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ADSORPTION ON NEUTRAL POLYSTYRENE RESIN

A SIMPLE METHOD FOR EXTRACTION OF 2,4-DINITROPHENYL DERIVATIVES FROM AQUEOUS SOLUTION AND FOR DECOLORATION OF PROTEIN HYDROLYSATES*

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SUMMARY

DNP-Derivatives of amino acids, peptides, and hexosamines, as well as dinitrophenylhydrazones are strongly adsorbed on Porapak Q from aqueous solutions. Adsorption of DNP-amino acids is strongest at low pH values and shows, in low salt concentrations, a minimum near pH 9. Addition of salts strongly increases their adsorption also in alkaline medium. The effect of "salting out on adsorbent" cannot be described by the general Setschenow equation and is influenced by the nature of the salt. In 1 *M* solutions of alkali halogenides at pH 8.8, the salting-out effect (DNP-cysteic acid on Porapak Q) is influenced much more by different anions than by different cations: $F > Cl > Br \gg J$ and $Li > Na \geqslant K \geqslant Cs$.

The adsorption isotherm of DNP-cysteic acid in 0.1 *N* HCl–0.1 *M* NaCl is nonlinear (linear capacity less than 0.6 %). The "logarithmic capacity" determined from the Freundlich isotherm is only about 1%, the total capacity being about 1 mmole/g Porapak Q. This nonlinearity suggests a heterogeneity of the adsorbent surface.

Adsorption of free phenylalanine is weak and practically not influenced by pH and salt concentration.

Procedures are described for adsorption of DNP-derivatives from acid hydrolysates and from dinitrophenylation mixtures. Application for decoloration of urine hydrolysates and extraction of other nonpolar compounds (steroids, dyes) is suggested.

INTRODUCTION

Liquid–liquid extraction procedures of small samples are time-consuming and difficult to automize for routine analysis. An alternative exists in adsorption on a column packed solid phase. Such columns can be handled much easier in series and avoid difficulties with emulsions which often cause delays in liquid–liquid extraction. Adsorption on charcoal is a well-known means for removing organic materials from

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aqueous solution. However, its adsorptive properties are insufficiently reproducible. Even small molecules, *e.g.* 2,4-dinitrophenylamino acids, may be bound irreversibly.

In a previous publication[1] we described a method for recognizing peptide patterns in human urine. Part of this method is the adsorption of DNP-peptides from aqueous solution on Porapak Q®. The observation that the adsorption of DNP-cysteic acid was not well reproducible led me to investigate the adsorption behavior of 2,4-dinitrophenyl (DNP) derivatives more thoroughly. The results will be described here.

Porapak Q® is a porous co-polymer of styrene and ethylvinylbenzene cross-linked with divinylbenzene*. The yellowish-white beads are of strong physical structure and available in controlled particle size and high internal porosity (specific area about 500 m^2/g). The material has found wide application as the stationary phase in gas chromatography[2,3], in chromatography with high-pressure gases or supercritical fluids[4], and in TLC of aromatic hydrocarbons[5] as suggested by JANÁK[6,7].

Recently, another neutral polystyrene resin, Amberlite XAD-2, was applied for the extraction of steroids and steroid glucuronides from aqueous solution[8,9], a technique first described by BRADLOW[8]. Unfortunately, this material is at present only available in relatively coarse beads.

MATERIALS AND PROCEDURES

Porapak Q®, 150–200 mesh, (Waters Associates, Framingham, Mass., U.S.A.) was swelled in ethanol and washed thoroughly with water and buffer.

All solvents and materials were of highest purity grade available. DNP-Derivatives of protein amino acids were purchased from Serva Entwicklungslabor, Heidelberg (G.F.R.); the DNP-derivatives of citrulline, cysteic acid, galactosamine, glucosamine, dipeptides, and tripeptides were synthesized using standard techniques[1,10,11] and thin-layer chromatography for purity control.

