THIN-LAYER CHROMATOGRAPHY ON CYCLODEXTRINS

by

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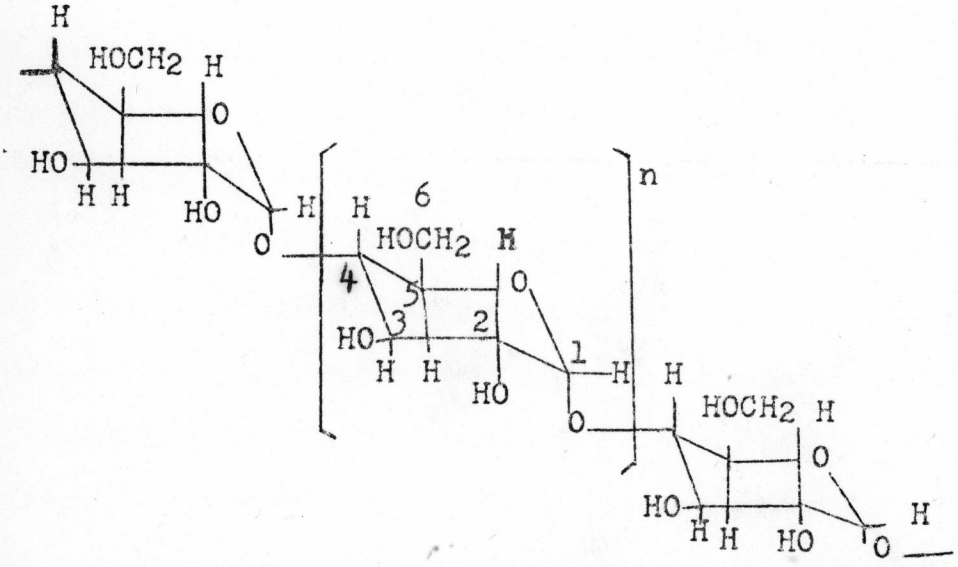
I. Introduction

A. Nature of α- (alpha) and β- (Beta) cyclodextrins

Cyclodextrins (also called Schardinger dextrins or cycloamyloses) are a homologous group of cyclic saccharides, composed of six or more α- D- glucopyranose units linked 1 → 4. They are obtained from the degradation of starch by an amylase of Bacillus Macerans. N.M.R.(1) and X-Ray(2) studies have established the C-1 Chair conformation for the glucose units in the cyclodextrins. The general formula of the cyclodextrins may be expressed as follows:



(n=4 for α- cyclodextrin and n=5 for β- cyclodextrin)

Each molecule of the cyclodextrins is doughnut-shaped and has the primary and secondary hydroxyl groups crowning opposite ends of its cavity, with H-3 and H-5 directed toward its interior and H-1, H-2, and H-4 located on its exterior. The interior of the cavity is mainly composed of the equally distributed bridging acetal oxygen atoms. Depending on the number of glucose units in the molecule, the rings of the cyclodextrins have different internal diameters. The diameters of the molecules of α- (6- glucose units), and β- (7- glucose units), based on the Stuart models (3), which are in good agreement with the X-ray studies, are 6, and 8 A° (angstrom) respectively.

The cavity of the cyclodextrin is a region of "high energy" and is capable of entrapping molecules such as aromatic compounds, paraffins, and carboxylic acids, as well as noble gases, into its hydrophobic center. Many labile drugs such as vitamin A and unsaturated fatty acids are stabilized by inclusion with the cyclodextrins. The interaction between the cyclodextrin (the "host") and the "guest" molecule is believed to be a combination of inclusion formation and other intermolecular attractive forces. Hydrogen bonding (4) and Van der Waals forces (5) are claimed to be the main forces in holding the cyclodextrin complexes together. The included molecule reacts via non-covalent bonding with the atoms lining and rimming the

cavity. Generally, the inclusion complexes are stable in aqueous medium but are less stable in non-aqueous medium due to the competition for the cavity between the guest molecule and the non-aqueous solvent.

Because of the fixed shape and size of the cyclodextrin cavity, the cyclodextrins have been found useful as models for studying the primary step of enzyme catalysis (6) or antigen-antibody reactions (7) The manner in which cyclodextrins increase or decrease rates of reactions is analogous to the "lock and key" theory of enzymatic catalysis proposed by Fischer. The extent of inclusion and the crystalline structure of cyclodextrin complexes depend strongly on the structure of the guest molecules. Previous study (8) has shown the importance of the structure of the guest molecules to the degree of interaction where inclusion formation is a major contributing factor.

Takeo and Kuge (10) have reported that the guest molecule has an influence on the crystal structure of the α- cyclodextrin complex, presumably because of the smaller size of the cavity of α- cyclodextrin. On the contrary, the intruded molecule has played no significant role in the crystal structure of the β- cyclodextrin complex, which may be attributed to the larger hole of β- cyclodextrin. Cramer, et. al (11) have studied the formation of inclusion compounds of α- cyclodextrins in aqueous solutions and

strongly confirmed the stereospecificity of the complex formation process.

Interaction of cyclodextrins with pharmaceuticals has been investigated (9, 12) and it has also been found that interaction mechanisms other than inclusion may be involved in the case of molecules which are too large to be included within the cyclodextrins but still can interact with the cyclodextrins. Tutt and Schwartz (13) have used β- cyclodextrin as a model for penicillinase and correlated the similarity between the cycloheptaamylose- catalyzed hydrolysis of penicillins and enzyme- catalyzed hydrolysis of the same compounds.

In summary, the cyclodextrins have proved to be useful in many aspects of studies by their properties of forming inclusion complexes or clathrates with both organic and inorganic molecules. This phenomenon leads to the stabilization of autooxidation-sensitive drugs (i.e., by reduction of a rate), selectivity control by altering the relative rates of the compounds (such as in the chlorination of anisole (14)), changes in physical properties (i.e., increasing or decreasing solubilities of the compounds), or alteration of intermolecular forces (i.e., hydrogen bonds, which are thought to be responsible for adsorption in chromatographic systems).

B. Prior Chromatographic Uses of Cyclodextrins

α- cyclodextrin has been used successfully as a spray reagent for detecting fatty alcohols, acids, esters, and monoglycerides on paper chromatograms (15). Utilizing α- cyclodextrin with iodine will reveal the presence of monoglycerides as white spots, whereas the background is violet due to the α- cyclodextrin-iodine complex. The substitution of iodine by solute molecules is responsible for disappearance of the violet color in the solute zone. The same method has been used by Green and Stumpf (16) to demonstrate the inhibition by cyclodextrins on the action of potato phosphorylase. With paper chromatograms containing inhibiting saccharides, the solute zones are revealed as white spots against a light blue background after spraying the chromatograms with a mixture of phosphorylase and α-D-glucopyranosyl phosphate, and diluted iodine solution, successively.

Hoffman (17) has reported the column chromatographic separations of nucleic acid components on β- cyclodextrin gels. The interaction between nucleic acid derivatives and the gels is the same as that for cyclodextrin molecules in solution. The gels have also been found useful in the separation of vitamins, perfumes, and aromatic amino acids. Owing to their heat-stability, acylated cyclodextrins have been found suitable as stationary phases in comparative

G.L.C. (18). β-cyclodextrin acetate, propionate, butyrate and valerate have been used as stationary phases for gas-liquid chromatography of α-olefins, alcohols, aldehydes, esters, aldehyde-esters, and diesters, and it was found that β-cyclodextrin valerate is the best for separation and resolution of homologous fatty esters.

C. Plan of Study

As previously described, the cyclodextrin is capable of being a "host" for the "guest" molecule, thus forming an inclusion compound or clathrate. The interaction is presumed to involve non-covalent bond formation between the "host" and the "guest". The deciding factor in this unusual complexing formation is the steric compatibility of the guest molecule with the cavity inside the cyclodextrin. The mode of interaction of cycloheptaamylose with barbiturates has been studied by means of the P.M.R. (12) method. The formation constant for phenobarbital is the highest, relative to aliphatic side chain barbitals, because of the perfect fit of the phenyl ring into the cycloheptaamylose cavity. The models constructed (19) of p-t-butylphenyl acetate and m-t-butyl phenyl acetate have revealed the position of the phenyl ring in the cavity of the cyclodextrins as accomodating its plane parallel to the axis of the cavity.

The inclusion feature of cyclodextrins has induced some remarkable changes in physico-chemical properties; for example, it was found that the solubilities of organic acids, when associated with α- and β- cyclodextrins, increase, even for acids which are not prone to form such complexes. The less soluble the acid is in water, the greater is its percent increase in solubility. The selectivity control of cyclodextrin has been demonstrated clearly as in the case of chlorination of anisole (14,20). The cyclodextrin- OH groups are first altered to -OCl by reaction of HOCl. The ortho position of an anisole molecule within a cyclodextrin mixed complex is blocked, leaving the para position free to be attacked by the chlorine atom which has been delivered from the cyclodextrin-OCl groups. Also an increase in fluorescence can be observed (11) when A.N.S. (1-anilino-8- naphthalene sulfonate), which has a character of exhibiting fluorescence in organic solvents, forms an inclusion complex with cyclodextrins. Because of the larger cavity of β- cyclodextrins, the naphthalene sulfonate groups can be enclosed, thus giving a larger increase in fluorescence, as relative to the smaller cavity of α-cyclodextrins in which only the aniline group can be included.

As yet, few applications of cyclodextrins in the analytical field have been made despite their interesting

properties of forming an inclusion complex with the guest molecule. It has been suggested (22) that the cyclic dextrins might be employed as stationary supports by coupling with inert and insoluble materials such as cross-linked dextrans or cellulose. Modified cyclodextrins (i.e., acylated cyclodextrins, cyclodextrin gels), as well as pure cyclodextrins, have been exploited successfully as analytical agents, as outlined above, (i.e., a spray reagent in paper chromatography, stationary supports in column chromatography, or in comparative G.L.C.)

The utilization of cyclodextrins as stationary phases in T.L.C. is the aim of this work. According to Connors (23), a lack of correlation between R_{fx} and R_{fy} values in a plot of the R_{fx} values against the R_{fy} values, when R_{fx} is the R_f value of a compound in system X and R_{fy} is the R_f value of the compound in system Y, will give the maximum information for the qualitative analysis of the compound. Therefore, a goal in selecting chromatographic systems is based on the degree of independence of R_f values between any two systems. In order to achieve uncorrelated sets of data, system X and system Y should be selected as much different as possible. The difference between the two systems might be obtained by either varying the stationary phases or the developing solvents used.

The stationary phases which have been taken into consideration in this study are silica gel G, α-cyclodextrin, β-cyclodextrin, and mixtures of α- and β-cyclodextrins in various proportions. As we have seen, an inclusion within the cyclodextrin cavity will alter the physico-chemical properties of the compounds (i.e., solubilities, rate of diffusion, and extent of adsorption). The migration rates of the molecules occluded within the cyclodextrin molecules might be retarded, thus resulting in lower R_f values. Moreover, since inclusion is a fairly specific process, the R_f values will perhaps be influenced in a selective way dependent upon complex formation. On the basis of this prediction, we hope that using cyclodextrins as stationary phases will help in identifying unknown compounds by the T.L.C. method, and may lead to unusual selectivity effects.

