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CHROMATOGRAPHIC BEHAVIOUR OF SOME INDOLE ACIDS ON A SEPHADEX COLUMN IN WATER AND IN THE PRESENCE OF SALTS

E. MARKLOVÁ and I. M. HAIS

Department of Pediatrics, Faculty of Medicine, and Department of Biochemistry, Faculty of Pharmacy, Charles University, Hradec Králové (Czechoslovakia)

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SUMMARY

The adsorption of indole-3-acrylic acid, [3-(indol-3-yl)acryloyl]glycine, tryptophan, indole-3-acetic, indole-3-propionic and indole-3-butyric acids and [3-(indol-3-yl)acryloyl]glycine ethyl ester on Sephadex G-10 from water increases in this order. With the exception of the ester, the adsorption of the indoles, particularly those of indole-3-acrylic acid and [3-(indol-3-yl)acryloyl]glycine, is enhanced in salt solutions. Similar sequences have been obtained in thin-layer chromatography on Sephadex G-10 and G-50 and on cellulose. Pre-treatment with pyridine (followed by acetic acid) of Sephadex G-10 (but not G-50) causes increased adsorption of the indoles (except for tryptophan) in water. In buffer, this applies only to the ester. The possible role of anion exclusion is discussed.

INTRODUCTION

During the elaboration of an analytical procedure for indolylacryloylglycine in urine[1], the compound was found to be strongly sorbed on a column of Sephadex G-10 in a medium of higher ionic strength, but was readily eluted with distilled water. In the present paper experiments are described which were designed to explain this observation.

EXPERIMENTAL

Materials

trans-Indole-3-acrylic acid (IAcrA) and *trans*-[3-(indol-3-yl)acryloyl]glycine (IAcrGly) were gifts from the Department of Pharmacognosy of the University of Bonn, G.F.R. *trans*-[3-(Indol-3-yl)acryloyl]glycine ethyl ester (IAcrGlyEE) was prepared by Dr. E. Kasafírek (Research Institute for Pharmacy and Biochemistry, Prague, Czechoslovakia). Indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-butyric acid (IBA), tryptophan (Trp), benzoic acid, hippuric acid and cinnamic acid were all obtained from Lachema, Brno, Czechoslovakia. *trans*-Imidazole-

4(5)-acrylic (urocanic) acid was donated by Compagnie Française des Matières Colorantes, Gattefossée, France.

The following materials were also used: Sephadex G-10, G-15, G-25 and G-50 (Pharmacia, Uppsala, Sweden); aluminium-backed cellulose "Lucefol QUICK" foils for thin-layer chromatography (Sklárny, Kavalier, Czechoslovakia); phosphate–citrate buffer, pH 7.1 (58.9 g of $Na_2HPO_4 \cdot 12\ H_2O$, 3.7 g of citric acid and 0.2 g of NaN_3 per litre; $I = 0.427\ M$ according to ref. 2); phosphate–citrate buffer, pH 8.3 (71.5 g of $Na_2HPO_4 \cdot 12\ H_2O$, 0.21 g of citric acid and 0.2 g of NaN_3 per litre; $I = 0.582\ M$ according to ref. 2); Tris–hydrochloric acid buffer, pH 7.2 [22.1 g of tris-(hydroxymethyl)aminomethane and 442 ml of 0.2 *N* HCl per litre]; Van Urk reagent [1 g of 4-dimethylaminobenzaldehyde, 50 ml of HCl (36%) and 50 ml of ethanol]; pyridine (Lachema).

Procedure

A column (5 × 1.8 cm I.D.) was prepared from 5 g* of Sephadex G-10 (or other types when stated) from which fines had been removed by decantation. The column was then washed with 50 ml of 0.1 *N* NaOH, 50 ml of 0.1 *N* HCl and 50 ml of distilled water. 50 μg of the indole acid under investigation in 0.5 ml of water were then placed on the column and the column was washed with distilled water. Fractions of 5 ml were collected, and their absorbance was recorded on a Unicam SP 500 spectrophotometer at the respective absorbance maxima, *i.e.*, at 323 nm for IAcrA, IAcrGly and IAcrGlyEE, and at 280 nm for IAA, IPA, IBA and Trp (light path, 1 cm). Elution curves of the individual substances are shown in Fig. 1. IAcrA and IAcrGly were eluted before the indole-3-alkanoic acids, which followed in the order of increasing molecular weight (IAA, IPA and IBA), *i.e.*, the reverse of the order obtained on simple gel filtration (molecular sieving). Tryptophan was eluted close to IAA.

