A NOVEL DIAZONIUM-SULFHYDRYL REACTION IN THE INACTIVATION OF YEAST ALCOHOL DEHYDROGENASE BY DIAZOTIZED 3-AMINOPYRIDINE ADENINE DINUCLEOTIDE

by

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LIST OF ABBREVIATIONS

AAD	3-Aminopyridine adenine dinucleotide
AADP	3-Aminopyridine adenine dinucleotide phosphate
ADPR	Adenosine diphosphoribose
MAP	1-Methyl-3-aminopyridinium chloride
NEM	N-Ethylmaleimide

INTRODUCTION

In recent studies [1], NAD was chemically converted to 3-aminopyridine adenine dinucleotide (AAD)[1] through the Hofmann hypobromite reaction. The chemical, spectrophotometric and fluorimetric properties of AAD were reported. As an analog of NAD, this dinucleotide was shown to be a coenzyme-competitive inhibitor of several NAD-requiring enzymes [1]. It was further observed that the 3-aminopyridine moiety of AAD could be diazotized by reaction with nitrous acid and the resulting diazonium chloride could be azo-coupled with N-1-naphthylethylenediamine to form an azo dye. The investigation of diazotized AAD as a site-labeling reagent for dehydrogenases revealed a first order irreversible inactivation of yeast alcohol dehydrogenase, the rate of which was decreased by the presence of NAD [1]. Spectrophotometric analysis of yeast alcohol dehydrogenase totally inactivated by diazotized AAD and extensively dialyzed indicated the presence of four AAD residues per molecule of enzyme or one per catalytic site. Although a site-specific inactivation was demonstrated, identification of the amino acid residue modified in the inactivation process was not achieved in these earlier studies.

The chemical reactions of aromatic diazonium compounds were among the earliest reactions to be studied intensively. In 1894, Hantzsch first applied physical organic principles to interpret product formation in reactions of aromatic diazonium compounds with

[1]See List of Abbreviations, page x.

hydroxide, cyanide and sulfite ions [2]. Diazo coupling reactions fall under the general class of electrophilic aromatic substitutions [3]. Aromatic diazonium ions behave as dibasic Lewis acids. But in contrast to other dibasic acids, the first acidity constant is smaller than the second [4]. The diazonium group is especially interesting in view of its being the most strongly electron-attracting substituent known [5].

The reactions between diazonium compounds and proteins were first studied by Pauly in 1904 [6] using diazotized sulfanilate in alkaline solution. The colored products formed were compared with reactions involving individual amino acids. Tyrosine and histidine were the only ones that gave color reactions. Subsequently, tyrosyl and histidyl derivatives of diazotized p-arsanilic acid were isolated [7]. When proteins were treated with excess diazotized p-arsanilic acid, the azoproteins formed contained more than the stoichiometric amounts of arsenic than were accountable by total amount of tyrosine and histidine present [8]. It was suggested that phenylalanine, tryptophan, proline and hydroxyproline may also have reacted. Kapellar-Adler and Boxer in 1936 [9] reported the preparation of such derivatives. In 1957, Howard and Wild [10] carried out experiments reacting diazonium derivatives of aniline, p-arsanilic acid and p-diphenylylamine with amino acids. They found that triazenes were formed with glycine, glycylglycine and ε-aminocaproic acid. They suggested that bis(diazo)amino linkages could be formed with the ε-amino groups of lysyl residues in proteins.

Since the first diazonium protein modification experiments, reactions have been carried out with equine serum, bovine serum albumin, ovalbumin, fibrinogen, insulin, pepsin, chymotrypsin, casein, gelatin, edestin, fibroin and many others [10-12]. However, the reactions were mostly not well defined, and the reaction products quite heterogeneous.

Since 1917, immunologists have taken particular advantage of the diazonium reagents to augment the antigenic properties of proteins and for the production of particular antigenic determinants. Landsteiner [13] introduced small organic compounds of widely different structures on to antigens through the azo linkage. The antigenicity of the substituted proteins was observed to be related to the presence of the small substituent groups. With the aid of the coupling reactions, it has been possible to explore the tremendous range of antibody specificity and to elucidate the groupings that play a prominent role in natural antigens. This was facilitated by the many compounds that could be diazotized and readily linked to the proteins at moderate temperatures and mild pH's without causing denaturation. Probably thousands of azoproteins have been prepared since. Generally, the whole serum rather than a purified protein was conjugated. A more refined study was carried out by Metzger et al. [14]. Two antihapten-antibody systems directed respectively to p-azobenzene arsonate and 2,4-dinitrophenyllysyl determinants were obtained. These were then labeled respectively by p-(arsonic acid)-benzene diazonium fluoroborate and p-nitrophenyl diazonium

fluoroborate. Spectrophotometric assays of azotyrosyl and azohistidyl derivatives for both systems gave evidence that the azo labels were similarly attached to one or more tyrosyl residues in the binding sites of the antibodies with high degrees of specificity. These observations led to the important conclusion that both the heavy and light chains of antibodies are involved in antigen-antibody interactions.

The biochemical studies of soluble alcohol dehydrogenase preparations from yeast were first made in 1933 [15]. Subsequently, the role of NAD as a coenzyme in the reaction was elucidated [16]. The reaction as a reversible oxidation of alcohol by NAD to aldehyde was formulated by von Euler et al. [17]:

$$RCH_2OH + NAD^+ \rightleftharpoons RCHO + NADH + H^+$$

Alcohol dehydrogenase was crystallized by Negelein and Wulff from brewer's yeast [18] as the first successful crystallization of a pyridine nucleotide dependent dehydrogenase. Currently, the most widely used purification scheme is the method of Racker [19] using baker's yeast.

Many physical and chemical properties of yeast alcohol dehydrogenase have already been determined [20]. Hayes and Velick [21] from sedimentation velocity and diffusion measurements calculated a molecular weight of 150,000 for the enzyme. A value of 151,000 was obtained by Kagi and Vallee in 1960 [22]. The crystalline enzyme showed only one peak in ultracentrifugation studies. Keleti [23] reported that yeast alcohol dehydrogenase was homogeneous as

determined by solubility curve studies. However, electrophoretic studies revealed two distinct components [21]. The one having an isoelectric point of 5.4 was catalytically active. The inactive second component varied between 6-60% of total protein concentration depending upon the state of purification [21]. Yeast alcohol dehydrogenase showed a normal protein absorption spectrum [21], with an extinction coefficient $E^{1\%}_{280\ nm} = 12.6$. The dispersion constant indicated a high content of helix structure [24].

Yeast alcohol dehydrogenase is quite unstable at pH values below 6.0 and above 8.5, and is very sensitive to heavy metal ions [20]. It may be dialyzed and lyophilized without loss of activity [25].

The amino acid composition of this enzyme has been determined by Wallenfels and Arens [26], and is listed in Table I, page 6. The active enzyme is tetrameric, consisting of four identical subunits each of molecular weight 36,000 [22]. The enzyme contains four zinc atoms and four coenzyme binding sites per molecule [21] with sulfhydryl groups necessary for activity [27].

TABLE I

Amino acid composition of yeast alcohol dehydrogenase

Amino Acid Residue	Moles/150,000 g Protein
Alanine	123.5 ± 1.4
Arginine	31.7 ± 2.0
Aspartic Acid	125.4 ± 3.4
Cystine/2	38.0
Glutamic Acid	111.9 ± 1.1
Glycine	150.2 ± 4.0
Histidine	39.6 ± 1.5
Leucine } Isoleucine	189.1 ± 7.1
Lysine	93.0 ± 1.4
Methionine	18.1 ± 0.5
Phenylalanine	68.2 ± 1.0
Proline	50.6 ± 1.3
Serine	75.4 ± 4.0
Threonine	58.6 ± 2.4
Tryptophan	27.1
Tyrosine	51.1 ± 5.1
Valine	149.4 ± 4.4
Amide-NH_2	73.7
Total	1400.86

LITERATURE REVIEW

Although the reactions of diazonium compounds with amino acid residues have been under study for many years, there is as yet no comprehensive review in this area. More recently, many diazonium derivatives have been applied to biochemical studies, and there is a need to compile and evaluate these data. In this section, the spectral studies of amino acid azo derivatives as well as biochemical studies using diazonium reagents will be summarized. A few reviews [28] are available on the general chemistry of aromatic diazonium compounds. However, the chemical complexities of these reactions have only been elucidated recently [2]. The basic theory which can formulate an interpretation of diazonium-sulfhydryl reactions will be presented. The synthesis, study and application of the active site specific 3-aminopyridine dinucleotides capable of diazotization have been reported only recently and will be dealt with here in some detail. Finally, although the chemistry of naturally occurring thiols are well documented [29, 30], and the sulfhydryl groups in yeast alcohol dehydrogenase have been discussed in many articles, it is necessary to point out the areas especially relevant to the present study as well as future possibilities in relation to diazonium-sulfhydryl reactions.

Spectral Studies of Amino Acid Azo Derivatives

Most of the diazonium protein modification experiments have been

studied by observing spectral changes without isolation of products. However, the spectral properties of only the azo derivatives of tyrosine, histidine and lysine have been reported in some detail.

Tabachnick and Sobotka [31] reacted the diazonium derivatives of arsanilic, sulfanilic and p-aminobenzoic acids with tyrosine, histidine, ε-aminocaproic acid and the N-acetyl derivatives of these amino acids. The experiments were carried out at 5° in 0.01 M borate buffer of pH 9.3-9.5. At pH 6.2, the monoazobenzene derivatives obtained for N-chloroacetyltyrosine and N-acetylhistidine showed absorption maxima at 325 and 370 nm respectively. The absorption maxima and molar extinction coefficients of these derivatives at pH 6.2 and in 0.1 N NaOH are listed in Table II, page 9. The bisazobenzenearsonic acid derivatives of N-chloroacetyltyrosine and ε-aminocaproic acid have been prepared. Their spectral characteristics are listed in Table III, page 10. The suggested chemical structure for bis(p-azobenzenearsonic acid)chloroacetyltyrosine [31] is shown in Figure 1, page 12. The pentazene structure suggested for bis(p-benzenearsonic acid diazo)ε-aminocaproic acid [31] is shown in Figure 2, page 14. It was thought that in the presence of free amino acids, the α-amino group could compete for the diazonium ions [31]. Thus N-acetyl derivatives were used to provide for more reliable spectra.

In a subsequent study [11], the diazo coupling reactions with native and acetylated bovine serum albumin were studied spectrophotometrically. To overcome the overlap of absorption spectra of

TABLE II

Spectral properties of monoazobenzene derivatives of N-chloroacetyltyrosine and N-acetylhistidine

Diazo reagent based on	Monoazo-chloroacetyltyrosine				Monoazo-acetylhistidine			
	pH 6.2		0.1 N NaOH		pH 6.2		0.1 N NaOH	
	λ_{max}	$\varepsilon \times 10^{-3}$	λ_{max}	$\varepsilon \times 10^{-3}$	λ_{max}	$\varepsilon \times 10^{-3}$	λ_{max}	$\varepsilon \times 10^{-3}$
Arsanilic acid	325 nm	21.6	330 nm	14.2	370 nm	21.6	420 nm	22.3
			490 nm	11.0				
Sulfanilic acid	325 nm	22.1	330 nm	14.0	370 nm	22.4	423 nm	23.4
			490 nm	11.5				
p-Aminobenzoic acid	325 nm	20.0	330 nm	13.9	370 nm	21.1	420 nm	21.7
			488 nm	10.7				

TABLE III

Spectral properties of bisazobenzenearsonic acid derivatives

Derivatives	pH 6.2			0.1 N NaOH		
	λ_{max}	$\varepsilon \times 10^{-3}$	color	λ_{max}	$\varepsilon \times 10^{-3}$	color
Bis(p-azobenzenearsonic acid)-chloroacetyltyrosine	330 nm	35.6	yellow	325 nm	26.0	purple
	420 nm	11.4		545 nm	17.5	
Bis(p-benzenearsonic acid diazo)ε-aminocaproic acid	363 nm	34.8	faint yellow	378 nm	30.8	light yellow

FIGURE 1.

