High Efficiency Coupling of Diazonium Ions to Proteins and Amino Acids*

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Azoproteins, obtained by the coupling of diazonium ions with the histidyl, tyrosyl, and lysyl groups of proteins, have continued to be materials of utility since their early investigation (1). The ease with which proteins can be modified by this procedure, the variety of aromatic amines that can be used, and the correlation that can be made between the chemical structure of the introduced group and the properties of the resulting azoprotein are responsible for the continued interest in these substances for studies of the relationship between chemical structure and biological activity of proteins. However, side reactions have been responsible for a low over-all efficiency of the coupling reaction, i.e. percentage of diazonium ion coupled. This is due primarily to a susceptibility of the diazonium ion to hydrolysis. The methods generally employed do not readily lend themselves to use with tracer amounts of aromatic amines containing 14C or tritium. In this paper a method is presented that permits the incorporation of radioactively labeled aromatic amines with high efficiency.

One of the reactions with α-amino acids and diazonium ions leads to the formation of an unstable diazoamino compound with the α-amino group. Because this reaction results in loss of diazonium ion and deamination of the amino acid, chloracetyl derivatives of the amino acid have been used for the preparation of azo derivatives (2). A method in which unmodified amino acids bound to cation exchange resins are used for the preparation of azo derivatives is also presented in this paper.

EXPERIMENTAL PROCEDURE

14C Radioactivity—Estimations were made with samples dried on aluminum planchets, in a Nuclear-Chicago gas flow counter, model D-47, with mica window, equipped with scaling unit model 161A, sample changer model C-110B, and printing timer model C-111B.

Absorption Spectra—A Beckman DK-2A ratio recording spectrophotometer was used with 1-cm cells. The molar extinction coefficient, ε, was calculated from readings in a Beckman DU spectrophotometer with 1-cm cells.

Chromatography—Benzene-acetic acid-isopropyl alcohol-water, 4:2:2:1, was the solvent system used in descending chromatograms on Whatman No. 1 filter paper.

Materiæ—Hydrochloric acid and sodium nitrite were reagent grade. p-Aminobenzoic acid-carboxyl-14C was obtained from California Corporation for Biochemical Research. Other nonradioactive amines were obtained from Eastman Kodak. "Special-absolute-pure" phenol (National Aniline Division), l-tyrosine, l-histidine monohydrochloride, and l-lysine monohydrochloride (California Corporation for Biochemical Research), and bovine albumin powder Fraction V (Armour Pharmaceutical Company) were used in coupling reactions. Analytical grade AG-50W-X1 and AG-50W-X8, 50 to 100 mesh, prepared from Dowex 50 resin and Dowex 1W-X8 (Bio-Rad Laboratories, Richmond, California), Duolite CS-101 (Chemical Process Company, Redwood City, California), and Sephadex G-25, medium grade (Pharmacia, Uppsala, Sweden) were used in the preparation or purification of products.

RESULTS

Diazotization—Diazotization was carried out between 0° and 5° in an ice bath. At least 3 equivalents of HCl and 1 equivalent of NaNO2 per equivalent of aromatic amine were used. In order to hasten diazotization and maintain an excess of hydrogen ion for maximum stability of the diazonium ion during its formation, the acid strength was increased 100-fold when tracer amounts of aromatic amine were used. For example, 100 mc of p-aminobenzoic acid-carboxyl-14C, corresponding to 0.02 meq, were dissolved in 2 ml of 1 n HCl and 7 ml of distilled water. To this mixture 1 ml of 1 M NaNO2 was added with continuous stirring. Under these conditions diazotization is complete in less than 5 minutes. The course of the diazotization reaction was followed by coupling aliquots of the reaction mixture with phenol and measuring the absorbance in a Klett-Summerson photometric colorimeter.

Formation of Diazonium-Cation Exchange Resin Compound—When diazotization was complete, a cation exchange resin was added with continuous stirring.

