ACTA CHEMICA SCANDINAVICA 3 (1949) 231—246

Some Adsorption Experiments with Amino-Acids and Peptides, Especially Compounds of Tryptophan

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We describe here some model experiments carried out in the hope of devising methods for the separation of peptides containing different numbers of aromatic amino-acid residues from one another and from similar purely aliphatic compounds. Such methods would be useful for analysing the complicated mixture of peptides of leucine, valine and tryptophan encountered in partial hydrolysates of gramicidin [1], the more so because peptides containing tryptophan residues do not show very striking differences of behaviour from corresponding leucine or valine compounds in partition chromatographic procedures so far described [2, 8]. Adsorption procedures employing charcoal seemed promising for the purpose, in view of its powerful selective adsorption of tryptophan and other aromatic amino-acids (Tiselius [3, 4]). This effect has been utilized by various authors for group separations of free aromatic amino-acids (for bibliography see Martin and Synge [5], Tiselius [6], Tiselius, Drake and Hagdahl [7], Fromageot, Jutisz and Lederer [25]).

It did not seem too much to hope that, using charcoal adsorption columns, a group separation of the peptides in a partial hydrolysate might be achieved as follows: —

A. Peptides devoid of aromatic residues.

B. Peptides with one aromatic residue per molecule.

C. Peptides with two or more aromatic residues per molecule.

Such a separation procedure might have a wide field of use in the study of proteins and peptides.

At one stage an alternative possibility was suggested by Moore and Stein's [8] observation of anomalous retardation, especially of aromatic amino-acids,

on starch partition chromatograms. We found that similar effects occur on filter paper, and by developing filter paper chromatograms with water, it was possible to demonstrate retardation of tryptophan (travelling as a well-defined spot having R_F 0.6—0.7) relative to such aliphatic amino-acids as leucine and valine, which moved as spots near the water front ($R_F = 1$). If Traube's rule were obeyed, tryptophyltryptophan should be still more strongly adsorbed, and this was found to be the case, it giving a somewhat elongated streak (R_F 0.25—0.45), permitting ready differentiation and separation of tryptophan and tryptophyltryptophan in mixtures. However, it soon became apparent that the desired group separation could not be realised by simple adsorption chromatography on paper or starch, since the addition of such solvents as ethanol (which are necessary for rendering peptides of the type to be studied sufficiently soluble in aqueous media) led to marked alteration in the relative R_F values of the various tryptophan compounds (see Experimental Section). The adsorption effects on paper do not seem to be strong enough to predominate over the changes in the solubilities of the various compounds (*cf.* Cohn and Edsall[9], Tiselius[10]) and/or incipient 'partition' effects caused by the addition of ethanol. The changes of this type thus appear to be rate-determining. In this way the relative positions on paper chromatograms of tryptophan and tryptophyltryptophan are actually reversed on passing from water to 60% *v/v* aqueous ethanol as developing solvent.

With adsorption on charcoal, effects due to modifying the solvent do not dominate the adsorption effects, and the relative behaviour of the different classes of compound is not greatly altered. (Compare the separations in the Experimental Section with water and with 50% *v/v* aqueous ethanol). Accordingly we recommend the use of charcoal for group separations of this type, especially with unknown compounds. Nevertheless, for particular separations, adsorption chromatography on starch or paper using elution development by aqueous media may prove advantageous, particularly in view of the ease of elution.

The separation of two mixtures of model substances has been mainly studied: (a) leucylglycine-glycyltryptophan; (b) leucine-tryptophan-tryptophyltryptophan. Clear-cut separations could be obtained in both cases, and it seems fairly well established that the content of aromatic residues per molecule is the dominant factor, and other effects subsidiary. Thus, although insertion of a peptide bond (glycine $\longrightarrow$ glycylglycine) or lengthening of an aliphatic chain (glycine $\longrightarrow$ leucine, glycylglycine $\longrightarrow$ leucylglycine) leads to marked increases in the adsorption by charcoal (Tiselius[3, 4]) such effects are not very noticeable in the series (tryptophan $\longrightarrow$ glycyltryptophan $\longrightarrow$ leucyltryptophan). Indeed, all three of these compounds were found to have similar retention volumes in adsorption on charcoal from aqueous solution, and