Structure of bis(p-azobenzenearsonic acid)chloroacetyltyrosine

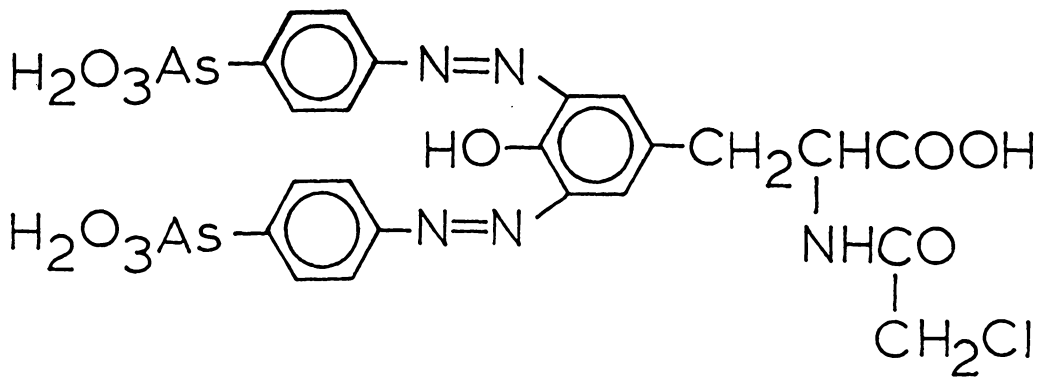
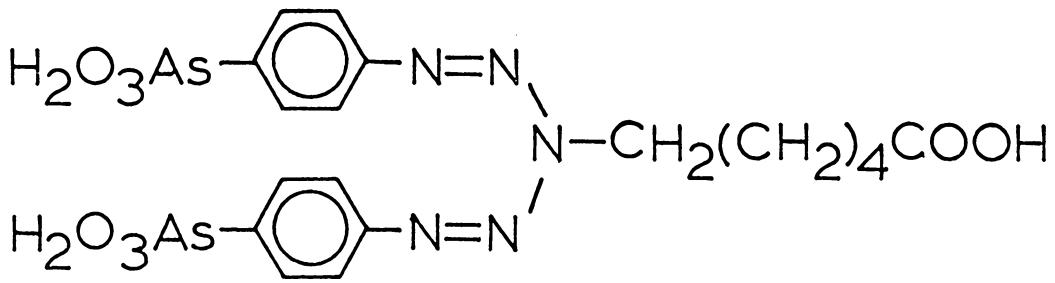

H2O3As
N=N
HO
CH2CHCOOH
NHCO
CH2Cl
H2O3As
N=N

FIGURE 2.

Structure for bis(p-benzenearsonic acid diazo)ε-aminocaproic acid


H2O3As
N=N
N
CH2(CH2)4COOH
H2O3As
N=N

azotyrosyl and azohistidyl derivatives, a simultaneous equation based upon absorbance at 460 nm and 500 nm was used to determine the amounts of each derivative. The protein bound arsenic was also quantitated. At pH 9 and 10, azotyrosine and azohistidine accounted for about 50% of the bound arsenic in native bovine serum albumin. With the amino groups blocked in acetylated bovine serum albumin, practically all the bound arsenic was found as azotyrosine and azohistidine under the same conditions. However, at pH 8, only about 50% of the bound arsenic could be attributed to azotyrosine and azohistidine irrespective of the degree of acetylation. The authors suggested that this may reflect a relatively greater degree of azo-coupling with other amino acids such as tryptophan and arginine [11].

Higgins and Harrington [32] studied the coupling reactions of diazotized sulfanilic acid with histidine and tyrosine. The spectral data were obtained without isolation of products. The spectral constants were similar to the monoazo derivatives obtained by Tabachnick and Sobotka [31]. Using these data, various groups studied the reactions of serum albumin [33], fibrinogen [34], and insulin [35] with diazonium salts. They reported that the reactions were not confined to the tyrosyl and histidyl residues. Subsequently, they demonstrated the reactions of ε-amino, guanidino and imino groups with diazonium compounds [12]. The products of reactions of lysine, arginine and proline with diazotized sulfanilic acid showed absorption maxima at 363, 356 and 317 nm, respectively.

The spectra of the monoazo and bisazo derivatives of tyrosine are pH dependent [11, 36]. The pK of the phenolic hydroxyl of monoazotyrosine is approximately 8.8, that of bisazotyrosine is near 8.2. Both derivatives decomposed when heated strongly in acid. The pK of monoazohistidine is approximately 7.6. Histidine and tyrosine residues in proteins react with diazonium reagents at similar rates. Bisazohistidine is usually formed at lower molar ratios of diazonium to protein than is bisazotyrosine. Azohistidines are also unstable in hot concentrated acids. Thus both azotyrosines and azohistidines are determined after acid hydrolysis only by comparison with the unmodified protein hydrolyzate in terms of the decrease in tyrosine and histidine content.

The colored products obtained when histidine and tyrosine were treated with a diazonium reagent formed the basis of several colorimetric procedures for determining these compounds. In the case of proteins, a large excess of reagent is often needed to insure complete reaction. However, this results in high blank values due to decomposition of diazonium ions to phenols followed by formation of azophenols. This problem is not encountered when diazonium-1-H-tetrazole is used. The latter has made possible the development of more accurate colorimetric procedures for the quantitation of both histidyl and tyrosyl residues in proteins [37]. The spectral properties of several such azotetrazole derivatives are listed in Table IV, page 17.

TABLE IV

Spectral properties of some azotetrazole derivatives of amino acids

Compound	λ_{max}	$\varepsilon \times 10^{-3}$
Azotetrazole-N-acetylhistidine	360 nm	11.5
Bisazotetrazole-N-acetylhistidine	480 nm	21.0
Azotetrazole-N-acetyltyrosine	478 nm	5.2
Bisazotetrazole-N-acetyltyrosine	548 nm	14.1

Biochemical Studies with Diazonium Reagents

Quite a few problems in the biochemistry of proteins have been tackled with various diazonium reagents. However, in every case, the assumption has always been that either tyrosyl, histidyl or lysyl residues were involved.

Affinity labeling studies with diazonium compounds were carried out with the acetylcholine receptor of electroplax synaptic membrane [38]. The compound p-(trimethylammonium)benzene diazonium fluoroborate was shown to specifically and irreversibly label the acetylcholine receptor sites. The authors suggested that either tyrosyl, histidyl or lysyl residues might be involved. In the presence of dithiothreitol the diazonium compound became a reversible activator. The enzyme acetylcholinesterase was similarly studied with p-(trimethylammonium)benzene diazonium fluoroborate [39]. It was found that the reagent bound with two classes of sites. One was the anionic site of the active center, the other was the noncatalytic anionic site. After exposure to dithiothreitol, the diazonium compound became a reversible inhibitor. Tyrosine was suggested to be the amino acid group involved in these reactions. Further studies with bovine acetylcholinesterase [40] indicated that irreversible inhibition occurred at 10^{-7} to 10^{-5} M diazonium ion concentration. Protection was effected by the reversible inhibitor phenyltrimethylammonium chloride. However, even with 2,500-fold or more of free histidine at pH 6, the rate of inactivation was only slowed down

partially. A similar degree of irreversible inhibition was obtained with p-nitrobenzene diazonium fluoroborate, but the p-acetoxy analog was ten times weaker [41]. It was considered that strong electron-withdrawing groups such as p-(trimethylammonium) and p-nitro groups promoted the positively charged diazonium group to form a covalent bond with an appropriate amino acid residue.

In experiments with native ribonuclease [42], gradual increases in the concentration of diazonium-1-H-tetrazole revealed that histidyl residues were attacked. The four histidyl residues coupled with the reagent in discrete steps. In the denatured enzyme, no such differential reactivities were observed.

Diazonium-1-H-tetrazole was also employed in studies of carboxypeptidase A [43]. At low concentrations of reagent, tyrosyl and lysyl residues were modified. A 200% increase in esterase activity correlated with the modification of a single tyrosyl residue while the peptidase activity was not affected. At higher reagent concentrations, the peptidase activity decreased progressively with loss of one histidyl residue. Prior acetylation of tyrosyl residues resulted in the formation of monohistidyl-carboxypeptidase retaining only esterase activity. This suggested that the histidyl residue may be important to peptidase but not esterase activity. In the presence of the competitive inhibitor β-phenylpropionate, the changes in activities were prevented [44]. The coupling with tyrosyl residues was also used as a probe for the chemical environment of the modified residues. When carboxypeptidase A was coupled with diazotized

arsanilic acid, a characteristic circular dichroic spectrum with multiple extrinsic Cotton effects between 300 and 600 nm indicative of arsanilazotyrosine was obtained [45]. Tyrosine involvement was confirmed by amino acid analysis and measurement of protein bound arsenic. The latter experiments further indicated the modification of lysine. Other carboxypeptidase derivatives, namely, O-acetyl-N-succinyl-, nitro- and azotetraazolylcarboxypeptidase were also modified with diazotized arsanilic acid. The results were consistent with the formation of arsanilazotyrosine as reflected in the visible circular dichroic spectra. The binding of a substrate, glycyl-L-tyrosine; or an inhibitor, β-phenylpropionate; or the removal of catalytically essential zinc markedly altered the spectrum in a way chararacteristic for each.

The histidyl residues of glucose dehydrogenase from A. oryzae [46] have been examined with diazonium-1-H-tetrazole coupling. The effects on methylene blue catalyzed photo-oxidation, metal inactivation and pH rate profiles have been reported. The inactivation was prevented by prior presence of substrate. Since the inactivation affected only the reduction of FAD by glucose while re-oxidation of $FADH_2$ by vitamin K_3 was unimpaired, it was concluded that probably a histidyl residue at the glucose binding site was modified.

The modification of α-chymotrypsin by diazotized sulfanilic acid, diazobenzene, 3-diazoquinoline, α- and β-diazonaphthalene at 4° was investigated [47]. α-Diazonaphthalene was the most inhibitory,

causing 90% inhibition in 20 min at 40-fold excess and pH 8.0. Inhibitory effects of the diazonium compounds increased with a decrease in K_i values of the corresponding amines. The enzyme was protected by the competitive inhibitor, β-phenylpropionic acid. The compound p-diazo-N-acetyl-L-phenylalanine methyl ester was synthesized and used as an active site directed irreversible inhibitor [48]. Spectral analysis indicated that azotization occurred in tyrosyl and lysyl residues. Two kinds of modified enzymes were obtained, the inactive and active modified enzymes. In both cases, amino acid analysis of lysine, histidine, arginine, aspartic acid, serine, alanine, methionine, isoleucine, tyrosine, phenylalanine and tryptophan indicated that only lysine and tyrosine contents had decreased after modification of the enzyme.