When, after the introduction of the sample, the column was washed with 30 ml of the pH 8.3 buffer followed by 150 ml of the pH 7.1 buffer, the sequence of elution

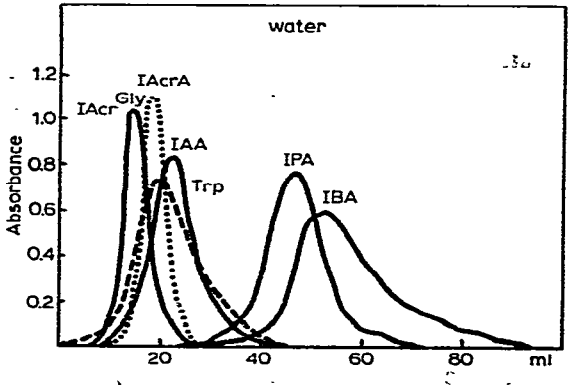


Fig. 1. Elution curve of indole acids on Sephadex G-10. Elution with water was started immediately after introduction of the sample. Measured at 323 or 280 nm; light path, 1 cm.

* Weighed as supplied by Pharmacia.

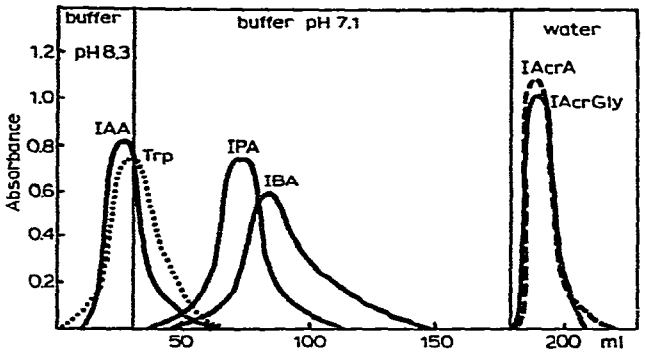


Fig. 2. Elution curves of indole acids on Sephadex G-10. Eluents as indicated in the diagram.

of the indole-3-alkanoic acids and of tryptophan remained unchanged (IAA, Trp, IPA and IBA) although their elution volume increased. IAcrA and IAcrGly were sorbed very strongly (Fig. 2).

The behaviour of some other cyclic carboxylic acids was studied under comparable conditions. All of the substances investigated, namely benzoic, hippuric, cinnamic and imidazoleacrylic (urocanic) acids, were readily eluted (Fig. 3).

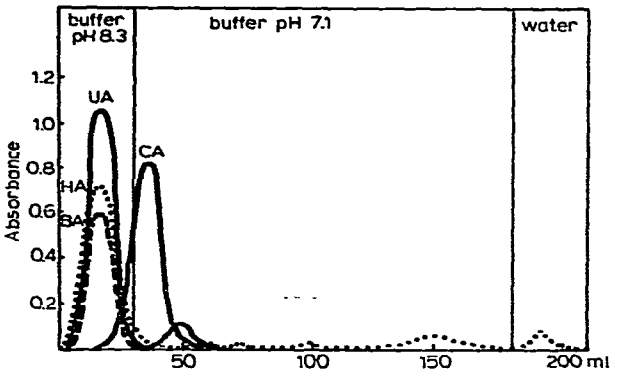


Fig. 3. Elution curves of benzoic (BA), hippuric (HA), cinnamic (CA) and urocanic acids (UA) on Sephadex G-10. Measured at 280 nm. Additional peaks are attributed to impurities in the UA and HA samples.

