# A General Method for the Specific Isolation of Peptides Containing Modified Residues, Using Insoluble Antibody Columns*

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ABSTRACT: A method is described for the specific isolation of peptides containing modified residues from selectively modified proteins. The method makes use of the affinity of antibodies specific to the group introduced by the modification. Antibody–Sepharose conjugates were thus used as a column for sequestering specifically and in high yields the peptides which contained the modified residue. These peptides can be eluted from the antibody–Sepharose columns under conditions which dissociate antigen–antibody bonds. This procedure enables the isolation of a modified peptide in essentially one step. Amino acid analysis of the isolated peptides allows the localization of the modified residue in the amino acid sequence of the protein. Two examples were chosen to illustrate this method. (1) Arsanilazotyrosyl-containing peptides were isolated from arsanilazo-*N*-succinylcarboxypeptidase A by anti-azobenzenearsonate antibodies. The modified tyrosyl residues in carboxypeptidase A were found to be in positions 19, 248, and 277. (2) A DNP-lysyl-containing peptide was isolated from mono-DNP bovine pancreatic ribonuclease by anti-DNP antibodies. The modified lysyl residue in ribonuclease was found to be in position 41 in agreement with previous findings.

Selective chemical modification of proteins is an important tool in studying their structure–function relationship (for reviews, see Cohen, 1968, Vallee and Riordan, 1969, and Shaw, 1970). The precise localization of the modified residue in the amino acid sequence is a prerequisite for the assessment of this relationship in a protein. The major difficulty in the localization of the modified amino acid is the isolation of the peptide which contains the modified residue. This is due mainly to the following. (a) The modifying reagent often reacts with several residues in various yields and the digest of the protein will therefore contain several modified peptides, each present in less than molar amounts. (b) The conventional methods for isolation of peptides require laborious and time-consuming steps, and each may cause a reduction in the final yield of the peptide. Therefore, it is desirable to have a method which will enable a one-step isolation of the modified peptide from the peptide mixture derived from a modified protein. Such a method must rely on an agent which will possess affinity only for the group introduced by the modification.

Recently we described a method for the isolation of modified peptides from affinity-labeled proteins (Givol *et al.*, 1970). In that method the native protein, having affinity to the ligand used for affinity labeling of the protein itself, was utilized for specifically sequestering the ligand-bound peptide. This method, which is limited to cases of affinity labeling, can be extended to many other chemical modifications if antibodies, specific to the modified moiety, are to be used as the isolating agent. Specific antibodies with high affinity can be raised against almost any small molecule (Landsteiner, 1936; Pressman and Grossberg, 1968) and they are therefore suitable regents for the isolation of modified peptides which have such small molecules attached to them.

We chose two examples to illustrate the applicability of this method: (1) the isolation of arsanilazotyrosyl peptides from arsanilazocarboxypeptidase A (Kagan and Vallee, 1969) employing anti-Ars[1] antibodies; (2) the isolation of the DNP-lysyl peptide from mono-DNP-ribonuclease (Hirs *et al.*, 1965) employing anti-DNP antibodies.

The isolation of the modified peptides can, in principle, be performed in solution (Thorpe and Singer, 1969; Weinstein and Shaltiel, 1970), but it is greatly facilitated by the use of insoluble antibody–Sepharose columns (Givol and Rotman, 1970), employing affinity chromatography (Cuatrecassas *et al.*, 1968) as the major technique. The high capacity of such an antibody column, the relatively high affinity of antibodies for a ligand and the fast rate of hapten–antibody interaction make this method a very useful tool for one-step isolation of modified peptides from the entire peptide mixture of a protein digest.

## Materials and Methods

*Antigens and Antibodies.* DNP-BSA and DNP-OV were prepared according to Eisen *et al.* (1953), using 2,4-dinitrobenzenesulfonate in a weight ratio of 1:10 to protein. Ars-BSA and Ars-RSA were prepared by reacting the protein with the diazonium salt of *p*-arsanilic acid, according to the procedure described by Tabachnick and Sobotka (1959, 1960). Antisera were raised in goats by injecting 2 mg of the antigen (DNP-BSA or Ars-BSA) in complete Freund's adjuvant at multiple intradermal sites. The antibody content of the serum was determined by a precipitin reaction with the cross-reacting modified proteins (DNP-OV and Ars-RSA for anti-DNP and anti-Ars, respectively). Anti-DNP sera contained 2.5–3 mg of antibody/ml and anti-Ars sera contained 0.9–1.2 mg of antibody/ml. Antibodies were purified on Sepharose immunoadsorbents as follows. Anti-Ars serum was adsorbed with 4-(*p*-aminophenylazo)phenylarsonic acid–Sepharose conjugate. After 1-hr incubation the immunoadsorbent was washed with PBS, on a sintered-glass funnel, until the absorbance at 280

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[1] Abbreviations used are: Ars, arsanilazo; FDNB, 1-fluoro-2,4-dinitrobenzene; BSA, bovine serum albumin; OV, ovalbumin; RSA, rabbit serum albumin; PBS, 0.01 M sodium phosphate–0.14 M NaCl (pH 7.4); CPD, carboxypeptidase; Gdn·HCl, guanidine hydrochloride.