Triose phosphate isomerase was inactivated by limited amounts of diazotized sulfanilic acid 10^{-5} to 10^{-4} M with the diazonium concentrations ranging from 0.5 to 5 times the molar concentration of the enzyme [49]. The enzyme was partially protected by dihydroxyacetone phosphate, and twice the amount of diazonium reagent was needed to render 50% inactivation. As there was no absorption maximum above 320 nm in the spectrum of the modified enzyme, discrediting the involvement of histidyl or tyrosyl residues, the authors suggested that lysyl residues were involved.

Deoxyribose-5-phosphate aldolase was inactivated by diazotized p-nitroaniline [50]. Substrate protection by deoxyribose-5-phosphate suggested that the inactivation occurred at the active site.

The histidyl or tyrosyl residues were again suggested as the possible amino acid residues being modified.

Neutral subtilopeptidase of Bacillus subtilis was treated with 50-fold excess of p-nitrobenzene diazonium fluoroborate at pH 6.5 [51]. It was found that the caseinolytic activity was reduced, while activity towards the synthetic substrate CBZ-Gly-L-Leu.NH_2 was increased. β-Diazonaphthalene fluoroborate at pH 8.5 to 10, also exerted a similar effect. When compared with model compounds, the spectral data indicated that azo coupling occurred exclusively with tyrosyl residues. Amino acid analysis indicated a decrease in the tyrosine content after modification. When 3-diazoquinoline fluoroborate was used, the activity was not affected in a similar way. It was suggested that in this case, the lysyl residues were involved [51].

The inhibition of coupling factor activity of chloroplast membranes by diazonium compounds were studied recently [52]. Diazotized sulfanilic acid was found to inhibit both non-cyclic and cyclic photophosphorylation. The uncoupling effect was shown by the increased rate of basal electron flow and loss of the ability of ADP to stimulate electron flow. The calcium-dependent ATPase activity of the coupling factor was also found to be severely inhibited.

Chemistry of Aromatic Diazonium Compounds

Aromatic primary amines react with nitrous acid in acidic solution to give diazonium ions, $R\text{-}\overset{+}{N}\equiv N$ [53]. The electrophilic

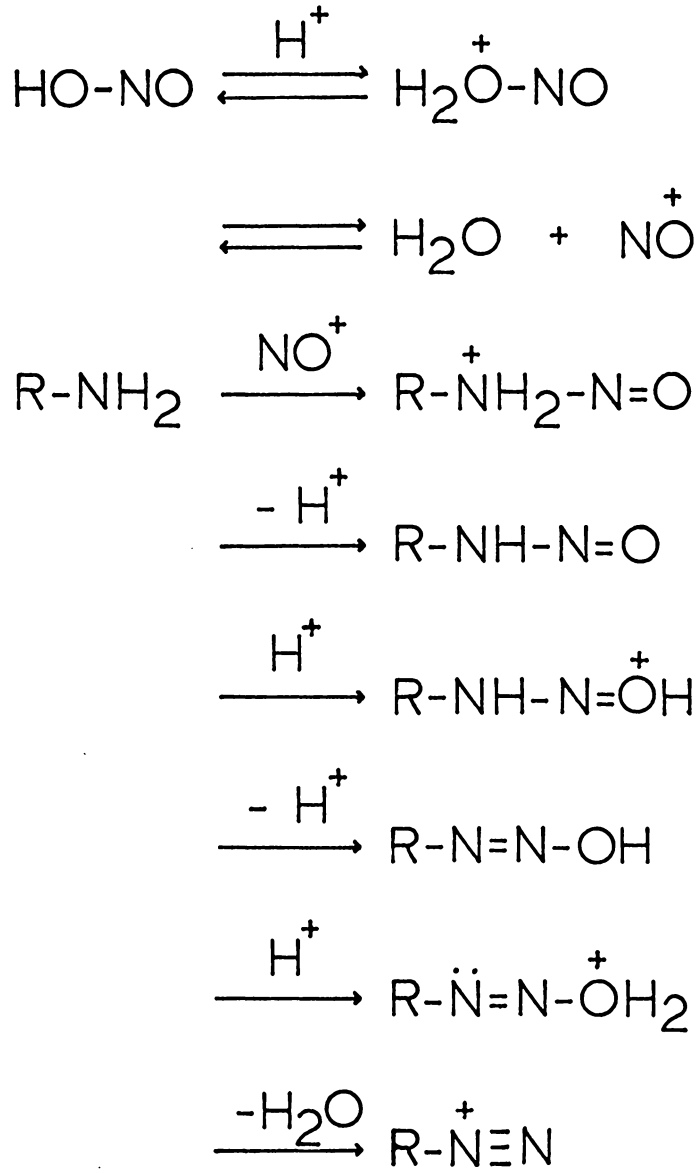
nitrosonium ion attacks the nucleophilic nitrogen of the amine, which is followed by a series of prototropic shifts with the final elimination of water. The mechanism of the reaction is summarized in Figure 3, page 25.

Aromatic diazonium ions are moderately stable in aqueous solution at low temperatures. Diazotization is normally carried out by adding an aqueous solution of sodium nitrite to a solution or suspension of the amine hydrochloride in an excess of hydrochloric acid cooled in an ice bath. The rate of addition is controlled to keep the temperature below 5°. Addition is continued until a slight excess of nitrous acid is reached. Amines that are substituted in the aromatic nucleus with electron-attracting groups are less easily diazotized, as the nucleophilicity of an amine nitrogen is reduced by partial withdrawal of the unshared electron pair into the nucleus. Acetic acid can be used as a suitable reaction medium in these cases, and even 2,4,6-trinitroaniline can then be diazotized.

Most reactions employing diazonium salts are conducted in solution. However, when anions of low nucleophilic power are present, diazonium compounds may be isolated as salts. The diazonium chloride or sulfate solution can be treated with alkaline fluoroborate to precipitate the insoluble diazonium fluoroborate $ArN_2^+BF_4^-$. An alternative method of preparation is to treat the amine hydrochloride with an organic nitrite and acetic acid in ether solution. The diazonium salt formed is insoluble in ether and is obtained as a precipitate.

FIGURE 3.

Mechanism of diazonium ion formation from an aromatic primary amine and nitrous acid


HO-NO
H+
H2O-NO
H2O + NO+
R-NH2
NO+
R-NH2-N=O
- H+
R-NH-N=O
H+
R-NH-N=OH
- H+
R-N=N-OH
H+
R-N=N-OH2
-H2O
R-N≡N

The reactions of aromatic diazonium ions may be classified into seven types [2]. These are summarized in Figure 4, page 28.

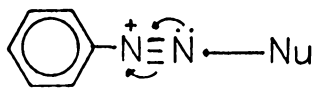
In Type I reactions, nucleophiles attack at the β nitrogen. The possibilities for the formation of cis and trans isomers can be considered by treating the aromatic diazonium ion as analogous to a monoarylacetylene [2]. As illustrated in Figure 5, page 30, two types of transition states for the aromatic diazonium ion-nucleophile intermediate are possible. If the transition state is reactant-like (early on the reaction co-ordinate), repulsive interaction between the nucleophile and aryl nucleus would be small as the Nu-N_β distance is still large. Thus the larger repulsion of the lone electron pair at N_β with the aryl nucleus favors a cis attack of nucleophile on N_β. If the transition state is product-like (late), the Nu-N_β distance is short. The stronger repulsion is that between the nucleophile and aryl nucleus, resulting in formation of the trans isomer. Experimental results indicated that transition states of reactions with nucleophiles containing π-electron systems (e.g., aromatic nuclei) are more product-like than for reactions with simple anions (e.g., OH^-, CN^- and N_3^-) [54].

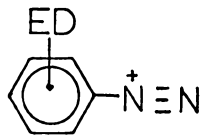
In Type IV reactions, dediazoniation is effected by nucleophilic aromatic substitution. In addition to hydrolysis and substitution by halide ion, diazonium salts can act as arylating agents in heterolytic arylation of aromatic compounds [55].

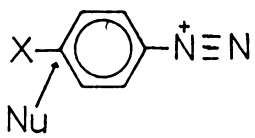
In Type VI reactions, a homolytic cleavage of the C(1)-N_α bond occurs. Experimental investigations seemed to indicate multiple

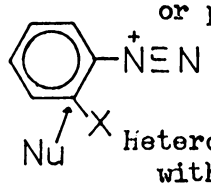
FIGURE 4.

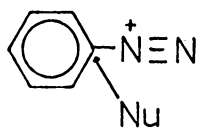
Type reactions of aromatic diazonium ions

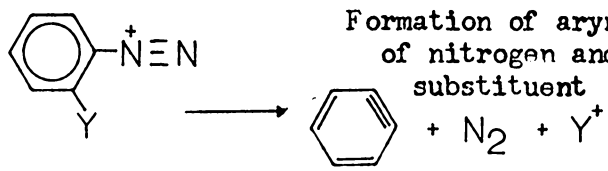
TYPE I.  Reactions with nucleophiles at the β nitrogen

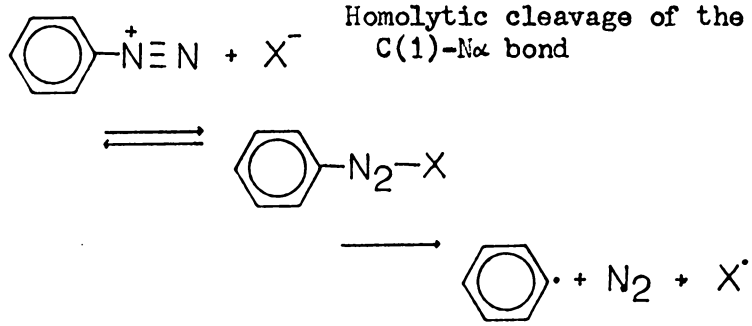
TYPE II.  Formation of complexes with electron donors

TYPE III.  Substitution of nucleofugal leaving groups in the ortho or para position of aryl nucleus



