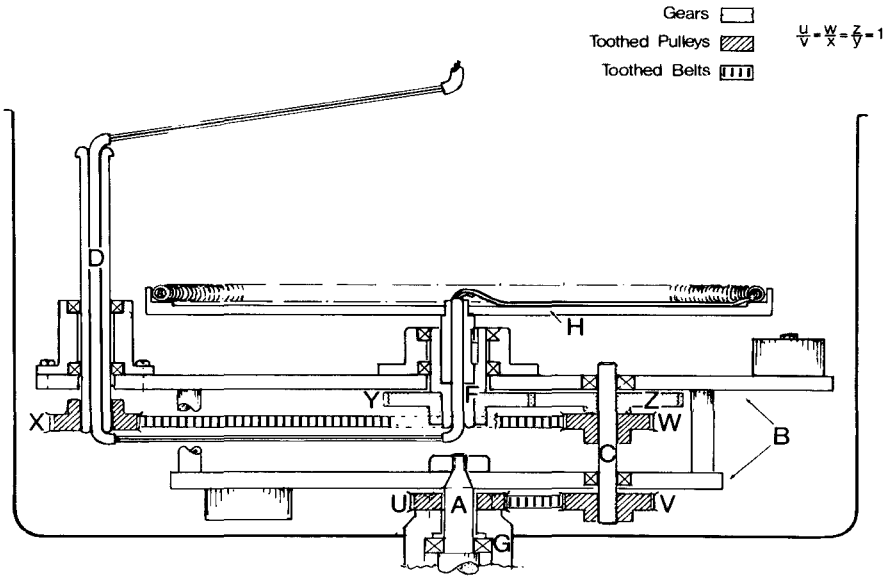

FIGURE 3

Diagram showing assembly of toroidal coil and seal-less connections. The drive shaft (A) rotates the carriage (B). The gear system (C) is constrained to rotate in planetary motion. Likewise the tube retaining shaft (D) is also constrained to rotate in planetary motion due to the toothed belt link between pulleys (X) and (W). The rotor shaft (F) will rotate in the opposite direction relative to shafts (C) and (D) due to the gear link between (Y) and (Z).

The current rotor is shown in figure 3, mounted in the bowl of a Beckman J6 centrifuge. This centrifuge is particularly suited to the installation of the rotor as the drive shaft is not integral with the motor, and can easily be replaced by a modified drive shaft with a stationary tooth pulley for the toroidal coil rotor.

The points of maximum stress on the input and output leads occur at the bottom of the rotor shafts and at the inlet to the centrifuge bowl, due to their tumbling and rolling motion. Wear

is minimised by mounting the inlet and outlet leads in a Tygon tubing sheath, rounding the exit holes of the shafts and applying a light covering of silicon grease. Tube life in excess of 250 hours (15,000,000 cycles) is common. It is important to ensure that the tension in the leads is not excessive or wear rates will dramatically increase. The rotor is dynamically balanced in two planes by counterweights.

The Operating System

The operating system is shown schematically in figure 4. A roller peristaltic pump (Gilson minipulse 2) is used to pump the phases from the reservoirs into the toroidal coil. The proportion of lower phase flow (α) is regulated by the relative sizes of the peristaltic tubing. A counter-flow, fine bore mixing chamber ensures efficient mixing of the phases. A 4-way slider valve (Altex) is used to inject the sample (loop volume, 1 ml). A fraction collector (Gilson TDC 80), in drop count mode, collects the eluent in 1.5 ml plastic centrifuge tubes (Ependorf).

THEORY

The fluid dynamics of two phase flow have been extensively studied for gas-liquid systems but for liquid/liquid systems still remain obscure, particularly when complicated by enhanced gravity and unconventional geometrical constraints.

A typical coil cross-section was constructed in Perspex, mounted on a specially adapted rotor and the behaviour of the phase system under the action of enhanced gravity observed with a stroboscope. The cross-section of the coil is shown in figure 5a-d. The coil is initially filled (a) with the lower phase followed (b) by the upper, lighter phase. The upper phase displaces the lower, heavier phase until it reaches point P1 when it cascades much like a waterfall to form (c) a new interface at Q1. Further flow of upper phase again displaces the lower phase in the next coil until new interfaces

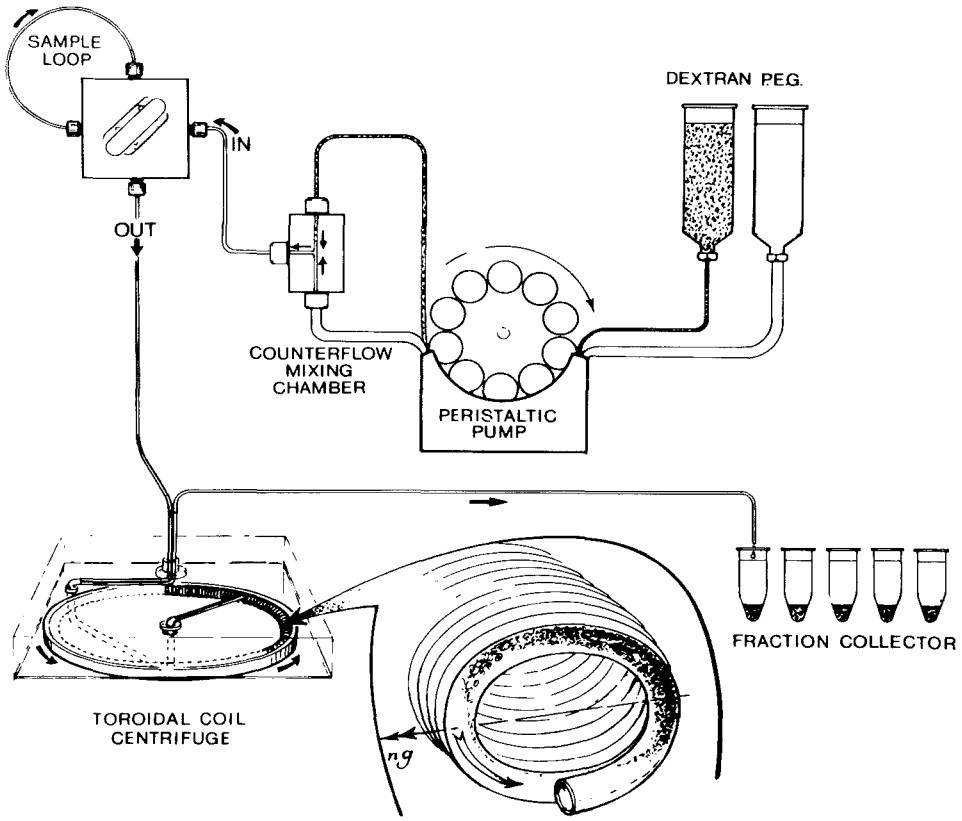

FIGURE 4

Schematic layout of the toroidal coil centrifuge operating system.

are formed (d) at Q2 and P2. The segment of lower phase between P1 and Q2 is left as the retained phase while the upper phase continues to stream through it. Mixing takes place at and below the interface Q2 relative to the acceleration vector (shown arrowed in 5a).

In principle either phase can be the mobile phase. If the heavy phase is mobile the cascade would occur on the other side

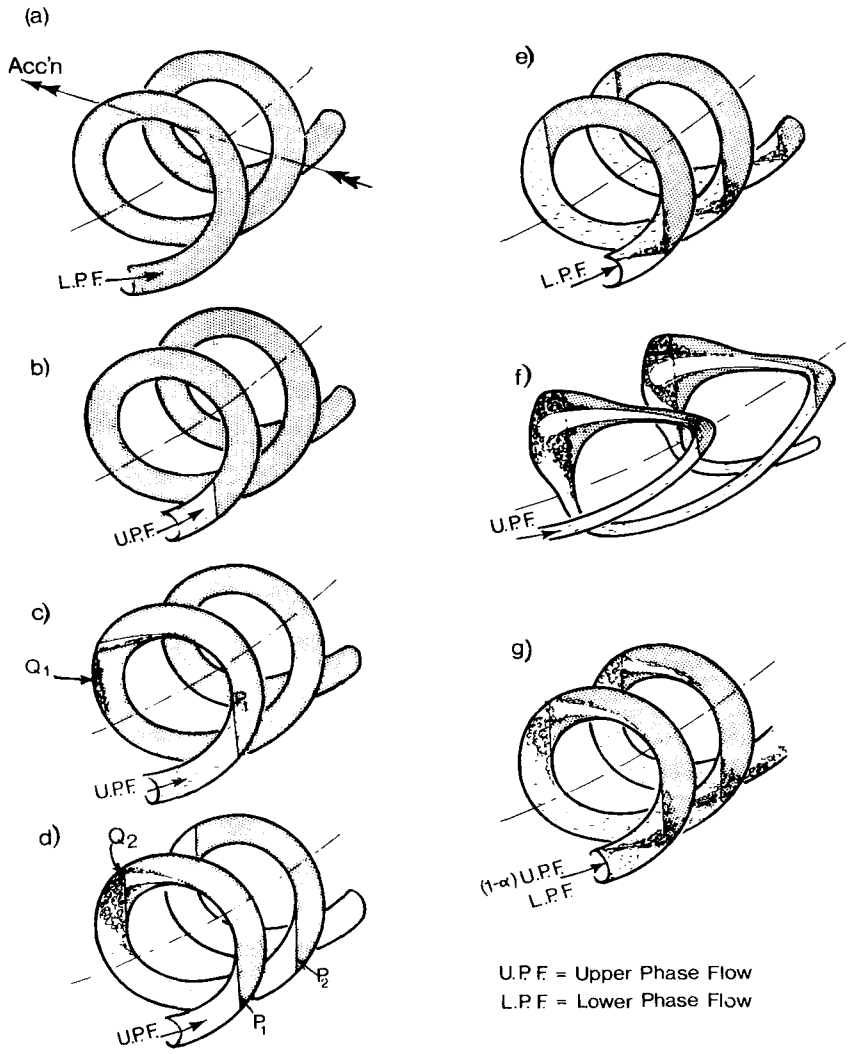

FIGURE 5

Diagram illustrating flow of 2 phase solutions through the helical coil. For detailed explanation see text. The shaded area represents the heavier dextran phase.

of the coil with mixing at and above the P interfaces (5e). It can be seen that mixing for light phase flow is limited to the Q interface areas and consequently is non-optimal. This can be improved by modifying the geometry (5f) or by flowing both phases at differing flow rates (5g). This has the advantage of avoiding "dead" zones in the retained phases giving mixing at P and Q interfaces and avoiding long retention times for low partition material.

If a number of linked coils are initially filled with the lower phase and the upper phase then pumped in, each coil will in turn approximately half fill with the upper phase displacing the excess lower phase through the coil system. The final result will be a retained phase, a mobile phase and mixing in each coil determined by the flow rate and the acceleration field.

The elution times of samples with varying partition coefficient will now be examined. The nomenclature is given in Appendix I.

For upper phase flow continuity (figure 6)

$$(1-\alpha)V_f = u_u A(1-\beta)$$

$$\therefore\ u_u = \frac{V_f\,(1-\alpha)}{A\ (1-\beta)} \qquad \text{--------- (1)}$$

For lower phase flow continuity :-

$$\alpha V_f = u_1 A\beta$$

$$\therefore\ u_1 = \frac{V_f \alpha}{A\ \beta} \qquad \text{--------- (2)}$$

Solute Partitioning

From partition theory, assuming adequate mass transfer between the phases; the mean linear velocity for a solute of partition coefficient (k) will be u_k where :-

$$u_k = u_u\left(\frac{k'}{k'+1}\right) + u_1\left(\frac{1}{k'+1}\right)$$

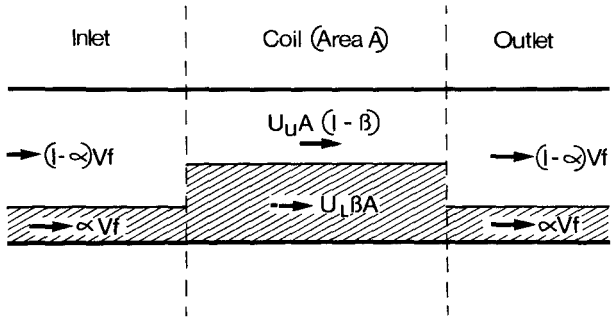

FIGURE 6

Representation of two-phase flow characteristics through the toroidal coil centrifuge. The inlet flow (V_f) is a mixture of lower phase flow (αV_f) and upper phase $(1-\alpha)V_f$ by arrangement. The volume flow through the coil will depend on the respective linear flows (u_u) and (u_l) and the relative proportion of the phases in the coils which can be considered constant in the steady state.

and $k = k'\beta/(1-\beta)$

$$\therefore u_k = \left[\frac{u_u k(1-\beta) + u_l \beta}{k(1-\beta) + \beta}\right] \quad \text{--------- (3)}$$

The time taken for solute of partition coefficient (k) to pass through the coil of length V^*/A will be V^*/Au_k and the volume eluted (V_k), taking into account the inlet and outlet tube volumes, will be :-

$$V_k = \frac{V_f V^*}{Au_k} + V_{in} + V_{out} \quad \text{--------- (4)}$$

Substitution of u_k from (3) and u_u and u_l from (1) and (2) gives :-

$$V_k = \left[\frac{k(1-\beta)+\beta}{k(1-\alpha)+\alpha}\right] V^* + V_{in} + V_{out} \quad \text{--------- (5)}$$

As a check on equation (5), a solute with a partition coefficient of unity should be treated by the system as though it was filled with a single solution, and hence the elution volume should equal the system volume. Substitution of k = 1 into equation (5) verifies this, giving :-

$$V_{k=1} = V^* + V_{in} + V_{out} \quad \text{---------} \quad (6)$$

Other useful markers are :-

$$V_{k=\infty} = \left[\frac{(1-\beta)}{(1-\alpha)}\right] V^* + V_{in} + V_{out} \quad \text{---------} \quad (7)$$

and $$V_{k=0} = \frac{\beta}{\alpha} V^* + V_{in} + V_{out} \quad \text{---------} \quad (8)$$

as they mark the beginning and end of sample elution from the point of injection.