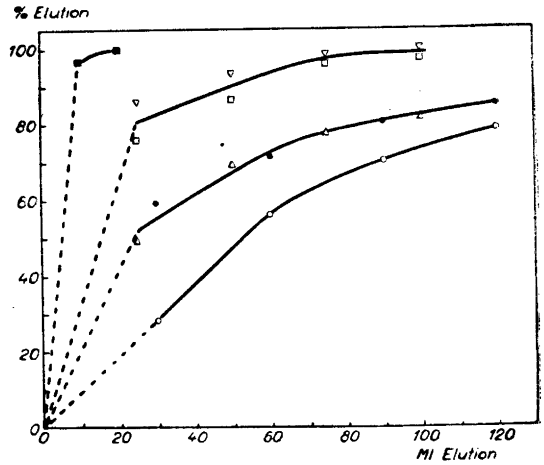

Fig. 1. Elution curves for DL-*leucylglycine from charcoal columns under various conditions (see text).*

○ *Untreated charcoal (water)*

●△ *Two batches St 2 charcoal (water)*

▽□ *Two batches St 6 charcoal (water)*

■ *St 6 charcoal (0.2 % w/v aqueous phenol)*

a mixture of the three showed no definite separation of fronts during frontal analysis, either in the presence or absence of phenol. By contrast, tryptophyltryptophan was found to be much more strongly adsorbed on charcoal than tryptophan, mixtures of the two substances giving well defined 2-step diagrams on frontal analysis, and the differences in adsorption became still more marked in the presence of phenol, which acts as a displacing-eluting agent. These observations lend strength to the view that the different components of tyrocidine recognised by Synge and Tiselius [11] (*cf.* Pedersen and Synge [12]) differ chiefly in the number of tryptophan residues per molecule. Certain eluting or displacing agents, namely stearic acid, phenol and cetylpyridinium bromide have been found of value for refining the separations obtained. It is convenient to discuss their use separately, in view of the different methods of introducing them. In many of our experiments stearic acid and phenol have been used concurrently with good results.

Stearic acid. This had been found useful as a displacing-eluting agent in experiments with tyrocidine on charcoal in ethanol solution [11]. The present experiments were done with aqueous solvents, and it was found practical to adsorb known amounts of stearic acid on to the charcoal in bulk from ethanol

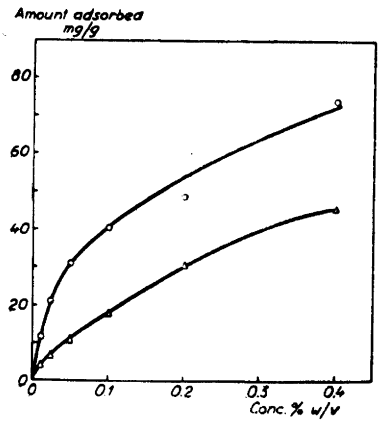

Fig. 2. Adsorption isotherms for aqueous DL-*valylglycine on charcoal before and after treatment with stearic acid.*
○ *Untreated charcoal*
△ *St 6 charcoal*

solution, and then to wash away the ethanol with water. There was no sign of elution of this adsorbed stearic acid from the charcoal except where high concentrations of ethanol and phenol were used on the chromatograms.

The charcoal treated with stearic acid showed better elution characteristics than untreated charcoal, as is apparent from the recoveries at different stages of elution development shown in the Experimental Section. This effect is reflected at least in part by a change in the form of the adsorption isotherms, illustrated by the adsorption isotherms for valylglycine on treated and untreated charcoals, (see Fig. 2). Presumably the stearic acid blocks adsorption centres at which very intense or irreversible adsorption would otherwise occur.

A further useful effect of the stearic acid treatment for the present work was apparent from a study of its effect on the retention volumes for a number of relevant compounds (see Table 2). The adsorption of the aliphatic compounds is depressed to a uniformly greater extent (40—55 %) than that of the aromatic compounds (18—27 %). The selectivity of the charcoal for the purpose in hand is thus increased, while at the same time specificity of adsorption within the two groups is not markedly affected. It seems therefore that pretreatment of charcoal with stearic acid can be recommended as a routine procedure when working with amino-acids and peptides in aqueous media (*e.g.* for such separations as those referred to immediately below). Similar effects were noted by Tiselius and Hahn [13] in adsorption separation of saccharides on charcoal pre-treated with ephedrine.