TYPE IV.  Heterolytic dediazoniations with nucleophile

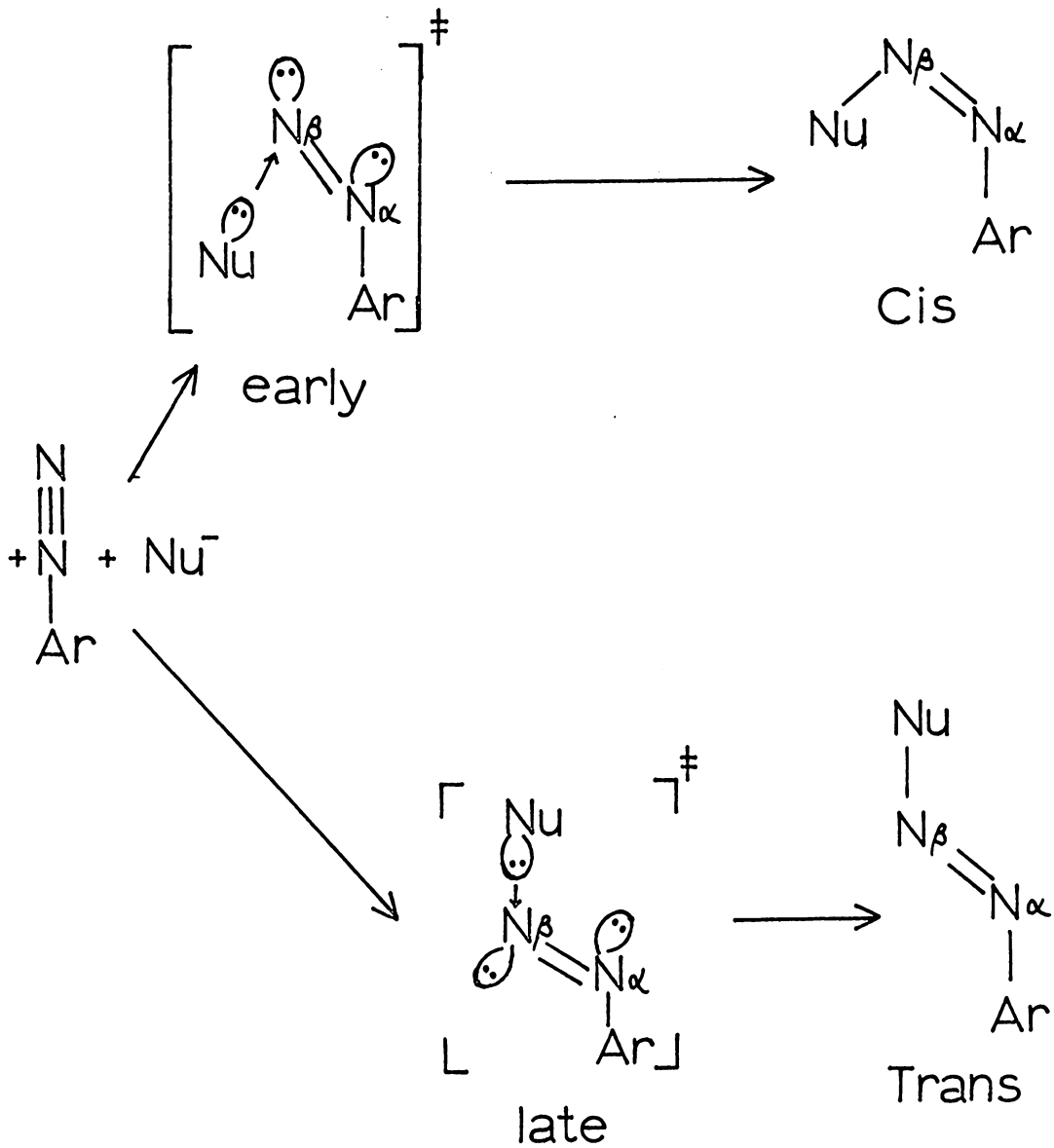
TYPE V.  Formation of arynes by loss of nitrogen and an ortho substituent

TYPE VI.  Homolytic cleavage of the C(1)-Nα bond

TYPE VII. Photolytic dediazoniations

FIGURE 5.

Cis and trans isomer formation in diazonium Type I reactions


Nβ
Nα
Nu
Ar
‡
early
Cis
N
+N
Nu⁻
Ar
Nu
Trans
late

pathways. However, recent examples [56] revealed that homolytic and heterolytic reaction mechanisms may resemble each other more closely than previously thought. The observations may be explained by the concept of a nucleofugal homolytic leaving group. This involves an attack by a nucleophile X^- on the β nitrogen of the diazonium ion and its release as a radical $X^{\bullet}$ by the fragmentation of the C(1)-N_α and the N_β-X bonds. The product $X^{\bullet}$ is a more efficient "gegen-radical" for reacting with the intermediate of the subsequent homolytic aromatic substitution.

The rates of reaction of the different amino acid residues histidine, tyrosine and lysine all increase with increasing pH [57]. The optimum reaction rate of each are reported to be near pH 9. At higher pH's the reaction becomes slowed down by the competitive formation of azohydroxides and their corresponding salts:-

$$Ar\text{-}\overset{+}{N}\equiv N + OH^- \rightleftharpoons Ar\text{-}N=N\text{-}OH \rightleftharpoons Ar\text{-}N=N\text{-}O^- + H^+$$

During azo coupling, tyrosine reacts first to the monoazo derivative and then with excess reagent to the bisazo derivative. Histidine also yields the monoazo and bisazo derivatives. Lysine reacts to form the triazene and pentazene derivatives. All these derivatives are unstable to heat and concentrated acids, decomposing apparently with the evolution of nitrogen. A few other amino acid residues in proteins were also reported to react slowly with diazonium compounds, but products of such reactions have not been well characterized.

Dinucleotide Derivatives of 3-Aminopyridine

In the investigation of dehydrogenase-catalyzed reactions, the participation of pyridine nucleotide coenzymes has been studied in many different ways. The use of pyridine nucleotide coenzyme analogs containing pyridine bases other than nicotinamide has successfully yielded important information concerning dehydrogenase-catalyzed processes. Recently, AAD was synthesized for use in dehydrogenase studies [1]. Since 3-aminopyridine is highly fluorescent, the incorporation of this nitrogen base into dinucleotide form provides an oxidized analog of NAD that can be used for fluorescence titration of dehydrogenases and other pyridine nucleotide-requiring enzymes. Also, since 3-aminopyridine is a diazotizable aromatic primary amine, the possibilities for diazotization of AAD for use as a site-labeling reagent for dehydrogenases can be considered.

The synthesis of AAD was achieved through the direct chemical modification of NAD [1]. Optimized conditions of the Hofmann hypobromite reaction [58] of the amide function produced AAD in a 68% yield. The compound was isolated by quasilinear gradient elution with ammonium formate solution from a Dowex AG 1-X8 formate column. Salt was removed by repeated extraction of the lyophilized product with absolute ethanol. The product gave a fluorescence spot by thin layer chromatography on cellulose with the solvent 0.1 M acetic acid-95% ethanol (1:1 by volume), R_F = 0.54. Alkaline hydrolysis and thin layer chromatographic analysis indicated two products identical with

3-aminopyridine and adenosine diphosphoribose. Snake venom phosphodiesterase cleavage of AAD gave products identical with AMP and 3-aminopyridine mononucleotide. Functional group quantitation indicated the expected ratio of amine:adenine:phosphate:ribose as 1:1:2:2. Elemental analysis of carbon, hydrogen and nitrogen was consistent with the molecular formula of 3-aminopyridine adenine dinucleotide monoformate monohydrate, molecular weight 733.52. Absorption maxima at 257 and 331 nm were observed for AAD with molar extinction coefficients of 19.5 and 3.09 x 10^3 cm^{-1} M^{-1}, respectively. Fluorescence spectra were characterized by an excitation maximum at 320 nm and an emission maximum at 402 nm.

As an analog of NAD, AAD was observed to inhibit seven NAD(P)-dependent enzymes. These were namely, yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, beef heart lactate dehydrogenase, beef muscle lactate dehydrogenase, glucose-6-phosphate dehydrogenase, pig heart malate dehydrogenase and bull semen NADase [1]. In all cases, the inhibition was shown to be competitive with respect to the pyridine nucleotide coenzyme employed.

When AAD was diazotized with nitrous acid, the absorption spectrum at pH 7.0 indicated the disappearance of the 331-nm absorption of AAD, and an appearance of a new 280-nm shoulder. Diazotized AAD was shown to be an irreversible inactivator of yeast alcohol dehydrogenase [1]. Supporting evidence for selective inactivation included: (a) low concentrations of reagent rapidly inactivated the enzyme; (b) four molecules of diazotized AAD were

bound per tetrameric molecule of enzyme; (c) NAD protected against the inactivation; (d) diazotized 1-methyl-3-aminopyridinium chloride inactivated the enzyme at only one tenth the rate of diazotized AAD at the same molar concentration. The effect of diazotized AAD concentration on the rate of inactivation appeared to be a sigmoidal relationship, which was interpreted to be related to the conversion of diazotized AAD to a form incapable of involvement in enzyme inactivation [1].

3-Aminopyridine adenine dinucleotide phosphate (AADP) was prepared from NADP and 3-aminopyridine through the pig brain NADase-catalyzed pyridine base exchange reaction [59]. The product was characterized by thin layer chromatography in two solvent systems. Functional group quantitation indicated that 3-aminopyridine, adenine, phosphate, ribose and ammonium ion were in a ratio close to 1:1:3:2:3. Elemental analysis of carbon, hydrogen and nitrogen suggested the compound to be 3-aminopyridine adenine dinucleotide phosphate triammonium monoformate trihydrate. Alkaline hydrolysis yielded 3-aminopyridine quantitatively. The absorption spectrum was identical to that of AAD, with absorption maxima at 257 and 331 nm, and molar extinction coefficients 19.4 and 2.92 x 10^3 cm^{-1} M^{-1}, respectively. The excitation maximum for fluorescence is 331 nm with an emission maximum at 420 nm.

Inhibition studies [59] indicated that AADP is an effective inhibitor of the NADP-requiring enzymes, yeast glucose-6-phosphate dehydrogenase, yeast glutathione reductase, bovine liver glutamic

dehydrogenase and yeast 6-phosphogluconate dehydrogenase. Inhibition by AADP was competitive with respect to NADP with yeast glucose-6-phosphate dehydrogenase, yeast 6-phosphogluconate dehydrogenase and yeast glutathione reductase. However, bovine liver glutamic dehydrogenase was inhibited noncompetitively. The NADP-requiring pig heart isocitrate dehydrogenase was not inhibited by AADP. The NAD-requiring dehydrogenases, yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, beef heart lactate dehydrogenase, beef muscle lactate dehydrogenase and pig heart malate dehydrogenase were likewise not inhibited by AADP. With bull semen NADase, substrate competitive inhibition by AADP was observed.

Fluorescence titration of yeast glucose-6-phosphate dehydrogenase indicated tight binding of two moles of AADP per dimeric form of enzyme. This was interpreted as the binding of one mole of AADP per catalytic subunit of the enzyme. AADP was also found to serve as a fluorimetric substrate for snake venom nucleotide pyrophosphatase [59].

When AADP was diazotized, the spectral changes were similar to those observed in the diazotization of AAD. Azo-coupling of diazotized AADP with N-1-naphthylethylenediamine or 2-naphthol was possible at acidic pH's, but no reaction occurred above pH 6.0 [59]. At pH 7.0, diazotized AADP inhibited yeast glucose-6-phosphate dehydrogenase and yeast 6-phosphogluconate dehydrogenase reversibly, being competitive with respect to NADP. However, no inhibition was observed with yeast glutathione reductase or pig heart isocitrate dehydrogenase.

Chemistry of Naturally Occurring Thiols

The thiols in cells may be grouped into three categories, low molecular weight thiols, non-enzyme proteins and enzymes. The low molecular weight thiols [29] include hydrogen sulfide, alkane thiols, cysteine, homocysteine, dihydrolipoate, ergothioneine, coenzyme A and glutathione. Most of the sulfhydryl compounds in nature are derivatives of L-cysteine, and glutathione is the most abundant non-protein thiol. Non-enzyme protein thiols include most cytoplasmic proteins [29], such as g-actin and myosin, plasma membrane proteins and structural proteins. Many enzymes contain sulfhydryl groups, which may be concerned directly with catalysis, binding of substrates, binding or regulation at allosteric sites or binding of subunits.