For equilibrium studies 300 mg of swelled Porapak Q was filled in a glass column of 10 mm I.D., provided with a glass disk, and was washed with buffer. 10 ml of sample solution were repeatedly (10 times) run through the column. The extinction of the solution was measured before and after equilibration, with or without previous dilution. The result was corrected for outer column volume (correction factor $f = 1.05$).

EXPERIMENTAL

Basic conditions for adsorption on neutral polystyrene resin

Adsorbent. Porapak Q must be wetted in ethanol for at least 15 min and the ethanol must then be removed completely by several water washings. A resin which was not wetted with an organic solvent does not adsorb at all. The ethanol-treated resin may be stored in distilled water for at least two weeks without significant change in its adsorption properties**.

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* Porapak Q contains keto groups. See under *Recovery*.

** 300 mg of Porapak Q pretreated with ethanol and stored in water for 16 days was washed with 0.2 *M* phosphate buffer (pH 7) and equilibrated with 0.3 mg of 2,4-DNP-cysteic acid sodium salt in 10 ml of the same buffer. The resin adsorbed 49% of the DNP-derivative, compared with 50% when the resin was washed with ethanol and buffer immediately before the experiment.

Swelling in a nonpolar solvent like xylene followed by short ethanol and long water washings does not change the adsorption capacity.

Solvent. The sample solution and the washings must be aqueous and should not contain organic solvents. Depending on the substances to be adsorbed, a few percent of ethanol or acetone may be permissible but the adsorption capacity of the resin may decrease considerably. On the other hand, a low pH and high salt concentration favor the adsorption of dinitrophenyl derivatives, as will be seen below.

Effect of pH

The influence of pH on the adsorption of several 2,4-dinitrophenyl derivatives and of phenylalanine on Porapak Q is shown in Fig. 1. Adsorption is strongest at low pH values. While the influence of pH on the adsorption of the free amino acid phenylalanine is rather small, all dinitrophenyl derivatives investigated show a clear adsorption minimum near pH 9 in 0.1 *M* buffer solutions. The amphoteric DNP-arginine, containing a strongly basic guanidino group, is adsorbed strongest, the sulfonic acid DNP-cysteic acid is bound weakest. Surprisingly, even DNP-cysteic acid can also be adsorbed quantitatively in alkaline medium on a short column provided a high salt concentration is applied. There is a second adsorption maximum near pH 11 in 1 *M* carbonate buffer as indicated in Fig. 1. It is interesting to note that BRADLOW[8] found no influence of pH on the adsorption of steroids on Amberlite XAD-2.