Particle Partitioning

The partition ratio (G) is defined as the ratio of particles in the upper phase to those at the interface between the phases. Assuming that the interface is retained with the lower phase, then the linear velocity of a particle with partition ratio (G) is given by :-

$$u_G = u_u\left(\frac{G}{G+1}\right) + u_l\left(\frac{1}{G+1}\right) \quad \text{---------} \quad (9)$$

The volume eluted can be calculated by substituting for u_u and u_l in (9) and proceeding as for solutes to give

$$V_G = \left[\frac{\beta(1-\beta)(G+1)}{\beta(1-\alpha)G+\alpha(1-\beta)}\right] V^* + V_{in}+V_{out} \quad \text{-----(10)}$$

Note the elution volumes for G = ∞ and G = 0 are the same as for solutes in equations (7) and (8) while $V_{G=1}$ is given by :-

$$V_{G=1} = \left[\frac{2\beta(1-\beta)}{\beta(1-\alpha)+\alpha(1-\beta)}\right] V^* + V_{in}+V_{out} \quad \text{-----(11)}$$

Calculation of Retention Volume (βV^*)

While all the above formulae are useful in determining the elution volumes if partition coefficients or ratios are known or alternatively to determine these partition values if they are not, the proportion of lower phase retained (β) still has to be determined in some way. This can be done in one of two ways. The first is at the beginning of a run: if the coil is initially filled with lower phase and a measuring cylinder is used to collect the displaced lower phase (V_E) then this value can be used to calculate the amount of lower phase left in the coil. Elution of the first amount of upper phase is equivalent to considering the elution volume of the $K = \infty$ peak in equation (7) so that $V_E = \left[\frac{(1-\beta)}{(1-\alpha)}\right] V^* + V_{in} + V_{out}$ rearranging

$$\beta = 1 - \left[\frac{(1-\alpha)\{V_E - (V_{in} + V_{out})\}}{V^*}\right] \text{----- (12)}$$

Alternatively at the end of run, the retained dextran phase can be pumped out giving :-

$$V_p = \alpha(V_{in} + V_{out}) + \beta V^*$$

Rearranging :-

$$\beta = \frac{\left[V_p - \alpha(V_{in} + V_{out})\right]}{V^*} \text{--------- (13)}$$

In practice the average value of β from the two equations gives the most reliable values.

MATERIALS AND METHODS

Phase System Preparation

Dextran T500 (batch FD16027) was obtained from Pharmacia (Uppsala, Sweden) and Breox poly(ethylene-glycol) 6000 from Hythe Chemicals (Southampton, England). Stock solutions of the polymers were used to prepare phase mixtures (150 g) containing 3.3% (w/w) dextran T500, 5.4% (w/w) poly(ethylene glycol) 6000, 10 mM sodium phosphate-phosphoric acid, pH 7.4, 0.26 M sucrose,

0.05 mM Na_2EDTA, ph 7.4 and 1 mM ethanol. When the phase system had cooled to $4^{o}C$, it was shaken and allowed to settle overnight before separating the two phases. This phase system is an adaptation of that used by Morris & Peters (6) for fractionation of rat-liver homogenate. The purpose of the ethanol was to minimise inactivation of the catalase (9).

Sample Preparation

Non-fasted male Sprague-Dawley rats (150-200 g) were stunned and killed by cervical dislocation. The liver was immediately removed and 0.5 g perilobular tissue was minced with a razor blade. The minced tissue was then disrupted in a Dounce homogeniser (Kontes Glass Co., Vineland, NJ, USA) in 10 ml of ice-cold poly(ethylene-glycol)-rich upper phase, from which 0.4 g of water had previously been evaporated, with nitrogen, to allow for the water content of the liver tissue. Homogenisation was standardised with 10 strokes of a loose-fitting (type A) pestle, followed by 10 strokes of a tight-fitting (type B) pestle. Fibrous material was removed by passing the homogenate through a 50 micron nylon mesh. Upper phase, 0.9 ml, containing homogenate, together with 0.1 ml of lower phase, was used as the sample for partition experiments.

Running Procedure

The coil was initially filled with dextran-rich lower phase. The centrifuge was then set to rotate the toroidal coil at 1000 rev/min. The mobile phase, comprising 94% poly(ethylen-glycol)-rich upper phase well mixed with 6% dextran-rich lower phase, was pumped into the coil at 14 ml/hr and the eluent collected in 1 ml fractions. When the system reached equilibrium, i.e. when the eluent also contained 6% dextran-rich lower phase, the sample was injected by means of a 4-way slider valve (Altex). After collection of about 40 x 1 ml fractions, the coil was brought to rest and the contents of the coil pumped out with

water. The volume ratio was analysed by measuring the height of the total phase and interface in each of the fractions. The fractions were stored at -20°C for subsequent analysis. All equipment was in the 4°C cold room.

Analytical Methods

Sucrose (0.25 M, 0.15 ml) was added to each fraction before analysis to give a single phase. The distribution of organelles was determined by assaying (10) the following marker enzymes in alternate fractions: N-acetyl-β-glucosaminidase (lysosomes); neutral-α-glucosidase (endoplasmic-reticulum); lactate dehydrogenase (cytosol); γ-glutamyl-transferase (plasma membrane).

RESULTS

Figure 7 compares the fractionation of rat-liver homogenate by both a 17 transfer counter current partition apparatus (6) and a toroidal coil centrifuge. Comparison of the two techniques is complicated by the fact that CCD is a discrete process conventionally transferring upper-phase to the right, whereas TCC is an elution process where the upper-phase elutes first, hence inverting the partition spectrum. For the purposes of comparison, the counter-current distribution has been reversed to give upper-phase transfer from right to left. The fractions are compared on an equal volume basis giving a counter current distribution of 18 fractions (23.4 ml) and a toroidal coil distribution of 36 fractions (36 ml). Note that theoretical markers have been placed on both graphs. The counter-current experiment markers are based on a theory outlined by Albertsson (11), while those for the toroidal coil experiment are derived from equations (6), (7), (8) and (11).

Both results show that the organelle distribution curves are highly reproducible and qualitatively similar. The toroidal coil results show a significant improvement in

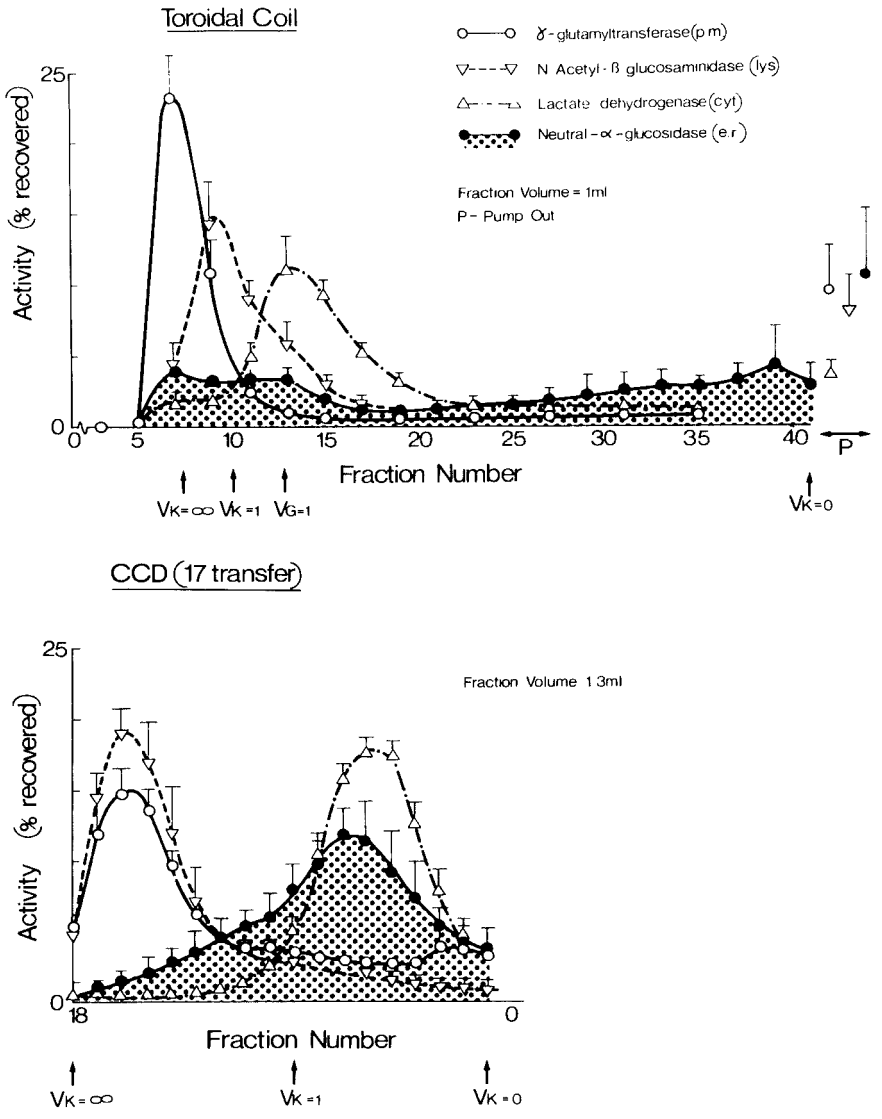

FIGURE 7

Analytical subcellular fraction of rat-liver homogenate by (a) 17 transfer discrete counter current apparatus (6) and (b) continuous toroidal coil centrifuge. Results show mean ± SD for (n) experiments with discrete CCD (n=3) and continuous TCC (n=7). The enzyme activities are given with the organelles in parentheses. Recovered enzyme activities range from 70-90%.

resolution, with a partial separation of lysosomes from plasma membrane and a clear separation of the cytosol peak from endoplasmic reticulum. Separation of plasma membrane from endoplasmic reticulum is achieved by both techniques although this is more complete for the toroidal coil experiment. The fact that the k = 1 elution point is biased towards the point of sample elution in the toroidal coil fractionation largely contributes to the shift of the soluble cytosol peak to the left.

When the CCD experimental results are compared with 17 transfer theoretical binominal distributions, the plasma membrane, lysosome and cytosol peaks are essentially single components, apart from small secondary peaks for plasma membrane and lysosomes at low k, and for cytosol at high k. The endoplasmic reticulum distribution shows signs of heterogeneity, with a major component at low k and one or possibly two components at high k. These are resolved in the toroidal coil distributions whereas the subsidiary lysosome components only appear as a shoulder and the plasma membrane component is not found.

The subsidiary peaks of the lysosomes and cytosol distribution are shifted to the left due to the asymmetrical partition distribution. This has the effect of concentrating the subsidiary cytosol peak and merging the lysosome one with the major peak. The absence of the plasma membrane subsidiary peak is anomalous, but could be explained by the small quantity of plasma membrane eluted at pump out. Any components affected by sedimentation in this way would necessarily be large and possibly anomalies from the homogenisation process.

Note that lysosomes and plasma membrane components are resolved by the toroidal coil experiment in contrast to the counter-current distribution experiment where they appear to have identical modal partition coefficients.

DISCUSSION

The data in this paper indicates that resolution of the principle subcellular organelles of rat-liver homogenates can be achieved by toroidal coil chromatography. In particular, marked heterogeneity of endoplasmic reticulum and its resolution from plasma membrane is demonstrated. These organelles, having very similar size and density characteristics, are not readily separated by density-gradient centrifugation techniques (12). Resolution of the various organelles is enhanced, compared with discrete counter-current partition experiments reported previously (6). The fact that the enzyme distributions are qualitatively similar for both processes and that the elution profiles are within the bounds of the theoretical markers, confirms a TCC separation based on partition. The assumption that the interface is retained with the lower phase is shown to be valid by the retention of the endoplasmic reticulum in the coil. The early elution of the plasma membrane peak compared to the $V_{K=\infty}(V_{G=\infty})$ marker may be due to locally high β values. The theoretical predictions assume a constant value of β, and analysis of the phase proportions in each fraction shows evidence of lower phase carry-over between fractions 5-8 which could produce early elution of the high partition peaks.

The shift of the soluble cytosol peak to the left in the TCC distribution would be expected from the marked shift to the left of the theoretical k=1 marker. Likewise, the shift of the G=1 marker to the left in the TCC distribution emphasises the fractionation between the low partition and high partition components of the endoplasmic reticulum peak, which is only just noticeable in the CCD distribution. Low partition components are therefore fractionated more efficiently than high partition ones. The reverse would be true if the dextran-phase was used as the mobile phase. Such a procedure could form the basis for subfractionating the plasma membrane and lysosome peaks.

The equipment described is moderately simple to construct and the toroidal coil rotor can be adapted for use in several commercially available centrifuges. The equipment has been routinely used for 3 years and for in excess of one hundred experiments. The seal-less method of connections between rotor and fraction collector/pump has proved particularly useful. It avoids heating problems, organelle/cell damage and technical failures, characteristic of many centrifuge seals. In addition, the same rotor can be used for particle separation, working on the same principle as a low speed zonal rotor. The toroidal coil will permit a large number of partitions between the two phases. The present rotor has approximately 550 turns and this represents a considerable improvement on existing two-phase partition machines (11), which are labour intensive and involve lengthy procedures. It would be relatively simple to mount two or more concentric toroidal coils on the rotor, in order to compare two separate samples under otherwise identical conditions. Direct comparison of the organelle separations achieved by the 17 transfer discrete partition apparatus with the same tissue source, enzyme analytic methods and essentially the same two-phase polymer systems indicates that, although the latter technique yields enhanced resolution, less than optimal separation was achieved for the greatly increased number of transfers expected within the toroidal coil. In other words the number of theoretical plates was less than expected. The reason for this is not clear but it is likely that complete equilibration between the two phases is not reached for each loop of the coil. Analysis of the peak shape suggests that mixing in the toroidal coil centrifuge is only about 5-10% efficient. There is thus clearly significant scope for improvement. Current experiments are aimed at enhancing organelle separation by varying such parameters as rotor speed, flow rate, phase mixture composition and relative proportions of the two phases and coil geometry. Similarly, a more detailed study of theoretical models for phase partition in the toroidal

coil centrifuge should help in the design of more effective separation procedures and these are currently in progress.

The effectiveness of the toroidal coil centrifuge has been demonstrated in comparison with an equivalent enhanced gravity CCD technique. If coil efficiency can be improved, then the process could become a powerful organelle separation technique which is particularly suited to automation.