Sulfhydryl enzymes have been described in most of the subgroups of oxidoreductases. A comprehensive list of such enzymes is compiled in Table V, pages 37 and 38. There is much variation in the total number of free sulfhydryl groups per molecule of enzyme, ranging from 1 to 36. There is no general relationship of the reactivity of sulfhydryl groups with enzyme activity. Thus the alcohol dehydrogenases [60, 61] and beef heart lactate dehydrogenase [63] lose their activities when their fast reacting sulfhydryl groups are titrated, whilst with malate dehydrogenase [64] sulfhydryl titration leads to activation. In isocitrate dehydrogenase [66], only two of the five reactive sulfhydryl groups are required for activity, whereas in catalase [83] none is involved. Substrate protection of sulfhydryl

TABLE V

Occurrence and properties of sulfhydryl groups of the oxidoreductases[a]

E. C. No.	Substrate	Source	Total SH	SH reactivity	References and comments
1.1.1.1	Alcohol	Horse liver	24	2F 2I	(60) fast SH reactivity
		Yeast	36	4F	(61) independent of pH.
1.1.1.26	Glyoxylate	Spinach	4	4F sI	(62)
1.1.1.27	Lactate	Beef heart	7	4F 4I	(63) self reactivation.
1.1.1.37	Malate	Heart muscle	15	4F 3,4A 8I	(64) NEM inactivates but
		Heart supernatant	6	3F 2N	(65) not iodoacetate.
1.1.1.42	Isocitrate	Pig heart	13	5M 2I	(66)
1.1.1.43	6-phosphogluconate	Bacteria	9	4M 4I	(67)
1.1.3.9	Galactose	Mold	1	3F	(68) SS reduction involved.
1.1.99.5	Glycerol-1-P	Liver	10	10F	(69)
1.2.1.12	Glyceraldehyde-3-P	Rabbit muscle	14	11F 3S 2,4I	(70) acyl-enzyme form?
		Lobster muscle	19	4F 4I	(71)
		Yeast	6		(72)
1.2.4.1	Pyruvate	E. coli	8	2F	(73)
1.4.3.3	D-amino acids	Hog kidney	12	7M 7I	(74) disulfides inhibit.
1.6.4.2	Glutathione (ox)	Yeast	8		(75)
1.6.4.3	Lipoate	Pig heart	4,7		(76)
1.6.4.-	Thioredoxin	Bacteria	2		(77)
1.6.99.3	NADH	Microsomes	4	3M 1I	(78)
1.6.99.4	Ferredoxin	Chloroplasts	4		(79)
1.8.4.-	Protein SS/SH exchange	Beef liver	3	1I	(80)

[a]The following abbreviations are used: A, active; F, fast; I, inhibit; M, moderate; N, no effect; s, some; NEM, N-ethylmaleimide.

TABLE V (Continued)

E. C. No.	Substrate	Source	Total SH	SH reactivity	References and comments
1.9.3.1	Cytochrome a	Beef heart	7		(81)
1.10.3.3	Ascorbate	Plant juice	12		(82)
1.11.1.6	Hydrogen peroxide	Erythrocytes	15	6N	(83)
1.13.1.2	Catechol	Bacteria	12	4M 4I	(84) slow reactivation.
1.14.1.5	Imidazole acetate	Bacteria	2	1I	(85)
1.-.-.-	Luciferase	Firefly	7	2I	(86)
1.1.1.17	Mannitol phosphate	Bacteria	s	sI	(87)
1.1.1.31	β-Hydroxy-isobutyrate	Kidney	s	sI	(88)
1.1.1.50	3-α-Hydroxysteroid	Liver	s	sI	(89)
1.2.1.3	Aldehydes	Liver	s		(90)
1.2.3.2	Xanthine	Various	s	sI	(91) mercurials inhibit.
1.3.99.1	Succinate	Various	s	sI	(92) disulfides inhibit.
1.4.1.2	Glutamate	Bird liver	s		(93) SH at allosteric site?
1.4.3.4	Monoamines	Mitochondria	s	sI	(94)
1.5.1.2	Pyrroline-5-carboxylate	Liver	s	sI	(95)
1.5.1.3	Folate	Bacteria	s		(96)
1.6.6.3	Nitrate	Bacteria	s	sI	(97)
1.6.6.-	Nitro groups	Bacteria	s	sI	(98)
1.6.99.3	NADH	Mitochondria	s	sI	(99)
1.8.4.-	GSH/Co A exchange	Rat liver	s		(100)
1.10.3.1	Tyrosine	Mushrooms	s		(101) NEM inhibits.
1.11.1.6	GSH	Rat liver	s	sI	(102)
1.13.1.3	Protecatechuate	Bacteria	s	sI	(103) SH binds Fe?
1.13.1.5	Homogentisate	Liver	s	sI	(104) SH binds Fe?
1.13.-.-	Tryptophan	Bacteria	s	sI	(105) SH binds Fe?
1.13.-.-	Indole	Leaf	s	1I	(106) SH binds Fe?
1.14.2.1	Dopamine	Adrenals	s	sI	(107)
1.14.-.-	Proline	Chick embryo	s	sI	(108)

groups has been demonstrated in some oxidoreductases, suggesting that the sulfhydryl groups may be close to the active sites. Additional evidence for the direct involvement in catalysis of sulfhydryl groups is obtained from studies utilizing electron spin resonance, spectrofluorimetry or isolation of presumed enzyme-bound intermediates. Such studies have been reported for the alcohol dehydrogenases [60, 61], lactate dehydrogenase [63], glyceraldehyde-3-phosphate dehydrogenases [70-72] and the disulfide reducing enzymes [80]. In other oxidoreductases, such as imidazole acetate oxygenase [85], the sulfhydryl groups are concerned only with binding of substrates. Other sulfhydryl groups may be involved in binding at allosteric sites. Thus in glutamate dehydrogenase [93], some sulfhydryl groups become nontitrable when allosteric activators or inhibitors are present. In enzymes that require metal ions or cofactors for activity, these metal ions or cofactors may be bound directly through the sulfhydryl groups, as in the case of D-amino acid oxidases [74].

Each of the main groups of transferases with the exception of alkyl and acyl transferases contains sulfhydryl enzymes. Free sulfhydryl groups per molecule of enzyme vary from 2 to 68. Titration of fast reacting sulfhydryl groups inhibits pyridoxamine pyruvate transaminase [109] and ATP-creatine phosphotransferase [110], but activates phosphofructokinase [111], and is without effect on phosphorylase b [112]. In some cases, reactivity of the sulfhydryl groups is affected when binding sites are occupied. Thus with aspartokinase [113], all 18 sulfhydryls become nontitrable when

threonine is added as an allosteric inhibitor. In aspartate transaminase [114] and carbamyl phosphate synthetase [115], the binding of substrates increases the reactivity of sulfhydryl groups towards mercurials. In aspartate transcarbamylase [116], phosphorylase b [112] and hexokinase [117], there is a slow separation of subunits after titration with mercurials, which can be reversed by adding an excess of simple thiols to reconstitute the active enzymes.

In hydrolases, apart from fructose diphosphatase [118] and urease [119], the sulfhydryl enzymes have only a small number of sulfhydryl groups. Proteolytic enzymes from plants such as papain [120], chymopapain [121], ficin [122] and bromelain [123] have one essential sulfhydryl group per molecule, and there is evidence that these sulfhydryl groups are directly involved in catalysis. Fructose diphosphatase [118] is an enzyme with a complex allosteric mechanism. Its activity is doubled when two of the 20 sulfhydryl groups are titrated with mercurials. An eight fold activation is attained when homocystine is used to block the sulfhydryl groups by mixed disulfide formation. However, in the case of urease [119], the enzyme activity is unaffected by titration of up to 27 of its fast reacting sulfhydryl groups.

Some of the sulfhydryl enzymes of lyases have a large number of sulfhydryl groups, but are inactivated when only a few are titrated. Examples include ribulose diphosphate carboxylase [124] and the citrate cleavage enzyme [125].

Only a small number of isomerases and ligases are reported to

contain sulfhydryl groups. In phosphoglucomutase [126], all of the sulfhydryl groups are titrable without loss of activity. Six ligases reported to possess sulfhydryl groups are concerned with the formation of acyl-RNA intermediates [127, 128], illustrating one point where sulfhydryl groups are involved in protein synthesis.

Sulfhydryl groups can participate in anionic, cationic and free radical reactions [29]. The reactivity of the thiolate anion is much greater than is accountable by its basicity, as it is one of the strongest biological nucleophiles. This is probably due to the polarizability of the sulfur electrons and the availability of empty d-orbitals, thus making d-orbital overlap possible. The pH dependence of these reactions is related to ionization of both reagent and sulfhydryl. In the case of mercaptide formation and alkylation, the rate increases with increasing pH, indicating that the thiolate anion RS^- is more reactive than the unionized thiol RSH.

The sulfhydryl groups in polythiol proteins often vary widely in reactivity. The differential reactivities can be explained as follows. Three factors are important to sulfhydryl groups in the native enzyme [129], steric, electrostatic and the state of ionization. Steric effects can impede the reaction if the reagent is unable to approach the sulfhydryl group located in a pit or crevice or within the protein structure. Electrostatic effects due to the electric field of surrounding groups can affect the nucleophilicity of the sulfhydryl group. Thus a pKa of 7.2-8.5 generally indicates that the sulfhydryl group is near a positive charge, a value of 8.5-9.2 indicates that

there is no electric field, and a value of 9.2-10.2 indicates that it is near a negative charge. The electric field will also discourage reaction with those reagents that have like charges. The state of ionization of the sulfhydryl group, whether thiol or thiolate, is also important. When the preferential form is not significantly present at the experimental pH, the reaction will be appreciably slowed.

Sulfhydryl Groups in Yeast Alcohol Dehydrogenase

The relationship of the cysteinyl residues of yeast alcohol dehydrogenase to substrate and coenzyme binding and catalytic activity has been studied with a variety of sulfhydryl reagents. The enzyme was first described as a sulfhydryl enzyme in 1935 by Wagner-Jauregg and Moeller [27]. In 1937, Dixon [130] reported that iodoacetate at a concentration of 3×10^{-4} M could inhibit yeast alcohol dehydrogenase but not eleven other dehydrogenases. Results of sulfhydryl titrations varied from case to case. Barron and Levine [131] obtained a value of 25.5 sulfhydryl groups per mole of enzyme with iodosobenzoate titration, and 19.9 per mole of enzyme by amperometric silver titration. Glutathione partially reversed the iodosobenzoate inhibition. Hoch and Vallee [132] obtained 22.2 and 24.7 sulfhydryl groups per mole of enzyme in two separate experiments with silver nitrate. Ethanol and NAD did not show significant protection in these experiments. Snodgrass *et al.* [133] demonstrated that to achieve 50% inhibition of the enzyme activity, 13.1 molar excess silver nitrate or 23.4 molar excess *p*-hydroxymercuribenzoate had to be used. They found that there

was no zinc ion dependence for the glutathione reversal of p-hydroxymercuribenzoate inhibition of yeast alcohol dehydrogenase. However, Wallenfels and Sund [134] reported that both zinc ion and glutathione were necessary for the reversal of inhibition. Whitehead and Rabin [135] conducted p-hydroxymercuribenzoate titration experiments with the enzyme and obtained values of 7 and 12 sulfhydryl groups per mole of enzyme. After the enzyme was inactivated by iodoacetamide, p-hydroxymercuribenzoate titration revealed that four sulfhydryl groups per mole of enzyme had reacted with iodoacetamide [135]. When iodoacetate was used, as many as eight sulfhydryl groups per mole were reacted when the inhibitor concentration was high. The coenzymes NAD and NADH both protected yeast alcohol dehydrogenase against iodoacetamide and iodoacetate inactivation, but ethanol had no such effect. Whitehead and Rabin [135] suggested that iodoacetamide reacted with one sulfhydryl group per monomeric enzyme unit. The rate constant of alkylation of thiolate ions in model compounds with iodoacetamide was estimated as 800 M^{-1} sec^{-1}. The reactive sulfhydryl groups in yeast alcohol dehydrogenase exhibited a lower but significant reactivity. The rate constant was around 0.4-0.65 M^{-1} sec^{-1} under comparable conditions. The alkylation reaction was pH dependent between pH 4 and 10. It was suggested that the reactive sulfhydryl groups were hydrogen bonded to imidazole or other basic groups [135].