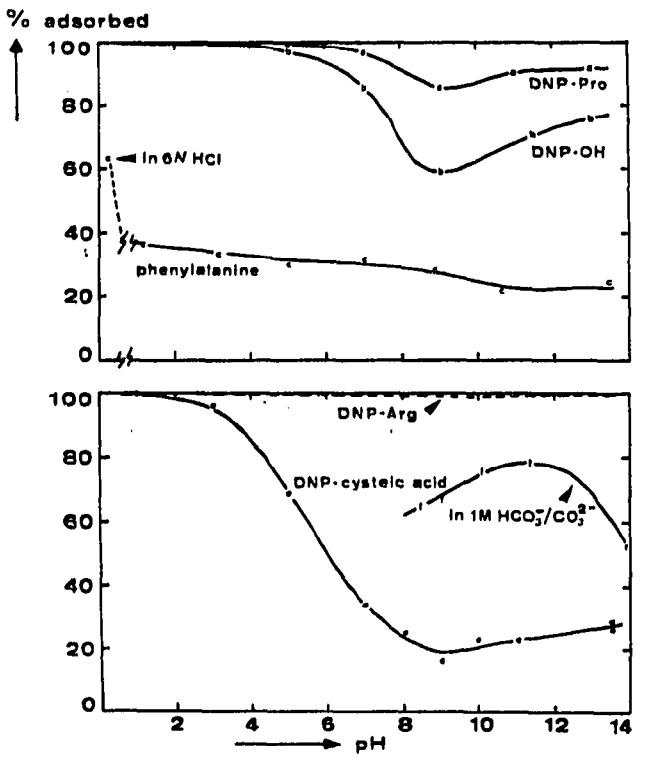


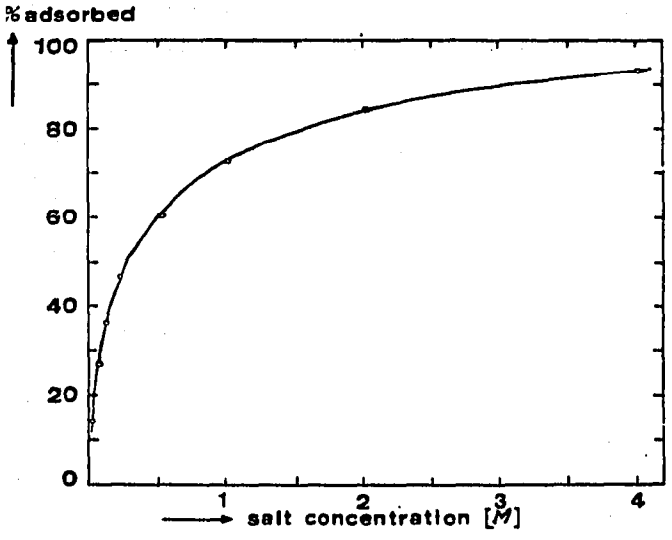
Fig. 1. Effect of pH on the adsorption of phenylalanine and DNP-derivatives on Porapak Q in 0.1 *M* (and 1 *M*) buffers. Note the weak influence of pH on the adsorption of phenylalanine and the strong on the adsorption of DNP-derivatives with a minimum near pH 9 in 0.1 *M* buffers and a further maximum near pH 11 in 1 *M* carbonate buffer. Arg: arginine; DNP-OH: 2,4-dinitrophenol. 0.1 *M* buffers used: 0.1 *N* HCl (pH 1); 0.1 *M* sodium citrate–HCl (pH 2–5); 0.1 *M* sodium–potassium phosphate (pH 6–8); 0.1 *M* glycine–KOH (pH 9–14).

Effect of salts and non-electrolytes

A very pronounced influence of salt concentration on the adsorption of dinitrophenyl derivatives on Porapak Q was found. This is shown in Fig. 2 for DNP-cysteic acid at pH 8.8. The effect of "salting out onto adsorbent" was shown for all DNP-derivatives investigated but not for the free amino acid phenylalanine. The general phenomenon of salting out (for a discussion see, *e.g.*, ref. 12) usually can be described by the Setschenow equation

$$\log \frac{S_0}{S} = \log \frac{f}{f_0} = k_s C_{salt}$$

where S_0 and S, f_0 and f refer to the solubility and the activity coefficient of the substance in water and salt solution, respectively, k_s is the salting out constant, and C_{salt} means salt concentration. This relation is based, among others, on the fact that the activity coefficient of a pure solid is constant. The presence of an adsorbent and the fact of a nonlinear adsorption isotherm (see below) alters the situation in so far as the activity coefficient of the adsorbed substance is no more constant but depends on the substance concentration. Hence, a plot of the logarithm of the solubility against the