nm was less than 0.05, and the antibodies were eluted by incubation of the Sepharose conjugate for 1 hr with 0.3 M *p*-arsanilic acid (brought to pH 8.0 with NaOH) in PBS. The eluted antibodies were dialyzed for 3 days against PBS to remove the hapten until the ratio of absorbance at 280/250 nm was 2.8. Anti-DNP antibodies were similarly purified using DNP-OV–Sepharose conjugate as the immunoadsorbent. The adsorbed antibodies were eluted by 0.1 M acetic acid followed by adjusting the solution of the eluted antibodies to pH 5 and dialysis against PBS. The yields of eluted antibodies were 100% for anti-Ars and 85% for anti-DNP.

*Preparation of Sepharose Conjugates.* This was performed by a modification of the procedure described by Axen *et al.* (1967). Sepharose 4B was washed on a sintered-glass funnel with water. The washed Sepharose (100 g wet weight) was suspended in water (300 ml) and solid CNBr (10 g) was added to the suspension. The pH of the solution was brought to pH 11 with 5 N NaOH and kept between pH 10.8 and 11.2 for 8 min by the addition of NaOH. Continuous stirring during the reaction assured complete dissolution of the CNBr during the first 5 min. The reaction was terminated by filtration and washing with water. The activated Sepharose was added to the proteins or hapten which were in solutions of 0.1 M $NaHCO_3$ (pH 8) in the following ratios of wet weight of Sepharose to weight of protein or hapten 30:1 in the case of antibodies, 15:1 in the case of antigens, 600:1 in the case of the hapten 4-(*p*-aminophenylazo)phenylarsonic acid. The concentration of protein in the solutions varies from 0.5 to 5 mg per ml in different preparations. The suspension was stirred slowly at 4° for 16 hr and then washed until no more absorbance was detected in the filtrate. The yields of binding as estimated from the absorbance in the solutions before and after coupling to Sepharose were 95% for the antibodies, 70–80% for the antigens, and 14% for the hapten.

Previously we reported the preparation of anti-DNP–Sepharose conjugate in the presence of a low-affinity hapten (DNP-OH) to protect the antibodies (Givol and Rotman, 1970; Givol *et al.*, 1970). This was found unnecessary, however, and the reaction performed without protection of the antibody sites yielded comparably good preparations. The antibody–Sepharose conjugate containing 15–20 mg of antibodies/ml of packed Sepharose was washed with PBS, suspended in a known volume of PBS, and stored at 4°. The capacity of these conjugates were tested in a small column, prepared in Pasteur pipets with known amounts of antibody–Sepharose conjugate, using [$^{14}$C]$\epsilon$-*N*-DNP-lysine for anti-DNP–Sepharose and *N*-benzyloxycarbonyl(*p*-azobenzenearsonate)-L-tyrosine ($\epsilon^{M}_{325}$ 21,600, Tabachnik and Sobotka, 1959) for anti-Ars–Sepharose. Anti-DNP–Sepharose can adsorb 1.3 moles of [$^{14}$C]$\epsilon$-*N*-DNP-lysine/mole of bound antibody and anti-Ars–Sepharose can adsorb 1.2 moles of arsanilazotyrosine/mole of bound antibody. These results were reproducible and demonstrate that the coupling of the antibodies to Sepharose did not result in significant loss of activity. In the same way the conditions for elution of bound haptens or modified peptides, as well as the regeneration of binding capacity after each elution were tested. These details will be given in the Results section.

The operation of the antibody–Sepharose column was as follows: an amount of antibody–Sepharose conjugate, estimated to bind all the modified peptides present in the digest, was packed into a small column with sintered glass (No. 1) at the bottom. A small amount of Sephadex G-25 (coarse) was layered on the top of the column (4–5 mm height). The column was washed with 0.1 M $NH_4HCO_3$ to avoid salt in the unadsorbed peptide fraction, and the enzymic digest, after inactivation of the enzyme, was applied to the column. The column was washed with 0.1 M $NH_4HCO_3$ until the absorbance of the fractions was less than 0.02 and 0.2 at 280 and 215 nm, respectively. Elution of the adsorbed peptides was performed with 1 M $NH_4OH$ for Ars peptides, and 6 M Gdn·HCl for DNP-Lys peptides, according to the findings in preliminary trials with small amounts.