The formation of an insoluble compound by exchange may be represented by Equation 1, which shows exchange with a sulfonated resin of the Dowex 50 type (R—SO3H), and Equation 2, which shows exchange with a resin of the Duolite type (R—COONa). The diazonium ion in both cases is derived from auxillin.

\[
\begin{align*}
&1) \text{N}^{+} + \text{Cl}^{-} + \text{HCl} + \text{NaCl} + 2 \text{RSO}_3 \text{H} \rightarrow \text{N}^{+} \text{RSO}_3 \text{Na} + 3 \text{HCl} \\
&2) \text{N}^{+} + \text{Cl}^{-} + \text{HCl} + \text{NaCl} + 2 \text{RCOO} \text{Na} \rightarrow \text{N}^{+} \text{RCOO} + 3 \text{NaCl}
\end{align*}
\]
The sodium form of the Duolite-type resin is used because of the high hydrogen ion concentration and high affinity of this type of resin for hydrogen ions. Two equivalents of ion exchange resin are required because of the presence of sodium ion or hydrogen ion, which also undergo exchange.

The course of exchange was followed by determining the amount of free diazonium ion at intervals after the introduction of the resin. Free diazonium ion prepared from aniline was measured by coupling with phenol. Fig. 1 shows the results for both resins when 2 meq of resin were used with 1 equivalent of diazonium ion in a total volume of 10 ml. The reaction with R—COONa was very slow after an initial rapid exchange of 45% of the capacity of the resin. The reaction with R—SO₃H, however, proceeded rapidly and became equilibrated at 99% of its theoretical capacity.

In order to study the relationship between the equilibration value and the amount of resin used, different amounts of R—SO₃H were mixed with 1 meq of diazotized aniline and allowed to reach equilibrium. Fig. 2 illustrates the result. It is possible to remove 99.95% of the free diazonium ion obtained from aniline from solution by using a 4-fold excess of R—SO₃H. A 2-fold excess leaves only 0.25% of the diazonium ion unexchanged.

The exchange reaction with tracer amounts of diazotized p-aminobenzoic acid-carboxyl¹⁴C shows a similar time course; equilibrium is reached within 15 minutes. However, owing to the presence of the anionic carboxyl group, proportionally larger amounts of ion exchange resin are required for complex formation. Fig. 3 illustrates the relationship between the amount of diazotized p-aminobenzoic acid-carboxyl¹⁴C free in solution and the amount of R—SO₃H added. The mixture in which exchange was observed contained 8 × 10⁻⁴ meq of diazotized p-aminobenzoic acid-carboxyl¹⁴C, 0.1 meq of NaCl, and 0.1 meq of HCl in a total volume of 10 ml. Free diazonium ion was determined by measuring the amount of ¹⁴C in dried, diluted aliquots. An amount of R—SO₃H equivalent to almost a 5-fold excess with respect to the sodium ion leaves almost 5% of the diazonium ion free in solution. When 100 µg of p-aminobenzoic acid-carboxyl¹⁴C, corresponding to 0.02 meq, are diazotized by a mixture of 2 meq of HCl and 1 meq of NaNO₂, 10 meq (25 ml) of Dowex 50W-X1 are used. This amount of R—SO₃H leaves only 2.5% of the labeled diazonium ion free in solution.

The exchange reaction has been observed with the following diazotized aromatic amines: m-aminobenzoic acid, anthranilic acid, methyl p-aminobenzoate, ethyl p-aminobenzoate, p-arsanilic acid, sulfanilic acid, p-aminohippuric acid, p-aminophenylacetic acid, p-anisidine, p-toluidine, p-aminophenol, aniline, 1-naphthylamine, N-methyl-p-phenylenediamine, N,N'-diethyl-p-phenylenediamine, p-phenylalanine, sulfanilamide, N,N'-diethylsulfanilamide, 4'-aminoacetophenone, 4'-aminoacetanilide, p-nitroaniline, p-chloroaniline, p-bromoaniline, and p-iodoaniline. The diazonium ions obtained from all of these amines except p-arsanilic acid and sulfanilic acid can be exchanged without excessive amounts of R—SO₃H. The two excepted compounds contain strongly anionic groups that make proportionally larger amounts of R—SO₃H necessary.

Following exchange, the resin with the bound diazonium ion can be washed repeatedly in a sintered glass filter funnel with distilled water at 0–5°C. Excesses of nitrous and hydrochloric acid are removed by the washing process. The hazard of alteration of proteins by deamination due to traces of residual nitrous acid in the diazonium ion preparation is eliminated. In addition, the washing procedure permits the use of stronger conditions for diazotization of more resistant amines.