Since the present work was done, Weiss [14] has described the treatment of charcoal with fatty acids, and higher alcohols. In addition to ion exchange and 'secondary adsorption effects' (*cf.* Steenberg [15]) Weiss observed diminution of the adsorption affinity of the treated charcoal for 5-aminoacridine.

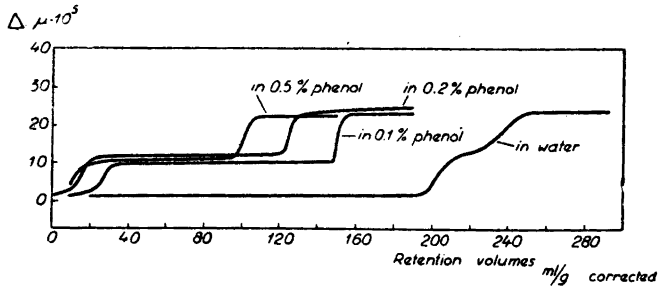

Fig. 3. Frontal analysis diagrams for 0.1 % w/v glycyl-L-tryptophan on St 6 charcoal columns in the presence of water and varying concentrations of phenol.

Phenol and cetylpyridinium bromide. Tiselius [6, 16] used phenol as a displacing agent in displacement analysis of mixtures of aliphatic peptides on charcoal. Frontal analysis experiments (see Fig. 3) on the effect of phenol on the adsorption of tryptophan showed that such low concentrations as 0.1—0.5% *w/v* had effects on the retention volumes for tryptophan, glycyltryptophan and leucyltryptophan very slight in comparison with their displacement-elution effects on purely aliphatic peptides. Similarly, higher concentrations of phenol, up to 5 % *w/v*, that had marked eluting effects on tryptophan (*cf.* Schramm and Primosigh[17], Tiselius, Drake and Hagdahl [7]) were found to have relatively slight effects in eluting tryptophyltryptophan (Table 3). Tentative evidence has been obtained that cetylpyridinium bromide (which, with similar substances, has been shown by Tiselius and Hagdahl[18] to be a useful agent for displacing tryptophan from charcoal) has very little power to elute tryptophyltryptophan from charcoal. Indeed, no satisfactory agent for this purpose has so far been found.

It is perhaps worth commenting briefly on the rationale of chromatographic development in which the solvent passing through the column is repeatedly changed or in which increasing concentrations of an eluting agent are incorporated in the solvent. This procedure (sometimes called 'flowing' chromatography) formed part of Tswett's original technique, and has been widely used for as long as chromatography has been practised. Although in some respects it is a cruder technique than the procedures of frontal analysis, displacement development and elution development (without change of solvent) it may be regarded in essence as the introduction of a displacing agent whose affinity for the adsorbent is intermediate between those of the substances which it is wished to separate. One of these is displaced, and the other is not. The development of the column would, in the absence of the displacing agent,

proceed more slowly and with considerable tailing of the first band into the second—perhaps with some displacement of the first substance by the second, but with no clear gap between them. The effect of introducing the intermediate displacing agent is to introduce a space between the two substances which it is wished to separate, filled by the displacing agent, which can then easily be separated from either substance by other means (with phenol, by volatilisation). The process is analogous to the introduction of extraneous substances to form azeotropes or as carriers in distillation of mixtures that are otherwise difficult to fractionate on account of similarity of boiling point or the small amount of material in relation to the apparatus.

The adsorption behaviour of tryptophan and its compounds on charcoal does not appear to be markedly affected by pH. In several experiments tryptophan hydrochloride was used instead of tryptophan. Cl' ions (as HCl) were found to pass through the columns without significant retardation, the retention of the tryptophan being unaffected by the presence of the acid.

We conclude that charcoal adsorption procedures suggested by the experiments described here may prove generally useful for group separations involving aromatic compounds.