TABLE I: Amino Acid Composition of Ars-succinylcarboxypeptidase A, Succinylcarboxypeptidase A, and Carboxypeptidase A.[a]

| Amino Acid | Ars-succinyl-CPD | Succinyl-CPD | CPD |
|---|---|---|---|
| Asp | 29.8 | 28.5 | 29 |
| Thr | 21.0 | 22.1 | 26 |
| Ser | 29.5 | 26.8 | 32 |
| Glu | 26.3 | 24.0 | 25 |
| Pro | 9.5 | 10.3 | 10 |
| Gly | 24.2 | 22.4 | 23 |
| Ala | 20.0 | 20.0 | 20 |
| ½-Cys | 1.6 | 1.4 | 2 |
| Val | 14.8 | 13.9 | 16 |
| Met | 2.8 | 3.0 | 3 |
| Ile | 16.6 | 15.8 | 20 |
| Leu | 21.2 | 20.5 | 23 |
| Tyr | 16.9 | 18.7 | 19 |
| Phe | 15.8 | 15.7 | 16 |
| Lys | 13.0 | 12.9 | 15 |
| His | 7.9 | 8.0 | 8 |
| Arg | 10.7 | 11.2 | 11 |

[a] The composition was determined after 24 hr of 6 N HCl hydrolysis at 110°. No correction for destruction or for low recovery of amino acids was made. Ars-succinyl-CPD and succinyl-CPD were a gift of Drs. B. Vallee and J. F. Riordan. The data on CPD are based on the amino acid sequence (Bradshaw *et al.*, 1969). The results are expressed in terms of residues per mole of protein.

*Modified Proteins.* *p*-Arsanilazo-*N*-succinylcarboxypeptidase A (Ars-CPD) was a kind gift from Drs. B. Vallee and J. F. Riordan. This sample was prepared by the reaction of succinyl-CPD with *p*-azobenzenearsonate at pH 9.5 and it is therefore different in this respect from the preparation described by Kagan and Vallee (1969). The amount of azotyrosine and azohistidine in this preparation was calculated from the visible spectrum using the simultaneous equation given by Tabachnik and Sobotka (1959). The amount of protein used in this determination was determined by amino acid analysis. The spectral data indicate the presence of 1.65 Ars-tyrosine and 0.2 Ars-histidine per mole of CPD. The amino acid analysis of the succinyl-CPD and the Ars-succinyl-CPD are given in Table I. It is shown that the amount of tyrosine in the Ars-CPD is lower by 1.8 residues from that of succinyl-CPD. It is not clear why the values obtained for lysine are lower than theoretical (Table I) but no decrease of lysine was observed in the Ars-CPD relative to succinyl-CPD. Since the coupling with *p*-azobenzenearsonate was performed on the succinyl enzyme, we assume that no modification of lysine did occur. This was also confirmed by the spectrum of the isolated peptides (see Results).

Mono-DNP-ribonuclease was prepared according to Mur-

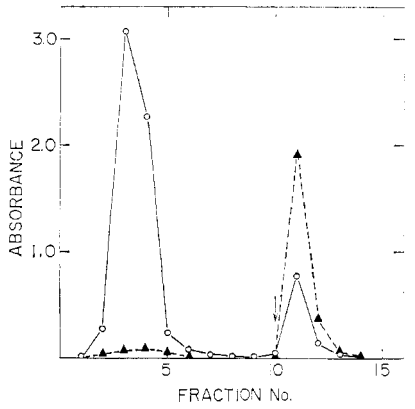


FIGURE 1: Fractionation of Ars-peptides derived from subtilisin digest of Ars-CPD on anti-Ars–Sepharose column. A digest of 0.5 μmole of Ars-CPD was applied to a 1 × 3.4 cm anti-Ars–Sepharose column (containing 0.44 μmole of antibody). After washing with 0.1 M $NH_4HCO_3$ elution was started by 1 M $NH_4OH$ (arrow). Fractions of 3.2 ml were collected. (○) Absorbance at 280 nm; (▲) absorbance at 325 nm after neutralization with $CO_2$.

dock *et al.* (1966). Phosphate-free bovine pancreatic RNase (40 mg) was reacted at pH 8 with FDNB (240 mg) for 12 min. The reaction was terminated by acidification with HCl to pH 2.5 and the excess reagent was extracted with benzene. The DNP-protein was lyophilized, dissolved in water, and chromatographed on IRC-50 column as previously described (Murdock *et al.*, 1966). The fraction corresponding to mono-DNP(Lys-41)-RNase was collected and used in this study. This DNP-RNase has an equal absorbance at 280 and 360 nm which indicates (after subtracting the contribution of the DNP group to absrobance at 280 nm) 0.9 mole of DNP group per mole of RNase. The amino acid composition of the modified RNase and of the parent protein used for dinitrophenylation is given in Table II. This analysis demonstrates the disappearance of one lysine residue.

TABLE II: Amino Acid Composition of DNP-RNase and the Parent Protein used for Dinitrophenylation.[a]

| Amino Acids | DNP-DNase | RNase[b] | RNase[c] |
|---|---|---|---|
| Asp | 15.0 | 15.3 | 15 |
| Thr | 8.7 | 9.0 | 10 |
| Ser | 14.1 | 13.2 | 15 |
| Glu | 11.8 | 12.0 | 12 |
| Pro | 4.1 | 3.8 | 4 |
| Gly | 3.2 | 3.1 | 3 |
| Ala | 12.0 | 12.0 | 12 |
| ½-Cys | 6.4 | 7.0 | 8 |
| Val | 8.3 | 8.6 | 9 |
| Met | 3.3 | 2.7 | 4 |
| Ile | 2.7 | 2.2 | 3 |
| Leu | 1.8 | 2.0 | 2 |
| Tyr | 5.5 | 5.6 | 6 |
| Phe | 3.0 | 3.0 | 3 |
| Lys | 9.1 | 10.3 | 10 |
| His | 3.9 | 3.8 | 4 |
| Arg | 4.0 | 4.0 | 4 |

[a] Composition was determined after 24 hr of 6 N HCl hydrolysis at 110°. No correction for destruction or for low recovery of amino acids was made. The results are expressed in terms of residues per mole of protein. [b] The protein used for dinitrophenylation. [c] Based on data from Dayhoff (1969).