**Stability of Diazonium-Cation Exchange Resin Compound**

The stability of the diazonium ion under the resulting condition of low hydrogen ion concentration was studied at 0–5°C and at room temperature, 23°C. After thorough washing with distilled water, preparations containing 2 meq of resin with 1 meq of diazotized aniline bound by exchange were covered with 10 ml of distilled water and kept at either of the above mentioned temperatures. These preparations were mixed at frequent
resin and phenol produced from hydrolysis by the addition of 0.4 meq of NaOH. After 194 hours at 23°, an accumulation of 0.003 meq of p-phenylazophenol could be detected. However, a reddening of the resin and evolution of nitrogen gas suggested that further diazonium ion was being lost as a result of hydrolysis and coupling within the resin. At the end of the period of observation, 19½ hours, 1 meq of phenol was added followed by a very slow addition of 1 meq of NaOH with constant stirring. The yield of p-phenylazophenol was 90.7% of theoretical. Therefore, a total of 9.3% of the diazonium ion was lost at room temperature in 104 hours. The preparations maintained at 0-5° were examined in an identical manner. No detectable release of diazonium ion or hydrolysis to phenol could be detected. The resin remained essentially unchanged in color, and only a rare bubble of nitrogen was observed. The yield of p-phenylazophenol on addition of phenol and NaOH did not deviate detectably from the theoretical. Therefore, no detectable loss of diazonium ion occurred at 0-5° in 194 hours.

**Coupling Reaction**—A generalized expression of the coupling reaction is given in Equation 3. In practice, there is an initial lag before the appearance of colored product because of the exchange of Na+ for H+ on the excess R-SO3H. If the NaOH is added too rapidly with insufficient mixing, hydrolysis of the diazonium ion within the resin becomes marked, and a reddening of the resin due to the formation of p-phenylazophenol from the diazonium ion becomes marked, and a reddening of the resin is observed. The proper addition of base results in no discoloration of the resin.

\[
\text{NHN}_2\text{NO}_2\text{SR} + \text{OH}^- + \text{NaOH} \rightarrow \text{HN} = \text{N} + \text{OH}^- + \text{RSO}_3\text{Na} + \text{H}_2\text{O}
\]

The coupling reaction with proteins proceeds in essentially the same way. The histidyl, tyrosyl, and lysyl groups of the protein appear to be the portions of the molecule most involved. The amino acid composition of bovine serum albumin (3) indicates 18 histidyl, 19 tyrosyl, and 58 lysyl groups per molecule of BSA. It is unlikely that all of these groups are readily substituted, since some at least may be expected to be sterically hindered owing to their position in the molecule. In addition, the stability of the product formed by coupling appears to vary. The azo derivative of the histidyl and tyrosyl groups is more stable than the pentazene compound formed with the ε-amino group of lysine (4). The ease with which these three amino acid residues can be substituted should be dependent upon the hydrogen ion concentration and related to the pK values of the different groups. The coupling reaction is dependent upon another effect of the hydrogen ion concentration. The susceptibility of the diazonium ion to hydrolysis increases with increasing pH. Therefore, the efficiency of coupling of diazonium ion to protein may be expected to depend on the relative number of free diazonium ions and activated groups on the protein, and the rates of the coupling reactions and destructive hydrolytic reaction of the diazonium ion. The concentration of free diazonium ion is itself dependent upon the rate of exchange of sodium ion for diazonium ion in the resin. The efficiency of coupling may be expressed as a percentage of the diazonium ion which becomes coupled to the protein by a stable linkage.

Fig. 4 illustrates the relationship between efficiency of coupling and hydrogen ion concentration. An aliquot containing 0.4 meq of Dowex 50W-X4 treated with \(8 \times 10^{-4}\) meq of diazotized p-aminobenzoic acid-carboxyl-14C and 11.2 mg (1.6 \(\times 10^{-4}\) mmole) of BSA was constantly stirred and rapidly adjusted to the different pH values indicated by the addition of 1 N NaOH. The coupling reaction was allowed to continue for 3 hours with occasional correction of the very small changes in hydrogen ion concentration by the addition of NaOH. After 18 hours, the resin was removed by filtration through sintered glass, and the protein was dialyzed against running tap water for 18 hours followed by gel filtration with Sephadex G-25. While dialysis removed the bulk of the side products, further purification to constant levels of radioactivity is accomplished by gel filtration. The protein samples were analyzed for 14C content. The highest efficiency of coupling under the conditions of the experiment was observed between pH 5.6 and 9.0. At lower hydrogen ion concentrations, the hydrolysis of the diazonium ion results in decreased efficiency of coupling. At a hydrogen ion concentration close to pH 9.7, the increased activation of one of the protein groups may somewhat compensate for the loss of diazonium ion caused by hydrolysis.