EXPERIMENTAL

Technique

Amino-acid and peptide preparations. With the exception of tryptophyltryptophan (see below), the preparations studied had all been characterised, and conformed to the usual criteria of purity, including homogeneity in paper chromatograms. Optical rotations of optically active compounds fell in the accepted range. The peptide specimens were the same as those studied by Synge [19], with the addition of glycylglycylglycine (kindly given by Mr. N. W. Pirie), D-leucyl-L-tryptophan (synthesised according to H. Fischer [20]) and D-leucylglycylglycine (Hoffmann La Roche).

Preparation of tryptophyltryptophan by partial hydrolysis of tryptophan anhydride. 600 mg of L-tryptophan methyl ester (prepared according to Abderhalden and Kempe [21]) was heated overnight in a sealed evacuated tube at 180°. The crystals melted at first to a colourless liquid which gradually became more viscous and yellower. By morning the hot tube contained rosettes of crystals which subsequent work indicated to be *tryptophan anhydride.* These could be recrystallised with poor recovery from methanol or ethanol, but this did not readily eliminate impurities running slowly on *n*-butanol-H_2O paper chromatograms, and giving a positive Ehrlich colour reaction (possibly tryptophan polypeptide esters). Tryptophan anhydride gives a positive Ehrlich

reaction and runs fast under the same conditions. The slow-moving impurities were precipitated by dissolving the melt in 20 ml hot ethanol, cooling, and adding 200 ml ether. The resulting precipitate was filtered off after storing the mixture at 0° overnight. The filtrate was almost free from Ehrlich-positive impurities. It was evaporated to dryness *in vacuo*, and dissolved in 15 ml glacial acetic acid. 15 ml 10 *N* HCl was added, and the mixture was kept for 10 days at 35° in a sealed evacuated vessel. Preliminary experiments had shown that after this time little or no unchanged anhydride remained. Paper chromatography (*n*-butanol-H_2O) showed trytophyltryptophan to be the main Ehrlich-positive component of the mixture, while some free tryptophan had been formed. Some impurities giving yellow-green colours with Ehrlich's reagent, and moving more slowly than tryptophan on the chromatograms, were also present. Further hydrolysis with hot HCl caused complete hydrolysis, with disappearance of the tryptophyltryptophan and an increase in free tryptophan (for quantitative data see below).

The crude 10 day partial hydrolysate was evaporated to dryness *in vacuo*, and the syrupy, partly crystalline reddish-brown product was redissolved in 50% *v/v* aqueous acetic acid (30 ml). This solution contained 2.24 mg N/ml (Kjeldahl-Friedrich), of which 3.2 % was carboxyl-N (by ninhydrin-CO_2 reaction — see below) and 20.4 % was amino N (Van Slyke, 4 min reaction time). After hydrolysis in 5 *N* HCl for 24 h in a sealed evacuated tube at 110°, the carboxyl-N rose to 23% of the total N. Suitable quantities of the solution were evaporated to dryness *in vacuo* before being used in the adsorption experiments. The solution was assumed to contain 1.05 mg tryptophan and 10.7 mg *tryptophyltryptophan* ($C_{22}H_{22}O_3N_4$) per ml (both as the respective hydrochlorides). In the frontal analysis, a low step, due to the free tryptophan, was always observed running ahead of the main front. Other impurities were undoubtedly present, which fluoresced in ultra-violet light on the paper chromatograms, but did not give a positive Ehrlich reaction. Presumably racemisation had also occurred in the course of the preparation.

Preparation of charcoal. A single batch of charcoal ('*Carbo activ*') was used throughout this work, made from '*Carbo activatus purus siccus*' (Merck, Darmstadt) by washing, drying, mixing and storing exactly according to Claesson's [22] directions.

Conduct of charcoal adsorption experiments. Many of the adsorption runs on columns were followed with optical control in the interferometric apparatus of Tiselius and Claesson according to the general procedure described by Claesson [22]. In some experiments, particularly elution experiments, the packed filters were used without connecting them to the interferometer. In most experiments other than simple frontal analyses, whether or not the inter-

ferometer was used, the effluent fractions were subjected to further confirmatory tests or to analyses. Unless otherwise stated, volumes of solvent passing the filters are given 'corrected'. Experiments were carried out at 25°. A. R. phenol was used throughout.

Ninhydrin-CO_2 determinations were done according to Van Slyke *et al* [23], using 100 mg pH 4.7 citrate buffer and 50 mg ninhydrin in water at a total volume of 3 ml.