*Enzymes.* Trypsin (TPCK treated), chymotrypsin, carboxypeptidase A, lysozyme, and bovine pancreatic ribonuclease were purchased from Worthington. Subtilisin-type Carlsberg (subtilopeptidase A) was obtained from Novo Industries, Copenhagen.

Digestion with enzymes (1:50, w/w ratio of enzyme to protein) was performed in 0.1 M $NH_4HCO_3$ at 37° for 4 hr unless otherwise specified. Subtilisin or chymotrypsin digestion was stopped by acidification to pH 2.5 for 20 min. Trypsin was inhibited by soybean trypsin inhibitors (Worthington).

*Materials.* Sepharose 4B and Sephadex G-25 were purchased from Pharmacia, Uppsala. FDNB and dansyl chloride were from Fluka, *p*-arsanilic acid from Eastman Kodak, and 4-(*p*-aminophenylazo)phenylarsonic acid from Aldrich. *N*-Benzyloxycarbonyl-(*p*-azobenzenearsonate)-L-tyrosine was prepared by the coupling of the diazonium salt of *p*-arsanilic acid with *N*-benzyloxycarbonyl-L-tyrosine according to Tabachnick and Sobotka (1959). The product was purified on DEAE-cellulose equilibrated in 0.1 M $NH_4HCO_3$, employing a gradient from 0.1 M $NH_4HCO_3$ to 0.5 M $NH_4HCO_3$. The first peak (orange material) was collected and was found to correspond to the monoazoarsanil derivative of tyrosine (Tabachnick and Sobotka, 1959).

*Analytical and Other Methods.* Amino acid analyses were performed as described by Moore and Stein (1963). Values reported are uncorrected for losses during hydrolysis. Ars derivatives were destroyed by hydrolysis (Kagan and Vallee, 1969) and their content was estimated from the absorption at 325 nm, in neutral pH, of the sample before hydrolysis, using $\epsilon_{325}^{max}$ of 21,600 (Tabachnik and Sobotka, 1959). N-Terminal residue of peptides was determined by dansyl chloride (Gray, 1967) and the dansylamino acids were identified by thin-layer chromatography on polyamide sheets (Woods and Wong, 1967). C-Terminal residue was determined by identification of the dansylamino acid after digestion with carboxypeptidase A (12 hr in 0.1 M *N*-ethylmorpholin acetate, pH 8). N-Terminal Ars-Tyr did not give a detectable spot after dansylation and hydrolysis, presumably due to destruction during the acid hydrolysis.

High-voltage electrophoresis on Whatman No. 3MM papers was run at pH values of 3.5 and 6.5. Performic acid oxidation was performed as described by Hirs (1968).

Absorbance of peptide mixtures was read at appropriate wavelengths using Zeiss PMQII spectrophotometer.

## Results

*Localization of Arsanilazotyrosyl Residues in p-Arsanilazo-N-succinylcarboxypeptidase A.* Ars-CPD (0.5 μmole in 5 ml of 0.1 M $NH_4HCO_3$) was digested with subtilisin for 3 hr at 37°. The reaction mixture was acidified with 2 N HCl to pH 2.5 in order to inactivate subtilisin and after 20 min it was neutralized with 3 N NaOH. This digest was applied to anti-Ars–Sepharose column (1 × 3.4 cm containing 0.44 μmole of bound antibody). The elution pattern of this column is shown in Figure 1. About 95% of the arsanilazo peptides were adsorbed by the column. Preliminary experiments showed that the adsorbed yellow peptides could be eluted from the column with either the uncolored hapten arsanilic acid (0.3 M) or with 1 M $NH_4OH$ (pH 11.7). Since it was found difficult to quantita-