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REFERENCES

1 Ito Y., Counter Current Chromatography, Jl. Biochem. Biophys. Methods, 5, 105-129 (1981)

2 Craig L.C., Comprehensive Biochemistry, 4, Elsevier, Amsterdam/London/New York (1962)

3 Snyder L.R. & Kirkland J.J., Introduction to Modern Liquid Chromatography, 2nd Ed, Wiley Interscience, New York, 323-348 (1979)

4 Ito Y. and Bowman R.L., Countercurrent Chromatography with the Flow-through Centrifuge without Rotating Seals, Anal. Biochem., 85, 614-617 (1978)

5 Sutherland I.A. and Ito Y., Cell Separation Using Two-Phase Polymer Systems in Nonsynchronous Flow-through Coil Planet Centrifuge, Anal.Biochem., 108, 367-373 (1980)

6 Morris W.B. and Peters T.J., Microanalytical Partition of Rat-Liver Homogenates by Poly(ethylene glycol)-Dextran Counter-current Distribution, Eur. J. Biochem., 121, 421-426 (1982)

7 Heywood-Waddington D., Sutherland I.A., Morris W.B. and Peters, T.J., Subcellular Fractionation of Rat-Liver Homogenates using Two-Polymer Phase Systems in a Toroidal Coil Centrifuge, submitted for publication (1983)

8 Ito Y., Suaudeau J. and Bowman R.L., New Flow-Through Centrifuge Without Rotating Seals Applied to Plasmaphoresis, Science, 189, 999 (1975)

9 Chance B., The Reactions of Catalase in the presence of the Notatin System, Biochem. J., 46, 387-402 (1950)

10 Smith G.D. and Peters T.J., Analytical Subcellular Fractionation of Rat-Liver with Special Reference to the Localisation of Putative Plasma Membrane Marker Enzymes, Europ. J. Biochem., 104, 305-311 (1980)

11 Albertsson P.A., Partition of Cell Particles and Macromolecules, Wiley Interscience, New York (1971)

12 Beaufay H., Amar-Costesec A., Thines-Sempoux D., Wibo M., Robbi M. and Berthet J., Analytical Study of Microsomes and Isolated Subcellular Membranes from Rat-Liver, J. Cell. Biol., 61, 213-231 (1974)

APPENDIX I

Nomenclature

A	=	Coil cross-sectional area
G	=	partition ratio for particles
k	=	partition coefficient for solutes
k'	=	apparent partition coefficient (corrected for volume ratio)
u_l	=	mean linear velocity of lower phase
u_G	=	mean linear velocity of particle with partition ratio G
u_k	=	mean linear velocity of solute with partition coefficient k
u_u	=	mean linear velocity of upper phase
V^*	=	Total volume of toroidal coil
V_E	=	Elution volume
V_f	=	Volume flow into coil
V_p	=	Volume lower phase pump out

V_k	=	Elution volume of compact with partition coefficient k
V_{IN}	=	Volume of inlet tube
V_{OUT}	=	Volume of outlet tube
α	=	proportion of lower phase in total flow
β	=	mean proportion of lower phase retained in coil

Subscripts

G	=	particle partition ratio G
k	=	solute partition coefficient k
l	=	lower
u	=	upper

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TOROIDAL COIL COUNTERCURRENT CHROMATOGRAPHY IN THE AFFINITY PARTITIONING OF NICOTINIC CHOLINERGIC RECEPTOR ENRICHED MEMBRANES

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ABSTRACT

A variation on the aqueous polymer phase partition method, affinity partitioning, has proved suitable for the preparative scale purification of binding site enriched membrane fragments. The full resolving potential of the affinity partitioning technique often requires the utilization of multiple extraction procedures such as countercurrent distribution. In this report, we evaluate the combination of a newly developed countercurrent purification technique, toroidal coil chromatography, with affinity partitioning. This approach provides an efficient method for purification and characterization of membrane bound nicotinic cholinergic receptors. The relative merits of the toroidal coil chromatography technology and the more conventional thin-layer countercurrent distribution techniques are compared.

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INTRODUCTION

Affinity chromatography, which relies upon the bio-specific binding properties of enzymes, receptors and other binding proteins to accomplish separation, has proven highly useful in the purification of soluble proteins or membrane bound proteins, once they are solubilized by the action of detergents. On the other hand, the application of affinity chromatography to the fractionation of intact cells and cell particles has met with several difficulties including problems in achieving specific retention onto affinity matrices and the converse difficulty of eluting bound cells or membrane fractions without the use of denaturing conditions. These problems appear to be due to the inherent limitations of the solid affinity matrices. Affinity partitioning (1-4) is a promising new affinity separation technique based upon the phase partition method for purification of biological particles in aqueous polymer phase systems (5), thus avoiding the problems associated with solid affinity matrices.

Aqueous polymer two-phase systems provide a gentle non-denaturing milieu for cells, subcellular particles and enzymes; near complete recoveries of biological activities are routinely achieved after distribution of heterogeneous biological material among the two aqueous phases and the interface between them. The phase systems are formed upon mixing solutions of water-soluble polymers, e.g. poly(ethylene oxide) [poly(EtO)] and dextran, a polymer pair most frequently used for such separations.

Several physical chemical parameters of proteins and cell particles are known to correlate with and, therefore, presumably influence their distribution among the phases and the interface. In the case of subcellular particles, these parameters include a particle's charge density (6), lipid composition (7), and tendency to interact with the constitutent polymers of the phase system (8). The latter parameter may be turned to advantage by synthesizing ligand-polymers that specifically interact with biological

particles containing biospecific binding sites. The two-phase systems can then be used to purify membranes enriched in specific binding sites in much the same way that affinity chromatography has been utilized to purify soluble proteins.

Typically, the specific ligand is covalently attached to one of the polymers that make up the phases, e.g to the ends of the linear poly(EtO) molecule. This variation on the phase partition method, designated affinity partitioning, has proved useful in the purification and characterization of Torpedo electroplax membranes enriched in nicotinic cholinergic receptor sites (3,4). In affinity partitioning, binding of a ligand-polymer to membrane fragments possessing specific binding sites influences their distribution to a degree that varies directly with the binding density per surface area. Thus, subcellular particles, which are often intrinsically heterogeneous, may be purified on the basis of their specific binding site content.

In purifying receptor enriched membranes, affinity partitioning has advantages over affinity chromatography where often, biological particles may be bound irreversibly to the affinity matrix. This is likely due to the multivalent character of an affinity matrix bead, which usually contains multiple covalently bound ligands arrayed such that simultaneous binding of matrix ligands to multiple cell surface sites occurs. In affinity partitioning, binding of ligand-poly(EtO) is essentially non-cooperative and ligand-polymers are generally reversibly bound and readily removed by centrifugation (4). The virtues of the affinity partitioning technique, when applied to the purification of subcellular particles, are similar to those of affinity chromatography when applied to the purification of soluble proteins: the binding of ligand-polymers provides specificity for the separation technique.

With both conventional and affinity aqueous phase partitioning techniques, purification is based upon differential distributions of the constituents in a mixture among the three

compartments of the two phases, i.e., the poly(EtO)-rich phase, the dextran-rich phase and the interface. If the constituents differ greatly in their distribution, then substantial purification may be achieved by performing a single or a few extractions, which are easily performed manually; but more often, the full separatory potential of the phase partition method may only be approached by performing multiple extractions using the countercurrent distribution (CCD) technique (5). Usually, adequate separation requires thirty or more transfers, which are best performed with an automated CCD apparatus.

The typical CCD apparatus designed for the purification of antibiotics or peptides in organic-aqueous phase systems is less suitable for aqueous polymer phase systems. This is because the settling times for aqueous polymer phase systems are substantially longer than for aqueous-organic phase systems. Since the settling time varies directly with the thickness of the phase layers, a useful approach for decreasing the phase settling time is to utilize thin phase layers with a proportionally greater cross-sectional area. Albertsson introduced a thin-layer CCD apparatus (9) that incorporates the required features in the form of two circular Plexiglas plates. As discussed below, the thin-layer CCD technology may have substantial limitations when applied to the affinity partitioning of membrane fractions.

A radically different approach was first developed (10) to increase the effectiveness of fractionation based upon distribution between aqueous-organic phase systems. A helically wound tube is located circumferentially on a spinning disc. The radial acceleration field holds the heavier phase stationary in the outer part of the coil, while the lighter phase is pumped through the stationary phase such that mixing occurs. A sample injected with the mobile phase travels through the coils at a rate dependent upon its distribution between the phases. This process, Toroidal Coil Chromatography (TCC), is analogous to a continuous form of

liquid-liquid chromatography, but without the associated disadvantages of a solid support. Alternatively, it can be considered as a continuous form of CCD as opposed to a discrete one.

The application of TCC to separations using polymer phase systems has so far been limited to gradient techniques. The initial success of these studies suggested that the TCC technology might offer substantial advantages for the purification of membranes fragments or subcellular particles when compared with the thin-layer CCD technique, both in terms of speed and efficiency of the separation and the avoidance of nonspecific interactions of particles with the Plexiglas surface. This paper describes the application of the TCC technique to the separation of subcellular particles containing nicotinic cholinergic receptors prepared from the electroplax of Torpedo californica, and provides a direct comparison with the results obtained using conventional CCD technology.

METHODS

Affinity Partitioning of Torpedo Membranes

Membranes enriched in nicotinic cholinergic receptor from Torpedo californica electric organ were partially purified by sucrose density gradient centrifugation (11) with the initial homogenization media containing EDTA and phenylmethylsulfonyl fluoride as described previously (4). For all experiments, membranes were analyzed using the nicotinic cholinergic ligand-polymer, hexaethonium-poly(ethylene oxide), [Et_3N-Me_6-NEt_2-poly(EtO)]. Single phase system extractions, countercurrent distribution, and membrane prelabeling with ^{125}I-α-bungarotoxin were performed as described previously (4).

Toroidal Coil Centrifuge Configuration

The toroidal coil rotor was constructed on a principle which eliminates the use of rotating seals as described by Ito and

Bowman (10,12). The coil was prepared by winding 18-gauge PTFE tubing (i.d. 1.07 mm) around a 5 mm diameter cylindrical nylon core. The core was formed into a 36 cm diameter torus and connected to the sample loop and a fraction collector, as shown in Figure 1. The dead volume from the end of the sample loop until the beginning of the coil was 0.4 ml. The sample coil contained a total volume of 8.5 ml, and the output dead volume was 0.7 ml. The toroidal coil rotor was mounted in a PR-2 International centrifuge located in a cold room (3.5°C). The centrifuge refrigeration system was set to maintain the chamber air temperature to within 0.5°C of the cold room temperature. In initial experiments, the cycling of the centrifuge refrigeration system was monitored, using a thermistor placed within the centrifuge chamber. The period of the refrigeration system cycle was 11 min with a 0.3°C variation in chamber air temperature.

Phase systems for eluting the TCC were prepared with an LKB Ultrograd gradient maker using a control valve (Fig. 1). This arrangement allowed variation of phase system ligand-polymer composition using an additional valve not shown and the inclusion of controlled amounts of dextran-rich bottom phase emulsified in the poly(EtO)-rich top phase. The mixing device on the LKB Ultrograd produced a fine emulsion containing the ratio of bottom phase suspended in top phase indicated in the Figure Legends.

Sample Loading and Analysis of Results

Operation of the TCC was begun by filling the coils at 1 x g with bottom phase. The rotor was then brought up to 1,200 rpm, and pumping of the emulsion commenced at a flow rate of 14 ml/hr. Prior to connecting the gradient maker output to the TCC, the LKB Ultrograd system was initiated until a uniform flow composition was achieved.

Membranes (0.36 mg protein containing 460 pmol receptor and prelabeled with ^{125}I-αBgt 45nCi, 0.22 pmol) were added to a volume

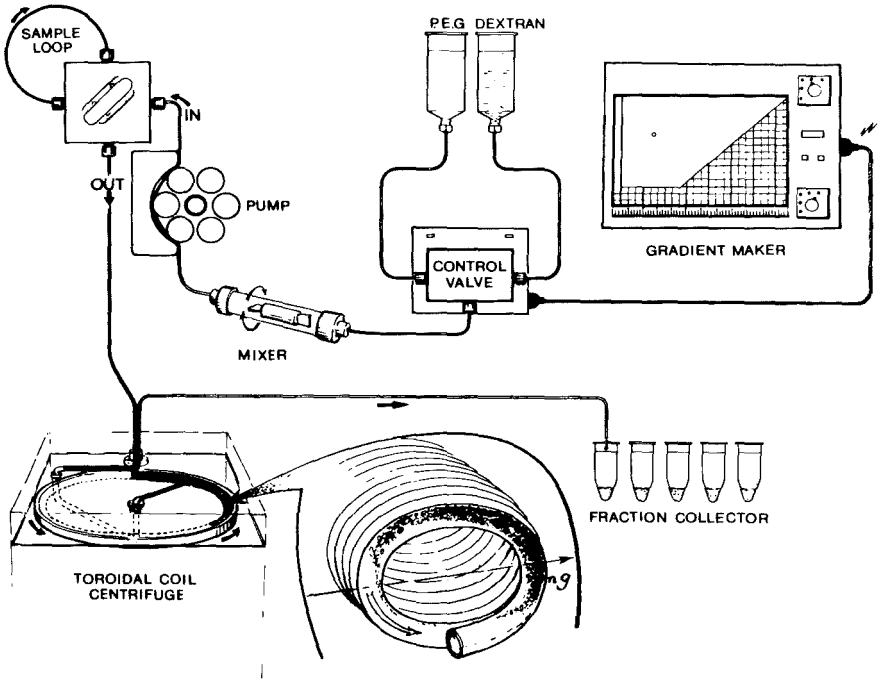

FIGURE 1

Toroidal Coil Chromatography Operating System

Schematic layout of operation system showing the method for generating an emulsion of the bottom (dextran enriched) phase in the upper (poly(EtO) enriched) phase. The enlargement of a single coil unit illustrates the retention of bottom phase in the outer segment of the coil, while the top phase percolates through it.

of the emulsion prepared by removing 20% of the water by evaporation under a stream of N_2 yielding the identical polymer composition but a sodium phosphate concentration at 2 mM higher than the phase system. The sample was suspended in the emulsion (0.5 ml) and loaded into the sample loop. The ratios of top phase to bottom phase volumes (V_r) were analyzed by measuring the height of the total phase volume and the interface in each of the frac-

tions (70 drops or 1.33 ml/fraction), which were collected in 1.5 ml plastic centrifuge tubes (Sarstedt No. 72.696). The relationship between the heights and the phase volumes was calibrated, and the conversions made to determine the phase volumes.

^{125}I-αBgt labeled membranes were counted in a Biogamma II counter for 5 min. Protein analysis of the fractions was performed by a modification of the Bradford (13) protein assay method. To minimize the degree of precipitation of membranes and phase components, 600 µl aliquots of effluent from the TCC were preincubated with 200 µl 0.5 M H_3PO_4 at 50° for 3 hr prior to addition of 530 µl 1:2 diluted Bradford reagent (Bio-Rad). Blanks and protein standards contained the identical phase system polymers and salt composition as the individual TCC fractions. The blank absorbance was observed to drift 0.025 OD over the measurement of forty fractions, and a proportional correction was made prior to calculation of the protein values per fraction.

RESULTS

TCC Elution Procedures

In earlier applications of the TCC, utilizing aqueous-organic solvent systems in the fractionation of dinitrophenylated amino acids, elution of the top phase (aqueous) through the bottom phase (organic solvent), proved suitable for achieving excellent separation (10). The elution of retained bottom phase with pure top phase was also utilized in the separation of various strains of *E. coli* in aqueous polymer phase systems. The various strains were separated by eluting with a gradient prepared with pure top phases containing varying concentrations of NaCl, which yielded fractionation on the basis of cell surface charge composition (14). The latter application employed a dextran-poly(EtO) phase system further from the critical point of the phase diagram than has proved useful in affinity partitioning applications (4).