In this work, the R_f values of series of compounds are plotted between two appropriate systems. These systems are selected to be different in either their developing solvent or stationary phases employed or in both. The dissimilarity between the two systems can be generalized into three cases: (1) the stationary phases are the same but the developing solvents are different; (2) the stationary phases are different but the developing solvent are the same; (3) the stationary phases and the developing solvents are both different. The various plots obtained then permit

choice of the systems giving the greatest randomness in the distribution of points, thus rendering the most satisfactory way the identification of the compounds by means of the T.L.C. method. The selectivity induced by cyclodextrin stationary phases also permit certain separations to be easily made. The advantages and the disadvantages of utilizing cyclodextrins as stationary phases in T.L.C. will be discussed in further detail in the discussion section. It was hoped that this work might be one of the useful applications of the cyclodextrins in the analytical field.

II. Experimental

A. Materials Used

Silica Gel G (Mallinckrodt) was analytical grade and was used directly.

α-cyclodextrin tetrahydrate (Aldrich, Lots #011747, 091147) was used directly. α-cyclodextrin from the Pierce Chemical Co. and Eastman Co. was used in some experiments. β-cyclodextrin (Nutritional Biochemicals Corporation, Lots #2390,5022) was used directly. Also β-cyclodextrin from the Aldrich Co. was used in some parts of the experiments.

L-leucine, DL-serine, L-cystine, L-aspartic acid were all from the Aldrich Chemical Co. DL-treonine, Hydroxy-L-proline, L-proline, DL-tryptophan, L-cysteine mono hydrochloride, DL-methionine, DL-phenylalanine, L-tyrosine, DL-lysine mono hydrochloride, and DL-arginine mono hydrochloride were obtained from the Nutritional Biochemicals Corp. and were used directly. DL-alanine, L-glutamic acid (both from Eastman), glycine (Mallinckrodt), DL-valine, histidine (both from the Matheson Coleman and Bell Co.) were used as received.

Ninhydrin (Aldrich), mercuric oxide (Merck), diphenyl carbazone (Aldrich), sodium nitrite (Mallinckrodt), N-(1-napthyl) ethylenediamine dihydrochloride (Eastman) were reagent grade and were used directly.

Sulfanilamide N.F., sulfaguanidine (Mann Research Laboratories), sulfathiazole (Merck), and sulfisoxazole (West-Ward Inc.) were used as received.

Phenobarbital U.S.P. (Mallinckrodt), cyclopentenyl allyl barbituric acid, mephobarbital U.S.P., pentobarbital, amobarbital U.S.P., diallyl barbituric acid, butabarbital, secobarbital U.S.P., hexobarbital, aprobarbital N.F. (all from Z. D. Gilman, Inc.) were used directly. Barbital (Z. D. Gilman, Inc.) was recrystallized twice from distilled water; m.p. 188-190° (lit: 190°-(24))

2-Butanone, ethanol (both from Mallinckrodt), ammonium hydroxide, benzene (both from Baker Analyzed Reagent), concentrated hydrochloric acid, concentrated sulfuric acid, mineral oil, and glacial acetic acid (all from E. I. Du Pont Co.), were reagent grade. p-Dioxane (Aldrich), chloroform, n-butanol, ethyl acetate, acetone, and methanol (all from Mallinckrodt) were analytical grade reagents. Commercial hexane was distilled at atmospheric pressure; b.p. 60-68° (lit: 68.95° - (25))

All water used was deionized distilled water.

Apparatus

1. Coplin Jar (vertical slide staining jar) from A. H. Thomas Co. for developing microscope slides (25 mmx75mm)

2. Rectangular glass developing tank (4x12x10") with glass lid for developing T.L.C. plates (9x9" or 20x20 cms) or smaller.

3. Chromatosprayer (Scientific Manufacturing Industries) consists of replaceable container of freon propellant, removable 120 ml glass reagent bottle, and plastic atomizer head.

4. 10 microliter capacity Hamilton Microliter Syringe (Hamilton Co.) was used for applying the sample.

5. Chromato-Vue® (Ultra-Violet Products, Inc.) was used for viewing fluorescent chromatograms.

B. Procedures

1. Preparation of the layers

A layer of slurry was prepared according to Lees and De Muria (26). Thoroughly cleaned microscope slides (25x75mm) were taped with masking tape, in sets of four, end to end on a flat surface. A slurry of α- or β-cyclodextrin was prepared by triturating 5 g of cyclodextrin and 8 ml of water in a mortar. A suspension consisting of 3 g silica in 6 ml of water was used in preparing silica layers.

α-/β- cyclodextrin mixtures were prepared by the following procedure: the specified amounts of cyclodextrins (Table I), throughly blended while dry, were mixed with a given amount of water (also Table I) by a mechanical stirrer for 30 minutes and the mixture was then triturated until a smooth slurry was obtained.

Table I: Amounts of cyclodextrins and water used for preparation of α-/β- cyclodextrin mixture layers

Cyclodextrins (g)		α-:β- cyclodextrin ratio (w/w)	water added (ml)
α- cyclodextrin*	β- cyclodextrin		
1.61	1.5	1:1	5
1.07	2	1:2	5
0.75	2.1	1:3	4.5
0.54	2.5	1:5	5
2.15	1	2:1	5
2.26	0.7	3:1	4.5
2.69	0.5	5:1	5
3.00	0.4	7:1	5
2.89	0.3	9:1	5

*α- cyclodextrin tetrahydrate (Aldrich) was used and it was converted to α- cyclodextrin by a factor of 1.07 on molecular weight basis

The smooth slurry obtained was poured on the first slide of the set and smoothed by drawing a glass rod over the plates in a direction parallel with the taped edges. The tape supports the rod and determines the layer thickness. The cyclodextrin layers were dried at room temperature for 24 hr. The silica layers were dried at 100^{o} for one hour.

2. Application of the sample

The 10 microliter Hamilton Microliter Syringe was used to apply the sample to bound layers. The sample was spotted 1-2 cm. from the end of the plate so that the solvent level will be at least ½ cm. below the center of the spot. The diameter of the spot should not exceed 3 mm. Table II shows the selected application solvents and the amounts used in application of barbiturates, amino acids, and sulfa drugs. The sample sizes are approximately 5-10 μg (microgram).

Table II: Approximate amounts of sample to be applied to thin layers

Compound	Application solvent	Sample solution conc. (mg/ml)	Volume of sample applied (μl)
Barbiturates	$CHCl_3$	1	15-30
Amino acids	0.1NHCl	1	10-15
Sulfa drugs	acetone	1	6-8

3. Detection of the spots

Numerous reagents have been tabulated in the relevant literatures (27,28,29,30,31,32). The detection of amino acids on silica gel layers was carried out as follows according to Brenner et al (33). The carefully dried chromatogram was sprayed with the ninhydrin solution (0.3g ninhydrin+ 100 ml n-butanol + 3ml glacial acetic acid) and kept for 30 min. at 60° c or 10 min. at 110° c; for cyclo-

dextrin layers, the plate was heated to 40-50° c for 10 min. The amino acids appear as dots or spots and are immediately marked with a sharp pencil.

Mercuric Sulfate- diphenylcarbazone reagent was employed in detecting barbiturates compounds and is described below:

Solution a: Mercuric Sulfate: 5g mercuric oxide dissolved in 100 ml water + 20 ml conc. sulfuric acid (stock solution)

Solution b: water

Solution c: 0.1% diphenylcarbazone in ethanol

Equal volumes of solution a and b are mixed and sprayed on the dried plate. The white spots formed are marked, and the plate is then sprayed with solution c. Barbiturates give a bluish-violet color.

Another method is to examine the dried plates under U.V. light (254 and 360 nm) for fluorescent spots.

The detection of sulfonamide drugs was carried out via diazotisation and coupling with N-(1- naphthyl) ethylene diamine. The dried chromatogram was sprayed with 1% $NaNO_2$ in 1 N HCl, followed by 0.1% N- (1- naphthyl) ethylenediamine in ethanol. Sulphonamides were detected as reddish-purple spots. Also detection can be made by observing under U.V. light (254nm).

Results

I. Amino Acids

After having tried some fifteen solvents, the following two gave the best results:

(1) n- butanol:2-butanone:17% ammonia (5:3:1)

(2) n- butanol:glacial acetic acid: water (4:1:1)

Table III shows the R_f values of amino acids on silica gel layers. The migration rates of the amino acids (i.e. histidine, DL-lysine, and DL-arginine) are slower in solvent number 1 than in solvent number 2.

The R_f values of amino acids on α- and β-cylodextrin layers are compiled in Table IV For the chromatography of the amino acids on α-cylodextrin layers, the results obtained are not satisfactory, since most of the amino acids remain at the start. The R_f values of 18 amino acids are higher on β-cyclodextrin layers than on silica gel layers in the same solvents. It can be seen from Tables III and IV that L-leucine and DL-lysine are best separated on β-cyclodextrin layers in solvent number 1, but DL-tryptophan and DL-lysine are separated well on silica gel layers in solvent number 2.

As mentioned earlier, a lack of dependence between any two sets of data is the aim in selecting the chromatographic systems As illustrated in Fig. 1, the scattering of points increases somewhat when the R_f values of amino acids on β-cyclodextrin layers in solvent number 1 are

plotted against the R_f values of acids on silica gel layers in solvent number 2. Therefore, the resolution of the amino acids, provided by the systems in fig. 1, is expected to be the best.

Fig. 2 and 3 represent the cases where the stationary phases are the same but the developing solvents are different, and vice versa, respectively. The separations of pairs of amino acids and mixtures of 3 amino acids have been performed on β-cyclodextrin layers in solvent number 1. The corresponding R_f values are compiled in Tables V and VI. The R_f values obtained in Table V and VI are in good agreement with the R_f values in Table IV for each compound. Table VII contains the R_f values of the amino acids obtained on α-/β-cyclodextrin mixtures. The plots of the R_f values of 3 amino acids (i.e. L- leucine, DL- threonine, and L-proline) in solvent number 1 as functions of the amounts of α- cyclodextrins used in α-/β- cyclodextrin layers are shown in Fig. 4. It is clearly evident from Fig. 4 that L-leucine and DL-leucine are separated best on (7:1) α-/β- cyclodextrin layers with solvent number 1, whereas L-leucine and L-proline are separated well on (1:3) α-/β- cyclodextrin layers. As experimented on (1:1) α-/β- cyclodextrin layers, a separation between L- leucine and DL- threonine can be accomplished, though the difference in their R_f values is not as much as that obtained from their chromatography on (7:1) α-/β-cyclodextrin layers.