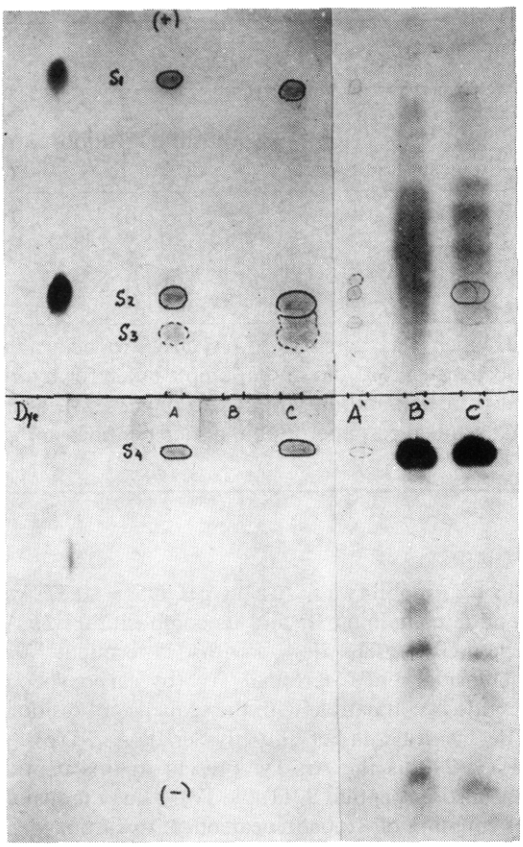


FIGURE 2: Paper electrophoresis of subtilisin digest of Ars-CPD. (A) Peptides eluted from antibody–Sepharose by 1 M $NH_4OH$. (B) Peptide mixture unadsorbed by the column. (C) Digest before antibody–Sepharose column. Electrophoresis was at pH 6.5 at 4 kV for 20 min. The dye on the left-hand side was a mixture of orange G, xylene cyanol FF, and basic fuchsin that served as visible markers during the run. The right-hand side of the electropherogram was photographed after staining with ninhydrin and the left-hand side was photographed without staining. The yellow peptides are marked.

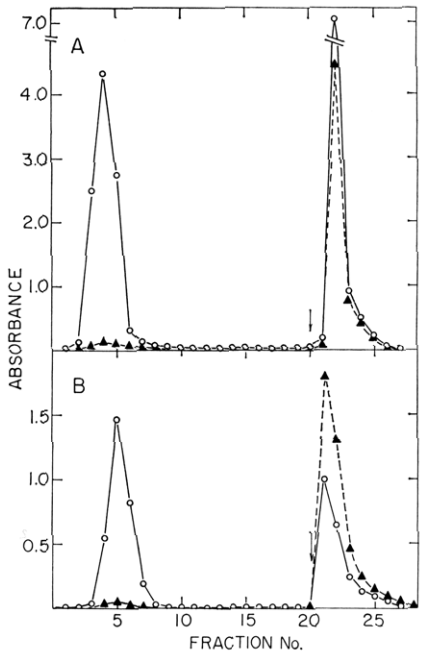


FIGURE 3: Fractionation of Ars-peptides derived from tryptic (A) and chymotryptic (B) digest of Ars-CPD. (A) A tryptic digest of 1.45 $\mu$moles of Ars-CPD was applied to a 1 $\times$ 11 cm anti-Ars–Sepharose column (1.4 $\mu$moles of antibody). (B) A chymotryptic digest of 0.72 $\mu$mole of the isolated tryptic Ars-peptides (A) was applied to a 2.2 $\times$ 3 cm anti-Ars–Sepharose column. Fraction volume was 3.5 ml. (○) Absorbance at 280 nm; (▲) absorbance at 325 nm.

tively remove the hapten from the eluted peptides, the elution was performed with 1 M $NH_4OH$. The elution can easily be followed by the movement of the pink boundary due to the change of color of the azo derivatives at alkaline pH.

The recovery of absorbance at 280 and 325 nm of the different fractions is given in Table III. After elution the column was washed immediately with PBS until the pH was neutral. The capacity of the used column was tested again and found to be 80–85% of the capacity of the column at the first use. No change in the capacity of the column was observed after the second cycle of adsorption and elution, and the column was used six times without a decrease in its capacity.

A comparative electropherogram of the enzymic digest and the different fractions eluted from the column is given in Figure 2. It is shown (a) that the 1 M $NH_4OH$ eluate contained only yellow Ars-peptides, (b) that the peptides in the eluate were present in the whole digest, and (c) that the unadsorbed fraction contained practically all the peptides present in the original digest except the yellow Ars-peptides. This indicates that the antibody–Sepharose column adsorbed all the Ars-peptides (96.5%, Table III) and only the Ars-peptides. The eluted Ars-peptides were separated by paper electrophoresis at pH 6.5 (Figure 2), and were eluted from the paper by water. The spectrum of the eluted peptides ($S_1$ to $S_4$) in 0.1 M NaOH clearly showed a peak at 480 nm indicating that they contain Ars-Tyr residue (Burton and Waley, 1967). The mobility, composition, and N-terminal residues of the different Ars-peptides, together with the position of the modified tyrosines inferred from these data, are given in Table IV. The relative recovery of each peptide was estimated from the absorbance of the eluted material at 325 nm. Some minor peptides which together accounted for 14.5% of the peptides eluted from paper were not analyzed. As is shown in Table IV, about 25% (not corrected for losses during elution from paper) of the labeled tyrosyl residues which yielded the peptide Ile-Ars-Tyr, could not be localized because there are three such sequences in the molecule (positions 19, 48, and 248, Bradshaw *et al.*, 1969). In order to obtain a larger Ars-peptide, a tryptic digestion was attempted (Figure 3A). The tryptic yellow fraction eluted by 1 M $NH_4OH$ from the antibody column did not

TABLE III: Adsorption and Elution of Ars-Peptides on Anti-Ars–Sepharose Column.[a]

| | Unadsorbed (%) | Eluate (%)[b] |
|---|---|---|
| Absorbance at 280 nm | 84 | 14 |
| Absorbance at 325 nm | 4.5 | 81 |

[a] A subtilisin digest of Ars-CPD containing 21.5 optical density units at 280 nm and 10.25 optical density units at 325 nm was applied to a 1 $\times$ 3.4 cm antibody–Sepharose column. [b] Elution was with 1 M $NH_4OH$. The readings at 325 nm were taken after bringing the pH to neutrality with $CO_2$.