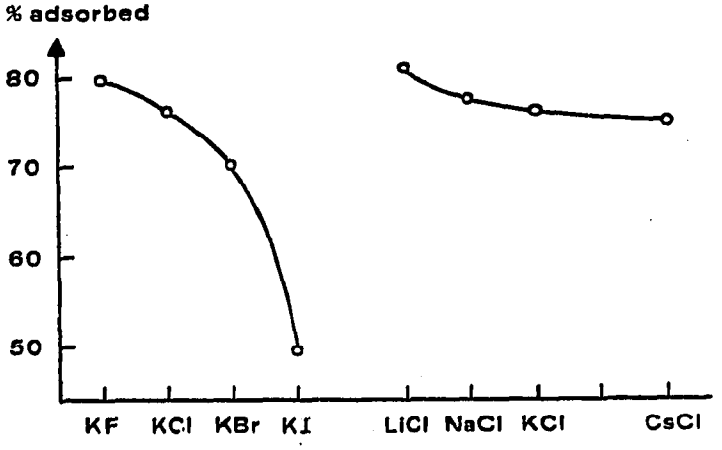


Fig. 2. Effect of sodium chloride concentration on the adsorption of DNP-cysteic acid (0.3 mg) on Porapak Q (300 mg) in 10 ml of 0.02 *M* glycine–KOH buffer pH 8.8 at 25°.

Fig. 3. Effect of 1 *M* potassium halides and of 1 *M* alkali chlorides on the adsorption of DNP-cysteic acid (0.3 mg) on Porapak Q (300 mg) in 10 ml of 0.05 *M* glycine–KOH buffer pH 8.8 at 22°.

salt concentration should not give a straight line. In fact, no straight line was obtained in the example described above (DNP-cysteic acid in sodium chloride solutions at pH 8.8 in presence of Porapak Q).

As may be expected, the nature of the salt is of some influence on the salting-out effect also in the presence of an adsorbent. A comparison of the alkali halogenides, applied as 1 *M* solutions at pH 8.8, shows that the nature of the cation is of minor influence (Fig. 3). The small lithium ion effects a somewhat stronger adsorption of DNP-cysteic acid than the other alkali cations. Similarly, the small fluoride anion

causes a stronger adsorption than the other halogenides; its effectiveness in comparison to the large iodide is striking. Obviously, an inversed influence is effected by anions and cations. This is suggested by Fig. 3, comparing the convex shaped curve of potassium halogenides (at the left) with the concave shaped curve of alkali chlorides.

Non-electrolytes do not favor adsorption of DNP-derivatives. As shown in Table I, 1 *M* solutions of glucose, urea, and especially of sucrose are able to desorb DNP-cysteic acid to some extent. As mentioned already, organic solvents such as alcohols and acetone, drastically decrease the adsorption of DNP-derivatives.

It is generally accepted that nonelectrostatic attraction from hydrophobic or van der Waals–London dispersion forces provides the driving forces for the binding of large ions to polymers. This is of importance especially if the solute is bearing a large hydrophobic group attached asymmetrically to the charged group, as in many DNP-derivatives, similar to detergents. However, in order to avoid the temptation to construct a *post facto* rationalization, it should not be tried to explain the above few observations in terms of hydrophobic forces, hydration energy, internal pressure of the solution, water structure, etc. Much more data would be necessary.

TABLE I

EFFECT OF POLAR NON-ELECTROLYTES AND SALTS ON ADSORPTION OF DNP-CYSTEIC ACID ON PORAPAK Q IN 0.05 *M* GLYCINE–KOH BUFFER pH 8.8 AT 22°

Concentration of additive	*DNP-cysteic acid adsorbed*[a] (%)
1 *M* Sucrose	12.0
1 *M* Urea	15.2
1 *M* Glucose	20.6
none	26.2
1 *M* Glycine buffer pH 8.8[b]	45.3
1 *M* NaCl	75.2

[a] 300 mg Porapak Q was equilibrated with 10 ml of buffer solution containing 0.3 mg DNP-cysteic acid sodium salt and additives tabulated.

[b] 0.1 *M* potassium glycinate–0.9 *M* glycine. In 0.1 *M* NaCl, about 40% would be adsorbed.

Adsorption isotherm at low pH

The adsorption of DNP-cysteic acid was measured as a function of sample concentration in 0.1 *N* HCl containing 0.1 *M* NaCl. The isotherm obtained is convex and similar to the Langmuir type. This would mean that monolayer adsorption rather than multilayer adsorption occurs. However, a bilogarithmic plot (Freundlich isotherm, see Fig. 4) shows a straight line only over a relatively small concentration range — up to about 12 μmole·g^{-1} — but with a remarkable steep slope ($n = 0.77$). A steep slope should mean high adsorption strength. Apparently the adsorbent surface is heterogeneous. Assuming a total adsorbent capacity of 10^3 μmole·g^{-1}, the adsorbing sites responsible for the lower linear part of the Freundlich isotherm (Fig. 4) adsorbe only about 1% of this amount ("logarithmic capacity").