Table III: R_f values of amino acids on silica gel layers (Ascending technique; development distance: 6cm)
Detection: Ninhydrin reagent

amino acid	[a] R_f in solvent number [b]		Width of spots(cm)	
	1	2	1	2
Neutral aliphatic amino acids				
L-leucine	0.31	0.75	0.8	0.5
Glycine	0.04	0.44	1	0.8
DL-alanine	0.08	0.57	0.9	0.8
DL-threonine	0.05	0.48	0.8	0.8
DL-valine	0.08	0.67	0.7	0.6
Isoleucine	0.09	0.72	0.6	0.6
DL-serine	0.07	0.37	0.5	0.5
Neutral heterocyclic amino acids				
Hydroxy-L-proline	0	0.28	-	0.6
L-proline	0	0.26	-	0.6
DL-tryptophan	0.56	0.81	0.7	0.5
Neutral thioaliphatic amino acids				
L-cystine	-	0.11	-	1.1
DL-methionine	0.16	0.19	0.6	0.6
L-cysteine	-	0.70	-	0.7
Neutral aromatic amino acids				
DL-phenylalanine	0.32	0.81	0.4	0.8
L-tyrosine	0.29	0.76	0.6	0.4
Acidic amino acids				
L-aspartic acid	-	0.59	-	0.6
L-glutamic acid	-	0.61	-	0.8
Basic amino acids				
Histidine	0.24	0.11	0.6	0.6
DL-lysine	-	0.09	-	0.7
DL-arginine	-	0.19	-	0.7

a The R_f values reported here are the average values from two determinations.

b solvent number 1= n-butanol:2-butanone:17% ammonia (5:3:1)
solvent number 2= n-butanol:glacial acetic acid:water (4:1:1)

Table IV: R_f values of amino acids on cyclodextrins layers (Ascending technique; development distance: 6 cm.)
Detection: Ninhydrin reagent

amino acid	β-cyclodextrin layers				α-cyclodextrin layers	
	R_f in solvent number[a][b]		Width of spots (cm)		R_f in solvent number	Width of Spots (cm)
	1	2	1	2	1	1
Neutral aliphatic amino acids						
L-leucine	0.76	0.92	0.7	0.7	0.09	0.5
Glycine	0.23	0.73	0.5	0.5	0.08	0.5
DL-alanine	0.49	0.88	0.9	0.7	0.04	0.4
DL-threonine	0.74	0.88	0.7	0.6	0.04	0.3
DL-valine	0.65	0.89	0.6	0.8	0.04	0.4
Isoleucine	0.65	0.92	0.6	0.6	0.07	0.5
DL-serine	0.40	0.80	0.6	0.6	0.05	0.4
Neutral heterocyclic amino acids						
Hydroxy-L-proline	0.25	0.89	0.4	0.5	0.04	0.3
L-proline	0.65	0.91	0.8	0.6	0.07	0.3
DL-tryptophan	0.71	0.91	0.9	0.3	0.05	0.3
Neutral thioaliphatic amino acids						
L-cystine	-	-	-	-	0.07	0.4
DL-methionine	0.70	0.92	0.4	0.6	0.04	0.4
L-cysteine	-	-	-	-	0.04	0.5
Neutral aromatic amino acids						
DL-phenylalanine	0.75	0.93	0.8	0.6	0.04	0.5
L-tyrosine	0.59	0.93	0.4	0.7	0.06	0.6
Acidic amino acids						
L-aspartic acid	-	0.68	-	0.6	0.04	0.4
L-glutamic acid	-	0.86	-	0.4	0.04	0.4
Basic amino acids						
Histidine	0.72	0.94	0.8	0.6	0.06	0.5
DL-lysine	0.13	0.65	0.5	0.4	0.10	0.6
DL-arginine	0.22	0.78	0.4	0.7	0.07	0.5

[a] The R_f values reported here are the average values from two determinations.

[b] solvent number 1 n-butanol:2-butanone:17% ammonia (5:3:1)
solvent number 2 n-butanol:glacial acetic acid (4:1:1)

Fig. 1 - A plot of the R_{fx} values of amino acids on silica gel layers in solvent number 2 *versus* the R_{fy} values of amino acids on β-cyclodextrin layers in solvent number 1 (data given in Tables III and IV)

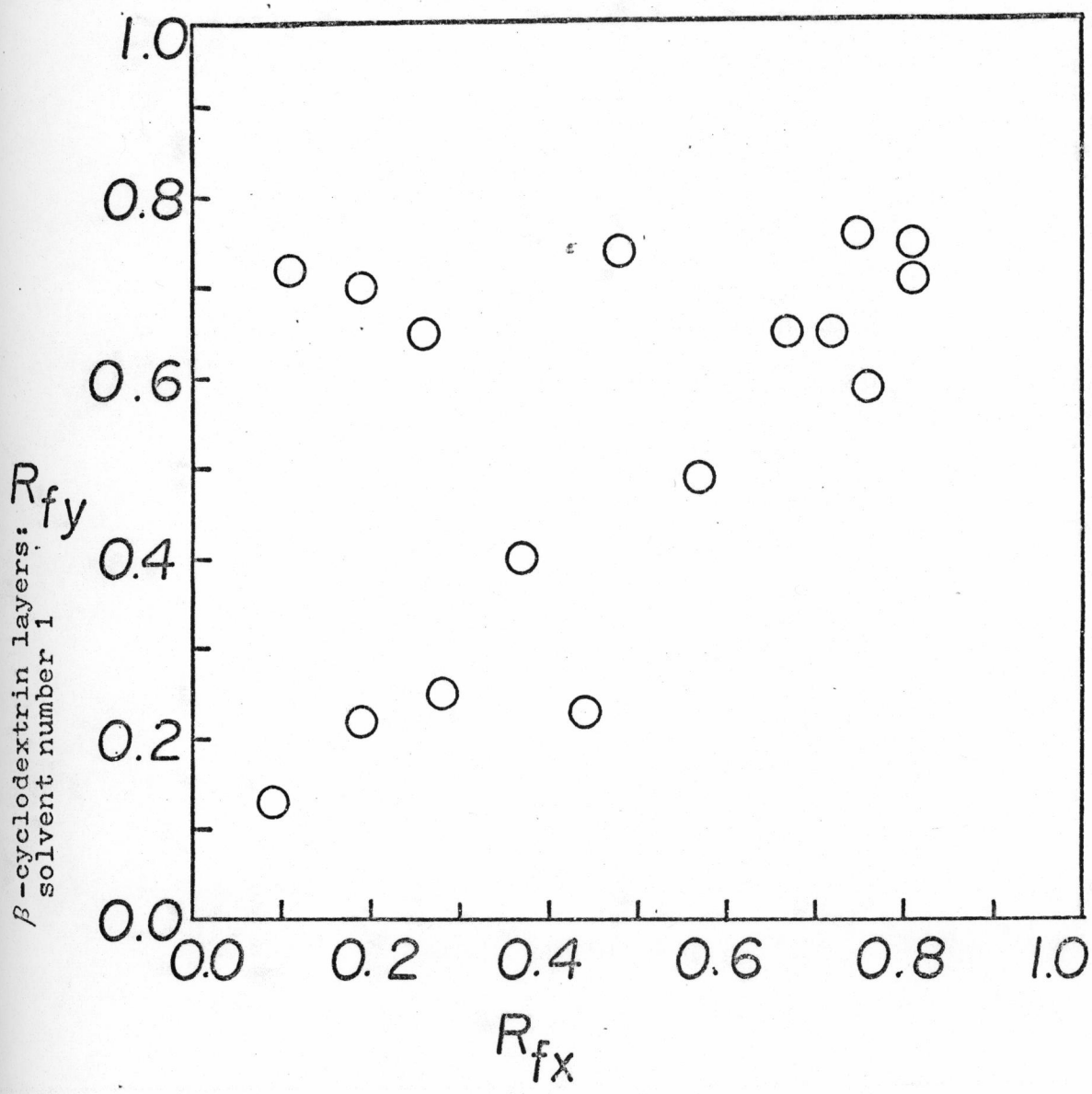

1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
R_{fy}
R_{fx}
β-cyclodextrin layers: solvent number 1
S.G. layers: solvent number 2

Fig. 2 - A plot of the R_{fy} values of amino acids on silica gel layers in solvent number 1 and in solvent number 2 (R_{fx}) (data given in Table III)

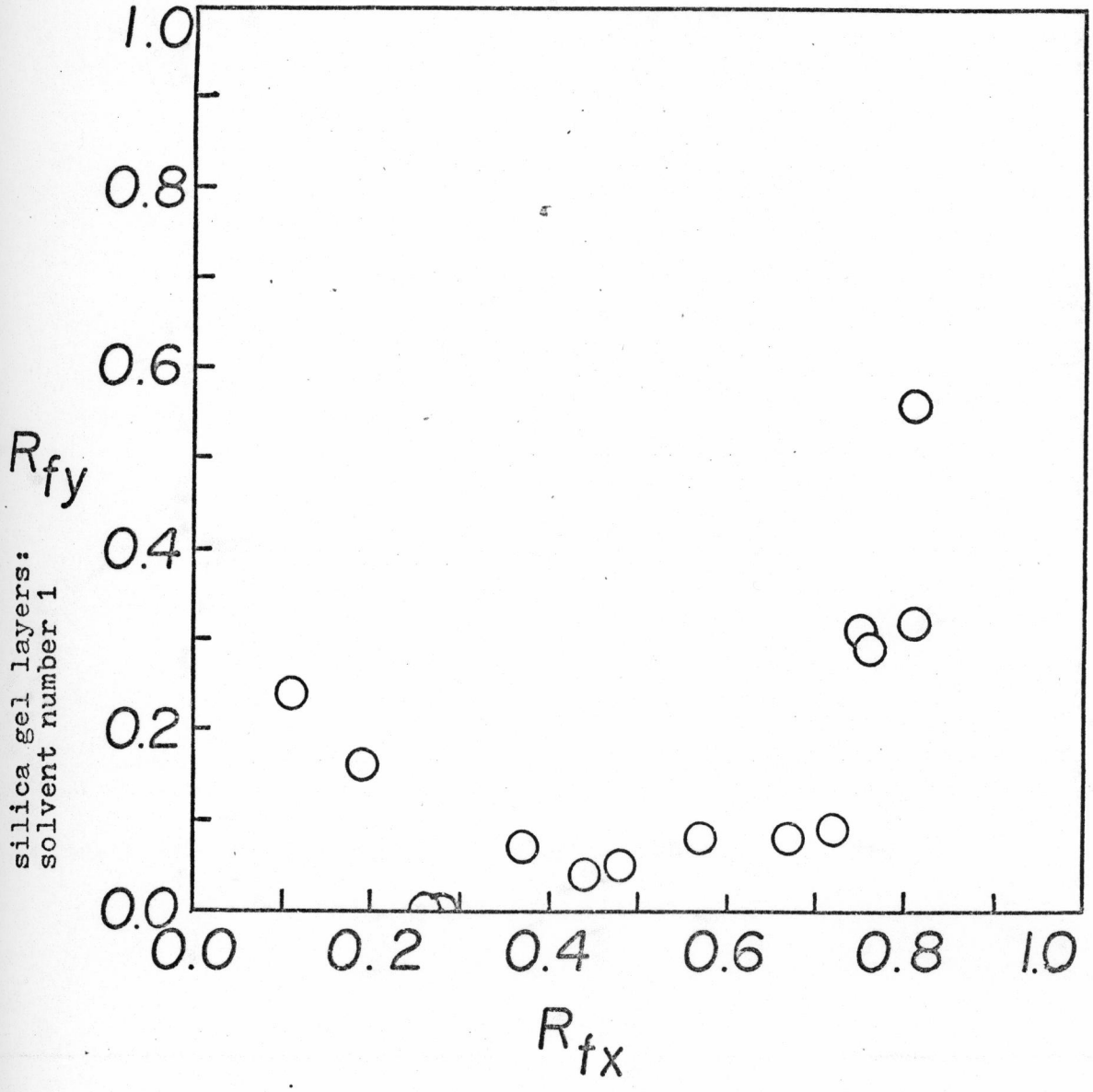

1.0
0.8
0.6
0.4
0.2
0.0
R_{fy}
silica gel layers:
solvent number 1
0.0
0.2
0.4
0.6
0.8
1.0
R_{fx}
silica gel layers:
solvent number 2