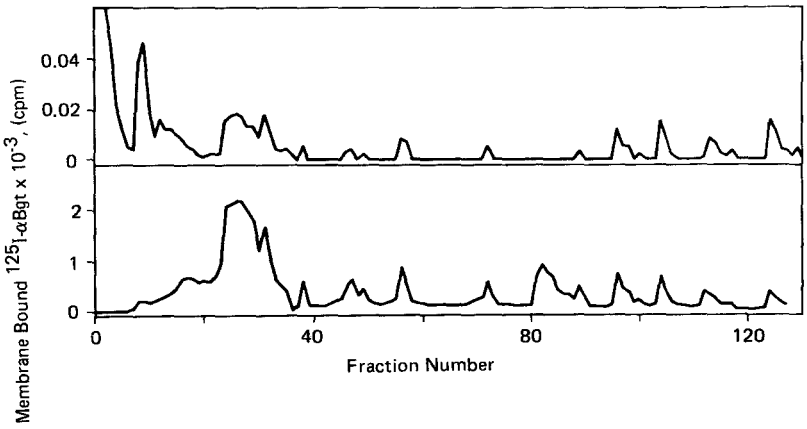

FIGURE 2

Chromatograms of Torpedo Membranes Obtained by Elution with Homogeneous Poly(EtO) Phase through Toroidal Coil

Torpedo membranes, enriched in nicotinic cholinergic receptor, were prelabeled with ^{125}I-αBgt at 0.05% of stoichiometric binding levels and loaded onto the TCC in pure poly(EtO)-rich phase. Elution was continued with pure top phase. An erratic profile of eluted bottom phase (plotted as $1/V_r$, bottom phase vol./top phase vol.) coincides with the elution of membrane bound cholinergic receptor. Phase system composition is the same as Figure 3.

In attempting to utilize a phase system close to the critical point, we typically observed results shown in Fig. 2 where the TCC apparatus was loaded with pure bottom phase and elution attempted by pumping pure top phase through the coil. Membrane fractions were eluted in a profile of narrow peaks that initially appeared to provide an exceptional degree of resolution of the membrane populations. Unfortunately, such fractionation profiles are not reproducible from one run to the next. An explanation for the erratic elution profiles was provided by measurements of the amount of bottom (dextran-rich) phase eluted in each fraction of the run (displayed as the volume ratio), which correlated remarkably well with the elution of membrane-bound ^{125}I-αBgt into

each fraction. This unpredictable "stripping" of the bottom phase was not due to temperature fluctuations, because the elution pattern did not correlate well with TCC refrigeration cycle of 11 min.

An intuitive and successful solution to this problem was the elution of the TCC with an emulsion containing dextran-rich phase dispersed in poly(EtO)-rich phase. In this configuration, elution of bottom phase was observed to be continuous due to the flow of some dextran phase through the system and erratic changes in the elution of dextran phase were obviated. In the absence of nicotinic cholinergic ligand-polymer (Fig. 3A), ^{125}I-αBgt bound to cholinergic receptor distributes into the bottom phase and the interface (4). Because the bottom phase is the stationary phase, the membrane fractions are retarded during their elution through the toroidal coil. In the presence of ligand-polymer (Fig. 3B), the distribution of ^{125}I-αBgt labeled membranes is shifted to the mobile [poly(EtO)-rich] phase, thus eluting in the breakthrough volume.

Comparison of CCD and TCC Separations

For comparison with the TCC results, parallel thin-layer CCD runs were performed. The same two phase system conditions displayed in Fig. 3 were used to provide a direct comparison of the two countercurrent extraction technologies (Fig. 4). Fractions from the TCC are eluted in reverse order to the conventional fraction numbering system commonly used to display CCD data. Thus, the higher transfer number fractions contain the membrane fragments that partition almost exclusively into the poly(EtO)-rich phase. A very apparent difference between the TCC and CCD profiles is the sharpness of breakthrough peak from the TCC, when compared with the analogous CCD peak. The symmetry of the CCD profiles is due to fact that such profiles can be modeled as binomial distributions; while the TCC is analogous to other forms of liquid chromatography and is best described by a Poisson distribu-

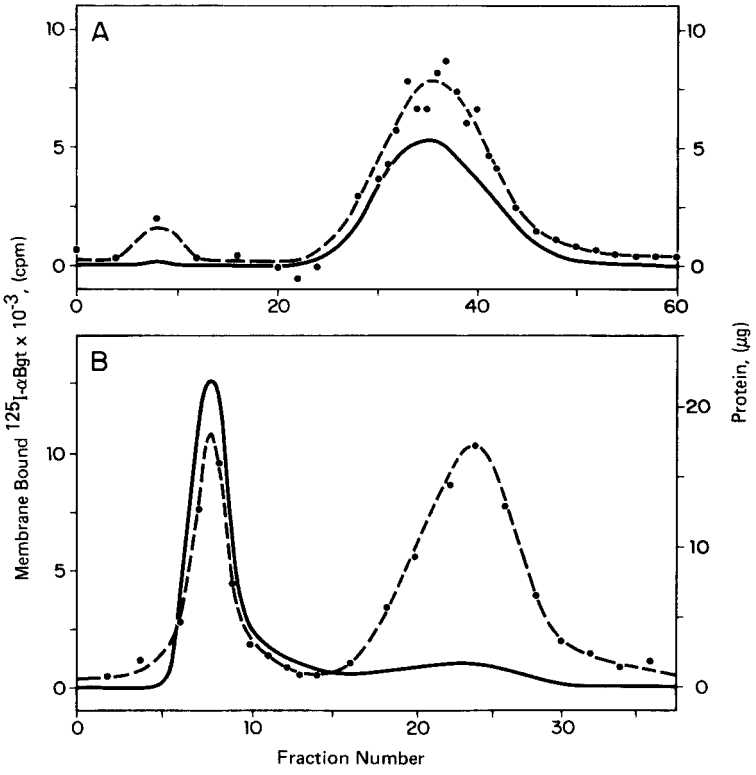

FIGURE 3

Chromatograms of Torpedo Membranes Obtained by Emulsion Elution through Toroidal Coil

Phase systems containing 4.6% (wt/wt) Dextran T-500 and 3.8% poly(EtO) 8000, 5 mM sodium phosphate pH 7.4, and 15 mM sodium chloride were prepared, the phases allowed to settle and the TCC system was loaded with bottom phase. Twenty-seven ml of emulsion containing dextran-enriched phase suspended in poly(EtO) rich phase [$1/V_r$ = 0.036, for A; 0.054, B] was then pumped through the rotating TCC apparatus. Torpedo membranes, suspended in the emulsion, were introduced into the emulsion flow at Fraction 1. Membrane bound ^{125}I-αBgt (——) was counted and protein assessments in the fractions performed (----). Profile A was observed with a phase system containing no ligand-poly(EtO), while B was obtained with a phase system in which 0.05% of the poly(EtO) was replaced with Et_3N-Me_6-NEt_2-poly(EtO). The flow rate was equivalent to 10 fractions/hr with sample loading as described in text.

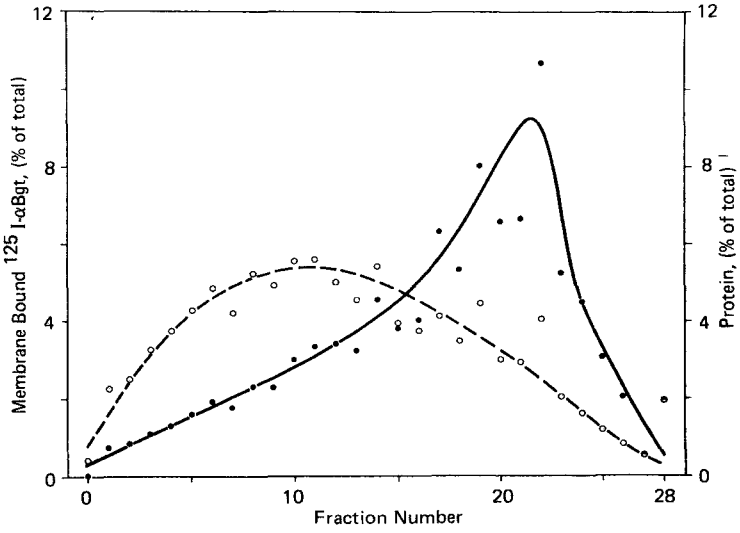

FIGURE 4

Thin-layer Countercurrent Distribution of Torpedo Membranes

Phase systems, containing 0.58% ligand-polymer, as described in Fig. 3, were used for CCD fractionation of membranes (1.8 mg protein). Profiles were determined by counting ^{125}I-αBgt (——) for receptor activity and Bradford assay (----) for protein. Note that membranes partitioning into the poly(EtO) enriched phase distribute into the higher CCD fractions, while during the TCC process, the order of fractionation is reversed.

tion. Both types of distributions approximate a Gaussian or normal distribution as the efficiency increases. A theoretical comparison (15) of the elution volumes of particles with differing partition coefficients shows that high partition components are concentrated in the early region of the TCC chromatogram, while for the CCD process the distributions are symmetrical.

Capacity of the TCC for Preparative Separation

The above results strongly suggest that affinity partitioning combined with TCC countercurrent chromatography provides a means

for the analysis of membrane heterogeneity, but its preparative scale utilization may be severely limited in the present configuration. However, it should be recognized that the inherent flexibility of the TCC would allow for modifications to increase its capacity. In order to provide a basis for further improvements in the technology, we were interested to determine the capacity of the present TCC coil configuration. The experiments described earlier were performed by loading 0.36 mg of protein (Fig. 3); upon application of 1.5 mg protein into the 0.5 ml sample loop of the TCC, essentially equivalent purification and TCC profiles were obtained. It is apparent that increasing the level of loading to as high as 3 mg/ml protein does not adversely influence the TCC process. Attempts to utilize higher protein concentrations in the sample loop were hindered, not by the capacity of the TCC, but rather by the difficulties in concentrating membranes samples.

DISCUSSION

Advantages of TCC and CCD Techniques: A Complementary Study

The thin-layer automated CCD technology provides substantially increased resolution over what may be achieved using manual countercurrent transfers. This apparatus has the virtue that mixing, transfer and separation of the phases is automated; however, loading and unloading of the phase systems is usually performed manually, and it is important to wash the plates extensively between runs. The choice of Plexiglas for construction of the thin-layer CCD apparatus was largely dictated by its water wettability properties, so that drop formation is avoided. It is also a suitable material for fabrication of the thin-layer CCD geometry, and, since it is transparent, the CCD process may be followed visually, especially in the separation of chloroplasts or erythrocytes. Another not so favorable property of Plexiglas thin-layer plates is that their brittleness makes them prone to impact damage and aging phenomena (small deformations) due to cycles of

contact with water or changes in temperature, making for a less effective seal between the chambers. The configuration of the thin-layer CCD plates limits the number of chambers to approximately two hundred, and results in extensive contact of biological particles with the Plexiglas surfaces.

Our experience in separation of electroplax membranes has indicated that nonspecific adsorption of membranes to the Plexiglas surface is a phenomenon that must not be ignored (4), especially when less than mg amounts of protein are loaded into the thin layer CCD apparatus. Such nonspecific adsorption phenomena could be due to deterioration of the surface properties of the Plexiglas during its normal use, but fortunately, this phenomenon was largely abolished by the addition of 2 mg/ml bovine serum albumin (BSA) to the phase systems (4). This, however, interferes with protein assays of the resulting fractions. In order to provide a direct comparison with the TCC technology, BSA was not added to phase systems used in the thin-layer CCD experiment illustrated in Fig. 4.

It is crucial for achieving a substantial affinity partitioning effect to utilize polymer systems near the critical point of the phase diagram (3,4), where the phases differ only slightly in composition and density. With these systems, the slight difference in densities and low interfacial tension between the phases lead to long settling times when compared to conventional aqueous polymer phase systems. Thus, high resolution CCD can take hours or even a day to achieve. Fortunately, the size of many subcellular structures is such that enhanced gravitation techniques can be considered to speed up the settling time of the phase system with minimal sedimentation effects on the sample itself. This, of course, would not be true for most cells. The simplest means to hasten phase separation is to centrifuge the phase system; this is especially useful when performing preparative scale separations involving a few extractions (4,16).

A second enhanced gravity approach is to place the thin-layer CCD apparatus in a centrifuge; increased leakage between the chambers is encountered, although these problems are now being approached with success (17).

A radically different approach was first developed (10) to increase the effectiveness of fractionation based upon distribution between phases (organic/aqueous or aqueous/aqueous polymer systems). In the latter application, the process is complicated by the viscous nature of the aqueous polymers used. The three most important factors for successful CCD separations are good mixing, settling and transfer. When these considerations are applied to the TCC process, settling and transfer are excellent with good retention of the stationary phase, but mixing can be poor as it is purely a function of flow phenomena (18). High flow will lead to good mixing, but at the expense of stationary phase retention. The retention volume is important in the fractionation of soluble constituents, which are not significantly retained in the interface; on the other hand, subcellular particles are significantly absorbed into the interface, and fractionation may depend primarily upon efficient contact of biological particles with the interface. In fact, Albertsson has shown for particles partitioning between the upper phase and the interface that the distribution is independent of the lower phase volume (5).

A theoretical advantage of the CCD technology is that the results of single tube partitioning experiments may be used to predict the countercurrent distribution behavior of presumably homogeneous particles. In turn, the results of CCD separations may be used to calculate the single tube distributions that should be obtained. Since a detailed understanding of partitioning of membranes into the interface can as yet not be translated into a membrane's TCC elution pattern. It is not clear just how single tube experimental results concerning the fraction of membranes that partition into the interface may be translated into a prediction of their behavior in the TCC; this is due to lack of

detailed knowledge of the geometrical considerations in separation of phases in the TCC coils. The empirically derived results displayed herein indicate that the membranes that partition only slightly into the phase system in single tube experiments are eluted early in the TCC run.

Future Improvements in TCC Technology

The TCC technique is a continuous flow separation technique and this facilitates the automation of the technique in a variety of ways. For example, it should be possible to adapt continuous flow protein and radioactivity analysis procedures for analyzing the membrane fractions. Also, the non-rotating seal platform used in the TCC design may accommodate multiple toroidal coils, facilitating simultaneous analysis of multiple samples or a single sample under several conditions.

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REFERENCES

1. Flanagan, S.D. and Barondes, S.H., Affinity Partitioning: A Method for Purification of Proteins using Specific Polymer-ligands in Aqueous Polymer Two-phase Systems, J. Biol. Chem., 250, 1484 (1975).

2. Flanagan, S.D., Taylor, P. and Barondes, S.H., Affinity Partitioning of Acetylcholine Receptor Enriched Membranes and their Purification, Nature, 254, 441 (1975).

3. Flanagan, S.D., Barondes, S.H. and Taylor, P., Affinity Partitioning of Membranes, J. Biol Chem. 251, 858 (1976).

4. Johansson, G., Gysin, R. and Flanagan, S.D., Affinity Partitioning of Membranes: Evidence for Discrete Membrane Domains Containing Cholinergic Receptor, J. Biol. Chem., 256, 9126 (1981).

5. Albertsson, P-Å., Partition of Cell Particles and Macromolecules, Almqvist and Wiksell, Stockholm, 1971, 2nd Ed.

6. Walter, H., Tamblyn, C.H., Levy, E.M., Brooks, D.E. and Seaman, G.V.F., Electrophoretic Mobilities of Human Peripheral Blood Lymphocytes Subfractionated by Partitioning in Two-polymer Aqueous Phase Systems, Biochim. Biophys. Acta., 598, 193 (1980).

7. Eriksson, E. and Albertsson, P-Å., The Effect of Lipid Composition on the Partition of Liposomes in Aqueous Two-phase Systems, Biochim. Biophys. Acta., 507, 425 (1978).

8. Johansson, G. and Westrin, H., Specific Extraction of Intact Chloroplasts using Aqueous Biphasic Systems, Plant Sci. Lett., 13, 201, (1978).