Sephadex gels possessing different degrees of cross-linking (G-50, 25, 15 and 10) were compared (all the columns were packed with 5 g of gel material). Fig. 4 shows that the adsorption of IAcrGly increased with increasing cross-linking (decreasing G numbers).

In order to check whether the buffers used had a specific effect in retarding IAcrA and IAcrGly, 300 ml of other eluents of higher ionic strength were used, namely 1 *N* NaCl and 0.1 *M* Tris–HCl buffer (pH 7.2). In both cases IAcrGly was desorbed when distilled water was used as eluent following the buffer. When the phosphate–citrate buffer (pH 7.1) was diluted, the elution volumes of both IAcrA and IAcrGly

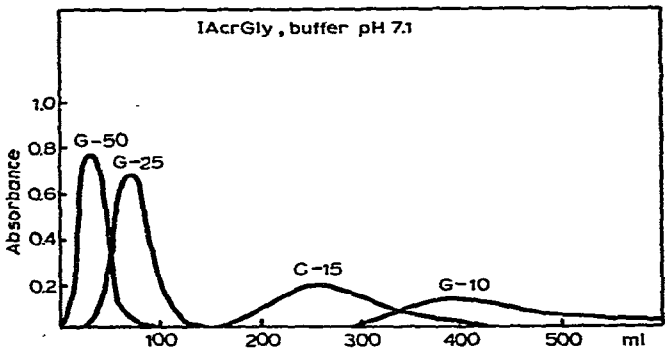


Fig. 4. Elution curves of IAcrGly on Sephadex G-10, 15, 25 and 50; pH 7.1 buffer throughout.

decreased. [When the buffer was diluted with water (1:4), the elution volume was 150 ml.]

When 2 *M* glucose or 1 *M* urea was used, the elution was somewhat retarded in comparison with distilled water, but the difference was very slight; the elution volumes of IAcrA and IAcrGly in these eluents were similar to that of IPA in phosphate–citrate buffer (see Fig. 2). This and the preceding experiment show that the buffer is not essential, but that the presence of any polar solute or higher osmotic pressure is not by itself sufficient to account for the effect which depends on ionic strength.

In order to investigate the role of the carboxyl groups, IAcrGly was compared with its ethyl ester (IAcrGlyEE). There was a very pronounced difference in elution behaviour in water: whereas the elution volume on Sephadex G-10 was 18 ml for IAcrGly (see Fig. 1), it was 450 ml for IAcrGlyEE (Fig. 5). No increase in adsorption was observed in a medium of higher ionic strength: the elution volume of IAcrGlyEE in the pH 7.1 buffer was 430 ml; this may be compared with the elution volume of 380 ml for IAcrGly (Fig. 4).

When the indole acids were subjected to descending TLC on Sephadex G-10 or G-50 using the phosphate–citrate buffer (pH 7.1) as the mobile phase, their order on the chromatogram corresponded to that in Fig. 2, IAA exhibiting the largest

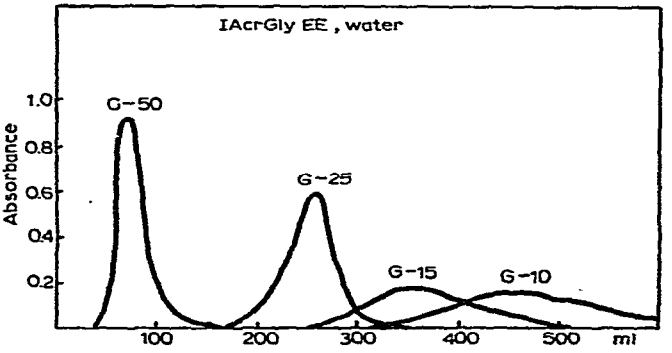


Fig. 5. Elution curves of IAcrGlyEE in water on Sephadex gels of different degrees of cross-linking; G-10, 15, 25 and 50.

elution volume. IAcrGlyEE had the lowest R_F value of all of the acids. Similar results were obtained by ascending chromatography on cellulose thin layers, using the same buffer as the eluent. The order of the R_F values essentially agreed with that on Sephadex G-10 (Fig. 6). Tryptophan was more retarded relative to the indole-3-alkanoic acids than on Sephadex G-10. With water as the mobile phase, the elution sequence also resembled that on Sephadex G-10 columns.