TABLE IV: Composition and Sequence Ars-Peptides Derived from a Subtilisin Digest of Ars-CPD.

| Peptide | Mobility[a] | Amino Acid Composition[b] | N-Terminal Residue | Suggested Sequence[c] | Possible Position of Ars-Tyr[c] | Yield (%)[d] |
|---|---|---|---|---|---|---|
| $S_1$ | −0.87 | $Asp_{1.0}$, Ars-$Tyr_{1.0}$ | | Tyr-Asp | 19 | 14.6 |
| $S_2$ | −0.37 | $Ile_{1.0}$, Ars-$Tyr_{1.3}$ | Ile | Ile-Tyr | 19 or 48 or 248 | 24.3 |
| $S_3$ | −0.30 | $Arg_{1.0}$, $Asp_{1.0}$, $Thr_{0.9}$, $Gly_{1.1}$, Ars-$Tyr_{1.0}$ | Asp | Asp-Thr-Gly-Arg-Tyr | 277 | 8.5 |
| $S_4$ | 0.0 | $Arg_{1.0}$, $Gly_{1.0}$, Ars-$Tyr_{1.1}$ | Gly | Gly-Arg-Tyr | 277 | 7.0 |

[a] Mobility was calculated *vs.* the mobility at pH 6.5 of aspartic acid which was taken as −1 relative to neutral amino acids. [b] The amount of Ars-Tyr in the peptides was estimated from the absorbance at 325 nm of the sample taken for hydrolysis. [c] According to the sequence published by Bradshaw *et al.* (1969). [d] Relative to the amount applied to paper as measured by absorbance at 325 nm. The total yield of eluted peptides was 64% of that applied to paper. Some minor peptides comprising about 10% of the absorbance were not analyzed.

move from the origin upon electrophoresis at pH 6.5, and the amino acid analysis could not be interpreted to implicate one single peptide. Hence, this yellow fraction isolated from the tryptic digest was further digested with chymotrypsin and applied to the antibody–Sepharose column (Figure 3B). The recoveries of the eluted Ars-peptides as measured by absorbance at 325 nm were 80 and 95% for the tryptic and chymotryptic peptides, respectively.

The eluted tryptic–chymotryptic peptides were separated by high-voltage electrophoresis at pH 6.5 to yield three major peptides:peptide $C_1$ (yield 13.3%) which was identical in mobility, composition, and yield to the peptide $S_1$ (Table IV), peptide $C_2$ (yield 13.8%), which had the composition Ars-$Tyr_{1.0}$,$Gly_{1.1}$ without detectable N-terminal residues and is compatible with the equence Tyr-Gly resulting from tryptic split at Arg-276, and chymotryptic cleavage at Gly-278 (Bradshaw *et al.*, 1969). This peptide must contain the same Ars-Tyr residue present in peptides $S_3$ and $S_4$ (Table IV). Peptide $C_3$ (yield 15.1%, mobility *vs.* Asp at pH 6.5 = 0.37) was the only peptide containing Ile and its composition was $Asp_{0.9}$,-$Ser_{1.5}$,$Glu_{1.1}$,$Gly_{1.9}$,$Ala_{1.0}$,$Ile_{2.0}$, with N-terminal Ile (by dansyl chloride) and C-terminal Trp (by carboxypeptidase). This peptide is compatible with the sequence of residues 247–257 (Ile-Tyr-Gln-Ala-Ser-Glu-Gly-Ser-Ile-Asp-Trp) which implies Tyr-248 as the Ars-Tyr present in this peptide and therefore also in peptide $S_2$ (Table IV). Thus the major positions of coupling of azobenzenearsonate to carboxypeptidase A are Tyr-248, Tyr-277, and Tyr-19. The data show that Tyr-248 was modified more extensively than the other tyrosyl residues.

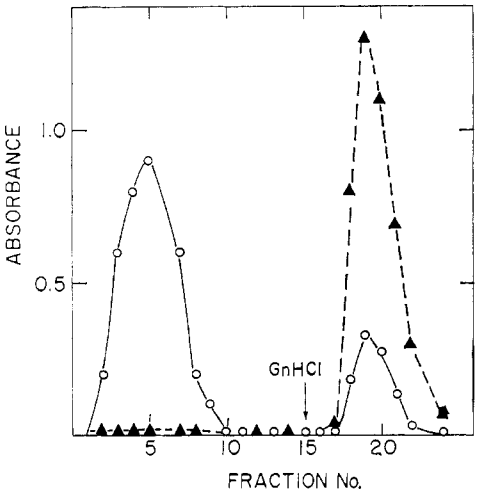


FIGURE 4: Fractionation of DNP-peptides derived from 41-DNP-RNase on anti-DNP–Sepharose column. A digest of 0.3 μmole of 41-DNP-RNase was applied to a 1.5 × 5 cm anti DNP–Sepharose column (containing 0.9 μmole of antibody). After washing with PBS the column was eluted with 6 M Gdn·HCl. Fractions of 1 ml were collected. (○) Absorbance at 280 nm; (▲) absorbance at 360 nm.