9. Albertsson P-Å., Thin-layer Countercurrent Distribution, Anal. Biochem., 11, 121 (1965).

10. Ito, Y. and Bowman, R.L., Countercurrent Chromatography with the Flow-through Centrifuge without Rotating Seals, Anal. Biochem., 85, 614 (1978).

11. Reed, K., Vandlen, R., Bode, J., Duguid, J. and Raftery, M.A., Characterization of Acetylcholine Receptor-rich and Acetylcholinesterase-rich Membrane Particles from Torpedo californica Electroplax, Arch. Biochem. Biophys., 167, 138 (1975).

12. Ito, Y., Suaudeau, J. and Bowman, R.L., New Flow-through Centrifuge without Rotating Seals Applied to Plasmaphoresis, Science, 189, 999 (1975).

13. Bradford, M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding, Anal. Biochem., 72, 248 (1976).

14. Sutherland, I.A. and Ito, Y., Toroidal Coil Chromatography, J. High. Res. Chrom. and Chrom. Res., 3, 171 (1978).

15. Sutherland, I.A., Heywood-Waddington, D. and Peters, T.J., Toroidal Coil Countercurrent Chromatography: A Fast, Simple Alternative to Countercurrent Distribution using Aqueous Two-phase Partition, J. Liquid Chromatogr., 7, 363 (1984).

16. Morris, W.B. and Peters, T.J., Microanalytical Partition of Rat-Liver Homogenates by Poly(ethylene glycol)-Dextran Countercurrent Distribution, Eur. J. Biochem., 121, 421 (1982).

17. Åkerlund, H-E., Apparatus for Countercurrent Distribution/ Multiple Sedimentation, Swedish patent 8101643-8 (1981).

18. Ito, Y., Countercurrent Chromatography, J. Biochem. Biophys. Meth., 5, 105 (1981).

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COUNTERCURRENT CHROMATOGRAPHIC SEPARATION OF BACTERIA WITH KNOWN DIFFERENCES IN SURFACE LIPOPOLYSACCHARIDE

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ABSTRACT

Countercurrent chromatography was used to separate cells of Salmonella typhimurium whose surfaces were identical except for the proportion of lipopolysaccharide molecules with long versus short chain polysaccharide chains. Large differences in partition properties resulted from slight differences in polysaccharide composition, and the results suggest that the physical state of the molecules, as well as their composition, can affect the partition of such cells.

INTRODUCTION

As is well known, countercurrent distribution with suitable polymer phase systems can separate particles on the basis of charge and hydrophobicity (1, 2). The recently described methods of countercurrent chromatography (3, 4) should provide a powerful method of separating particles that differ only slightly in the hydrophobicity and/or charge of their surfaces, and testing the effect of slight variation in the macromolecules of the cell

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surface on these properties. To test this possibility, we used Salmonella typhimurium, which normally produces a surface completely covered with lipopolysaccharide (LPS) molecules containing long carbohydrate chains [see (5) for review of LPS structure]. Mutants defective in UDP galactose-4-epimerase cannot synthesize galactose, a sugar required for synthesis of the complete polysaccharide of S. typhimurium, and therefore in the absence of added galactose will make LPS molecules with a very short chain polysaccharide. If galactose is then added, LPS molecules synthesized subsequently will have a long polysaccharide (6). Stendahl and coworkers (7-9), using countercurrent distribution, were the first to note that LPS determines the partition of S. typhimurium on countercurrent distribution, so that bacteria covered with long chain LPS partition as hydrophilic uncharged particles, and those covered with short chain LPS partition as hydrophobic, charged particles. They also found that growth of these mutant bacteria in a medium containing galactose resulted in a change in the elution properties of the cells from a hydrophobic charged particle to one which was hydrophilic and neutral (10). Since synthesis of LPS is continuous during steady-state growth of the bacteria, we reasoned that growth of mutant bacteria in the presence of galactose for various times should provide particles with defined percentages of long chain LPS, and thus defined proportions of both types of LPS on their surfaces. We would thus be able to test the resolving power of the countercurrent chromatography apparatus with particles of known, slightly different properties and also determine whether the distribution of the particles reflects solely their LPS composition.

We show herein that when we fractionate bacteria that have increasing percentages of long chain LPS, there are discontinuous changes in the position of these bacteria that do not reflect solely their percentages of surface constituents. Some bacteria containing as little as 20% long chain LPS fractionate at the position of bacteria containing 100% long chain LPS. We postulate

that the distribution of the LPS on the surface of the cell and not solely its composition can also affect partition in countercurrent chromatography.

MATERIALS AND METHODS

Reagents

[3,4,5-^{3}H]Leucine (110 Ci/mmol) was from New England Nuclear Co. (Boston, Massachusetts). [1-^{14}C]Galactose was from New England Nuclear Co. (56.5 mCi/mmol) or Amersham-Searle Co. (Arlington Heights, Illinois) (49.4 mCi/mmol). Proteose Peptone No. 3 and beef extract were from Difco Laboratories (Detroit, Michigan). Polyethylene glycol 6000 was obtained from Union Carbide Corp. (Oak Ridge, Tennessee) and dextran 500 from Sigma Chemical Co. (St. Louis, Missouri). All other reagents were from standard sources.

Growth of Bacteria

S. typhimurium G30, a mutant lacking UDP galactose-4-epimerase, was the kind gift of Dr. Paul Rick, Uniformed Services University of the Health Sciences, Bethesda, Maryland. It was grown at 37°C with shaking on proteose peptone beef extract medium (PPBE) (11) which had been depleted of residual galactose by allowing the bacteria to grow for several generations, centrifuging down the bacteria, and sterilizing the medium by filtration. Fresh bacteria that had been grown only on depleted PPBE were then used for the experiment. Galactose was added as indicated for each experiment. When galactose was to be added for only short times, the cells were previously grown for several generations in the presence of 0.01 mM D-fucose, which induces the synthesis of enzymes of galactose transport and metabolism. Radioactive galactose to label LPS and radioactive leucine to label protein were added as indicated for each experiment. Cells were grown to between 2 and 6 x 10^{8} bacteria per ml, as measured by OD_{530} after calibration of optical density to bacterial counts by standard methods. Cells were harvested by centrifugation at 8,000 x g for 5-10 minutes at 4°C in the presence of 0.005% NaN_3

to prevent further growth and metabolism. Samples were washed with 0.03 M Tris-chloride, pH 7.0, containing 0.005% NaN_3, and resuspended in the same buffer at 4 or 5 times the initial cell concentration. Fractions were often precipitated in trichloroacetic acid (5%) prior to counting in a scintillation counter; for all samples other than those labeled for < 3 minutes in galactose, total and acid-precipitable counts were virtually identical.

Apparatus

Countercurrent chromatography was performed with the most recent model of the nonsynchronous flowthrough coil planet centrifuge without rotating seals (4). The apparatus subjects the coiled column assembly to a planetary motion which consists of slow rotation (0-50 rpm) around its own axis and high speed revolution (500-1,000 rpm) around the central axis of the apparatus. The revolution produces a strong centrifugal force field (maximum 150 x g) while the slow rotation of the coil assembly provides efficient mixing of the two polymer phases and retention of the stationary phase in the coil. Both revolutional and rotational speeds of the coil are made independently adjustable to meet the requirements for the aimed separation. The coiled column was prepared from a single piece of 1 mm i.d. PTFE tubing (Zeus Industrial Products, Raritan, New Jersey) by winding it onto 6 units of 20 cm long, 0.6 cm o.d. stainless steel pipe cores in a series to make about 600 helical turns with a total capacity of approximately 15 ml. Both inlet (head) and outlet (tail) terminals of the coil were each connected to a piece of 0.4 mm i.d. PTFE tubing which was led to the outside of the rotor without the use of rotating seals. This seal-free rotor design makes the entire elution system leak-proof to provide a safeguard in separation of hazardous agents.

Preparation of Polymer Phase System

The polymer phase system employed in this study is based on the work of Stendahl et al. (7-9) and is composed of 6.2% (w/w)

dextran 500, 4.4% (w/w) polyethylene glycol 6000, 0.05 M Tris-chloride (pH 7.0), 10 mM potassium phosphate (pH 7.0), and 0.01% sodium azide. The solvent system was prepared by mixing equal volumes of the following two stock solutions (A and B) in a separatory funnel at room temperature. Stock solution A (double strength polymer solution) was prepared by dissolving 124 g of dextran 500 and 88 g of polyethylene glycol 6000 in 788 g of warm distilled water. Stock solution B (double strength buffer solution) was prepared by mixing 200 ml of 0.5 M Tris-chloride (pH 7.0), 200 ml of 0.1 M potassium phosphate (pH 7.0), and 20 ml of 1% sodium azide, in a 1-liter volumetric flask and adding water to bring the final volume to 1 liter. Stock solution A, because it forms two layers, should be thoroughly mixed into homogeneous suspension before each use. Strictly speaking, this method gives a concentration of each polymer slightly lower than the nominal value (on the order of 0.1%). However, it is simple and gives highly reproducible results.

Separation Procedure

Separation of bacterial cells was performed as follows: The coiled column is first filled with approximately equal amounts of the upper and the lower phases of the polymer phase system described above. This is done by delivering the solvents from a horizontally positioned syringe (20 ml capacity) containing 10 ml of each phase. After the filling process is completed, 1 ml of the sample suspension in equal amounts of the two phases is injected into the column through the sample port located at the pump outlet. Then the apparatus is run at 600 rpm combined with a slow column rotation of 5 rpm while the upper mobile phase is pumped into the column at a rate of 8.5 ml/h with an FMI Lab Pump (Fluid Metering Inc., Oyster Bay, New York). The effluent from the outlet of the column is collected with an LKB fraction collector to obtain 1 ml fractions for further analysis. After the desired number of fractions (30 fractions) are collected, the centrifuge run is terminated and the column contents (15 ml) are further

fractionated by eluting the stationary column with an equal volume mixture of the two phases with a syringe driver as described in filling the column. Then the column is emptied by pushing with air followed by washing with distilled water. The column is finally filled with 1 N NaOH and left overnight. This column cleaning procedure prevents adhesion of cells to the column wall, resulting in near 100% cell recovery.

RESULTS

Stendahl and coworkers devised a method for separating S. typhimurium that contain long chain LPS from those that contain short chain LPS on countercurrent distribution (7); in their work, the peaks containing the two types of cells were separated by 15 fractions. When we used their solvents to separate such cells by countercurrent chromatography, the cells containing long chain LPS distributed almost entirely into the mobile phase as expected and eluted out quickly from the column, but cells with short chain LPS remained in the column, even after chromatography was carried out for several days or 500 fractions were collected (data not shown). For this reason, it was necessary to modify the method: After the cells containing long chain LPS had been eluted and 30 fractions collected, the centrifuge run was terminated and the column contents containing retained cells were fractionated as described earlier. The results are shown in Fig. 1. Very sharp peaks, corresponding to cells containing long chain LPS (designated peak I, at left) and cells containing short chain LPS (designated peak II, at right) were obtained. There was no overlap, and 93-98% of the cells put into the column were recovered, as measured by recovery of radioactivity.

Having obtained a clear separation, we tested the effect of slight differences in the proportion of long chain LPS on the elution properties of the bacteria. During steady-state logarithmic growth at a given temperature, S. typhimurium synthesizes LPS continuously, and the ratio of LPS molecules to cell protein is known (12). When S. typhimurium G30 is transferred

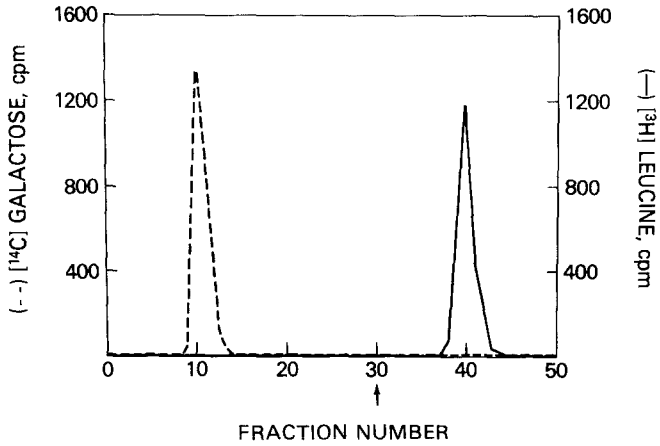

FIGURE 1. Separation of cells of S. typhimurium G30 containing long chain LPS from those containing short chain LPS by countercurrent chromatography. Cells were grown either without galactose and with [^{3}H]leucine (1 μCi/ml), or in the presence of 0.1 mM [^{14}C]galactose (1 mCi/mmol). The cells were harvested and washed, and 3 x 10^{9} cells of each were mixed and subjected to countercurrent chromatography as described. In this and other figures, the arrow on the bottom axis indicates the point at which the centrifuge run was terminated to start fractionation of the column contents. Also, the left peak will be referred to as peak I and the right peak as peak II. Identical results were obtained if the culture containing galactose was labeled with [^{14}C]leucine instead of [^{14}C]galactose.

from media lacking galactose, where it makes short chain LPS, to one containing galactose, all newly synthesized molecules of LPS have long chain polysaccharide, but pre-existing short chain LPS molecules are not modified or converted to long chain; furthermore, there is no turnover of LPS (13, 14). By growing this mutant for many generations in radioactive galactose, which labels only long chain LPS, and radioactive leucine, which labels protein and not LPS, we can measure the steady-state specific activity (galactose relative to leucine, i.e., LPS/protein) of bacteria completely covered with long chain LPS. It is then possible to calculate the proportion of long chain LPS relative

to total LPS in cells grown continuously in labeled leucine without galactose, and then given labeled galactose for various short lengths of time. LPS is made on the inner membrane and transferred shortly thereafter (within 1-2 minutes) to the outside of the outer membrane (13), so for all times of exposure to galactose except those less than ~ 3 minutes, the percent of labeled long chain LPS present in the organism will accurately reflect the percent of such molecules in the outer membrane. It is therefore possible to prepare Salmonella of known proportions of long and short chain LPS on their surface and to test the effect of these on the fractionation of the bacteria by countercurrent chromatography.

The results of such an experiment are shown in Fig. 2 and Table 1. After several generations of growth in the presence of [^{3}H]leucine and no galactose, [^{14}C]galactose was added and the cells harvested at 0 to 5 minutes thereafter. Table 1 shows the percent of long chain LPS relative to total LPS present on the surface of the bacteria. Figures are not given for 0-3 minutes, since at those early times a large proportion of the LPS is internal, having not been translocated to the outer membrane; however, by 4 minutes more than 85% of all galactose-containing LPS should be in the outer membrane.