Heitz et al. [136] have suggested that N-alkylmaleimide inactivation of yeast alcohol dehydrogenase involved reactions with the essential sulfhydryl groups. The positive chainlength effects of

the N-alkyl derivatives on the rate of inactivation indicated that the essential sulfhydryls were in the vicinity of a non-polar environment. It was also found that NADH was a better protecting reagent than NAD against N-ethylmaleimide inactivation. The lack of protection by pyridinium derivatives suggested that the negative charge on the enzyme which interacted with the quaternary nitrogen of the pyridinium ring of NAD was not the sulfhydryl group modified by N-ethylmaleimide. Polycyclic compounds, benzoquinolines and phenanthrolines, have been shown to interact at the hydrophobic region of the NAD binding site of yeast alcohol dehydrogenase. At 10^{-5} M, 2,7-dichlorofluorescein produced a 15% inactivation of the enzyme. Fluorescein mercuric acetate when administered at inhibitor to active site ratios of 1:1, 2:1 and 3:1 effected an irreversible inactivation of 60, 85 and 97%, respectively. Dialysis experiments and reversal by cysteine suggested that fluorescein mercuric acetate inactivated the enzyme by modifying the essential sulfhydryl groups.

There has been some consideration as to whether one or two sulfhydryl groups were involved at the active site of yeast alcohol dehydrogenase. Fluorescein mercuric acetate, p-hydroxymercuribenzoate, iodoacetate, iodoacetamide and N-ethylmaleimide are capable of completely inactivating the enzyme. At low concentrations, iodoacetate and iodoacetamide attacked only one sulfhydryl group per monomeric unit of enzyme for complete inactivation. However, at higher concentrations of iodoacetate, two sulfhydryl groups per monomeric unit of enzyme were modified. Mercurials and N-ethylmaleimide reacted

with two sulfhydryls per monomeric unit of enzyme. Wallenfels and Sund [137] suggested that the quaternary nitrogen of the oxidized nicotinamide ring of NAD and the uncharged nitrogen of the reduced nicotinamide ring of NADH both interacted with the same sulfhydryl, while a second sulfhydryl was attached to a zinc atom which interacted with the adenine ring of NAD or NADH. Van Eys and Kaplan [138] pictured only one sulfhydryl group linked through a positively charged adenine amino group of NAD or NADH. Heitz et al. [136] emphasized the importance of non-polar interactions in reactions of the sulfhydryl groups of this enzyme. They also argued that NADH protection against maleimide inactivation would suggest that at least one of the functionally important sulfhydryl groups of the enzyme was located close to the hydrophobic region of the coenzyme binding site [139-141]. More recently, Twu and Wold [142] used butyl isocyanate to study the sensitive sulfhydryl groups of yeast alcohol dehydrogenase. They reported that three sulfhydryl groups per molecule of enzyme were attacked during inactivation. From peptide analysis, the modified sulfhydryl groups were shown to be different from those derivatized by iodoacetamide [143]. Twu et al. [143] proposed that there were two distinct "essential" sulfhydryl groups per active site necessary for enzyme activity.

Plapp et al. [144] reasoned that N^1-(ω-bromoacetamidoalkyl)-nicotinamides should be bound at the pyridinium ring region of the NAD binding site of yeast alcohol dehydrogenase. After binding, the bromoacetamido side chain was inferred to be correctly placed as

capable of alkylating neighboring functional groups. N^1-(ω-Bromoacetamidoethyl)nicotinamide was shown to compete with NAD as an inhibitor of the enzyme. The binding of the ethyl derivative was promoted by ADP, and a cysteinyl sulfhydryl group was alkylated after binding. This could indicate that an essential sulfhydryl group was located near the pyridinium binding site. On the basis that yeast alcohol dehydrogenase showed strong coenzyme specificity, Eisel and Wallenfels [145] suggested that a relationship must exist between stereospecificity and dissymmetry of the protein in the active site. Consequently, sulfhydryl groups located in a dissymmetric environment should show stereoselective behavior towards asymmetric reagents. This was demonstrated to be true as the enzyme was inactivated ten times faster by D(+)α-iodopropionic acid than by the L(-)-antipode, and was also similarly true for the amide antipodes.

OBJECTIVES

A review of the literature suggested that there was a need to further investigate the reaction between diazotized AAD and yeast alcohol dehydrogenase. Although studies of diazonium reactions with proteins have been reported on many occasions, observations have been indirect and there is a lack of well defined results. The work of Fisher et al. [1] has indicated that AAD could be diazotized and the resulting diazonium chloride azo-coupled with N-1-naphthylethylene-diamine to form a dye. Evidence has been presented to support selective inactivation of yeast alcohol dehydrogenase by diazotized AAD. However, identification of the amino acid residue modified in the inactivation process was not achieved in these earlier studies.

Preliminary investigations using 5,5'-dithiobis(2-nitrobenzoic acid) revealed the loss of sulfhydryl groups during inactivation of yeast alcohol dehydrogenase. Thus it seemed of interest to initiate a detailed study on the nature of the reaction between diazotized AAD and sulfhydryl groups of this enzyme, and to identify the derivative formed during the enzyme inactivation process. It would also be profitable to evaluate the possibility of using diazotized AAD as a selective reagent for modification of functional groups of other dehydrogenases. According to Friedman [30], an ideal site labeling reagent should meet the following requirements: (a) it should selectively modify a specific functional group under mild experimental conditions; (b) the modified amino acid residue should survive acid

hydrolysis; (c) the amino acid derivative should elute in a convenient position, as a well-resolved peak in amino acid analysis; (d) the ninhydrin color yield of the derivative should be proportional to its concentration and (e) the derivative should contain an ultraviolet absorbing chromophore, which will permit its determination by another independent technique, namely ultraviolet spectroscopy. The possibilities for diazotized AAD to function in this capacity will be studied and analyzed especially in reference to yeast alcohol dehydrogenase.

It is generally considered that reactions of aryldiazonium derivatives with proteins invariably involve tyrosyl, histidyl or lysyl residues. However, in the case of diazotized AAD modified yeast alcohol dehydrogenase, the absence of absorption above 350 nm did not substantiate such reactions, but rather that other evidence suggested that the cysteinyl residue was probably involved. Thus the documentation of a reaction of a diazonium derivative with a sulfhydryl group can have far reaching implications for the study of diazonium modification of many other proteins. Therefore the possibilities of diazonium-sulfhydryl reactions of a variety of diazonium derivatives with some simple sulfhydryl compounds related to cysteine will be studied and analyzed.

INACTIVATION OF YEAST ALCOHOL DEHYDROGENASE BY DIAZOTIZED 3-AMINOPYRIDINE ADENINE DINUCLEOTIDE

Fisher et al. have demonstrated the selective inactivation of yeast alcohol dehydrogenase by diazotized AAD [1]. The investigation of diazotized AAD as a site-labeling reagent revealed a first order irreversible inactivation of the enzyme, the rate of which was decreased by the presence of NAD. Spectrophotometric analysis of the modified enzyme indicated the presence of four AAD residues per molecule of enzyme or one per catalytic site.

It is usually considered that azo-coupling reactions of aryldiazonium derivatives with proteins involve tyrosyl, histidyl or lysyl residues. Preliminary studies of diazotized AAD modified yeast alcohol dehydrogenase failed to provide any evidence of reactions with the above mentioned amino acid residues. However, 5,5'-dithiobis-(2-nitrobenzoic acid) titration suggested a loss of sulfhydryl groups during the inactivation process. The present study was initiated to investigate the nature of the reaction between diazotized AAD and sulfhydryl groups of yeast alcohol dehydrogenase and to identify the derivative formed during the enzyme inactivation process.

Experimental Procedures

Materials - Crystallized and lyophilized yeast alcohol dehydrogenase (alcohol: NAD oxidoreductase, E. C. 1.1.1.1.) was obtained from Sigma Chemical Company. Stock solutions of the enzyme

were prepared in 0.1 M sodium phosphate buffer, pH 7.0. NAD, L-cysteine, D(+)-galactosamine HCl, D(+)-glucosamine HCl, 5,5'-dithiobis(2-nitrobenzoic acid) and ninhydrin aerosol spray were also obtained from Sigma Chemical Company. Ultra pure guanidine HCl was obtained from Mann Research Laboratories. Constant boiling HCl, sequanal grade was obtained from Pierce Chemical Company. 3-Aminopyridine was obtained from Eastman Kodak Company and sublimed before use. Chromagram cellulose sheets containing fluorescent indicator were also obtained from Eastman Kodak Company. Amino acid calibration mixture Type 1 was obtained from the Spinco Division of Beckman Instruments, Inc. Dowex AG 1-X8 chloride form, 200-400 mesh and Bio-Gel P2, 200-400 mesh were obtained from BioRad Laboratories. Zipax-SCX packing was obtained from E. I. Du Pont De Nemours and Company, Inc. Dialysis membrane was obtained from Union Carbide Corporation.

The compound AAD was prepared by means of the Hofmann hypobromite reaction according to Fisher et al. [1]. The product was purified by quasilinear gradient (mixer: 1 liter of H_2O; reservoir: 1 liter of 1.0 M LiCl) from a column (height/diameter = 10) containing 0.6 eq of Dowex AG 1-X8 chloride form, 200-400 mesh. The effluent was monitored at 260 nm, and all fractions of the major peak with absorbance > 2 were pooled and lyophilized. The resulting powder was repeatedly extracted with absolute ethanol to remove LiCl, and then dried under vacuum over anhydrous $CaCl_2$.

Methods -

1. 5,5'-Dithiobis(2-nitrobenzoic acid) titration of sulfhydryl groups in native and modified yeast alcohol dehydrogenase. Titration experiments were carried out to characterize quantitatively the change in free sulfhydryl groups during complete inactivation of the enzyme. In both the titrations of native and modified enzyme, reactions were carried out at room temperature in 1 ml reaction mixtures containing 6 M guanidine HCl, 10^{-4} M 5,5'-dithiobis-(2-nitrobenzoic acid), and 0.1 M sodium phosphate buffer at pH 7.6. The absorbance at 412 nm was measured using a Zeiss PMQ II spectrophotometer against a blank containing reagents without the addition of enzyme. A standard curve was determined with cysteine. Three different concentrations of enzyme: 1, 1.65 and 3.3 x 10^{-5} M and a control without enzyme were used in the study.