*Isolation of DNP-Peptide from DNP-ribonuclease.* Performic acid oxidized DNP-ribonuclease (5 mg) was digested with trypsin for 8 hr followed by digestion with chymotrypsin for 8 hr at 37°. The yellow digest was applied to an anti-DNP–Sepharose column (1.5 × 5 cm). The elution pattern of this column is given in Figure 4. All the yellow material was adsorbed by the column and 85% of the original absorbance at 360 nm could be eluted by 6 M Gdn·HCl (Figure 4). After this treatment the antibody–Sepharose conjugate lost most of its activity and could not be used again. The 6 M Gdn·HCl eluate was chromatographed on a Sephadex G-25 column in 0.1 M acetic acid. The yellow material which emerged from the column before the guanidine peak was found to contain only one peptide. Amino acid analysis of this peptide (overall yield 78%) showed the composition $CyA_{1.0}$,$Asp_{1.0}$,$Thr_{0.9}$,-$Pro_{1.0}$,$Val_{0.9}$,$Phe_{1.0}$ with N-terminal cysteic acid. This peptide coresponds to residues 40–46 in ribonuclease (Cys-Lys-Pro-Val-Asn-Thr-Phe) and confirms the previous finding (Hirs *et al.*, 1965) that lysine-41 is the residue which is dinitrophenylated under the conditions employed.

## Discussion

Methods for separation and isolation of peptides are generally based on certain physical properties (charge, size, hydrophobicity, conformation, etc.) which vary quantitatively from one peptide to another. The method described in this communication takes advantage of the biological specificity of antibodies. It is based on the affinity of the antibody to a certain ligand attached to the peptide and is independent of the physical properties of the peptide itself. Only peptides which possess such a ligand will be specifically sequestered by

the antibodies and thereby removed from the entire peptide mixture.

The introduction of Sepharose as a convenient support for insolubilizing antibodies (Axen *et al.*, 1967; Wide *et al.*, 1967; Givol and Rotman, 1970; Givol *et al.*, 1970) and the improvement of various techniques for affinity chromatography (Cuatrecasas *et al.*, 1968) facilitate rapid and easy procedures for the isolation of modified peptides using our method. The high capacity of CNBr-activated Sepharose and the use of purified antibodies allows the employment of very small columns for the isolation of micromole amounts of the modified peptide.

The high rate of hapten–antibody interaction allows very high flow rates during the operation. The specific isolation of modified peptides by adsorption to and elution from an antibody column can be rapidly accomplished in 1–2 hr. In addition, the antibody–Sepharose column could also serve as a means for concentrating the specifically adsorbed peptide. Thus, the adsorbed peptides could be recovered in approximately the volume of the antibody–Sepharose column, regardless of the volume in which the peptides were applied to this column. On the other hand, the unadsorbed peptides contained all the unmodified peptides of the digest and can be used for other studies on the sequence of the protein.

The adsorption of most (about 95%) of the modified peptides from the peptides mixture could be accomplished under mild conditions (neutral pH and various ionic strengths). The elution of the modified peptides from the antibody–Sepharose column, however, required different conditions depending on the ligand as well as on the antibodies used. Thus, the Ars-peptides can be dissociated from anti-Ars–Sepharose by 1 M $NH_4OH$, whereas DNP-Lys peptides did not dissociate from anti-DNP–Sepharose unless 6 M Gdn·HCl (or 20% formic acid (Givol *et al.*, 1970) were used. We have found that 1 M $NH_4OH$, if it dissociates the antibody–hapten bonds, was the best solvent for elution of adsorbed peptides. After this treatment the column can be regenerated to almost full capacity and can be reused many times. In addition, almost no antibodies were released from the Sepharose (less than 0.1% of the bound antibodies) and the eluted peptides were obtained pure. Obviously, if the eluate contains several modified peptides, conventional methods should be used to further separate and purify them from one another. The use of 6 M Gdn·HCl caused substantial inactivation of the antibodies and although the conjugate can be used again with lower efficiency, we preferred to discard such conjugates after the first use. It should be noted, however, that the production of anti-DNP antibodies in great amounts is not difficult. A more serious problem was posed by the release of a small part of the bound antibodies by 6 M Gdn·HCl. This caused difficulty in subsequent steps of purification of the modified peptides. In the case of Ars-peptides (eluted by 1 M $NH_4OH$) high-voltage electrophoresis resulted in good separation of the eluted peptides whereas in the case of DNP-Lys peptides (eluted 6 M Gdn·HCl) it was necessary to first remove the eluted antibodies and Gdn·HCl on Sephadex G-25. Although the amount of antibodies released from the columns by 6 M Gdn·HCl is very small (2–3% of the bound antibodies), this is the major source of contaminants in the purified peptides, and this point is open for an improvement.