When the distribution of such cells on countercurrent chromatography is examined, the following observations were made. As expected, the position of cells within the first 2 minutes of exposure to galactose is identical to that of cells grown without galactose, since little of the new LPS would be on the surface of the cell. However, at the time when a significant proportion of the long chain LPS appears in the outer membrane (~ 2-3 minutes), two phenomena appear. First, heterogeneity appears in peak II, indicating that the presence of some molecules of long chain LPS are affecting the mobility of these cells. Second, a substantial proportion of the cells appear in the position of peak I, even though they differ only slightly in percent long chain LPS from the cells in later fractions. The change is especially striking

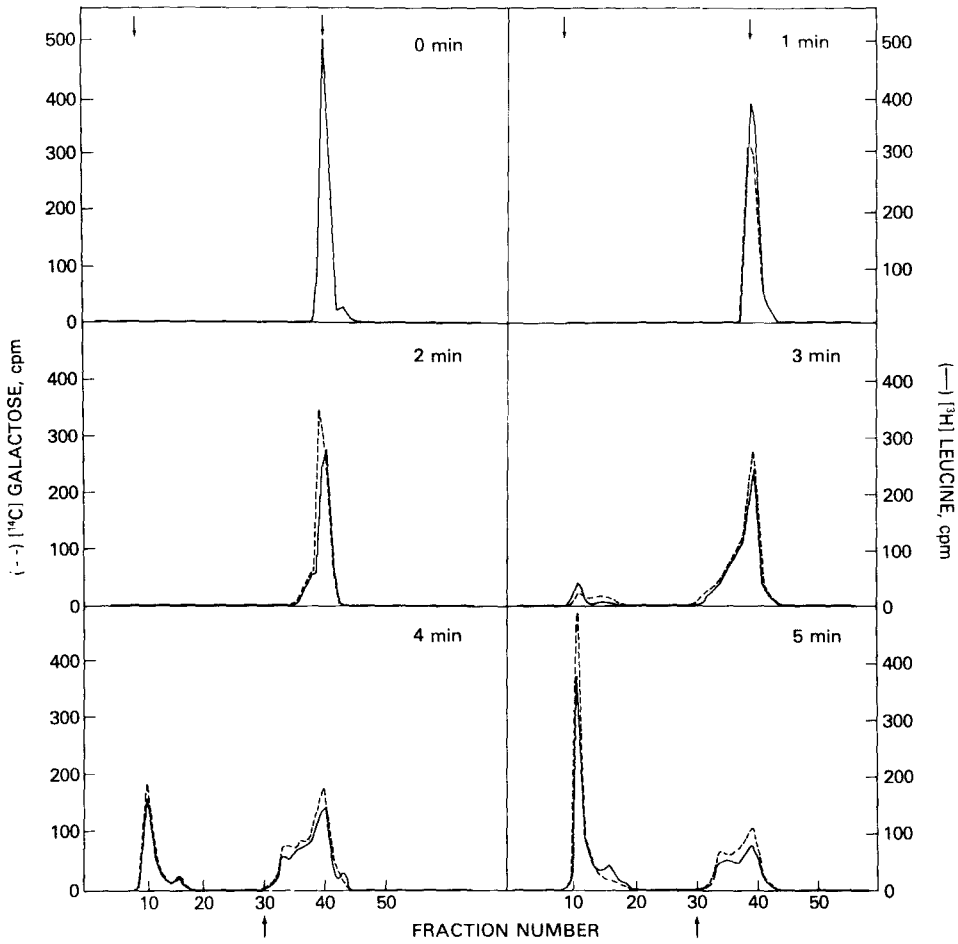

FIGURE 2. Separation of cells of S. typhimurium G30 grown 0-5 minutes with [^{14}C]galactose and continuously with [^{3}H]leucine. Cells were grown without galactose and with [^{3}H]leucine (1 µCi/ml) to a density of 3 x 10^{8}/ml and [^{14}C]galactose added (3 mCi/mmol). Samples were chilled and harvested at 0, 1, 2, 3, 4, and 5 minutes thereafter as described in Fig. 1 and Materials and Methods. The arrows at the top of the figure indicate the position of peaks I (left peak) and peak II (right peak).

TABLE 1

Properties of Cells Grown Varying Short Times in Galactose

Experiment shown in	Duration of exposure to galactose (min)	% Long chain LPS (relative to total) *	% Total cells in †	
			Peak I	Peak II
Figure 2	3	15	7	93
	4	19	31	69
	5	22	63	37
Figure 3	4	31	32	68
	5	35	56	44
	6	36	60	40
	7	39	67	33

*The percent of long chain LPS was calculated as described in the text.

†The percent of total cells in each peak was calculated by dividing the leucine cpm/peak by the total leucine cpm recovered in all fractions.

in comparing the 4-minute to the 5-minute sample: although the total percent of long chain LPS went up only 3% from 19% to 22% (Table 1), the percent of total cells in the area of peak I went from 31% to 63%. No significant difference in percent of long chain LPS is seen in peaks I and II at a given time point. These results suggest that a property other than total percent of long chain LPS affects the partition behavior of these cells in countercurrent chromatography.

To investigate these findings further, the experiment was repeated, except that samples were removed at 4 to 7 minutes after galactose addition (Fig. 3). In this experiment the percent of long chain LPS in the cells increased from 31% to 39% over the time period surveyed. Once again, countercurrent chromatography showed cells falling in three areas: the position of peak I, the position of peak II, and a series of shoulders forming a leading edge to peak II. Once again, there is a dramatic shift in percent of cells falling in peak I between 4 and 5 minutes of

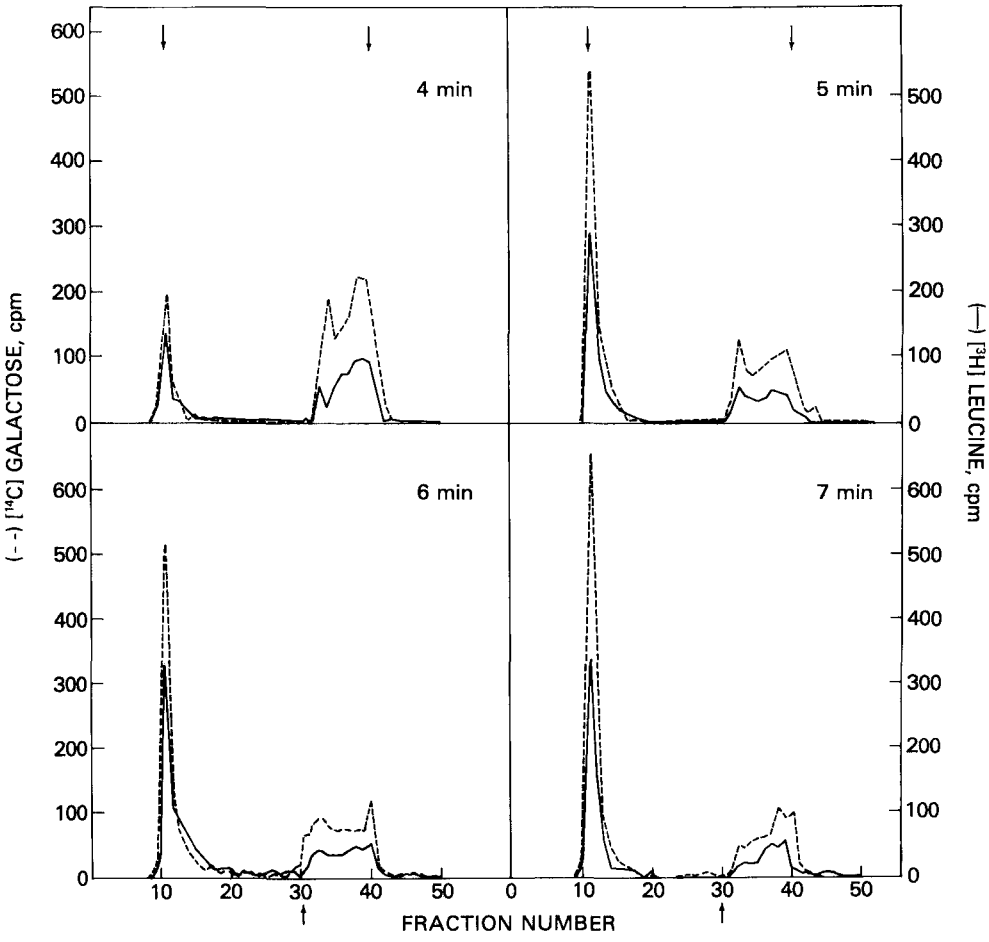

FIGURE 3. Separation of cells of S. typhimurium G30 grown 4-7 minutes with [14C]galactose and continuously with [3H]leucine. Procedure exactly as for Fig. 2 except that cells were harvested at 4, 5, 6, and 7 minutes after addition of galactose.

exposure to galactose (from 32% to 56%), and the percent of cells in peak I continues to increase, somewhat more slowly, over the next few minutes. As with the previous experiment, the percent of long chain LPS present in cells of peak I and peak II at a given time point are substantially the same. In another experiment (data not shown), approximately 90% of cells were in peak I after 11 minutes of exposure to galactose, and 100% in peak I after 20 minutes. These results confirm that some property of the cells other than actual percent of long chain LPS in the outer membrane affects their distribution in countercurrent chromatography.

DISCUSSION

The outer membrane of gram-negative bacteria and the synthesis of its LPS is diagrammed in Fig. 4. LPS is synthesized in the inner membrane (13) and is translocated to the outer membrane at a large number of points, after which it spreads over the surface (14-17). Mutants of S. typhimurium defective in UDP-galactose-4-epimerase provide an excellent tool for studying the effect of changes solely in LPS on the mobility of cells in countercurrent chromatography. When galactose is added to such mutants, the only change in the outer membrane is the addition during subsequent growth of LPS that contains long polysaccharide side chains instead of LPS with short chains. The total proportions of all outer membrane components stay the same. In general, only a tiny percentage of total LPS is found at one time in the inner membrane (perhaps 2%), and translocation of a newly synthesized molecule to the outer membrane is rapid (13, 16), so that at 37°C, 3-4 minutes after addition of labeled galactose to the S. typhimurium mutant, virtually all label will reflect molecules associated with the outer membrane. In contrast, spread of the LPS to cover the surface is much slower, taking 10-20 minutes to reach homogeneity (16-18). It is not known whether spreading of outer membrane molecules occurs from true lateral diffusion or from growth.

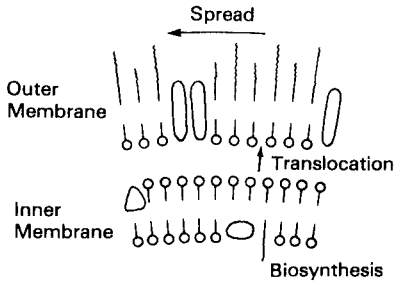

FIGURE 4. Diagram of structure of the outer membrane of S. typhimurium and biosynthesis of LPS. Symbols: ⌇ = LPS; ♀ = phospholipid; ○ = protein. The outer face of the outer membrane is the final destination of LPS, which is synthesized in the inner membrane, translocated to the outer membrane, and spreads to cover the surface. Translocation probably takes place at between 50 and 200 adhesion points between the two membranes (diagrammed by an arrow, but not pictured). It is not known whether spread occurs by diffusion within the bilayer or by growth or both. LPS may appear transiently on the outer face of the inner membrane and/or the inner face of the outer membrane during biosynthesis and translocation (not pictured).

We find with countercurrent chromatography that cells with virtually the same proportion of LPS molecules containing long chain LPS (in the range of 15-40%) can fractionate either in peak I (the position of cells covered entirely with long chain LPS) or in the position of peak II (the position of cells covered entirely with short chain LPS). The percentage of cells that fractionates in each peak changes during the period of 3-11 minutes after addition of galactose to the S. typhimurium G30 mutant. The changes in fractionation pattern do not reflect synthesis of LPS with a different average side chain length immediately after galactose addition, relative to cells grown continuously with galactose, since Palva and Mäkelä (19) showed that the distribution of side chain lengths in LPS was the same at 1 minute after galactose addition as in cells grown continuously with galactose. We can best explain these results by the following hypothesis:

Bacteria with low (15-40%) percentages of long chain LPS in their outer membrane can fractionate either in the position of

cells covered with long chain LPS or cells covered with short chain LPS depending on where the long chain LPS is in the outer membrane. For a few minutes after translocation to the outer membrane the long chain molecules are "hidden", perhaps because they may face inward rather than outward on the outer membrane, or perhaps because they may not have spread over the surface from the points of their insertion. Although we cannot as yet distinguish these hypothesis, it is certain that countercurrent chromatography provides a tool for separating bacteria on the basis of physical properties of their outer membrane that have not been detectable or separable by other means, and thus may be of great help in analyzing steps in membrane biogenesis.

REFERENCES

1. Walter, H. Partition of cells in two-polymer aqueous phases: a surface affinity method for cell separation. In Methods of Cell Separation (Catsimpoolas, N., ed.), Vol. 1, Plenum Press, New York, 1977, p. 307.

2. Walter, H., Krob, E. J., and Brooks, D. E. Membrane surface properties other than charge involved in cell separation by partion in polymer, aqueous two-phase systems. Biochemistry 15, 2959 (1976).

3. Ito, Y. Countercurrent chromatography. J. Biochem. Biophys. Methods 5, 105 (1981).

4. Ito, Y., Bramblett, G. T., Bhatnagar, R., Huberman, M., Leive, L., Cullinane, L. M., and Groves, W. Improved nonsynchronous flowthrough coil planet centrifuge without rotating seals: principle and application. Sep. Sci. Technol. 18, 33 (1983).

5. Lüderitz, O., Freudenberg, M. A., Galanos, C., Lehmann, V., Rietschel, E. H., and Shaw, D. H. Lipopolysaccharides of gram-negative bacteria. In Current Topics in Membranes and Transport. Membrane Lipids of Prokaryotes (Razin, S., and Rottem, S., eds.), Vol. 17, Academic Press, New York, 1982, p. 79.

6. Osborn, M. J., Rosen, S. M., Rothfield, L., and Horecker, B. L. Biosynthesis of lipopolysaccharide, I. Enzymatic incorporation of galactose in a mutant strain of Salmonella. Proc. Natl. Acad. Sci. U.S.A. 48, 1831 (1962).

7. Stendahl, O., Magnusson, K.-E., Tagesson, C., Cunningham, C., and Edebo, L. Characterization of mutants of Salmonella typhimurium by counter-current distribution in an aqueous two-polymer phase system. Infect. Immun. 7, 573 (1973).

8. Magnusson, K.-E., Stendahl, O., Tagesson, C., Edebo, L., and Johansson, G. The tendency of smooth and rough Salmonella typhimurium bacteria and lipopolysaccharide to hydrophobic and ionic interaction, as studied in aqueous polymer two-phase systems. Acta Path. Microbiol. Scand. Sect. B 85, 212 (1977).

9. Stendahl, O., Edebo, L., Magnusson, K.-E., Tagesson, C., and Hjertén, S. Surface-charge characteristics of smooth and rough Salmonella typhimurium bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. Acta Path. Microbiol. Scand. Sect. B 85, 334 (1977).

10. Stendahl, O., Tagesson, C., and Edebo, M. Partition of Salmonella typhimurium in a two-polymer aqueous phase system in relation to liability to phagocytosis. Infect. Immun. 8, 36 (1973).

11. Osborn, M. J., Gander, J. E., and Parisi, E. Mechanism of assembly of the outer membrane of Salmonella typhimurium. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. 247, 3962 (1972).