The dependence of the efficiency of coupling on the stability of the diazonium ion can be demonstrated in another way. Fig. 5 shows the effect of protein concentration on the efficiency of coupling. Aliquots containing 0.4 meq of Dowex 50W-X1 treated with \(8 \times 10^{-4}\) meq of diazotized p-aminobenzoic acid-carboxyl-14C and 5.6, 11.2, 22.4, and 44.8 mg of BSA were constantly stirred and rapidly brought to pH 9.0 by the addition of 1 N NaOH; they were maintained under these conditions for 18 hours. The protein was purified as before by dialysis and gel filtration. Fig. 5 shows that efficiency of coupling is not directly proportional to the number of amino acid groups available for coupling.

**Preparation of Azo Derivatives of Aromatic Amines and Amino Acids**—Coupling reactions with the diazonium compound and substances containing aromatic or aliphatic amino groups were studied with the use of a cation exchange resin. The reaction between a diazonium ion and an aromatic amine appears to proceed by formation of a diazamino compound with subsequent rearrangement (5). Aliphatic amines, particularly those in which the amino group is on the α carbon atom (as in α amino

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Fig. 4 (left). The effect of hydrogen ion concentration on the efficiency of coupling, i.e. the fraction of the diazonium ion that becomes coupled to the protein by stable linkage.

Fig. 5 (right). The effect of protein concentration on the efficiency of coupling, i.e. the fraction of the diazonium ion that becomes coupled to the protein by stable linkage.

1 The abbreviation used is: BSA, bovine serum albumin.
acids), form an unstable compound that decomposes with an evolution of nitrogen (5). The stability of aliphatic diazoamino compounds is lowest under conditions of high hydrogen ion concentration. Acetylated derivatives of α-amino acids have generally been used to avoid this kind of alteration of the amino acid and loss of diazonium ion.

The coupling reaction of diazonium ion with aniline was carried out by using a sufficient excess of R—SO₃H in allow both components of the reaction to be bound by exchange. If exchange of the diazonium ion and the coupling reaction proceed at greater rates than the exchange of the amine, a reasonable yield of the azo derivative without the formation of a diazo amino compound would be expected. Equation 4 represents coupling of diazonium ion with aniline.

When 1 meq of bound diazonium ion was coupled with 1 meq of bound aniline, the reaction proceeded without appreciable discoloration of the solution. Therefore, the release of aniline by exchange or neutralization of the protonated amino group was not appreciable. The resin reddened as the reaction progressed. At the end of the reaction, an excess of base was added. The product, mono(p-azobenzene)aniline, is highly insoluble in base, while the side products, phenol and azobenzenephenols, are base-soluble. The product on the resin was washed free of side products and finally eluted by exchange with 1 N HCl. The release of the product from the resin is slow. The use of ion exchange resins with low levels of cross-linkage facilitates release. No further purification or study of this derivative prepared with diazotized aniline was made.

Mono(p-azobenzene carboxylic acid) L-tyrosine was prepared in a similar manner. The solubility of tyrosine is 0.196 g per liter at 0° (5). However, the reaction involves tyrosine bound to the resin and not in free solution. Therefore, the reaction volume is limited only by the volume required to suspend the resin. The diazotization was carried out as before. p-Aminobenzoic acid, 10 meq (1.37 g), was dissolved in 30 ml of 1 N HCl, chilled, and diazotized by the addition of 10 ml of 1 N NaNO₂. The tyrosine was stirred with 60 meq of Dowex 50W-X4 (150 ml) until exchanged and chilled by the addition of ice. The diazotized p-aminobenzoic acid was added, and the mixture was stirred for 15 minutes. The resin becomes orange because of the formation of a salt composed of the amino acid and the diazonium ion. This salt is quite insoluble and can be formed in the absence of ion exchange resin. The resin was washed with 500 ml of ice water. Approximately 80 ml of 1 N NaOH were added dropwise with constant stirring. The mixture became red after the addition of 40 ml. The pH was raised to 11.0, and base addition continued until the pH remained constant at 11.0 for 1 hour.