Fig 3 - A plot of the R_{fx} values of amino acids on silica gel layers in solvent number 2 against the R_{fy} values of acids on β-cyclodextrin layers in the same solvent (data given in Tables III and IV)

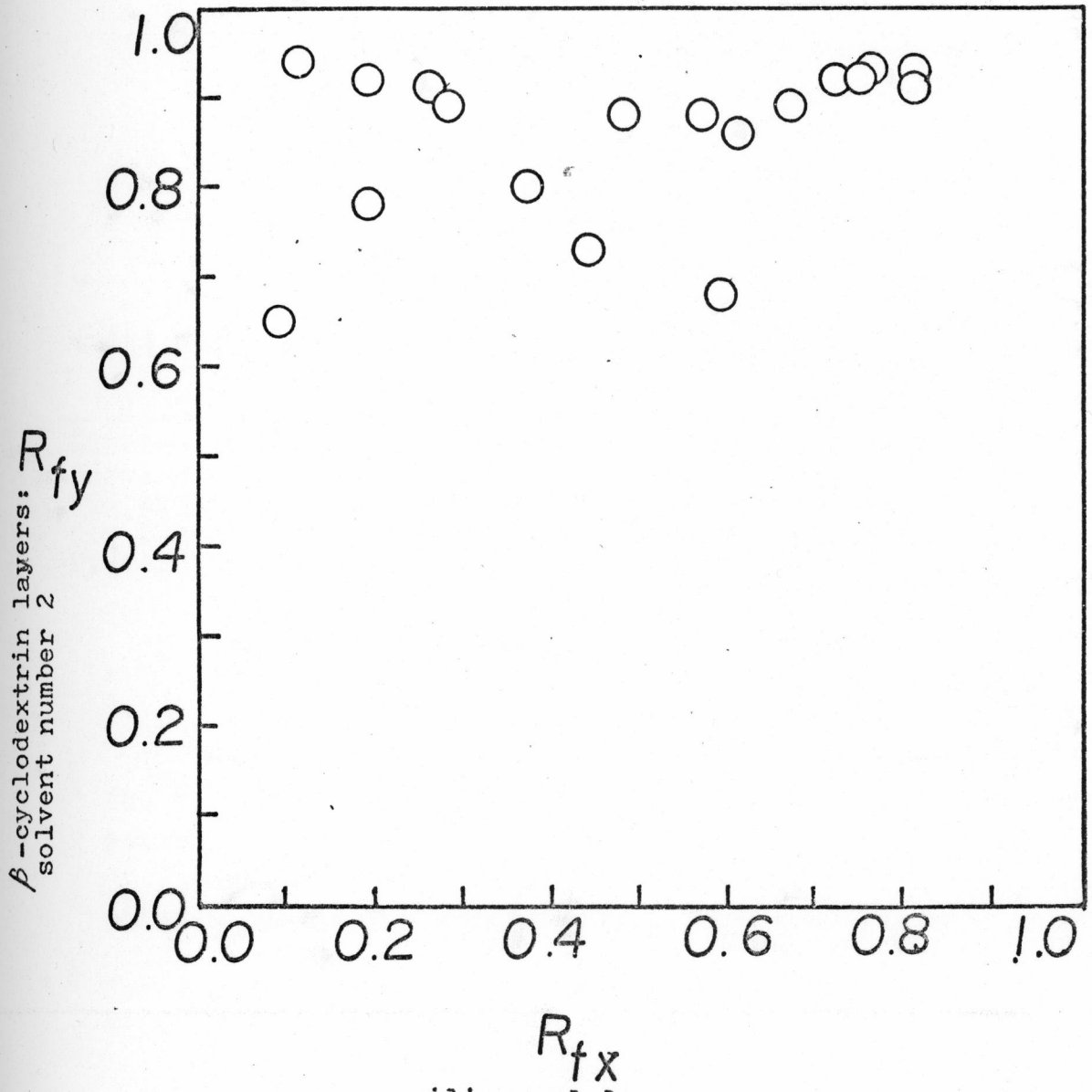

1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
R_{fy}
β-cyclodextrin layers:
solvent number 2
R_{fx}
silica gel layers:
solvent number 2

Table V - R_f values of amino acids in pairs of amino acids on β-cyclodextrin layers (Ascending technique; development distance:14 cm)
Detection: Ninhydrin reagent
Development time: 2.25 hrs.

amino acids	[a] R_f in solvent number [b] 1		Width of spots(cm) 1	
	spot[c] 1	spot 2	spot 1	spot 2
Glycine & DL- phenylalanine	0.38	0.79	0.6	0.7
Glycine & L-tyrosine	0.38	0.70	0.5	0.6
DL- arginine & DL- methionine	0.12	0.64	0.4	0.5
DL- lysine & DL- trytophan	0.09	0.66	0.5	0.7
Hydroxy-L- proline & L-leucine	0.06	0.70	0.4	0.6

[a] The R_f values given here are the average values from two determinations

[b] solvent number 1 = n-butanol:2-butanone:17% ammonia (5:3:1)

[c] The spots are numbered according to the order of amino acids written in each pair of acids

Table VI - R_f values of amino acids in mixtures of 3 amino acids on β-cyclodextrin layers (Ascending technique; development distance: 14 cm.)
Detection: Ninhydrin reagent
Development time: 2.35 hrs.

amino acids	[a] R_f in solvent number [b] 1			Width of spots (cm) 1		
	[c] spot 1	spot 2	spot 3	spot 1	spot 2	spot 3
Glycine & L-tyrosine & L-leucine	0.08	0.34	0.77	0.5	0.6	0.7
DL-lysine & DL-alanine & L-leucine	0.20	0.61	0.93	0.5	0.8	0.6
DL-arginine & DL-alanine & L-leucine	0.21	0.68	0.96	0.5	0.9	0.7
DL-arginine & L-tyrosine & L-leucine	0.27	0.86	0.95	0.5	0.5	0.8
DL-arginine & DL-alanine & DL-phenylalanine	0.24	0.65	0.96	0.5	0.9	0.9

[a] The R_f values given here are the average values from two determinations

[b] solvent number 1 = n-butanol:2-butanone:17% ammonia (5:3:1)

[c] The spots are numbered according to the order of amino acids written in a mixture of amino acids

Table VII - R_f values of amino acids on α-/β-cyclodextrin mixture layers, developing solvent number 1[b]
(Ascending technique: development distance: 6 cm)
Detection: Ninhydrin reagent

amino acid	[a] Rf on α-/β-cyclodextrin mixture layers in the weight ratio of									Width of spots on α-/β-cyclodextrin mixture layers in the weight ratio of								
	1:1	1:2	1:3	1:5	2:1	3:1	5:1	7:1	9:1	1:1	1:2	1:3	1:5	2:1	3:1	5:1	7:1	9:1
L-leucine	0.67	0.69	0.49	0.36	0.62	0.70	0.86	0.93	0.89	0.7	0.7	0.4	0.4	0.9	0.7	0.8	0.9	0.6
DL-threonine	0.49	0.50	0.35	0.30	0.63	0.58	0.76	0.65	0.63	0.5	0.5	0.6	0.6	0.8	0.3	0.4	0.3	0.4
L-proline	0.60	0.56	0.27	0.32	0.60	0.63	0.79	0.91	0.88	0.4	0.5	0.6	0.4	0.6	0.4	0.5	0.8	0.8

[a] The R_f values reported here are the average values from two determinations

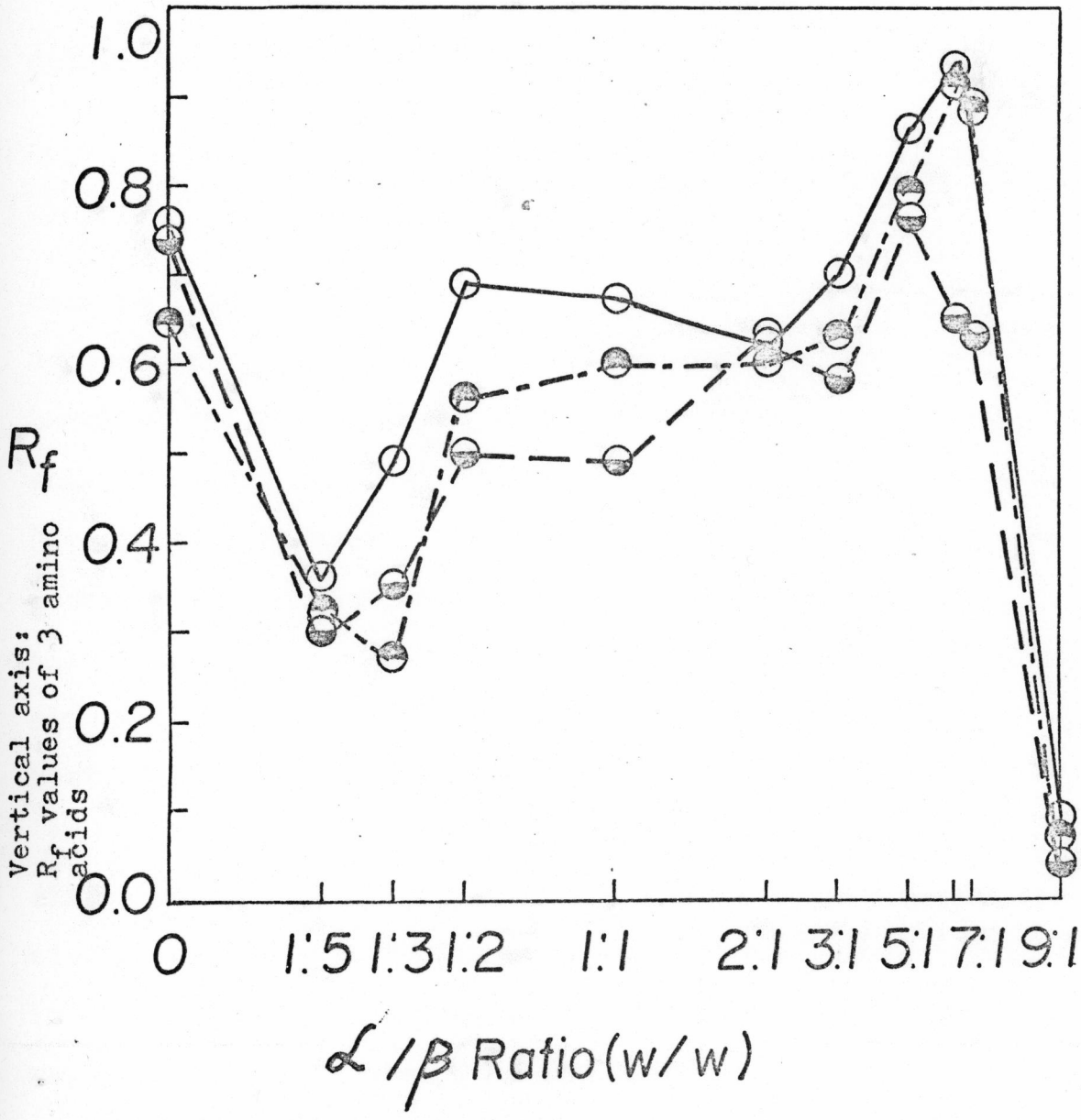
[b] solvent number 1 = n-butanol:2-butanone:17% ammonia (5:3:1)