12. Nikaido, H. Biosynthesis and assembly of lipopolysaccharide and the outer membrane layer of gram-negative cell wall. In Bacterial Membranes and Walls (Leive, L., ed.), Vol. 1, Marcel Dekker, New York, 1973, p. 131.

13. Osborn, M. J., Gander, J. E., and Parisi, E. Mechanism of assembly of the outer membrane of Salmonella typhimurium. Site of synthesis of lipopolysaccharide. J. Biol. Chem. 247, 3973 (1972).

14. Muhlradt, P. F., Menzel, J., Golecki, J. R., and Speth, V. Outer membrane of Salmonella. Sites of export of newly synthesized lipopolysaccharide on the bacterial surface. Eur. J. Biochem. 35, 471 (1973).

15. Muhlradt, P. F., Menzel, J., Golecki, J. R., and Speth, V. Laterial mobility and surface sensitivity of lipopolysaccharide in the outer membrane of Salmonella typhimurium. Eur. J. Biochem. 43, 573 (1974).

16. Kulpa, C. F., Jr. and Leive, L. Mode of insertion of lipopolysaccharide into the outer membrane of Escherichia coli. J. Bacteriol. 126, 467 (1976).

17. Bayer, M. E. Role of adhesion zones in bacterial cell surface function and biogenesis. In Membrane Biogenesis (Tszgaloff, A., ed.), Plenum Press, New York, 1975, p. 393.

18. Bayer, M. E. The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. In Bacterial Outer Membranes. Biogenesis and Functions (Inouye, M., ed.), John Wiley and Sons, New York, 1979, p. 167.

19. Palva, E. T., and Mäkelä, P. H. Lipopolysaccharide heterogeneity in Salmonella typhimurium analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Eur. J. Biochem. 107, 137 (1980).

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CELL SEPARATIONS ON THE COUNTERCURRENT CHROMATOGRAPH

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ABSTRACT

Separation of cells differing only subtly has been achieved by partitioning between the two phases formed by solution of dextran and polyethylene glycol in water. Cell populations which have related, but not identical, surface properties seldom exhibit sufficiently different partition behavior to be separated in a single extraction. In such cases, repeated partitions are carried out via countercurrent distribution or countercurrent chromatography to effect the separation. Potential advantages of countercurrent chromatography are its ease and rapidity of operation. In the present work we describe our approach to determining and possibly improving the efficiency of polymer phase partitioning by countercurrent chromatography.

INTRODUCTION

Modern biomedical research depends heavily upon effective techniques of separation and purification. An important prerequisite for studying the normal or pathological activity of any biological system, whether molecular or cellular, is the ability to isolate its components from one another so that they can be characterized, manipulated, and recombined under controlled

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conditions. Separation methods for molecular biology are well developed. However, the preparation of homogeneous, functionally specific populations of living cells remains one of the chief obstacles to progress in cell biology today. Among the most widely used techniques of cell purification are velocity sedimentation and isopycnic centrifugation, which operate entirely on the basis of such physical characteristics as cell size and density. Other separation methods employ the selective killing of unwanted cells within a culture, or the tendency of certain cells to attach themselves to solid substrates. While these are useful procedures in many cases, a number of biologically important cell types are not amenable to such separations. For the most part, the methods described lack the power to select a subpopulation of cells differing only subtly from contaminating cell types. Cell types may differ from one another only in the nature or amount of a single surface membrane constituent, for example, a characteristic that cannot be detected by the separation techniques in ordinary use.

A need exists for a cell purification technique that is able to sort a heterogeneous mixture of cells on the basis of specific, subtle features of the cell surface, and can do this for cells about which one may have very little specific biochemical information. A simple and sensitive method that shows promise of fulfilling these criteria is phase partitioning in aqueous polymer systems.

When aqueous solutions of two different polymers are mixed above certain concentrations they frequently form immiscible, liquid, two-phase solutions. Each of these phases usually consists of more than 90 percent water and can be buffered and made isotonic and hospitable to cells, organelles, and biomolecules by addition of low molecular weight species. If a cell or particle suspension is added to such a system, then shaken, the cells (upon re-equilibration) are frequently found to have partitioned unequally between one of the phases and the interface. This preferential partition behavior can be used as the basis of

a separation procedure for differing cell populations since partition in these systems is determined directly by cell membrane properties such as charge, lipid composition, and specific cell proteins (1,2).

The polymers typically used for phase partitioning are polyethylene glycol (PEG) and dextran. Solution of these polymers above certain concentrations (approximately 5% by weight for PEG-6000 and dextran-500,000) produces two, immiscible aqueous phases in which the top layer is PEG-rich and the bottom is dextran-rich. The concentration at which the two phases become immiscible is known as the critical point. By convention, the concentrations of dextran and PEG in a phase system are represented as a ratio; for example, a 5/4 system is one containing 5% dextran and 4% PEG. Some important physical characteristics of these phase systems are as follows: densities, upper 1.01 g/mL and lower 1.03 g/mL to 1.08 g/mL; interfacial tensions, 0.007 dyne/cm for a 6/4 system, 0.0031 dyne/cm for a 5/4, and 0.00046 dyne/cm for a 5/3.5 (1); and viscosities, upper 4 cP and lower from 44 cP for a 5.6/4 system to 27 cP for a 5/4 to 23 cP for a 5/3.5 (3). Density differences, interfacial tensions, and lower-phase viscosities decrease as polymer concentrations are lowered and approach the critical point; buffer variations also affect these same parameters.

Cell populations which have related, but not identical, surface properties seldom exhibit sufficiently different partition behavior to be separated in a single extraction. In such cases, many repeated partitions are carried out via countercurrent distribution (CCD) or countercurrent chromatography (CCC) to effect the separation. The usual automated device for performing repetitive partitioning is the Albertsson CCD apparatus (1). In this machine 60 to 120 thin vessels are arranged around the periphery of a disc. Each vessel is loaded accurately with top and bottom phases, and the sample is introduced into a small number (usually three) of the adjacent vessels. The entire disc is then shaken to promote mixing of the phases and partitioning of the sample.

After settling has brought about phase separation (typically 15-20 minutes) the disc is rotated so that top halves of the vessels are shifted to join with adjacent bottom halves. The interface and a small portion of the bottom layer are carried with the top vessel half, or alternatively, the interface and a portion of the top phase remain with the bottom half. The result of repetition of this process is a countercurrent movement of top and bottom phases and a consequent countercurrent distribution of the partitioned material. The prime limitations of CCD for polymer-phase partitioning are the laboriousness of accurately and individually loading the 60-120 vessels and the long delay necessitated for phase separation after each mixing (e.g., 60 transfers at 15 minutes per transfer equals 15 hours per run).

Phase partitioning of cells can also be performed by CCC with the potential advantage of avoiding the above limitations of CCD. However, injection of particles the size of cells (> 1 μM) into the typical countercurrent chromatograph in which a centrifugal force is applied to the medium results in the particles being pressed against the column walls and immobilized. This immobilization can be eliminated by applying a second, counter rotation to the coils as in the nonsynchronous coil planet centrifuge (NSCPC) of Ito, Figure 1. Ito and Sutherland have demonstrated that cells can be separated on this device (4-6). The major barriers to broad application of the NSCPC to these purifications are the lack of demonstrated broad applicability of the device and the lack of precise information about the efficiency of these separations. To date only a single work has been published on cell purifications by polymer phase partitioning on the NSCPC; this pioneering work was done by Sutherland and Ito, and involved separations of fresh red blood cells (RBC's) of sheep, human, and dog (6).

Unlike the Albertsson CCD device, the NSCPC has the capability to perform separations based upon cell size and sedimentation velocity in addition to the two-phase partitioning technique described above. In this mode, the coils of the NSCPC column are

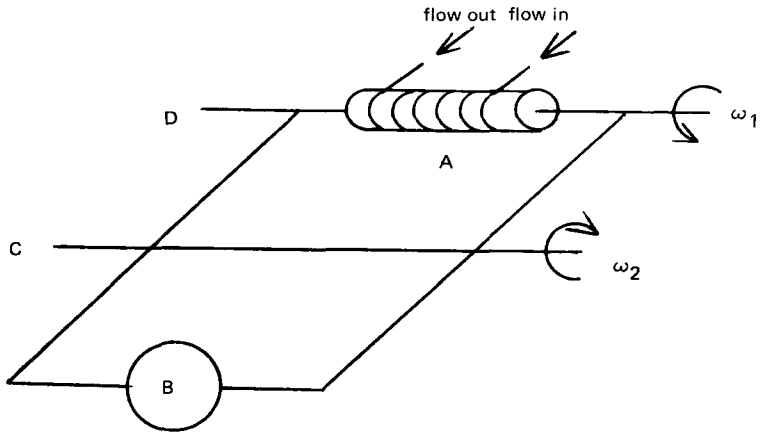

Figure 1. Schematic drawing of the Ito nonsynchronous coil planet centrifuge. A - helical coil (column), B - counter weight, C - central rotation axis, D - planetary rotation axis.

filled with a single solution of physiological saline or some similar isotonic eluent (5).

SEPARATION EFFICIENCY - INITIAL CONSIDERATIONS

Although Sutherland and Ito achieved good resolution in their separations, there is reason to suspect that polymer phase partitioning may be relatively inefficient on the NSCPC as compared to CCD. First, as described in the accompanying article by Sutherland and coworkers (7), polymer phase mixing in CCC is apparently inefficient. These workers have used an elegant apparatus to view PEG mobile phase streaming through dextran stationary phase with little mixing; what mixing does occur takes place at the interface where the PEG phase leaves the dextran phase, and is suggestive of the turbulence at the foot of a waterfall. Presumably this inefficient mixing is due to the high viscosity of the dextran phase.

The second consideration suggestive of inefficiency in the NSCPC derives from a theoretical consideration of cell partition-

ing and comes from the work of Brooks and coworkers (8). In this work an expression is derived relating the effects of factors such as interfacial free energies, interfacial potential, and surface charge density of particles on partitioning. Surprisingly, a bacterial partitioning experiment carried out to test this theoretical construct could only be explained if it was assumed that the temperature of the experiment had been equal to 10^5 Kelvin. Obviously, the expression fails to take account of some powerful randomizing factor. These workers suggest that this randomizing energy comes from the fluid shear stresses that occur during phase separation following phase mixing. If this conjecture is correct, then the high G field present in the NSCPC might be expected to enhance shear forces and cell randomization, leading to a reduction in the quality of separations achieved.

EXPERIMENTAL MEASUREMENT OF EFFICIENCY

Efficiency in the NSCPC can be determined by use of the Craig equations for CCD, eqs. 1-3, where p and q are the mole

$$(p + q)^n = 1 \tag{1}$$

$$K = p/q = p/(1 - p) \tag{2}$$

$$T_{n,r} = \frac{n(n - 1)(n - 2)\ldots(n - r + 2)}{(r - 1)!}(1/ K + 1)^n K^{r-1} \tag{3}$$

fractions of soluble sample in upper and lower phases, respectively, n is the number of transfers, r is the tube number, and $T_{n,r}$ is the amount of sample in the rth tube after n transfers. Insertion of the proper partition coefficient K and the proper n into eq. 3 permits reproduction of experimental sample distribution. Application of CCD equations to separations on the NSCPC is appropriate because of the manner in which the cell separations are performed. The column is initially filled with the more dense dextran stationary phase, rotation is begun and approximately one milliliter of mobile PEG phase is pumped onto the column. The sample, dissolved in mobile phase, is then injected and mobile phase is pumped for the remainder of the run. Rather than allowing the run to continue in this fashion until all cells have elu-

ted, the rotation is halted just before the sample reaches the end of the column, a technique found by Sutherland and Ito to optimize resolution (6). After spinning is stopped, the column contents (resembling a CCD distribution) are pumped from the machine and fractionally collected.

To evaluate the efficiency of phase mixing on the NSCPC in the absence of shear forces on particles, we have determined the distribution of albumin using the same protocol as used for cell separations (see Experimental), Figure 2. Note that the experimental curve can be reproduced effectively by eq. 3 assuming that $\underline{K} = 3.0$ and $\underline{n} = 20$. Thus under these conditions, the equivalent of 20 transfers (or mixing events) has occurred. However, since the NSCPC column contains 600 coils, almost all of which have been shown (see below) to retain some stationary phase throughout the run, this instrument should be able to deliver the equivalent of around 600 individual transfers ($\underline{n} = 600$). Apparently phase mixing under these conditions is indeed poor.

Another indication of poor mixing is provided by comparison of the theoretical $\underline{K}$ value of 3.0, Figure 2, with the experimental value of 0.38 for a single tube partition. Such a high theoretical $\underline{K}$ could result if the albumin, injected dissolved in the PEG mobile phase, moved through the column without interacting with the dextran stationary phase.

In applying the Craig equations it is necessary to include the volume ratios of stationary and mobile phases (V_S/V_m). For the sake of simplicity we have assumed this ratio is unity. In fact it varies throughout the column as revealed by pumping off the column contents (see V_S/V_m, Figure 2) but typically averages about 0.8.

Application of the Craig equations to cell separations is not entirely satisfactory, since the cell samples are heterogeneous in size and possibly in cell surface properties as well. It is important to recall that cell size affects retention time on the NSCPC. Consequently cells will be smeared across the column and provide a poor measure of the number of transfers involved. Also it is

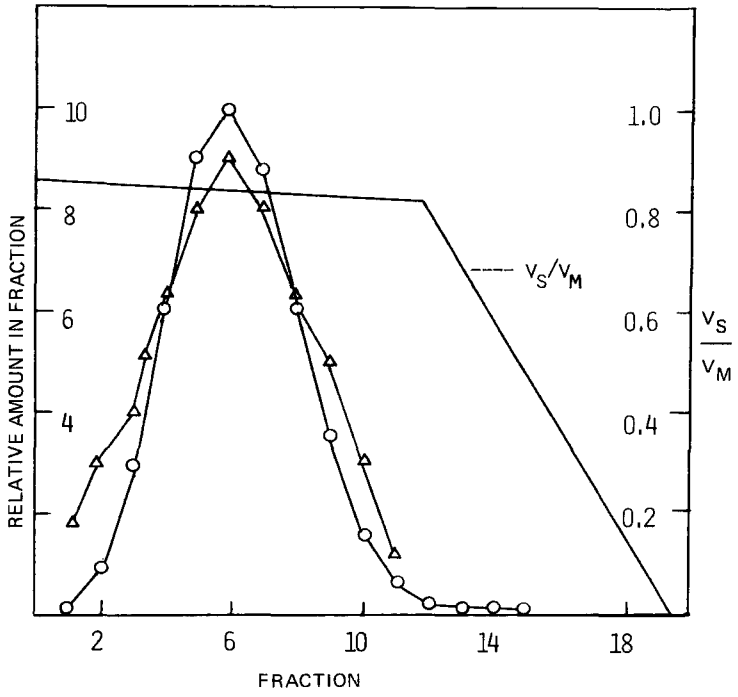

Figure 2. Theoretical (o) and experimental (Δ) curves for partitioning of albumin, and a plot of V_s/V_m as a function of fraction number (in phase system "D"). The calculated curve assumes $\underline{n}$ = 20 and $\underline{K}$ = 1.50; experimental $\underline{K}$ = 0.38.

important to note that cells are partitioned between the upper phase and the interface, and it is impossible at present to approximate the ratio (needed for eq. 3) of interface volume to mobile-phase volume in the heterogeneous environment of the countercurrent chromatograph. With these limitations in mind, application of eq. 3 to two passes of fresh sheep cells through the NSCPC with differing phase systems is shown in Figure 3. Note that the theoretical $\underline{K}$ value is greater than the measured value and further that $\underline{n}$ is rather small. It is interesting that a significant

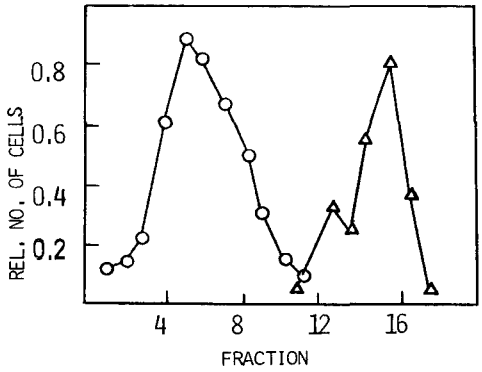

Figure 3. Plots for two runs of fresh sheep RBC's in phase system D (o) and phase system B (Δ). For D, $K_{exptl} = 0.31$, $K_{theor} = 0.80$ and $\underline{n} = 20$. For B, $K_{exptl} = 0.02$, $K_{theor} = 1.3$, and $\underline{n} = 30$. Theoretical curves using these parameters match the experimental curves well.

amount of band broadening occurs despite poor phase mixing, since under these circumstances one might expect the cells to remain in a tight band as they move through the machine. Possibly this broadening results from the increased shear forces characteristic of the NSCPC. On the other hand, as discussed above, it could result from the heterogeneity of the sample.