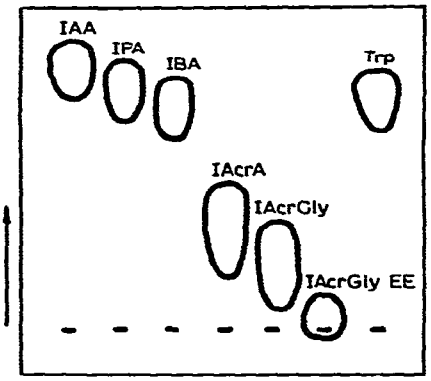


Fig. 6. Cellulose thin-layer chromatography on Lucefol Quick (Kavalier) layers using phosphate–citrate buffer (pH 7.1) as eluent. Time of development, 30 min; detection with the Van Urk reagent.

The question arose as to whether the relatively rapid elution of IAcrA and IAcrGly by water was due to the lower pH of distilled water which had not been boiled before being used as eluent and had a pH of *ca.* 6.5. For distilled water whose pH had been adjusted to 4.5 with HCl or to 8.3 with ammonia, practically no differences in elution volumes of IAcrGly and IAcrA were found, thus the difference between water and the buffer cannot be explained by the difference in pH.

It seemed interesting to compare IAcrA (mol. wt. 187) with a substance of a similar molecular weight, but devoid of the characteristic N-heterocyclic structure and conjugated bond system. Comparison with glucose (mol. wt. 180, elution with water) resulted in the following data:

	V_e	K_D
IAcrA	18	1
Glucose	14.8	0.68

where V_e is the elution volume and K_D is the distribution coefficient; for details of the calculation see ref. 3. This shows that IAcrA, unlike glucose, exhibits adsorption behaviour even in distilled water, although the adsorption is less pronounced than that of IAcrA in solutions of higher ionic strength or that of, for example, IPA in water (Fig. 1).

Prompted by the observations of Eaker and Porath[4] on the influence of pyridine washings on the chromatography of amino acids, experiments with pyridine-washed columns were carried out. The Sephadex G-10 (or G-50) column (*cf.* first

paragraph of the *Procedure*) was washed with a 1 *M* aqueous solution of pyridine for 24 h at a flow-rate of 20 ml/h. Subsequently, the column was washed with 0.2 *M* aqueous solution of acetic acid at the same flow-rate and using the same total volume as in the case of pyridine. The results are presented in Table I. In the case of Sephadex G-50, the pre-treatment with pyridine either had no effect or only slightly suppressed the adsorption (lower K_D values), except for Trp in the buffer. The same observation applies to the Sephadex G-10 columns with buffer as the mobile phase, except that the least polar substance, namely the ester IAcrGlyEE, was more strongly retained on pyridine-washed columns.

TABLE I

K_D VALUES OF INDOLIC ACIDS ON COLUMNS OF SEPHADEX G-10 AND G-50

W = In water; B = in phosphate–citrate buffer (pH 7.1); subscript P column washed with pyridine.

Compound	*G-10*				*G-50*			
	W	W_P	*B*	B_P	*W*	W_P	*B*	B_P
IAA	2.1	(19.0)*	4.1	4.1	0.7	0.7	0.75	0.6
IPA	4.8	(19.0)*	8.1	7.6	0.75	0.68	0.65	0.6
IBA	6.1	(19.0)*	9.5	9.3	0.8	0.55	0.7	0.6
Trp	2.3	1.6	2.1	2.1	0.6	0.5	0.65	0.75
IAcrA	1.1	(35.0)*	35.1	33.1	0.95	0.8	0.8	0.75
IAcrGly	0.9	(35.0)*	35.1	33.1	0.95	0.8	0.8	0.8
OAcrGlyEE	44.1	—	44.1	55.1	1.05	0.75	1.05	0.9

* Minimum values. The emergence of the respective solutes was not been observed even at the volumes indicated.