Isotherm linearity and linear capacity generally decrease for more strongly adsorbed compounds and for heterogeneous adsorbents. In the system investigated the linear capacity (10% deviation from straight line in a lin–lin plot, see Fig. 5)

amounts to about 6 μmole$\cdot$g^{-1}, or about 0.6 % of the total capacity, and thus is very small. This means that chromatography under such conditions would be unfavorable because of the tenacious tailing which is to be expected, and probably would lead to incomplete elution of the bands.

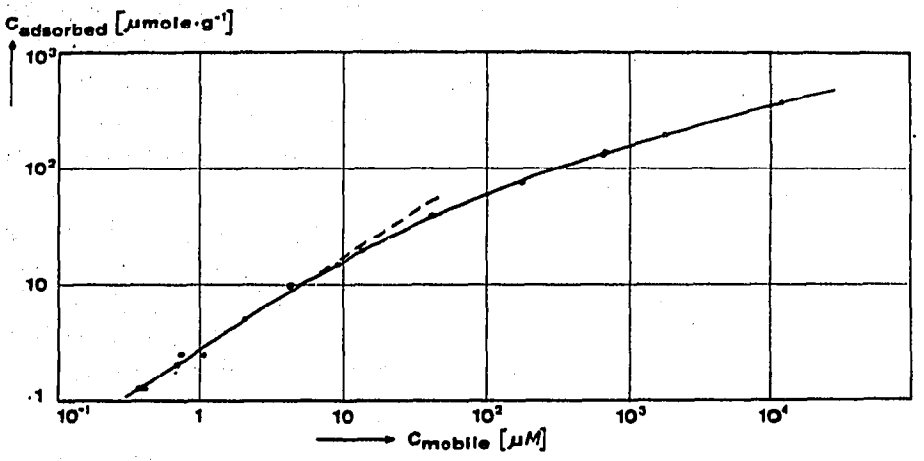


Fig. 4. Freundlich isotherm of DNP-cysteic acid on Porapak Q in 0.1 *N* HCl–0.1 *M* NaCl at 26°. The "logarithmic capacity" is only about 1% of the total capacity. The curve suggests surface heterogeneity of the adsorbent.

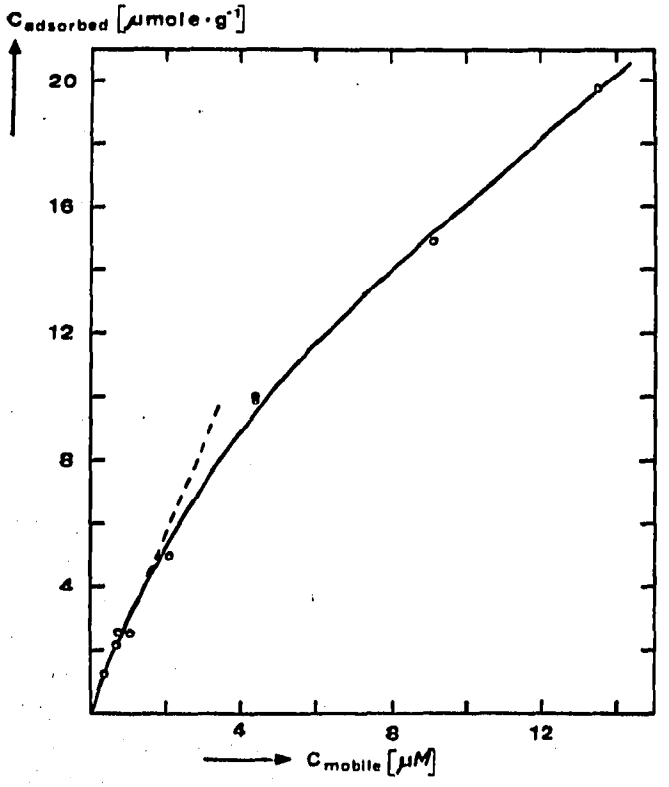


Fig. 5. Adsorption isotherm of DNP-cysteic acid in 0.1 *N* HCl–0.1 *M* NaCl at 26°. Note the non-linearity.