Ehrlich colour reaction for tryptophan and its derivatives. At least an equal volume of 0.1% *w/v* *p*-dimethylaminobenzaldehyde in 10 *N* HCl was added to the sample to be analysed (in water or acetic acid). It was found desirable to remove ethanol or phenol by prior evaporation to dryness. The resulting blue colour was allowed to develop for up to 24 h at room temperature (see also the following paragraph).

Paper chromatography. One-dimensional paper chromatography was much used throughout the work for qualitative and rough quantitative tests on the composition of effluents from the filters. The pairs of substances whose separation on charcoal was studied were to some extent selected so that they could be readily identified on paper chromatograms. The substances were commonly applied to the paper as 5 μl. portions of the 1% *w/v* solutions. The various amino-acids and peptides were detected on the paper chromatograms by spraying with ninhydrin in the usual way and also, for distinguishing or demonstrating the tryptophan derivatives, by spraying with 0.1% *w/v* *p*-dimethylaminobenzaldehyde in 10 *N* HCl. After spraying with this reagent the papers were hung up wet at 37° in a closed vessel over 10 *N* HCl for a few hours, during which time the tryptophan derivatives gave a permanent blue colour, which increased for some time after removal from the vessel and drying.

Tryptophyltryptophan coloured rather slowly under these conditions, taking several days after drying to reach its maximum intensity. This compound gave a very faint colour with ninhydrin on paper, compared with other peptides studied. Munktell OB filter paper was used throughout the present work. Semi-quantitative comparisons were made by running different known amounts of the same compound as parallel chromatograms on the same sheet of paper with the specimen it was desired to assay.

Behaviour of amino-acids and peptides on paper chromatograms in different solvents

In work with *n*-butanol-H_2O chromatograms it was found that the R_F values for tryptophan in relation to leucine and valine and for glycyltryptophan relative to leucylglycine were lower than those recorded by Consden,

Gordon and Martin [2, 24]. It is reasonable to attribute this to higher adsorptive capacity of the Munktell paper used in the present work compared with the Whatman paper used by Consden, Gordon and Martin. However, experiments with recent batches of Whatman no. 1 paper have given similar results to those recorded below for Munktell paper. Table 1 gives R_F values for representative compounds in various solvents.

Table 1. R_F values for a series of amino-acids and peptides on paper.

Authors	Consden, Gordon and Martin [2, 24]	Present work		
Paper	Whatman no. 1	Munktell OB	Munktell OB	Munktell OB
Solvent mixture	*n*-butanol-H_2O	*n*-butanol-H_2O	60 % *v/v* aqueous ethanol	water
Addition	cupron	HCN	—	—
DL-Leucine	0.38	0.40	—	1
DL-Valine	0.20	0.21	—	1
L-Tryptophan	0.35	0.27	0.5	0.6—0.7
DL-Leucylglycine	0.23	0.25	—	—
Glycyl-L-tryptophan	0.29	0.16	0.6	0.8
L-Leucyl-L-tryptophan ..	0.60	0.6—0.7	1	0.9
Tryptophyltryptophan ..	—	0.4—0.6	0.8—0.9	0.25—0.45

Some theoretical implications of the data recorded in Table 1 have been mentioned above. All the components listed except tryptophyltryptophan gave fairly compact spots. Tryptophyltryptophan under all conditions gave a more elongated spot, the R_F values given in the table defining its limits.

For routine differentiation of leucylglycine and glycyltryptophan in effluents from charcoal chromatograms, development with butanol-HCN was employed. For leucine, tryptophan and tryptophyltryptophan development was with water.

Treatment of charcoal with stearic acid and its effect on adsorption phenomena

50 g of *Carbo activ* was mixed with a solution in 200 ml ethanol of the amount of stearic acid (Kahlbaum) which it was desired to incorporate with the charcoal. After stirring at intervals for an hour, water was added gradually with stirring until the volume was 2 l. The charcoal was then allowed to settle from the clear supernatant, and was washed by decantation with ten

further changes of distilled water, after which it was filtered off and dried in a vacuum desiccator over H_2SO_4 and soda lime before storing. The resulting products looked in no way different from the untreated charcoal, and were, if anything, more readily wetted by water. Various batches were prepared containing 2% by weight of stearic acid on the weight of charcoal taken (St 2) and 6% (St 6) respectively.