With distilled water as the mobile phase and Sephadex G-10 columns, the sorption decreased only in the case of the amphoteric substance tryptophan (K_D decreasing from 2.3 to 1.6). In all of the other cases, an increase in adsorption was noted; for IAA, IPA and IBA the increase was greatly in excess of that which would be caused by the substitution of the buffer for water (compare columns W_P and B in Table I, G-10).

DISCUSSION

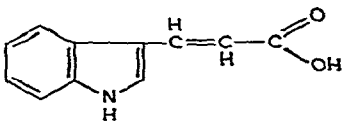
Adsorption sequence on Sephadex G-10 in water. It can be seen from Fig. 1 that indolic substances are eluted in approximately the reverse order of polarity, beginning with IAcrGly, IAcrA, and tryptophan, proceeding through the indole-3-alkanoic acids and ending with the ester. This would suggest "non-polar sorption" as the decisive factor. A decrease in their solubility in water is among the factors favouring adsorption of purines on Sephadex G-10 (ref. 5).

The molecular-sieve effect does not seem to contribute, with a possible exception in the order of IAcrGly and IAcrA. The extent of the planar conjugated double-bond system does not favour adsorption in this case, since the extent of adsorption of IAcrA was less than that of IPA.

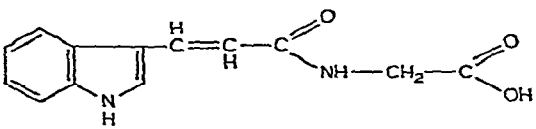
Adsorption on Sephadex G-10 in electrolyte solutions. All of the indole acids under investigation, with the exception of IAA, were more strongly retained in the

solution of higher ionic strength (buffer), but the effect was much more striking in the cases of IAcrA and IAcrGly. The elution volumes of these two compounds were very large indeed (Table I). The indole-3-alkanoic acids exhibited the same adsorption sequence as observed in water. This effect may belong to the class of "salting-out adsorption", a term coined by Tiselius[6,7]. Indolic substances were among those in which adsorption was favoured by salts; tryptophan and its peptides migrated more slowly on cellulose paper than other amino acids and simple peptides[8,9]. The classical concept of the salting-out effect stresses the competition for water of the ions of the salt with the polar groups of the solute and/or those of the sorbent[10].

According to this theory, the striking increase in elution volume for IAcrA and IAcrGly indicates that, if these compounds and/or the sorbent* are stripped of their hydration coat, the extended double-bond system conjugated with the indole nucleus will cause very strong adsorption on the cross-linked polysaccharide. In other words, the structure of the hydration envelope of the solute and possibly of the sorbent–water interphase is modified by the presence of salts in such a way as to allow stronger interaction between the polysaccharide and the planar heteroaromatic solute. In agreement with this conclusion, Prakash and Nandi[11] reported the enhancement of the hydrophobic effect by electrolytes in the case of aromatic amino acids chromatographed on Sephadex LH-20.



IAcrA



IAcrGly

In the present work, solutes which were non-electrolyte (glucose and urea), even in high concentrations, had a much smaller effect than salts in retarding the elution of IAcrA and IAcrGly. These results are also parallelled by the observations of Prakash and Nandi[11]**.

The chromatographic sequence was essentially similar on cellulose (TLC, see Fig. 6) with the buffer as eluent. Latinák[13] considered partition as a possible mechanism for the chromatography of aromatics on cellulose (paper) in aqueous salt solutions, suggesting that decreased solubility in the aqueous mobile phase might force the solutes into the aqueous stationary phase***. This explanation implies negative sorption, *i.e.*, at least partial exclusion, of salts by cellulose. "Negative sorption" of salts (ion exclusion) by Sephadex G-10 would have to be presumed if the hypothesis were applied to the latter column material.

The salting-out effect is of practical importance, since it facilitates the separation of IAcrGly (or IAcrA) from other solutes present in biological fluids[1].