Adsorption capacity

As is evident from Figs. 1 and 2 and Table I, the adsorption capacity for DNP-derivatives is a function of pH and of the concentration of salts and other components

in the solvent. From the Freundlich isotherm (Fig. 4) a remarkably high theoretical adsorption capacity of about 1 mmole DNP-cysteic acid per g Porapak Q (in 0.1 *N* HCl containing 0.1 *M* NaCl at 25°) can be calculated. In practice, however, a somewhat lower value is to be expected. For instance, 0.5 mmole DNP-cysteic acid per g resin could be quantitatively adsorbed on a column of 10 × 80 mm in 6 *N* HCl, and 0.4 mmole DNP-arginine per g resin in 0.1 *N* HCl.

Recovery

All DNP-derivatives investigated (except dinitrophenylhydrazine, see below) are eluted quantitatively (> 97 %) with acetone–water (80:20). Occasionally, however, traces of previously adsorbed samples can be detected in washings following desorption (memory effect). Therefore, storage of the resin in acetone–water and washing of the resin prior to use is recommended.

Dinitrophenylhydrazine, applied in acid solution, binds irreversibly to some extent. It reacts with keto groups which are present in Porapak Q and which can be demonstrated also by IR-spectroscopy. To avoid this irreversible binding, the keto groups of the polymer can be reduced to the alcohol by sodium borohydride in methanol or to the hydrocarbon by hydrazine hydrate–sodium ethylate in ethylene glycol at 190°. Amberlite XAD-2 is free of keto groups.

Cleaning of the resin

A resin contaminated by impurities, *e.g.* from a urine hydrolysate, is purified by washing the column with the strong detergent RBS-25* followed by washings with water and ethanol. This treatment does not change the adsorption properties of the resin. However, contamination by a denaturated protein may not be removed in all cases. For instance, cytochrome C, equilibrated with Porapak Q, could not be removed completely. As mentioned above, also dinitrophenylhydrazine, applied in 0.1 *N* HCl, binds irreversibly to Porapak Q.

APPLICATIONS

Adsorption of DNP-derivatives from acid hydrolysate

In a total hydrolysate of DNP-peptide or DNP-protein, free amino acids and DNP-amino acids usually are subjected to different methods of analysis. Therefore, separation of DNP-amino acids from the free amino acids is advantageous, especially if only low amounts are available. The following procedure may be used, scaled down for small amounts, if necessary. 2 ml hydrolysate containing up to 70 μmole DNP-amino acid in 6 *N* HCl are diluted tenfold with distilled water and slowly filtered through a small column filled with 300 mg Porapak Q, equilibrated with 0.1 *N* HCl. The resin is washed with 15 ml of 0.1 *N* HCl (or 5 % acetic acid). The combined eluates (fraction 1) containing the free amino acids are evaporated in vacuo. The DNP-derivatives adsorbed on Porapak Q are then eluted with 10 ml of acetone–water (80:20) and evaporated to dryness in a stream of nitrogen at 50° (fraction 2). As shown in Table II, all amino acids are found quantitatively in fraction 1. The only exception is phenylalanine**, which is recovered to 80 % in fraction 1, the other 20 % being eluted

* Available from Carl Roth, OHG, Karlsruhe, G.F.R.