Table 2 shows the retention volumes on charcoal before and after treatment with stearic acid for a number of substances in 0.1% *w/v* aqueous solution, as determined by frontal analysis. The data for untreated charcoal fall into a sequence very similar to that given by Tiselius [16] for 0.5% aqueous solutions on a different type of charcoal with different adsorption capacity. The present data extend this series to some hitherto unstudied peptides. The *diminution* of the originally very low adsorptive capacity for glycine indicates that the stearic acid treatment does not confer ion-exchanging properties on the charcoal, at least in respect of monoamino-monocarboxy-compounds (*cf.* Weiss [14], Steenberg [15]). The last column in the Table shows clearly the different extent to which aliphatic and aromatic compounds are affected by the stearic acid treatment.

The data for tryptophan and tryptophyltryptophan referring to frontal analyses as hydrochlorides are for break-through of the amino-acid or peptide fronts, demonstrated by steps in the interferometer curves and by the simultaneous appearance of a positive Ehrlich reaction in the effluent. The Cl' fronts appeared on the diagrams as low steps, almost without retardation; their nature was confirmed by testing the effluent with $AgNO_3$.

Procedure for study of the separation of leucylglycine and glycyltryptophan

A number of comparative experiments were carried out as follows: — A 250 π mm^3 filter was packed with charcoal (0.36—0.39 g) and washed thoroughly with water. Exactly 3.0 ml of an aqueous solution containing 11.7 mg DL-leucylglycine and 9.45 mg glycyl-L-tryptophan was then pressed into the filter from a micro-burette. The micro-burette was then replaced with a larger burette, supplying distilled water, with which development was continued. The effluent was collected in successive fractions of known volume (measured from the beginning of pressing in) which were analysed for N by the Kjeldahl procedure, Ehrlich tests having shown that at no stage had any glycyltryptophan emerged from the filter during the development with water. A check on the completeness of elution of the leucylglycine, revealed by the cumulative Kjeldahl figures (see Fig. 1) was afforded by substituting

Table 2. Retention volumes (ml/g) for 0.1 % w/v aqueous solutions of amino-acids and peptides on charcoal before and after treatment with stearic acid.

Charcoal	Carbo activ	St 2	% of retention vol. with untreated charcoal	St 6	% of retention vol. with untreated charcoal
Aliphatic, etc. amino-acids					
Glycine	0.9	0.6	67	—	—
DL-Alanine	1.1	—	—	—	—
DL-Valine	9.3	—	—	—	—
DL-Leucine	27	19.9	74	13.7	51
L-Proline	5.4	—	—	—	—
Aliphatic peptides					
Glycylglycine	5.3	—	—	3.2	60
Glycylglycylglycine	30	23	77	14	47
Glycyl-DL-alanine	7.4	—	—	—	—
DL-Alanylglycine	8.9	—	—	—	—
Glycyl-DL-valine	25	—	—	12.6	50
DL-Valylglycine	40	—	—	18	45
Glycyl-DL-leucine	60	—	—	32	53
DL-Leucylglycine	68	51.5	76	37	54
D-Leucylglycylglycine	96	—	—	—	—
Aromatic compounds					
DL-Phenylalanine	155	—	—	—	—
L-Tryptophan	235	177	75	171	73
do (as HCl)	—	—	—	187	—
Glycyl-L-tryptophan	300	246	82	222	74
D-Leucyl-L-tryptophan	258	—	—	212	82
Tryptophyltryptophan (as HCl)	—	—	—	290	—
Phenol	172	—	—	137	80

5% *w/v* aqueous phenol for the eluting water (*cf.* Tiselius, Drake and Hagdahl[7]). 30 ml of this eluted any remaining leucylglycine from the filter as well as 60—70% of the glycyltryptophan. The 5% phenol eluate was evaporated to dryness *in vacuo* and the amount of leucylglycine in it was determined semi-quantitatively by paper chromatography with *n*-butanol-H_2O (see above). The glycyltryptophan was recovered undegraded and unchanged as far as could be judged from the paper chromatograms, on which it always gave a simple spot.