Fig.4 - R_f values of 3 amino acids against the amounts of α-cyclodextrin employed in α-/β-cyclodextrin layers (data given in Tables IV and VII)

○ L-leucine

◒ DL-threonine

◒ L-proline



Horizontal axis: the amounts of α-cyclodextrin used in α-/β-cyclodextrin mixtures

II. Barbiturates

The following solvents were employed in the chromatography of barbiturates:

1. $CHCl_3$: acetone (9:1)
2. ethyl acetate:hexane:ammonium hydroxide (20:9:10)

The R_f values of 11 barbiturates on α- and β-cyclodextrin layers are compiled in Table VIII. The observation can be made from Table VIII that Hexobarbital and Phenobarbital can be separated on β-cyclodextrin layers with solvent number 2. The resolution of the barbiturates on β-cyclodextrin layers in solvent number 1 and 2 seem to be poor. The migration rate of Hexobarbital, Cyclopentenyl allyl barbituric acid, Pentobarbital, and Secobarbital are almost identical. For the chromatography on α-cyclodextrin, most of the barbiturates remain at the start.

Table IX lists the R_f values of barbiturates on silica gel layers in solvent number 1 and 2. The resolution of the barbiturates is rather poor, however a distinction is still possible. A plot of the R_f values of the barbiturates in solvent number 1 as functions of the R_f values of the barbiturates in solvent number 2, is illustrated in Fig. 5. From this plot, it can be seen that the data points are not well distributed; therefore, the resolution is anticipated to be poor. Fig. 6 presents a plot of the R_f values of the barbiturates on silica gel layers V.S. the R_f values

of the barbiturates on β-cyclodextrin layers, both developed in solvent number 2. The clustering of points, as can be seen from Fig. 6, leads to the unsatisfactory resolution of the barbiturates. Fig. 7 demonstrates a plot of the R_f values of the barbiturates on silica gel layers in solvent number 1 against the R_f values on β-cyclodextrin layers in solvent number 2. Yet the resolution of the barbiturates provided by the systems in Fig. 7 is not quite successfully accomplished.

The separation of the barbiturates is more apt to be achieved on α-/β-cyclodextrin mixture layers. From consideration of the R_f values in Table X, Barbital and Aprobarbital can be separated well on (1:1) α-/β-cyclodextrins stationary phases whereas the separation between Mephobarbital and Barbital can be achieved on (2:1) α-/β-cyclodextrin mixture. In the actual experiment, it is not practical to separate the mixture of the barbiturates in solvent number 2 because of the long developing time and because the solvent fails to run in a long distance thereby preventing the substances chromatographyed to be separated. It should be noted also that the barbiturates chromatographyed on cyclodextrin stationary phases can not be detected by observing under U.V. light.

Table VIII - R_f values of barbiturates on cyclodextrins layers (Ascending technique: development distance:6 cm)

Detection: Mercuric sulfate-diphenylcarbazone reagent

barbiturate	β-cyclodextrin layers				α-cyclodextrin layers	
	R_f[a] in solvent number[b]		Width of spots(cm)		R_f in solvent number	Width of spots(cm)
	1	2	1	2	2	2
Hexobarbital	0.93	0.93	0.6	0.6	0.01	0.9
Phenobarbital	0.91	0.69	0.9	0.7	0.02	0.8
Cyclopentenyl allyl barbituric acid	0.96	0.90	0.6	0.5	0.01	0.8
Mephobarbital	0.91	0.85	0.8	0.6	0.01	0.9
Pentobarbital	0.95	0.95	0.7	0.5	0.20	0.8
Amobarbital	0.89	0.82	0.7	0.9	0.10	0.9
Diallyl barbituric acid	0.94	0.77	0.6	0.8	0.05	0.7
Butabarbital	0.94	0.73	0.7	0.7	0.04	0.5
Barbital	0.96	0.73	0.7	0.7	0.04	0.5
Secobarbital	0.92	0.91	0.7	1.0	0.06	0.6
Aprobarbital	0.90	0.78	0.8	0.9	0.04	0.8

[a] The R_f values reported here are the average values from two determinations

[b] solvent number 1 = $CHCl_3$:acetone (9:1)

solvent number 2 = ethyl acetate:hexane:ammonium hydroxide (20:9:10)

Table IX - R_f values of barbiturates on silica gel layers (Ascending technique; development distance: 6 cm)
Detection: Mercuric sulfate-diphenyl-carbazone

barbiturate	R_f [a] in solvent number [b]		Width of spots(cm)	
	1	2	1	2
Hexobarbital	0.66	0.86	0.7	0.6
Phenobarbital	0.27	0.71	0.5	0.4
Cyclopentenyl allyl barbituric acid	0.37	0.77	0.5	0.3
Mephobarbital	0.61	0.92	0.6	0.4
Pentobarbital	0.44	0.86	0.6	0.5
Amobarbital	0.46	0.85	0.5	0.8
Diallyl barbituric acid	0.35	0.83	0.4	0.2
Butabarbital	0.37	0.84	0.5	0.5
Barbital	0.33	0.73	0.6	0.6
Secobarbital	0.48	0.92	0.6	0.4
Aprobarbital	0.39	0.76	0.6	0.5

[a] The R_f values reported here are the average value from two determinations

[b] solvent number 1 = $CHCl_3$:acetone (9:1)
solvent number 2 = ethyl acetate:hexane:ammonium hydroxide (20:9:10)

Fig. 5 - A plot of the R_{fx} values of barbiturates on silica gel layers in solvent number 1 against the R_{fy} values of barbiturates on silica gel layers in solvent number 2 (data given in Table IX)

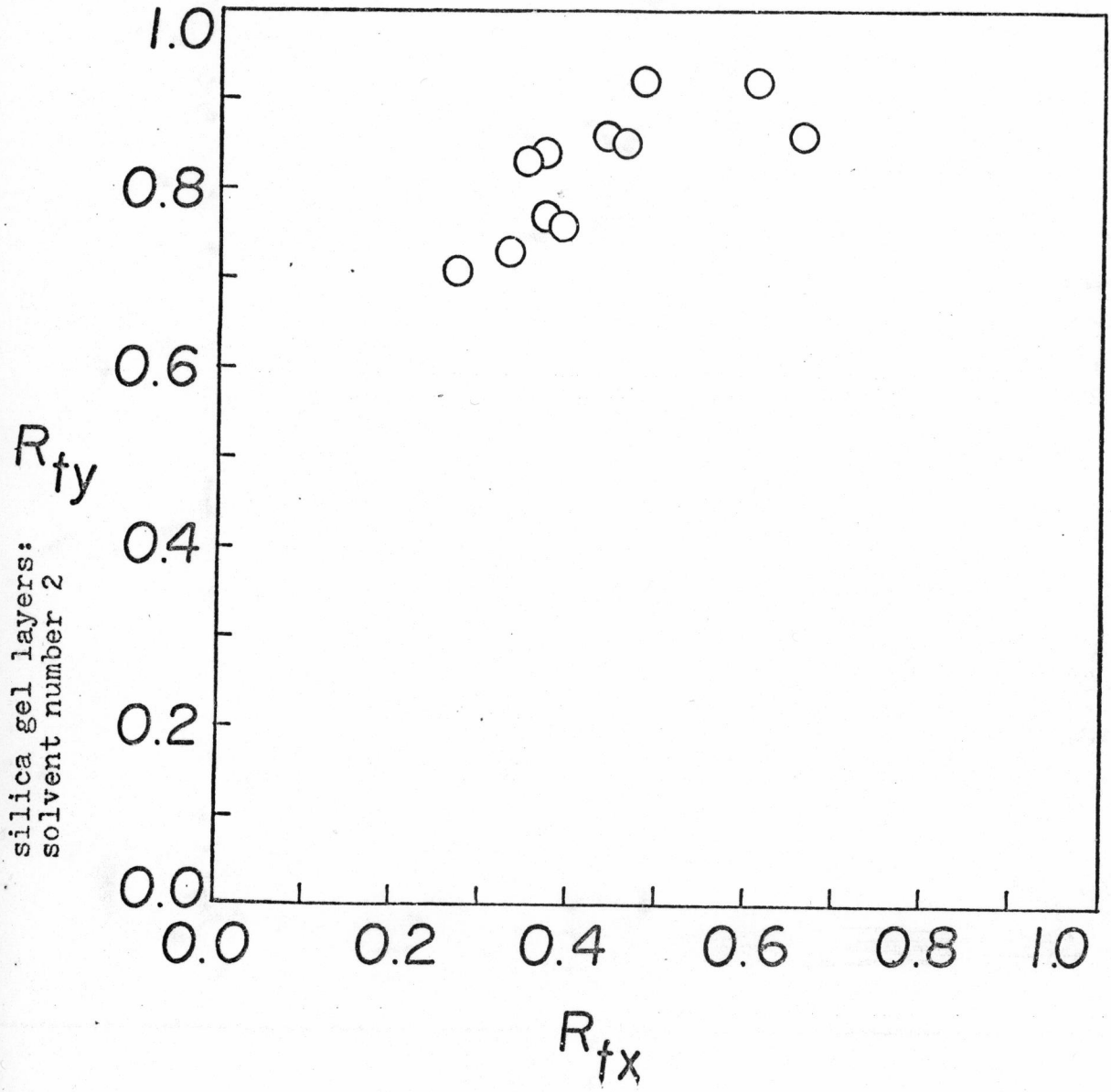

1.0
0.8
0.6
0.4
0.2
0.0
R_{fy}
silica gel layers:
solvent number 2
0.0
0.2
0.4
0.6
0.8
1.0
R_{fx}
silica gel layers:
solvent number 1