While the events occurring in the reaction on the addition of base are not completely stepwise, Equations 5 through 10 indicate the essential features of the process involved. Most of the diazonium ion appears to be in salt linkage with the amino acid prior to the addition of base owing to the low solubility of the amino acid diazonium salt. Any diazonium ion linked directly to the resin is displaced by NaOH, and the salt of the amino acid is encouraged early in the addition of base.

The product was removed from the resin by filtration through a coarse, sintered glass filter, and the resin was washed free of color. The product was freed of nonionic side products by stirring with 50-ml batches of Dowex 50W-X4 until no further color was removed from solution. Generally three treatments for 2 to 3 hours sufficed. The batches of resin were washed with large amounts of water until no further color could be removed. The resin was brought to pH 10.5 by the addition of 1 N NaOH, and the product was removed by filtration and precipitated as the hydrochloride by the addition of HCl. After collection by centrifugation and washing with water to remove salts and traces of unreacted tyrosine, the product was dried. The yield of the purified product was between 40 and 50%.

The product was chromatographically homogeneous in the benzene-acetic acid-water solvent system. In 0.1 M phosphate buffer, pH 6.0, at 330 μm, 14,900; at 485 μm, 9,700.

Fig. 6 shows the absorption spectra of the mono(p-azobenzene-carboxylic acid) L-tyrosine at pH 6.0 in 0.1 M phosphate buffer and in 0.1 N NaOH.

Mono(p-azobenzene-carboxylic acid) L-histidine was prepared in a similar manner. Purification involved separation of the
product from noncationic side products and the deaminated product, which remained cationic because of protonation of the imidazole group. The histidine derivative is less stable in acid than the tyrosine derivative. Treatment with Dowex 50 results in the loss of 2 nitrogens and 1 carbon from the molecule. The product was purified on a Dowex 1-X4 column in the chloride form. The solution of product and impurities was adjusted to pH 12.0 and passed through a 100-ml column of the anionic exchange resin. After washing with water, the product was eluted with 1 M NaCl at pH 9.0. The deaminated material remains on the column until the pH is lowered to 7.0. The noncationic materials are very difficult to elute from the column. The pH 9.0 eluate was adjusted to pH 3.5 by the addition of 1 M HCl, collected by centrifugation, washed with water to remove traces of unreacted histidine and salts, washed with 95% ethanol to remove traces of noncationic materials, dried, and weighed. The yield was generally about 40%. At pH 3.5 the product, which contains 1 molecule of water of crystallization but no chloride, was chromatographically homogeneous in the solvent.

**Table I**

<table>
<thead>
<tr>
<th>pH of coupling to BSA</th>
<th>Coupling efficiency*</th>
<th>Specific activity (c.p.m./mg)</th>
<th>$\Delta\text{Au}/\Delta\text{Au}$ in 0.1 N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>40</td>
<td>$6.6 \times 10^4$</td>
<td>1.3</td>
</tr>
<tr>
<td>9.5</td>
<td>40</td>
<td>$7.1 \times 10^4$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Expressed as percentage of diazonium ion coupled to protein.
system described above. \( \varepsilon \) at pH 6.0, at 375 m\( m \), 21,000; in 0.1 N NaOH at 440 m\( m \), 22,500.

\[
\text{C}_9\text{H}_4\text{N}_2\text{O}_4\cdot \text{H}_2\text{O}
\]

Calculated: C 48.5, H 4.6, N 22.0, O 24.9
Found: C 48.5, H 4.4, N 22.3, O 24.9

Fig. 7 shows absorption spectra of the mono(p-azobenzene-carboxylic acid) L-histidine at pH 6.0 and in 0.1 N NaOH.

The molar extinction coefficients are similar to those reported by Tabachnick and Sobotka (2). Their determinations were made on the reaction mixture and not on the purified derivatives of the nonacetylated amino acids. An important colored contaminant in reactions involving diazotized p-aminobenzoic acid would be expected to be 3-(p-azobenzene-carboxylic acid) 4-hydroxybenzoic acid. Fig. 8 shows the spectra of this material prepared from p-hydroxybenzoic acid and diazotized p-aminobenzoic acid. Small amounts of this material could markedly affect the extinction coefficient and may be responsible for the slightly higher values reported (2).