Two different modified proteins were chosen to illustrate the use of this method. Mono-DNP-RNase is known to contain a DNP group on lysine-41 (Hirs *et al.*, 1965). The DNP-peptide isolated, in this study, directly from the entire digest by the anti-DNP–Sepharose column was obtained pure after removal of the Gdn·HCl. The composition and N-terminal residue of this peptide agrees with a previous finding (Hirs *et al.*, 1965). In the case of Ars-CPD we were able to establish that the modification by azobenzenearsonate occurred at three different positions. However, most of the Ars group was localized on Tyr-248. A peptide containing this residue was previously isolated from CPD after pair-labeled iodination procedure (Roholt and Pressman, 1967). Recently, from X-ray analysis, Tyr-248 was implicated to be involved in the active site of the enzyme (Lipscomb *et al.*, 1970), and it was suggested that this was the residue nitrated in mononitrotyrosyl-CPD (Sokolovsky *et al.*, 1966). The results obtained by Kagan and Vallee (1969) in their study on the effect of nitration and arsanilation on the activity of CPD indicate that either nitration, or coupling with azobenzenearsonate affects the same tyrosine. Hence, our study provides support for these suggestions. Discussion on the involvement of the other two arsanilated tyrosines (residues 19 and 277) in the activity of CPD is beyond the scope of this paper. It is possible, however, that under more gentle conditions of arsanilation, where only one tyrosine will be modified, the Ars group will be found exclusively on Tyr-248. Our studies merely illustrate the use of this method to locate in a rapid and convenient way the position of the modified residues in the sequence of the protein.

The use of antibodies as a tool for the isolation of modified peptides can be extended to many more examples, since almost any chemical modification of proteins can form haptenic determinants which might provoke synthesis of specific antibodies (Landsteiner, 1936). Moreover, the use of such antibodies is not limited only to selective modification of proteins. If a specific modification of *all* the residues of the same kind (*e.g.*, tyrosine or lysine or tryptophan, etc.) can be performed, one can foresee the use of insoluble antibodies for the isolation of all peptides containing these residues (*e.g.*, all tyrosyl peptides, lysyl peptides, or tryptophyl peptides). This might be valuable in cases of proteins whose sequence is not known, or in sequencing work when overlapping peptides of certain enzymatic digest are required. We believe that the examples given in this study will help the introduction of such techniques into protein chemistry.

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# Gadolinium(III) as a Paramagnetic Probe for Proton Relaxation Studies of Biological Macromolecules. Binding to Bovine Serum Albumin*

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ABSTRACT: An investigation of the applicability of proton relaxation methods in studying metal ion binding to biological macromolecules using gadolinium(III) as a paramagnetic probe has been carried out. The binding of Gd(III) to bovine serum albumin has been studied. The longitudinal proton relaxation rate of water due to the paramagnetic Gd(III)–bovine serum albumin complex is enhanced relative to the ion in solution. Using the enhancement as an analytical parameter it was found that bovine serum albumin has four binding sites for Gd(III) with an apparent dissociation constant of $1.3 \times 10^{-4}$ M at 300°K. Conclusions regarding the mechanism of enhancement have been drawn from measurements at different frequencies and temperatures. The correlation time for the electron–nuclear dipolar interaction is dominated by the rotation of the complex for the aquo and cacodylate complexes of Gd(III), whereas for the Gd(III)–bovine serum albumin complex it is mainly the electron spin relaxation time.

The use of transition-metal paramagnetic ions as probes for magnetic resonance studies of biological macromolecules is well documented (Mildvan and Cohn, 1970; Cohn and Reuben, 1971). In this paper we present results of an investigation aimed to establish the extent of applicability of magnetic resonance methods in studies of the macromolecular environment of ions using a member of the lanthanide series, gadolinium(III), as the paramagnetic probe.

Inhibition of enzymatic reactions (Holten *et al.*, 1966, and references cited therein) and of calcium transport across membranes (Mela, 1969) by trivalent lanthanides has been reported. More recently it has been found that lanthanides may act as cofactors in the activation of trypsinogen by trypsin (Darnall and Birnbaum, 1970).

Among the paramagnetic transition-metal ions manganese(II) has been found to be most suitable as a probe for magnetic relaxation studies of macromolecular systems. From the magnetic resonance point of view its "analog" in the lanthanide series is gadolinium(III) which is also an *S*-state ion with high electronic spin ($S = 7/2$), relatively long electron relaxation time, and labile hydration sphere. Therefore gadolinium(III) was chosen for this investigation. Bovine serum albumin is a readily available protein known to bind metal ions (*cf.*, *e.g.*, Mildvan and Cohn, 1963). Preliminary experiments have shown that the longitudinal proton relaxation rate in aqueous solutions of $GdCl_3$ is enhanced in presence of albumin suggesting that gadolinium does bind to this protein.[1] Therefore bovine serum albumin was chosen as the macromolecule for this study. In conjunction with the albumin study and for comparison also studied were solutions of $GdCl_3$ in absence and in presence of tetramethylammonium cacodylate (dimethyl arsinate), which was used as buffer.

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[1] The preliminary experiments were carried out in collaboration with Dr. F. Lantelme.