2. Enzyme modification. The enzyme modification procedures were as follows. Diazotized AAD was prepared at 0-4°. To 0.25 ml of 60 mM AAD was added 0.1 ml of 2.0 N HCl and then 0.1 ml of 1.0 M $NaNO_2$. After 10 min, 0.1 ml of 2.0 M ammonium sulfamate was added slowly with stirring in order to destroy the excess nitrous acid. After an additional 10 min, the solution was adjusted to pH 7.0 by adding 0.1 ml of 2.0 N NaOH and 0.10 M sodium phosphate buffer, pH 7.0, and made to a total of 1.0 ml. One volume of the 15 mM diazotized AAD was incubated with one volume of 6.6 x 10^{-4} M yeast alcohol dehydrogenase for 15 min, and samples of the modified enzyme were used for the 5,5'-dithiobis(2-nitrobenzoic acid) titration.

3. Enzyme assays. The activities of the native and modified enzyme were assayed to ascertain complete inactivation. Yeast alcohol dehydrogenase activity was measured by the formation of NADH at 340 nm. The assay mixture contained 330 mM ethanol, 8 mM NAD, 16 mM sodium pyrophosphate, pH 8.8, in a total volume of 3 ml.

4. Kinetic data were obtained at 25° with a Beckman ACTA III spectrophotometer with I_0 suppression capabilities at the double beam mode. pH was measured at 25° with a Radiometer PHM 52 pH meter and and type 2020 C glass electrode.

5. Thin layer chromatography of the synthesized products was performed using Eastman Chromagram cellulose sheets containing fluorescent indicator. The solvent systems used were 0.1 M acetic acid-95% ethanol (1:1 by volume) and n-butanol-acetic acid-water (5:2:3 by volume). The spots were first detected by ultraviolet light, then sprayed with ninhydrin aerosol spray and developed for one hour at room temperature. The R_f values were then measured.

6. High pressure liquid chromatographic analysis of compounds was performed using a Varian 1000 liquid chromatograph. A strong cation exchange column with Zipax-SCX packing, 2.1 mm x 15 cm was used. 10 µl volumes of solutions were injected and the column was eluted with 0.005 M sodium citrate buffer pH 5.76 at a column pressure of approximately 100 psi and a flow rate of 75 ml per hour. The effluent was monitored at 254 nm. Elution times were recorded.

7. Elemental analysis. Elemental analysis for carbon, hydrogen and nitrogen was performed by the Chemistry Department, Virginia

Polytechnic Institute and State University. Samples were dried under vacuum over P_2O_5 overnight immediately before the analysis.

8. Melting point determinations. Melting points were determined with a Thermodyne hot stage microscope melting point apparatus without temperature correction, using a 0-360° range thermometer.

9. Ultraviolet spectra. Ultraviolet spectra of the compounds were obtained in 0.1 M sodium phosphate buffer, pH 7.0 by scanning from 900 to 200 nm with a Beckman ACTA III spectrophotometer against buffer as a blank. The absorption maxima were determined and the respective molar extinction coefficients calculated.

10. Infrared spectra. Infrared spectra of the compounds were obtained in KBr with a Beckman IR-5A infrared spectrophotometer. Samples of 0.1 mg were mixed with 10 mg of spectral grade KBr and pressed to a clear plate with a Beckman ram and die micropellet assembly at 125,000 psi. The KBr plate was then set in a NaCl condenser system in the light path of the spectrophotometer, and scanned from 2 to 16 μm.

11. Magnetic resonance spectra. Proton nuclear magnetic resonance spectra of compounds were obtained with 1.5% (w/v) solutions in D_2O. A model JEOL JMN-PS-100 nuclear magnetic resonance spectrometer was used. The instrument was operated at ambient temperatures at a frequency of 100 MHz for proton nuclei with a sweep width of 1080 cycles per second and peak areas were integrated.

12. Amino acid analysis. Amino acid analyses were performed with a model 121 Beckman automatic amino acid analyzer with system AA

computing integrator.

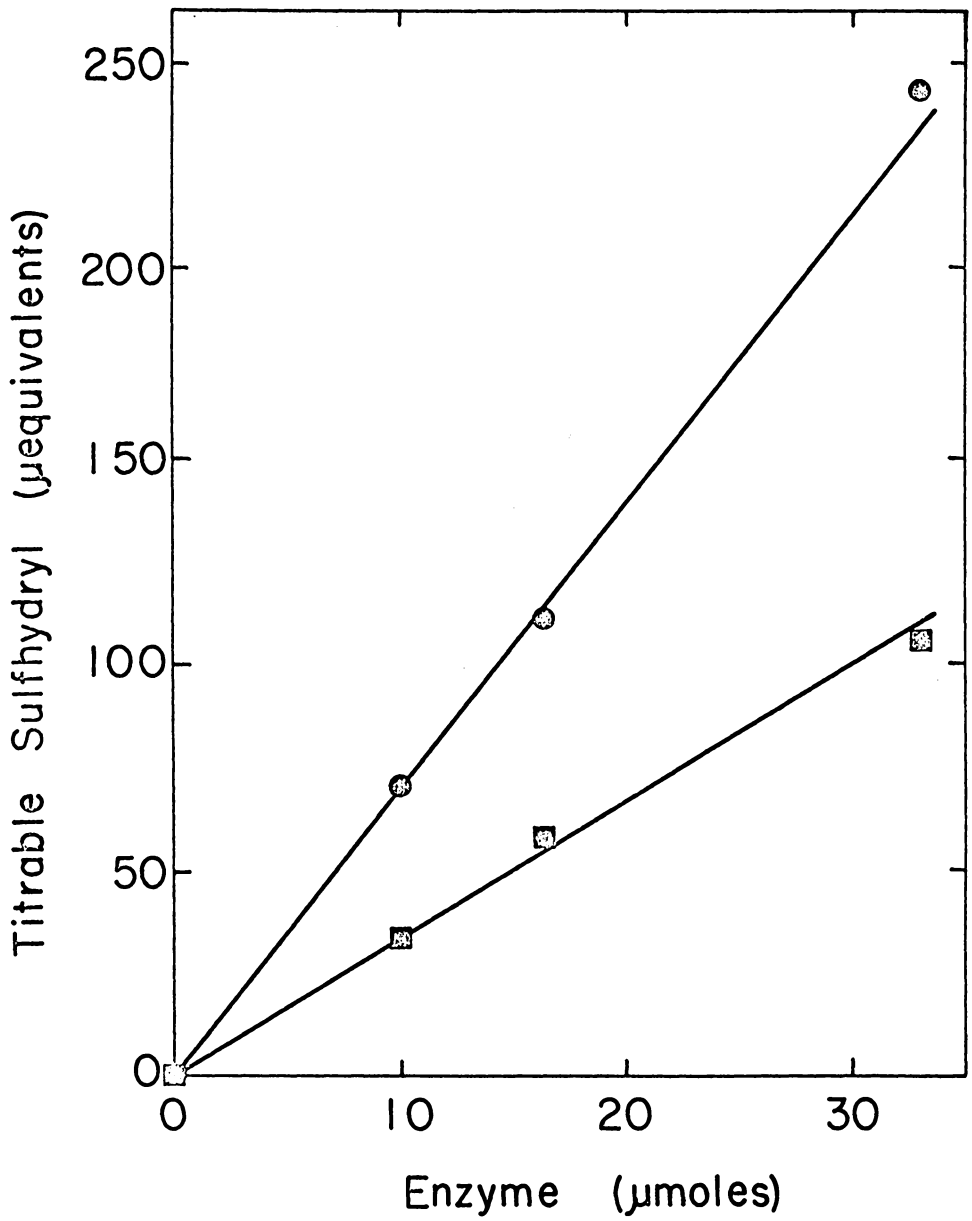
Results

5,5'-Dithiobis(2-nitrobenzoic acid) Titration of Sulfhydryl Groups in Native and Modified Yeast Alcohol Dehydrogenase - Enzyme activity assays indicated that the incubation of yeast alcohol dehydrogenase in 7.5 mM diazotized AAD as described (Methods, page 51) led to complete inactivation during the 15 min incubation period. The enzyme incubated in the absence of diazotized AAD showed no loss of catalytic activity. The fully active native enzyme and the completely inactivated enzyme were then compared for sulfhydryl content by the 5,5'-dithiobis(2-nitrobenzoic acid) titration experiments.

The standard 5,5'-dithiobis(2-nitrobenzoic acid) titration curve for cysteine under the experimental conditions agreed well with that reported by Ellman [146], the molar extinction coefficient at 412 nm being found to be 13,600 cm^{-1} M^{-1}. The results of the titration of sulfhydryl groups in native and modified yeast alcohol dehydrogenase for varying amounts of enzyme are shown in Figure 6, page 56. A linear relationship exists between the μequivalents of titrable sulfhydryl groups and the concentration (μmoles) of enzyme titrated. From the difference in the slopes of the curves for modified and native enzyme, it was calculated that the decrease in titrable sulfhydryl after complete modification by diazotized AAD was 3.5-4.0 equivalents of sulfhydryl per molecule of enzyme.

FIGURE 6.

5,5'-Dithiobis(2-nitrobenzoic acid) titration of yeast alcohol dehydrogenase before and after inactivation by diazotized AAD. Reaction mixtures contained 0, 1, 1.65 and 3.3 x 10^{-5} M native or modified enzyme, 6 M guanidine HCl, 10^{-4} M 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1 M sodium phosphate buffer, pH 7.6, in a total volume of 1 ml. For the enzyme modification, corresponding amounts of enzyme were inactivated with 7.5 mM diazotized AAD at pH 7.0 prior to the addition of guanidine HCl, 5,5'-dithiobis-(2-nitrobenzoic acid) and pH 7.6 buffer. Closed circles, native yeast alcohol dehydrogenase; closed squared, yeast alcohol dehydrogenase inactivated by diazotized AAD.