A more effective measurement of separation efficiency, and an evaluation of the factors involved in band broadening, may be obtained by direct comparison of cell experiments on the NSCPC and the CCD apparatus, since the number of transfers is known in CCD. Such experiments are currently in progress in our laboratory and that of D. E. Brooks (University of British Columbia).

IMPROVEMENT OF EFFICIENCY

The measurements of efficiency we have presented thus far are of course dependent on the specific machine and phase parameters utilized. It is important to explore the possibility of improving the efficiency of the NSCPC. Regarding machine para-

meters it is evident that increasing flow rate and reducing rotation rate results in rapid removal ("stripping") of the stationary dextran phase from the column. A flow of approximately 8 mL/hr and a rotation rate of approximately 750 rpm is ideal from this standpoint; changing the "small" rotation has no effect on stripping of stationary phase.

Since presumably the inefficient phase mixing on the NSCPC is largely influenced by the high differences in viscosity and density between the polymer phases and also possibly by variation in interfacial tension between polymer phases, one would suspect that varying the phase systems themselves would be likely to enhance phase mixing. We have preliminary evidence that this is the case. In figure 4 we present a comparison of RBC separations on the NSCPC under presumably identical conditions; experiment 4a was performed in our laboratory and experiment 4b was performed by Sutherland and Ito (6). It is important to note that the machine parameters are identical in the two experiments. In addition, the column on our machine is the same one which was used on the Sutherland-Ito machine. Our machine was constructed at NIH by Ito, who simply removed the column from his older instrument and transferred it to our newer instrument.

The differences between experiments 4a and 4b are obvious; the peak shapes and separation of 4b are far superior; in addition 4b reveals a different V_s/V_m pattern and different $\underline{K}$ values. Although the phase systems are supposedly the same, the difference between these two experiments lies in fact in the phase systems. Workers in the area of polymer phase partitioning are painfully aware of the difficulty in reproducing a particular phase system. Specific problems include variations in molecular weight distributions from batch to batch of the polymer, impurities, and determination of the water content of the hygroscopic polymer stock powders. Since the work of Sutherland and Ito (6), significant advances have been made in controlling these variables (see Experimental section for methods). (One problem which remains to be dealt with, however, is that of impurities). Discussions with

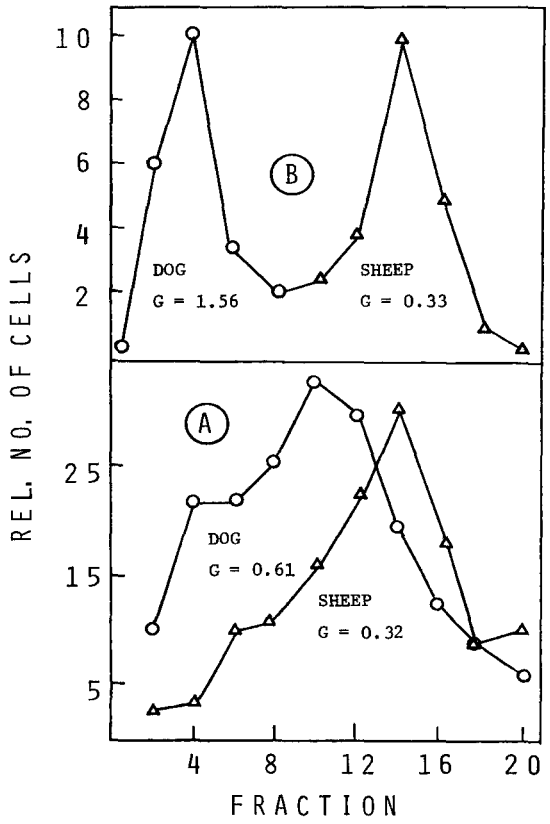

Figure 4. Separations of fresh sheep (Δ) and dog (o) as done by Sutherland and Ito (6) (plot B) and in our laboratory (plot A) using phase system "D".

Sutherland and Ito have made it evident that the water content of their polymers was not accounted for in their preparations of the 5/4 system used. If this is the case, we calculate that their phase system was in fact close to a 4.7/3.8 system. This concentration is nearer the critical point than the 5/4 system we used. Since interfacial tensions, phase-density differences, and phase-viscosity differences decrease as the critical point is approached,

phase mixing should be enhanced in a phase system close to the critical point. This effect could explain the superior separation results of experiment 4b. We are actively investigating this hypothesis with careful determination of polymer phase compositions.

EXPERIMENTAL

Polymer phase systems were prepared and separations on the NSCPC were performed as described previously (6). In a typical NSCPC run a large rotation rate of 750 rpm was used along with a small rotation rate of 10 rpm and a flow rate of 8 mL/min. PEG concentrations in stock solutions were determined by refractometry using an empirical relationship between refractive index and concentration derived with samples dried at 100°C for 24 hours; the PEG was also purified by precipitating from acetone with ethyl ether. Dextran concentrations in stock solutions were determined by polarimetry as described by Albertsson (1). PEG-6000 was purchased from Union Carbide as Sentry grade material. Dextran-500,000 was purchased from Pharmacia and used without purification. Phase system "B" contains 0.01M phosphate and 0.15M chloride, and system "D" contains 0.07M phosphate and 0.06M chloride (6). Partition coefficients were determined after phase settling had proceeded for 10 minutes rather than the three minutes used by Sutherland and Ito (6). Albumin concentrations were determined by absorbance at 280 ηM after dilution with water to assure a single phase system. A Coulter counter was used to determine cell numbers.

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REFERENCES

1. Albertsson, P. S., Partition of Cell Particles and Macromolecules, Wiley, New York, 1960.

2. Walter, H., in Methods of Cell Separation, Vol. 1, Catsimpoolas, N. D., Ed., Wiley, New York, 1977, p. 307.

3. Brooks, D. E., personal communication.

4. Ito, Y., Bramblett, G., Bhatnagar, R., Huberman, M., Leive, L. L., Cullinane, L. M., and Groves, W., Sep. Sci. Tech., 18(1), 33 (1983).

5. Ito, Y., Carmeci, P., Sutherland, I. A., Anal. Biochem., 94, 249 (1979).

6. Sutherland, I. A. and Ito. Y., Anal. Biochem., 108, 367 (1980).

7. Sutherland, I. A., Heywood-Waddington, D., and Peters, T. J., This Journal.

8. Brooks, D. E., Van Alstine, J., Sharp, K. A., and Bamberger, S., In preparation.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(2), 433 (1984)

ANNOUNCEMENT

The Chromatography and Separation Chemistry subdivision has been formed under the aegis of the American Chemical Society's Analytical Chemistry Division.

The subdivision has the following objectives:

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LC NEWS

ANALYSS oF BLEACHING & PULPING LIQUORS via ion chromatography has become a provisional TAPPI method (T-699 pm-83). The method is approved for simultaneous determination of hypochlorite, chlorite, chloride, and chlorate in bleaching liquors...and the simultaneous determination of sulfide, sulfite, sulfate, oxalate, and thiosulfate in white, green and black liquors. Other anions, such as fluoride, nitrite, nitrate and phosphate, can also be determined. Dionex Corp., JLC/84/2, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(2), 437-440 (1984)

LC CALENDAR

1984

JANUARY 17: "Process Scale HPLC," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

JANUARY 19-20: Workshop: "Low Dispersion Liquid Chromatography," The Free University of Amsterdam, The Netherlands. Contact: LDLC Workshop Office, Dept. of Analytical Chem., The Free University, DeBoelelaan 1083, 1081 HV Amsterdam, The Netherlands.

FEBRUARY 12-16: 14th Australian Polymer Symposium, Old Ballarat Travel Inn, Ballarat, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Dr. G. B. Guise, RACI Polymer Div., P. O. Box 224, Belmont, Victoria 3216, Australia.

FEBRUARY 19-22: Fourth Annual Congress for Recombinant DNA Research, Town & Country Hotel, San Diego, CA. Contact: Sherago Associates, Inc., 1515 Broadway, New York, NY, 10036.

FEBRUARY 20-22: International Symposium on HPLC in the Biological Sciences, Regent Hotel, Melbourne, Australia. Contact: The Secretary, Int'l Symposium on HPLC in the Biological Sciences, St. Vincent's School of Medical Research, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia.

FEBRUARY 22: First Annual Congress for Automation Scale-up, and the Economics of Biological Process Engineering, Town & Country Hotel, San Diego, CA. Contact: Sherago Associates, Inc., 1515 Broadway, New York, NY, 10036.

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MARCH 20: "New Developments in HPLC of Water Soluble Macromolecules," sponsored by the Chicago Chromatography

Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL: "HPLC of Water Soluble Polymers," a 2-day course (date to be announced) sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL 8-13: National ACS Meeting, St. Louis, MO. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

APRIL 17: "New Developments in TLC," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

JUNE 3-5: International Symposium on LCEC and Voltammetry, Indianapolis Hyatt Regency Hotel, Indianapolis, IN. Contact: The 1984 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

JUNE 10-14: 14th Northeast Regional ACS Meeting, sponsored by the Western Connecticut and New Haven Sections, at Fairfield University, Fairfield, CT. Contact:D. L. Swanson, American Cyanamid Co., Stamford, CT, USA.

JUNE 18-20: Second International Conference on Chromatography & Mass Spectrometry in Biomedical Sciences, sponsored by the Italian Group for Mass Spectrometry in Biochemistry & Medicine, Milan, Italy. Contact: Dr. A. Frigerio, via Eustachi 36, I-20129 Milan, Italy, or Dr. H. Milon, P. O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

JUNE 18-21: Symposium on Liquid Chromatography in the Biological Sciences, Ronneby, Sweden, sponsored by The Swedish Academy of Pharmaceutical Sciences. Contact: Swedish Academy of Pharmaceutical Sciences, P. O. Box 1136, S-111 81 Stockholm, Sweden.

AUGUST 26-31: National ACS Meeting, Philadelphia, PA. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

SEPTEMBER 10-14: Advances in Liquid Chromatography, including the 4th Annual American-Eastern European Symposium on LC and the Int'l Symposium on TLC with Special Emphasis on Overpressured Layer Chromatography, sponsored by the Hungarian Academy of Sciences' Chromatography Committee & Biological Research Center and the Hungarian Chemical Society, in Szeged, Hungary. Contact: Dr. H. Kalasz, Dept. of Pharmacology, Semmelweis University of Medicine,

P.O.Box 370, H-1445 Budapest, Hungary, or Dr. E. Tyihak, Research Inst. for Plant Protection, P.O.Box 102, H-1525 Budapest, Hungary.

OCTOBER 1-5: 15th Int'l. Sympos. on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, D-6000 Frankfurt Main, West Germany.

DECEMBER 10-12: "TLC/HPTLC-84: Expanding Horizons in TLC," Sheraton-University City, Philadelphia, PA. Contact: J. C. Touchstone, University of Pennsylvania, Dept. OB-GYN, 3400 Spruce Street, Philadelphia, PA.

DECEMBER 16-21: International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, sponsored by the Chemical Inst. of Canada, Chemical Soc. of Japan, and the American Chem. Soc. Contact: PAC CHEM '84, International Activities Office, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

1985

FEBRUARY 11-14: Polymer 85, Int'l Symposium on Characterization and Analysis of Polymers, Monash University, Melbourne, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Polymer 85, RACI, 191 Royal Parade, Parkville Victoria 3052, Australia.

APRIL 28 - MAY 3: 189th National ACS Meeting, Miami Beach. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

JULY 1-5: Ninth International Symposium on Column Liquid Chromatography, sponsored by the Chromatography Discussion Group and by the Royal Society of Chemistry's Chromatography & Electrophoresis Group, Edinburgh, Scotland. Contact: Prof. J. H. Knox, 9th ISCLC Secretariat, 26 Albany Street, Edinburgh, EH1 3QH, Great Britain.

SEPTEMBER 8-13: 190th National ACS Meeting, Chicago. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

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APRIL 6-11: 191st National Am. Chem. Soc. Mtng., Atlantic City, NJ. Contact: A. T. Winstead, ACS, 1155 16th Streeet, NW, Washington, DC, 20036, USA.

SEPTEMBER 7-12: 192nd National Am. Chem. Soc. Mtng., Anaheim,

Calif. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

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APRIL 5-10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

The Journal of Liquid Chromatography will publish announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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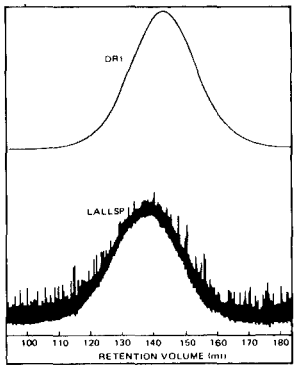

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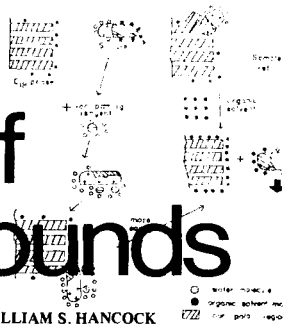

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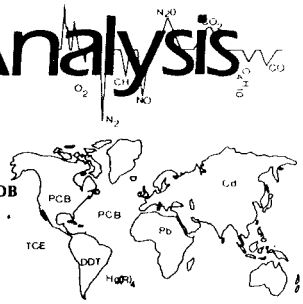

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