Fig. 6 - A plot of the R_{fy} values of barbiturates on β-cyclodextrin in solvent number 2 versus the R_{fx} values of barbiturates in the same solvent (data given in Tables VII and IX)

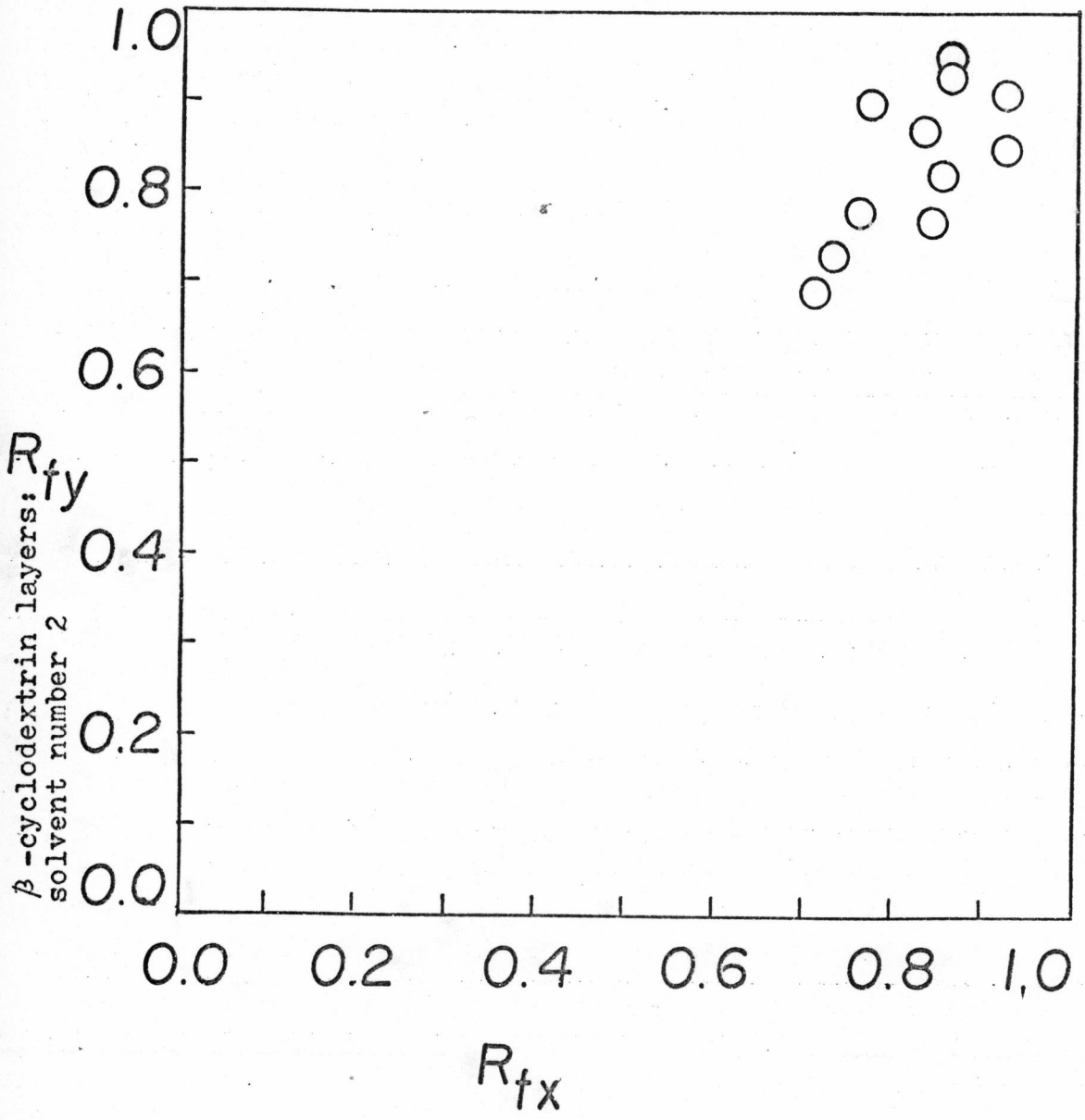

1.0
0.8
0.6
0.4
0.2
0.0
R_{fy}
β-cyclodextrin layers: solvent number 2
0.0
0.2
0.4
0.6
0.8
1.0
R_{fx}
silica gel layers: solvent number 2

Fig. 7 - A plot of the R_{fy} values of barbiturates on silica gel layers in solvent number 1 against the R_{fx} values of barbiturates on β-cyclodextrin layers in solvent number 2 (data given in Tables VIII and IX)

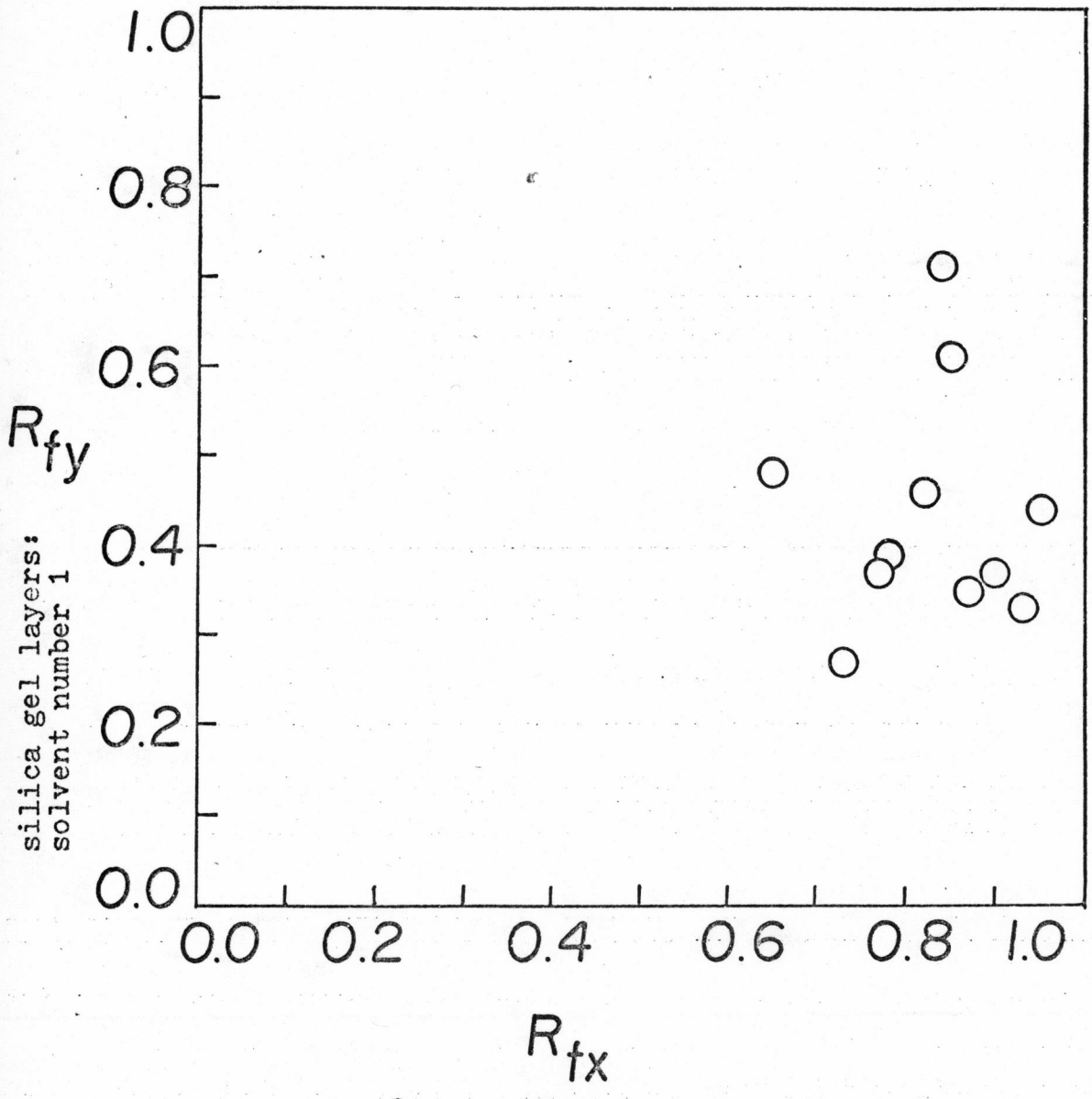

1.0
0.8
0.6
0.4
0.2
0.0
R_{fy}
silica gel layers:
solvent number 1
0.0
0.2
0.4
0.6
0.8
1.0
R_{fx}
β-cyclodextrin: solvent number 2

Table X - R_f values of barbiturates on α-/β-cyclodextrin mixture layers, developing solvent number 2[b]
Detection: Mercuric sulfate-diphenylcarbazone reagent