Elution behaviour of charcoal before and after treatment with stearic acid

The cumulative water elution curves for DL-leucylglycine according to the above procedure are plotted in Fig. 1 for charcoal of 5 different batches. The marked effect of treatment with stearic acid in improving the elution is manifest. The paper chromatograms on the phenol eluate, made after elution of St 6 columns with 90 ml of water indicated that as little as 0.5% of the leucylglycine used had remained uneluted.

Adsorption isotherms for charcoal before and after treatment with stearic acid

Adsorption isotherms were constructed from retention volume (frontal analysis) data obtained for a range of concentrations of DL-valylglycine in water both with untreated charcoal and with St 6 charcoal (*cf.* Claesson [22]). The data obtained are shown graphically in Fig. 2.

Retention volumes of tryptophan compounds: effects of phenol and ethanol

Retention volumes were ascertained for 0.1% *w/v* aqueous solutions of tryptophan and its compounds by frontal analysis on St 6 charcoal in the presence of varying concentrations of phenol. The procedure was modified as follows to deal with the presence of a second solute. The filter, packed dry with a known weight of charcoal, was washed with at least 3 times as much of the aqueous phenol solution as would be required to saturate the charcoal (see Table 1). A 0.1% *w/v* solution of the amino-acid or peptide to be studied (dissolved in the same phenol solution) was supplied from a burette, and frontal analysis with optical control was commenced in the usual way, except that the reference channel of the interferometer was filled with the appropriate aqueous phenol solution instead of pure water. Fig. 3 shows a family of curves obtained in this way for glycyl-L-tryptophan in the presence of different concentrations of phenol. The early step on each curve must represent a rise in concentration of phenol owing to its displacement by the glycyltryptophan. The Ehrlich reaction only became positive in the effluent with the second step in each curve. It seems from the curves that in each case the amount of phenol displaced is directly proportional to the amount of peptide adsorbed. The retention volumes, given by the position of the appropriate steps, are shown in Table 3. The figures show a marked difference between the behav-

iour of tryptophyltryptophan and the other tryptophan compounds containing only one tryptophan residue per molecule. These data served as an indication for the use of rather high concentrations of phenol (5% *w/v*) for separating tryptophan from tryptophyltryptophan (see below).

Table 3. Retention volumes for 0.1% w/v solutions of tryptophan compounds on St 6 charcoal in various aqueous phenol solutions (ml/g).

(The figures in brackets give the retention volume in a given phenol solution as a percentage of that in pure water).

Concn. of phenol in solvent (% *w/v*)	0	0.1	0.2	0.5	1.0%
L-Tryptophan	171	—	—	82 (48%)	—
do (as HCl)	187	—	—	103 (55%)	77(41%)
Glycyl-L-tryptophan	222	149 (67%)	124 (56%)	100 (45%)	—
D-Leucyl-L-tryptophan .	212	—	—	126 (59%)	—
Tryptophyltryptophan .. (as HCl)	290	—	—	250 (86%)	220 (76%)

Frontal analysis of mixed solutions of tryptophan, glycyltryptophan and leucyltryptophan (each 0.03% *w/v*) on St 6 charcoal both in water and in 0.5% *w/v* aqueous phenol gave no well-defined steps, nor did paper chromatography of the effluent fractions (*n*-butanol-H_2O with HCN) indicate any marked separation of the three compounds. On the other hand, frontal analysis of a mixture of tryptophan and tryptophyltryptophan (each 0.1% *w/v*) as hydrochlorides in water gave two well-defined steps (192 and 250 ml per g charcoal respectively). Paper chromatography (water) of the effluent fractions indicated clearly that the first step corresponded to tryptophan and the second to tryptophyltryptophan. When the same experiment was done, without interferometric control, in 50% *v/v* aqueous ethanol, paper chromatographic tests on the effluent indicated a retention volume for tryptophan of approx. 57 ml per g charcoal and for tryptophyltryptophan in the range 190—360 ml per g.

Phenol in the separation of leucylglycine and glycyltryptophan in aqueous solution

The separation procedure described above for these compounds was carried out on St 6 charcoal (previously saturated with 0.2% *w/v* aqueous phenol), 0.2% *w/v* aqueous phenol being used as solvent for applying the peptide

mixture and for the development. The elution data (Kjeldahl N. — see Fig. 1), showed that 99.7% of the leucylglycine had emerged in the first 20 ml of effluent. Development with this solvent was continued up to 50 ml. No leucylglycine whatsoever could be detected contaminating the glycyltryptophan in the further effluent obtained by elution with 30 ml 5% *w/v* aqueous phenol.