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* Hydration of the carboxyl group of the solute (isolated in case of IAcrGly) might have an effect since the adsorption of IAcrGlyEE was not enhanced by the presence of salt (*cf.* the final part of this Discussion).

** Sugar has a pronounced retardation effect on the elution of N-acetyltyrosine ethyl ester on Sephadex LH-25 (ref. 12).

*** *Cf.* also the possibility pointed out in ref. 12.

The effect of pre-washing with pyridine. In a study of the chromatography of amino acids on Sephadex G-10 columns, Eaker and Porath[4] observed variations due to the history of the column. The main source of variation was found to be the treatment with pyridine solutions which caused an increase in mobility of basic amino acids. Since the recovery of pyridine used for pre-washing was quantitative (or rather more than quantitative, *i.e.*, 104%), these workers suggested that pyridine was not retained by irreversible sorption, but that it removed an acidic compound which had acted as an exchange material, probably oleic acid liberated from a detergent used in the manufacture of the gel beads*. This prompted us to check the influence of pyridine pre-washing. We found a striking increase in adsorption of the anionic indoles when water was used as the mobile phase. As this enhanced adsorption was not observed when buffer was used as the mobile phase (*cf.* columns W_P and B_P, Table I, G-10), anion exchange is indicated: one might suppose that trace amounts of pyridine remain adsorbed on the gel matrix in spite of the elution with acetic acid.

The influence of cross-linking. Fig. 4 shows that the retention of IAcrGly in an electrolyte solution increases with the increasing degree of cross-linking from Sephadex G-50 to G-10 (ref. 14). A similar conclusion can be drawn for IAcrGlyEE in water (Fig. 5). The relative effects of the proportion of ether groups[15] (or of any other function derived from the cross-linking agent) on the one hand and of the close texture of the polysaccharide gel on the other are not known. Some workers[10,16,17] attribute the greater adsorption of aromatic compounds on Sephadex materials with increased cross-linking to the lower proportion of hydration water in the column material.

The potentional role of ion exclusion. It has been observed that some anions are less strongly retarded on Sephadex gels in water than expected[3,18]. This has been attributed to electrostatic repulsion by the carboxylate groups present in the dextran matrix. The effect was removed by salts. In our case this effect may be considered to explain why the adsorption of the acids, observed in electrolyte solutions and especially strong in the case of IAcrGly and IAcrA, was reduced in water, whereas there was little difference between buffer and water in case of the ethyl ester of IAcrGly.

Charge of identical sign on both the solute and sorbent gel is a condition for ion exclusion to occur. Distilled water which had been adjusted to pH 4.5 had the same effect as that adjusted to pH 6.5 or 8.3. The pK of indole-3-acetic acid is *ca.* 4.6 (ref. 19); those of IAcrA and IAcrGly are not yet known, but may be still lower. Thus a substantial proportion of the solute molecules is likely to bear a negative charge. If Sephadex had carboxyl groups in its sugar units, their pK might be approximately 3.5 (for gluconic acid[19], the pK is 3.56; the pK of glucuronic acid is 3.5**). The estimation of the pK of Sephadex G-10 is difficult because of the low content of acidic groups: Dr. Karlíček found 1.6 micro equivalents per gram of our sample (as supplied); between 2 and 10 micro equivalents per gram dry weight were found in the Pharmacia laboratory[20]. An apparent pK value of 6.5 estimated independently by direct titration in both laboratories may differ from the real value and accordingly does not de-

* According to recent information from Pharmacia, contamination of Sephadex G with oleic acid is unlikely.

** Values obtained by titration in the presence of excess of Na^+ by Drs. M. Polášek and R. Karlíček (Department of Analytical Chemistry, Faculty of Pharmacy, Hradec Králové), to whom thanks are due.

finitely rule out the possible effect of anion repulsion caused by the low proportion of acidic groups even at pH 4.5.

There are certain analogies between the salting-out and the anion-exclusion concepts: in the first case, salts would disturb a hydration coat which prevents the close contact between solute and adsorbent; in the second case the salts would remove electrostatic repulsion which prevents this contact.

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