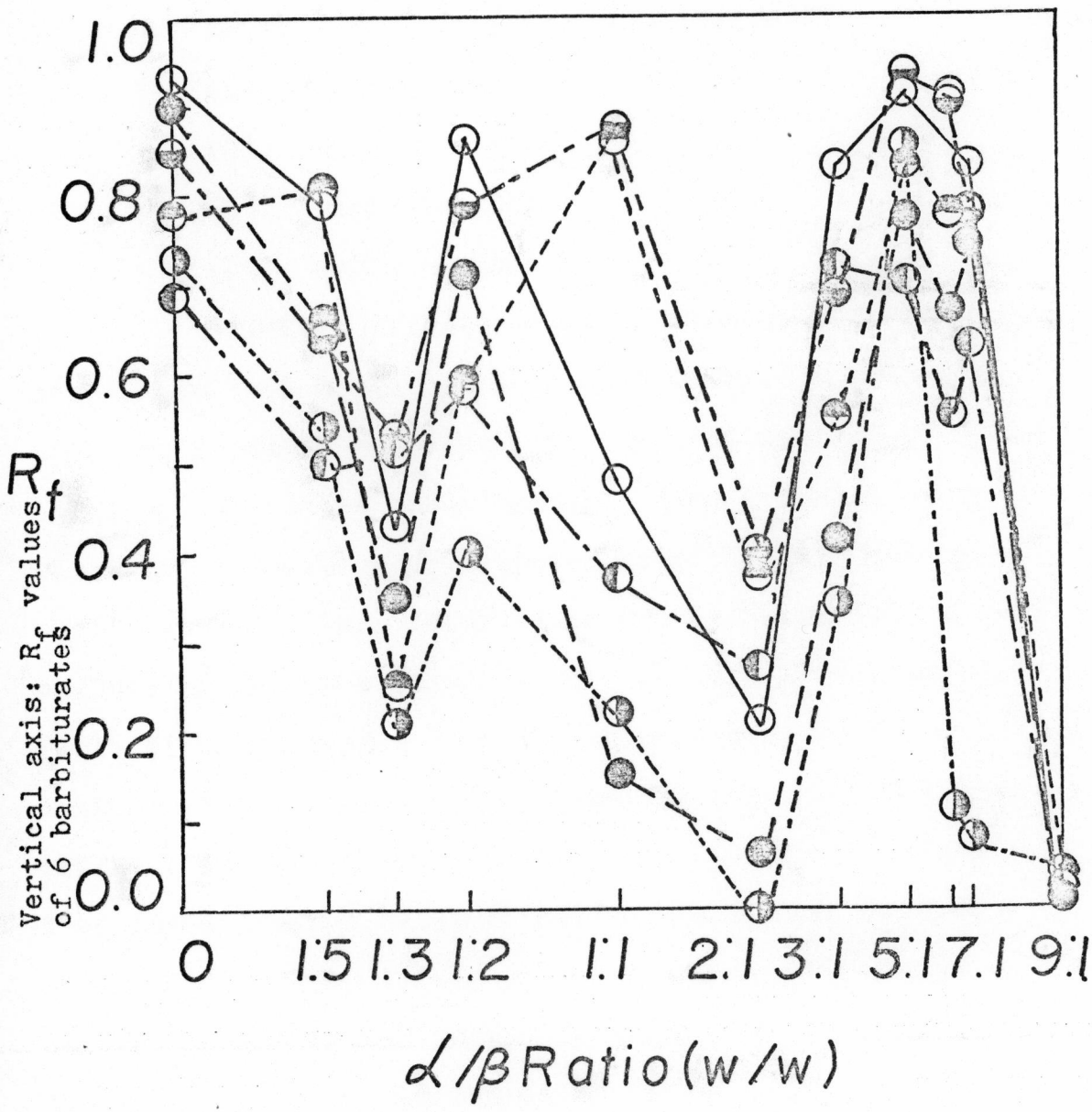
barbiturate	[a] R_f on α-/β-cyclodextrin mixture layers in the weight ratio of									Width of spots on α-/β-cyclodextrin mixture in the weight ratio of								
	1:1	1:2	1:3	1:5	2:1	3:1	5:1	7:1	9:1	1:1	1:2	1:3	1:5	2:1	3:1	5:1	7:1	9:1
Hexobarbital	0.48	0.86	0.43	0.79	0.21	0.83	0.91	0.91	0.83	0.6	0.6	0.4	0.6	0.7	0.4	0.9	0.9	0.6
Phenobarbital	0.37	0.58	0.51	0.50	0.27	0.72	0.70	0.55	0.63	1.0	0.8	0.6	0.6	0.7	0.5	0.8	0.4	0.7
Cyclopentenyl allyl barbituric acid	0.15	0.71	0.35	0.66	0.06	0.41	0.77	0.67	0.74	0.8	0.6	0.6	0.6	0.9	0.5	0.8	0.4	0.5
Mephobarbital	0.87	0.79	0.53	0.64	0.40	0.69	0.93	0.90	0.78	0.7	0.9	0.4	0.6	0.7	0.7	0.8	0.6	0.5
Barbital	0.22	0.40	0.21	0.54	0	0.34	0.85	0.11	0.08	0.8	0.6	0.8	0.6	0.8	0.5	0.8	0.6	0.5
Aprobarbital	0.86	0.59	0.25	0.80	0.37	0.55	0.83	0.78	0.78	0.8	0.6	0.5	0.6	0.7	0.5	0.8	0.5	0.8

[a] The R_f values reported are the average values from two determinations

[b] solvent number 2 = ethyl acetate:hexane:ammonium hydroxide (20:9:10)

Fig. 8 - R_f values of 6 barbiturates against the amounts of α-cyclodextrin employed in α-/β-cyclodextrin layers (data given in Tables VIII and X)

- Hexobarbital
- Phenobarbital
- Cyclopentenyl allyl barbituric acid
- Mephobarbital
- Barbital
- Aprobarbital



Horizontal axis: the amounts of α-cyclodextrin used in α-/ β-cyclodextrin mixtures

III. Sulfa drugs

Sulfa drugs employed are as follows:

(1). sulfanilamide

(2). sulfaguanidine

(3). sulfisoxazole

(4). sulfathiazole

Previous studies on amino acids and barbiturates have shown that utilizing α-/β-cyclodextrin mixtures as stationary phases can result in the good separation of the amino acids and the barbiturates. Hence, the investigation about the chromatographic behaviour of the sulfa drugs on cyclodextrins layers involved mainly α-/β-cyclodextrin mixture layers as stationary phases. The data given in Table XI are obtained from the chromatography of 4 sulfonamides on cyclodextrin layers in solvent $CHCl_3$:methanol (10:1). It can be observed from the R_f values in Table XI that the migration rates of sulfisoxazole and sulfaguanidine differ very little on α-cyclodextrin stationary phases, nevertheless; their distinction is still possible. Sulfaguanidine and sulfathiazole are separated particularly well on β-cyclodextrin layers. Table XII shows the R_f values of 4 sulphonamides on α-/β-cyclodextrin mixture stationary phases in solvent $CHCl_3$:methanol (10:1). The Rf values of the sulfa drugs against the amounts of α-cyclodextrin used in α-/β-cylodextrin mixture layers profile is shown in

Fig. 9. It is clearly evident from the plot that sulfanilanilamide and sulfaguanidine are best separated on (2:1) α-/β-cyclodextrin mixture layers. The R_f value of sulfisoxazole is significantly higher on (9:1) α-/β-cyclodextrin layers, thus making plausible the separation between sulfisoxazole and sulfanilamide.

A binary mixture of sulfathiazole and sulfaguanidine was separated on (2:1) α-/β-cyclodextrin mixture and the R_f values were in accord with the R_f values obtained from chromatographing each compound separately. On (9:1) α-/β-cyclodextrin mixture, sulfisoxazole and sulfanilamide can be separated as predicted from the R_f profile for mixed cyclodextrins. However, the separation of 3 sulphonamides were not accomplished because of the tailings of the spots even though the differences in the R_f values seem to permit the separations.

Table XI - R_f values of sulphonamides on cyclodextrins layers (Ascending technique: development distance: 6cm)

Detection: 1% $NaNO_2$ in 1NHCl-0.1%N-(1-naphthyl) ethylenediamine HCl in ethanol

Solvent: $CHCl_3$:methanol(10:1)

sulphonamide	β-cyclodextrin layers		γ-cyclodextrin layers	
	R_f [a]	Width of spots	R_f	Width of spots
sulfanilamide	0.21	0.9	0.50	0.8
sulfaguanidine	0.13 [b]	0.7	0.45 [b]	0.6
sulfisoxazole	0.60 [b]	0.7	0.60 [b]	0.9
sulfathiazole	0.55 [b]	0.8	0.49 [b]	0.7

[a] The R_f values reported here are the average values from the two determinations

[b] The tailing of spots is observed

Table XII - R_f values of sulfonamides on α-/β-cyclodextrin mixture layers.

Detection: 1% $NaNO_2$ in 1N HCl-0.1% N-(1-napthyl) ethylenediamine HCl in ethanol

Solvent: $CHCl_3$:methanol(10:1)

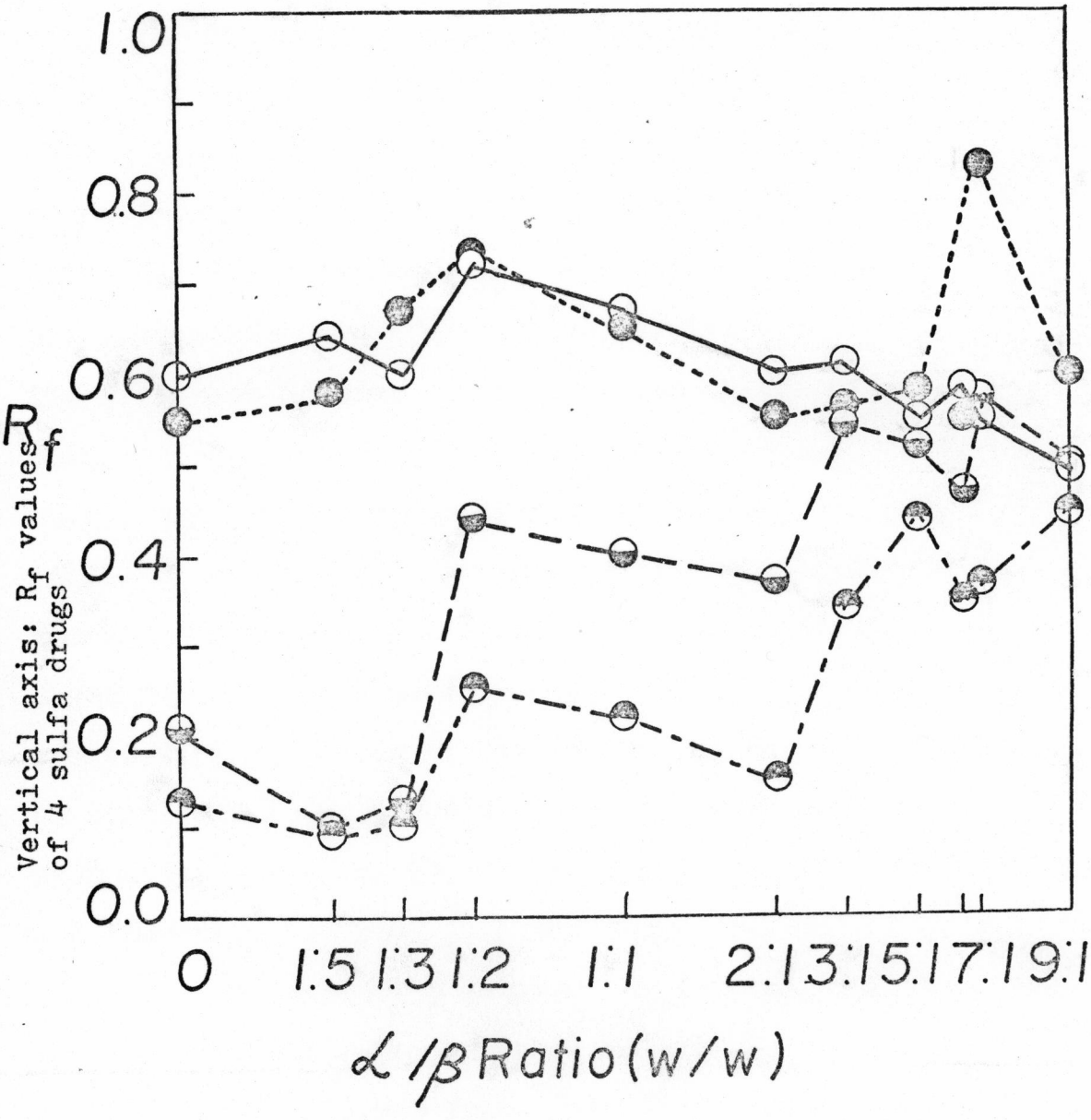
sulphonamide	R_f[a] values on α-/β-cyclodextrin mixture layers in the weight ratio of									Width of spots on α-/β-cyclodextrin in the weight ratio of								
	1:1	1:2	1:3	1:5	2:1	3:1	5:1	7:1	9:1	1:1	1:2	1:3	1:5	2:1	3:1	5:1	7:1	9:1
sulfanilamide	0.40	0.44	0.13	0.10	0.37	0.54	0.52	0.47	0.57	0.5	0.5	0.7	0.6	0.8	1.1	0.9	0.4	0.7
sulfaguanidine	0.22	0.25	0.10	0.09	0.15	0.34	0.44	0.35	0.37	0.5	0.4	0.6	0.6	0.7	0.8	0.8	0.4	0.6
sulfisoxazole[b]	0.65	0.73	0.60	0.58	0.55	0.56	0.58	0.55	0.83	0.6	0.6	0.4	0.5	0.7	0.7	0.6	0.6	0.8
sulfathiazole[b]	0.67	0.72	0.67	0.64	0.60	0.61	0.55	0.58	0.55	0.7	0.7	0.5	0.5	0.6	0.5	0.7	0.5	0.4