The pentazene derivative of lysine can be readily formed by the method used for histidine and tyrosine. Two milliequivalents of the diazonium ion were used. The product is very unstable and was purified rapidly to minimize loss by decomposition. The material was precipitated at pH 3.5 with HCl, washed with several water times to remove excess histidine, and finally freed of noncationic contaminants by washing with acetone, then dried. The yield was only 12%. The yellow product continues to decompose to a red material even in the dried condition.

\[
\text{C}_9\text{H}_4\text{N}_2\text{O}_4\text{Cl}
\]

Calculated: C 50.2, H 4.8, N 17.6, O 20.1, Cl 7.3
Found: C 51.7, H 5.0, N 16.5, O 19.5, Cl 7.3

The analysis was made 5 days after isolation. Decomposition in solution shows nitrogen evolution. The lysine derivative shows an absorption maximum at 370 m\( m \) both in 0.1 M phosphate at pH 6.0 and in 0.1 N NaOH. The instability of the material makes measurement of the extinction coefficient difficult. Fig. 9 shows the decrease in \( \varepsilon \) with time at 370 m\( m \) of the material at pH 6.0 and in 0.1 N NaOH. The study was carried out at a concentration of 1.26 \( \times 10^{-3} \) M with cells of 1-mm light path. The material was 7 days old at the time of the study and had already undergone some decomposition.

Preparation of Azoproteins—Two batches of BSA coupled to diazotized p-aminobenzoic acid at two different hydrogen ion concentrations are presented to illustrate the method and the properties of the resulting products. In both cases, 100 mc of p-aminobenzoic acid-carboxyl-\( ^{14} \text{C} \), equivalent to 0.02 mmole, were diazotized in a mixture of 2 ml of 1 N HCl, 1 ml of 1 M NaNO\( _2 \), and 7 ml of H\( _2 \)O between 0° and 5°. The diazonium ion was bound to 10 ml of Dowex 5OW-X1, 4.0 meq, by exchange and was washed free of excess HCl and nitrous acid. After washing, 97% of the \( ^{14} \text{C} \) remained bound to the resin. BSA, 150 mg dissolved in 3.0 ml of water, was added to the resin suspended in water, and 1 N NaOH was added very slowly from a microburette with constant stirring. One reaction mixture was brought to pH 7.5, the other to pH 9.5. The hydrogen ion concentrations were maintained for 1 hour by the addition of a small amount of NaOH. Diazotized, nonradioactive p-aminobenzoic acid (1 mmole) bound to Dowex 5OW-X1 was added to modify the protein further, and the pH was again adjusted to 7.5 and 9.5. Coupling was allowed to continue for 18 hours. The temperature was maintained between 0° and 8°, and the reaction mixtures were stirred continuously. After removal of the resin by filtration, the azoprotein preparations were dialyzed against running water for 48 hours and further purified by gel filtration through Sephadex G-25 until the \( ^{14} \text{C} \) content per mg of protein was constant. Figs. 10 and 11 show the absorption spectra of these two proteins, and Table I lists some of their properties.

The coupling efficiency is higher under these conditions because of the slower addition of base. The principal difference in the absorption spectra of the two azoproteins in 0.1 M NaOH is in the region of the spectrum between 400 and 600 m\( m \). The tyrosine derivative has a maximum at 485 m\( m \), as can be seen in Fig. 6. The histidine compound has a maximum at 460 m\( m \), as can be seen in Fig. 7. The ratio of absorbance at 400 m\( m \) to absorbance at 485 m\( m \) is 3.2 for the histidine derivative and 0.70 for the tyrosine derivative. A comparison with the ratios shown in Table I suggests that coupling at pH 7.5 results in a protein labeled less heavily in tyrosine than does coupling at a higher pH. Contributions to the absorption spectra made by azoamino compounds such as the lysine and glycine derivatives is difficult to evaluate. Coupling at pH 9.5 results in the formation of some material of low solubility and suggests that a certain amount of denaturation of the protein results either from maintenance at the high pH or from the more extensive modification due to coupling with a greater number of amino acid residues. Attempts at further characterization of the proteins as to the amount of coupling with different amino acids have been unsuccessful. The azo derivatives are degraded by acid hydrolysis.

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SUMMARY

Methods for the coupling of diazonium ions to proteins and amino acids at high efficiency are described. The methods involve the formation of a highly stable diazonium compound with ion exchange resins.

Coupling with the \( \alpha \)-amino acids, tyrosine and histidine, without deamination of the \( \alpha \)-amino group was accomplished by binding the amino groups to ion exchange resins. Absorption spectra of purified amino acid derivatives are presented and are used to interpret the absorption spectra of azoproteins prepared under two different conditions of coupling.

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