Phenol and cetylpyridinium bromide in the separation of leucine, tryptophan and tryptophyltryptophan in aqueous ethanol

The retention volume data for tryptophan and tryptophyltryptophan in 50% *w/v* aqueous ethanol given above suggested that the substitution of this mixture for water should, in elution development, favour the separation of tryptophan from tryptophyltryptophan. However, the marked lowering effect of the ethanol on the retention volume of tryptophan suggested that the separation of aliphatic compounds from tryptophan etc. might be impaired. This was found to be the case in the elution experiment described below, and accordingly we recommend that ethanol concentrations should be kept as low as solubility factors permit, at least during the stage of separating purely aliphatic peptides from aromatic ones. Conditions may thus be approximated to those described in the preceding paragraph for the separation of leucylglycine and glycyltryptophan.

A 500 π mm^3 filter was packed dry with St 6 charcoal (0.711 g) and was washed with 100 ml 0.1% *w/v* solution of phenol in 50% *v/v* aqueous ethanol. 15 mg each of D-leucine, L-tryptophan and tryptophyltryptophan (all as hydrochlorides) was then introduced by pressing in 3 ml of a solution of the mixture in the same phenol-ethanol-water mixture. Development was continued with this same solvent mixture. Successive portions of effluent (measured from the beginning of pressing in) were collected. Tryptophan broke through in the fraction 6—9 ml and Kjeldahl determinations showed that about 90% of the leucine had previously emerged from the column. When altogether 45 ml of the eluting mixture had passed, about 50% of the tryptophan had been eluted. Elution was continued with 75 ml of 5% *w/v* phenol in 50 % *v/v* aqueous ethanol. This eluted approximately a further 15 % of the tryptophan and a certain amount of stearic acid. Paper chromatograms (water) on this last eluted material showed that tryptophyltryptophan (and of course leucine) were completely absent from it.

In view of the successful results of Tiselius and Hagdahl[18] in eluting tryptophan from charcoal with cationic detergents, the same experiment was

repeated using as eluting agent 0.5% *v/v* 'Fixanol C' (a preparation of cetylpyridinium bromide kindly given by Imperial Chemical Industries Ltd., Manchester) in 50% *v/v* aqueous ethanol. This had the expected displacing-eluting effect on tryptophan, but no indication was obtained of the elution of tryptophyltryptophan even after the passage of 150 ml of the eluting solution through a filter previously saturated with a solution of 'Fixanol C' at the same concentration before adsorbing the mixture for analysis.

The presence of cetylpyridinium bromide in the eluates interfered with the recognition of tryptophyltryptophan by paper chromatography. However, control experiments showed that 'Fixanol C' did not interfere with the ninhydrin-CO_2 reaction of tryptophan and did not prevent the determination of tryptophyltryptophan by hydrolysing it with hot 6 *N* HCl in a sealed evacuated tube, evaporating the product to dryness, and carrying out ninhydrin-CO_2 determinations on the residue. A large excess of 'Fixanol C' was present throughout all these operations. In none of the eluate fractions was there noted an increase in the ninhydrin-CO_2 figure after applying the acid hydrolysis.

SUMMARY

Experiments are described which indicate that adsorption analysis on charcoal in aqueous solution can be used for the group separation of aliphatic from aromatic peptides, and for the further separation of the latter according to the number of aromatic amino-acid residues incorporated in a molecule. Separations of this kind should prove useful in analysing mixtures such as partial hydrolysates of gramicidin. Effects of stearic acid, phenol, ethanol, cetylpyridinium bromide and hydrochloric acid on the adsorption phenomena have been studied and in some cases utilised for improving the separations.

Some new data are presented on the relative roles of partition and adsorption phenomena in chromatography on filter paper.

We are grateful to Mr. S. Gerstedt and Miss Eileen Fallows for their technical assistance.

Part of the expenses for this investigation were defrayed by grants from the Swedish Natural Science Research Council.

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Received February 5, 1949.