[a] The R_f values reported here are the average values from two determinations

[b] The tailing of spots is observed

Fig. 9 - A plot of the R_f values of 4 sulphonamides versus the amounts of α-cyclodextrin used in α-/β-cyclo--dextrin layers (data given in Tables XI and XII)

- sulfanilamide
- sulfaguanidine
- sulfisoxazole
- sulfathiazole



Horizontal axis: the amounts of α-cyclodextrin used in α-/β-cyclodextrin mixtures

Discussion and Conclusion

In general, chromatography serves as a means for the resolution of mixtures and for the isolation and partial description of the separated substances. The separation and identification of substances by chromatographic procedures are based on differences in the physico-chemical properties of substances. As we have known, cyclodextrin, in regard to its inclusion forming properties, can induce some appreciable changes in physico-chemical properties. So it has been speculated that selectivity can be rendered by its effect on the R_f values, thus making certain separations plausible. The chromatography of barbiturates, amino acids, and sulfa drugs has been performed on α-, β- cyclodextrins and α-/β-cyclodextrin mixtures as stationary phases. For the sake of simplicity, the discussion will be focused on the two main areas, that is, the chromatography on pure cyclodextrins and the chromatography on mixed cyclodextrins.

As commonly practised the technique of thin-layer adsorption chromatography is likely to give variable results. In our studies, it was found that the standard deviation of the R_f values lay within the limits 0.01 and 0.05 provided that the cyclodextrin stationary phases employed were from the same batch. For different batches, the standard deviation may vary from 0.07 to 0.09. The factors affecting the reproducibility of the R_f values in T.L.C. have

been investigated by many workers (37-46). Henegger (37) stated that the activity of adsorbent, relying on the varying quality from batch to batch, influences the R_f values the most. The decrease in the capacity of the adsorbent can be seen as paralleling the reduction in activity; therefore, the individual grades must also behave quite differently in chromatography.

The relative humidity at the time of application of the samples and during developing, is also of decisive importance (39,40). The amount of moisture, directly related to its activity, has a very considerable effect on the R_f values since the readily adsorbed water molecule will block the active sites on the molecule. For the same reason, the migration rates are also inevitably affected by impurities present since the active sites of the adsorbent are hindered, thus decreasing the adsorption sites available for the substances chromatographed.

In particular for cyclodextrin stationary phases, for which the adsorptive properties are rendered by the fixed size and shape of the cavity, the adsorptive power will be affected most by any molecule (i.e. impurities, water molecule) within its cavity. In the actual experiment, it was found that cyclodextrins obtained from different companies, even for the same company but different batches, do not behave exactly the same.

Pataki (41) and many workers (42-44) confirmed the variability of R_f values with a variation in the thickness of the layer. Preparation of the layers by the procedure used in this study do not practically provide for uniform layers as would be obtained from the applicator. Nevertheless, in a small scale preparation and for the sake of economy, this method of layer preparation may furnish more or less uniform layers after sufficient practice provided that the reproducibility is not desired to be as good as that obtained from the applicator method. Other factors accounting for the deviation in the results are the degree of saturation or lack of saturation of the developing chamber atmosphere with the solvent vapors (37, 38, 45, 46) and the development distance together with the distance of the starting point from the surface of the solvent.

In the light of our studies, regardless of the latter factors, the factors encountered here dealt with are the activity of the cyclodextrins and the uniformity of the layers. As long as there is a careful preparation of the layer so as to have uniform thickness and control of the activity of the layer by standardized drying conditions and handling of the plate, good reproducibility of the R_f values will thus be obtained.

It is noticeable from the present data that the migration rates exhibited by the compounds under the same conditions are higher on β-cyclodextrin layers than on α-cyclodextrin layers. Several explanations can be derived from this result. The most plausible one is that the compound fits more tightly into the α-cyclodextrin cavity than the β-cyclodextrin cavity, which is apparently due to the smaller cavity of α-cyclodextrin. Consequently, the complexes formed by α-cyclodextrin, as relatively to that formed by β-cyclodextrin, are more stable since the forces holding the molecule within the smaller α-cyclodextrin cavity are stronger.

Postulations are made such that only the uncomplexed molecules are transported by the solvent and the adsorbed substances are bound to the surface of the cyclodextrins by van der waal forces or by hydrogen bonding formation. In extreme cases, the interaction can possibly lead to the alteration of the physico-chemical properties of the adsorbed molecule. As studied by Lach and Jack (8, 9), the complex formed between cyclodextrins and the organic compound either by inclusion or association, especially for the non-polar compound, increases its solubility in water as a function of cyclodextrin concentration. It may be said that the cyclodextrin can induce the non-polar molecule to become more polar by complexation process.

From the most general viewpoint, it can be said that compounds not exhibiting appreciable affinity for cyclodextrins will move relatively unretarded, whereas those recognizing the cyclodextrins will be retarded in proportion to the affinity existing under the conditions employed. The steric compatibility is claimed to be one of the factors in determining the degree of affinity occuring between the cyclodextrins and the compounds. Hence, the unusual low R_f values shown in the present data can presumably resulted from the greater affinity of the compound for the cyclodextrin than for the solvent, based on the assumption that only the uncomplexed form can be carried by the solvent.

For the chromatography on mixed cyclodextrins, it was shown that the separation of barbiturates, amino acids, and sulfa drugs can be accomplished on α-/β-cyclodextrin layers at some proportions. In the light of the reproducibility of R_f values, besides the factors accounting for the variability of the results on pure cyclodextrins, as previously described, the homogenity of mixing the cyclodextrins also has a pronounced effect on the R_f values. It was found that the standard deviation are within the limits of 0.04 to 0.08 for the chromatography on 1:5, 7:1 and 9:1 α-/β-cyclodextrins mixture, and it was found to be within the limits of 0.02 to 0.04 for the chroma-

tography on 1:1 α-/β-cyclodextrins mixture. From the consideration of the R_f values it may be said that the errors of mixing increase as parallel to the higher proportion of α-/ to β-cyclodextrins, and vice versa, in the mixture. Moreover, α- and β- cyclodextrins might complex with each other by the unperceived mechanism, thereby making it complicated and difficult to control the reproducibility of the R_f values.

In addition, it is observable from the R_f profiles for α-/β-cyclodextrin mixtures that the R_f values decrease as small amounts of α-cyclodextrin are added to the β-cyclodextrin, as in 1:5 α-/β-cyclodextrin ratios, since the α-cyclodextrin, being present in the α-/β-cyclodextrin mixture, will presumably complex with the compounds, thus retarding the migration rate of the molecule. Any small compounds, whose sizes are permissible for inclusion within both α- and β- cyclodextrins, will be definitely more accessible to complex with α-cyclodextrin due to the snug fit between α-cyclodextrin and the compounds. The molecule, being entrapped in the α-cyclodextrin cavity, will behave by attaching itself to the stationary phases, and its migration is eventually stopped while the solvent zone as a whole passes over. Consequently, the R_f values are decreasing significantly because of the factors explained above.

By contrast, the chromatography of the compounds on α-cyclodextrin stationary phases, in the presence of a small amount of β-cyclodextrin, as in 7:1 and 9:1 α-/β-cyclodextrin ratios, yield increasing R_f values for each particular compound. This phenomenon indicates that there might be some interactions involved, for instance, α- and β- cyclodextrin may interact with each other thus preventing α-cyclodextrin from complexing with the drug molecule. Yet the mechanism of this interaction is unknown, but some speculations can be derived from this finding. As we have known, both α- and β- cyclodextrins have the primary and secondary hydroxyl groups crowning opposite ends of the cavity; it is therefore conceivable that the interaction between α- and β-cyclodextrin undergo hydrogen-bonding formation between the hydroxy groups rimming the two cyclodextrin molecules. Accordingly, the retarding effect of α-cyclodextrin as a consequence of its inclusion forming with the compounds is attenuated in proportion to the lessened amount of α-cyclodextrin being present consequent upon the interaction between the α- and the β-cyclodextrin. Simply stated, there may be a competition between the β-cyclodextrin and the drug molecule for the α-cyclodextrin and this phenomenon may contribute to the peculiar chromatographic behaviours of the compounds found at 7:1 and 9:1 α-/β-cyclodextrins mixtures.

Another factor which is worthwhile considering is concerned with the solubility of the complexes formed between the cyclodextrins and the drug molecule, for instance, there may be a tremendous change in the solubility of the drug after complexing with the cyclodextrins. Hence, the migration behaviours of the drug molecules on the cyclodextrin stationary phases are altered by the appreciable changes in the solubility influenced by the complexation by the cyclodextrin. Furthermore, since the cyclodextrin is capable of forming complexes with many varieties of molecules, drug molecules as well as the components in the solvents employed are therefore equally accessible to inclusion formation with the cyclodextrins. This phenomenon will, in turn, lead to uncertainties in performing chromatography on α-/β-cyclodextrin mixtures or even on α- or β- cyclodextrin stationary phases alone due to the inconsistency of the solvent composition in running each chromatogram. At the molecular level, each molecule is progressing in a stop and go sequence dependent upon the perpetuating alteration in the solvent composition. On the other hand, performing chromatography in this situation, that is, in the different efficiency of the solvent, might yield somewhat better separation of the compounds. And this is in accord with the actual experiment as shown in the result section. In summary, the

factors described above may account in part for the chromatographic behaviour of the compounds on α-/β- cyclodextrin mixture or on α- or β- cyclodextrin stationary phases alone.

As a conclusion, chromatography on α-, β-cyclodextrins and mixed cyclodextrins permit the separation and partial description of substances whose separations are difficult to achieve on the customarily used silica gel layers. Yet the mechanism is still unperceived and the peculiar R_f profiles for the mixture of cyclodextrins can not be fully explained. Some speculations have been attempted in discussing the results obtained. Nevertheless, it appears that "inclusion chromatography" employing cyclodextrin stationary phases may be of general use in the separation of molecules which differ in any substituent groups suitable for inclusion in cyclodextrin cavities. Though the results are not yet satisfactorily reproducible and preparative conditions must still be refined, we hope that this study might be useful in exploiting the cyclodextrins as analytical agents.

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