** Tryptophan would also be adsorbed[14], but is destroyed in the foregoing acid hydrolysis.

TABLE II

ADSORPTION OF DNP-DERIVATIVES FROM ACID HYDROLYSATES

Percentage recovery of free amino acids after filtration through Porapak Q in 0.6 *N* HCl and washing with 0.1 *N* HCl (fraction 1) and 50% ethanol (fraction 2), respectively. Fraction 2 may contain DNP-derivatives. The test solution contained 2.5 μmole of each amino acid in 2 ml of 6 *N* HCl. Other details see text.

Amino acid	*Fraction 1*	*Fraction 2*
Asp	105	0
Thr	104	0
Ser	100	0
Pro	100	0
Glu	100	0
Gly	99	0
Ala	98	0
Val	95	0
$(Cys)_2$	101	0
Met	102	0
Ile	104	0
Leu	98	0
Tyr	95	0
Phe	80	17
Lys	105	0
His	99	0
Arg	98	0

in fraction 2 with the DNP-amino acids. It is interesting that phenylalanine is also strongly adsorbed on the basic ion-exchanger Zerolit H (containing a polystyrene matrix) under conditions used for separation of free amino acids and DNP-amino acids[13]. The above observation (Table II) is a direct indication that the adsorption of phenylalanine on such ion-exchangers is caused also by the matrix itself, *i.e.* by polystyrene.

Decoloration of urine hydrolysates

Decomposition products formed during acid hydrolysis of urine may interfere with assays of polar constituents based on colorimetry. The hydrolysate is easily decolorized by filtering through a small column of Porapak Q, pretreated with 0.1 *N* HCl. The column is washed afterwards with 0.1 *N* HCl or 5 % acetic acid. Aromatic compounds may be retained partially. In this case, the elution procedure described above (adsorption of DNP-derivatives from acid hydrolysates) is recommended. In any case it will be necessary to check and correct for losses and dilution in the standardized procedure. The resin may be cleaned with RBS-25, as described above.

Adsorption of DNP-amino acids and DNP-peptides after dinitrophenylation

As indicated in Fig. 1, DNP-derivatives can be adsorbed in alkaline medium on Porapak Q in a solvent of high salt concentration. Hence, it is possible to avoid the time-consuming extraction of DNP-derivatives after dinitrophenylation and to get one single fraction. In general, the conventional separation of "ether-soluble" and "acid-soluble" DNP-derivatives is not advisable because di-DNP-histidine and many DNP-peptides are spread over both fractions.

Adsorption from alkaline solution. To the reaction mixture, containing up to 100 μmole of DNP-derivatives and less than 10 % ethanol, 1 volume of 2 *M* NaCl is added. The mixture is filtered slowly through 1 g Porapak Q in a column, prewashed with 1 *M* NaCl. Then the resin is washed successively with 5 ml of 1 *M* NaCl, 2 × 5 ml of 1 *N* HCl, and 2 × 5 ml of 5 % acetic acid. The first 5-ml portion of the 1 *N* HCl is used previously to rinse the reaction vessel and then is transferred to the column. The DNP-derivatives are eluted with 10–20 ml of acetone–water (80:20). Again, small portions of this solvent are employed previously for rinsing the reaction vessel, and these washings are transferred quantitatively to the column.

Adsorption from acid solution. If the dinitrophenylation has been carried out with a large excess of fluorodinitrobenzene, it may be advantageous to remove first the excess reagent by ether extraction in alkaline medium. After removal of the remaining ether from the aqueous phase under *vacuo*, the solution is adjusted to pH $\leqslant 2$ with conc. HCl and filtered through the Porapak Q column. Vessel and Porapak are washed with 2 × 5 ml of 5 % acetic acid. The vessel is then carefully rinsed with small portions of acetone–water (80:20), these solution are transferred to the column, and the DNP-derivatives are eluted with acetone–water (80:20), totalling about 10–20 ml.

Further applications

Porapak Q was also shown to adsorb easily dinitrophenylhexosamines, dinitrophenylhydrazones, estrogens, and dyes from aqueous solutions. It is to be expected that the simple adsorption on neutral polystyrene resin may replace the liquid–liquid extraction of still other substances bearing a large nonpolar group.

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