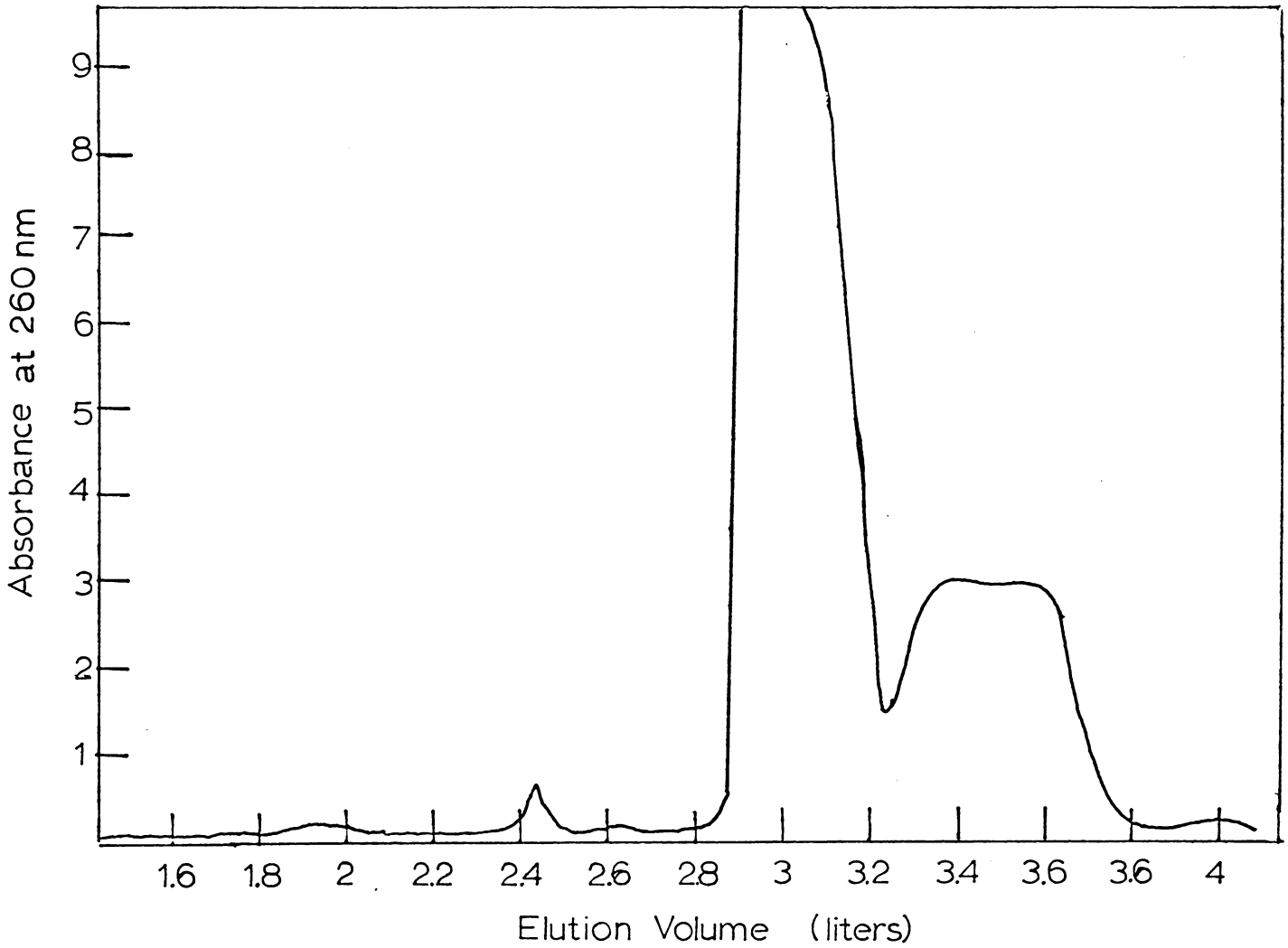

250
200
150
100
50
0
Titrable Sulfhydryl (μequivalents)
0
10
20
30
Enzyme (μmoles)

Synthesis of S-(3-Pyridyl)cysteine - Synthesis of S-(3-pyridyl)-cysteine was carried out as follows. Sodium nitrite (130 mg) and 3-aminopyridine (188 mg) were dissolved in 10 ml of ice-cold 1 N HCl and allowed to react for 10 min. Ammonium sulfamate (230 mg) was added and the solution stirred vigorously for 10 min. Cysteine (242 mg) was then added, with stirring continued for 15 min. All reactions were maintained at 0-4°.

Purification of S-(3-Pyridyl)cysteine - The solution mixture of the synthetic reaction was chromatographed on a Bio-Gel P2 column, 5 cm x 200 cm, and eluted with distilled water by gravity. Fractions were collected after an initial 750 ml were eluted. The effluent was monitored at 254 nm, and fractions that showed absorption were scanned from 350 to 200 nm. A major peak with a 250-nm absorption maximum was obtained upon elution. This occurred at an elution volume of approximately 3 liters, whilst the void volume of the column with reference to blue dextran was found to be 1.8 liters. Samples of the product from the major peak gave a single ninhydrin positive spot on thin layer chromatography with acetic acid-ethanol solvent system. A minor peak containing material with a 315-nm absorption maximum was also eluted in fractions collected 200 ml after the major peak. Thin layer chromatography of this material revealed two ninhydrin positive spots, the one with the lower R_f value corresponded to that of the major peak. The elution pattern of this chromatographic separation is shown in Figure 7, page 59. The major peak fractions were pooled,

FIGURE 7.

Elution pattern of S-(3-pyridyl)cysteine preparation from Bio-Gel P2 column chromatography. The solution mixture of the synthetic reaction was chromatographed on a Bio-Gel P2 column, 5 cm x 200 cm, and eluted with distilled water by gravity. Fractions of 20 ml each were collected after an initial 750 ml were eluted. The absorbance at 260 nm was recorded for each fraction.


Absorbance at 260 nm
9
8
7
6
5
4
3
2
1
1.6
1.8
2
2.2
2.4
2.6
2.8
3
3.2
3.4
3.6
3.6
4
Elution Volume (liters)

lyophilized and dried.

Chromatographic Analysis of Purified S-(3-Pyridyl)cysteine - The lyophilized product from the major peak of the Bio-Gel P2 column chromatography was subjected to thin layer chromatography on cellulose sheets. Results indicated a single ultraviolet quenching spot which was also ninhydrin positive. For the solvent system, 0.1 M acetic acid-95% ethanol (1:1 by volume), an R_f = 0.75 was obtained. For the solvent system, butanol-acetic acid-water (5:2:3 by volume), an R_f = 0.65 was obtained. Samples of purified S-(3-pyridyl)cysteine also showed a single elution peak in the high pressure liquid chromatograph when monitored at 254 nm. The elution time was found to be 1.2 min. When the amino acids phenylalanine and tyrosine were applied on to the liquid chromatograph, they were found to be eluted at different positions of elution times 1.6 and 4 min, respectively.

Elemental Analysis and Melting Point Determination of Purified S-(3-Pyridyl)cysteine - Assuming the formula for S-(3-pyridyl)cysteine to be $C_8H_{10}N_2O_2S$, the theoretical percentages of composition of carbon, hydrogen and nitrogen were calculated. Two experimentally determined sets of values were obtained from two different vacuum dried samples of S-(3-pyridyl)cysteine. The second sample had been treated further by an additional Bio-Gel P2 column chromatography. The values obtained are as follows:

Calculated:	C 48.47,	H 5.08,	N 14.13
Found:	C 48.25,	H 4.65,	N 13.69
	C 47.47,	H 5.06,	N 13.48

Purified S-(3-pyridyl)cysteine was found to melt at 183-5° (uncorrected) and with decomposition.

Ultraviolet Spectral Studies of S-(3-Pyridyl)cysteine - The ultraviolet spectrum of purified S-(3-pyridyl)cysteine in 0.1 M sodium phosphate buffer at pH 7.0 is shown in Figure 8, page 63. No absorption above 350 nm was observed. Absorption maxima were exhibited at 210, 250 and 280 nm with molar extinction coefficients of 15.7, 11.2 and 7.6 x 10^3 cm^{-1} M^{-1}, respectively.

Infrared Spectral Studies of S-(3-Pyridyl)cysteine - The infrared spectrum of S-(3-pyridyl)cysteine in solid KBr is shown in Figure 9, page 65. Prominent absorption bands were observed at 703 (s), 800 (m), 1025 (w), 1110 (w), 1200 (w), 1350 (m), 1400 (m), 1520 (m), 1600 (s), 3020 (s) and 3500 (s) cm^{-1}.

Nuclear Magnetic Resonance Studies of S-(3-Pyridyl)cysteine - The nuclear magnetic resonance spectrum of S-(3-pyridyl)cysteine in D_2O is shown in Figure 10, page 67. Proton absorptions were found to occur at τ 1.0, τ 1.5, τ 2.0, τ 5.6 and τ 6.0 with the corresponding proton integrations in a proportion of 2:1:1:1:2. The spectrum showed a complex spin-spin coupling pattern.

FIGURE 8.

Ultraviolet absorption spectrum of 1.33×10^{-4} M S-(3-pyridyl)cysteine in 0.1 M sodium phosphate buffer, pH 7.0.

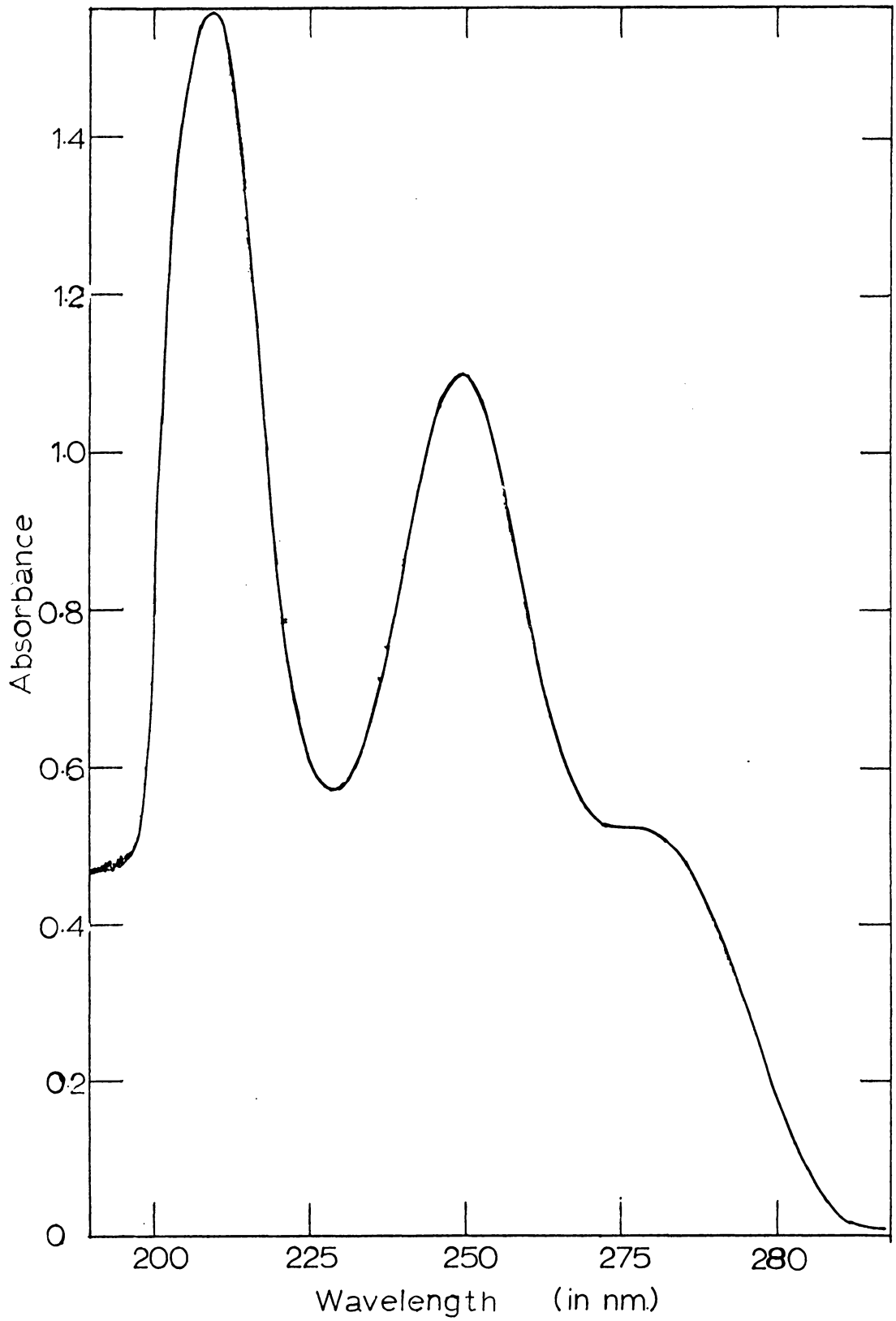

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Absorbance
200
225
250
275
280
Wavelength (in nm.)

FIGURE 9.

Infrared spectrum of S-(3-pyridyl)cysteine in KBr.

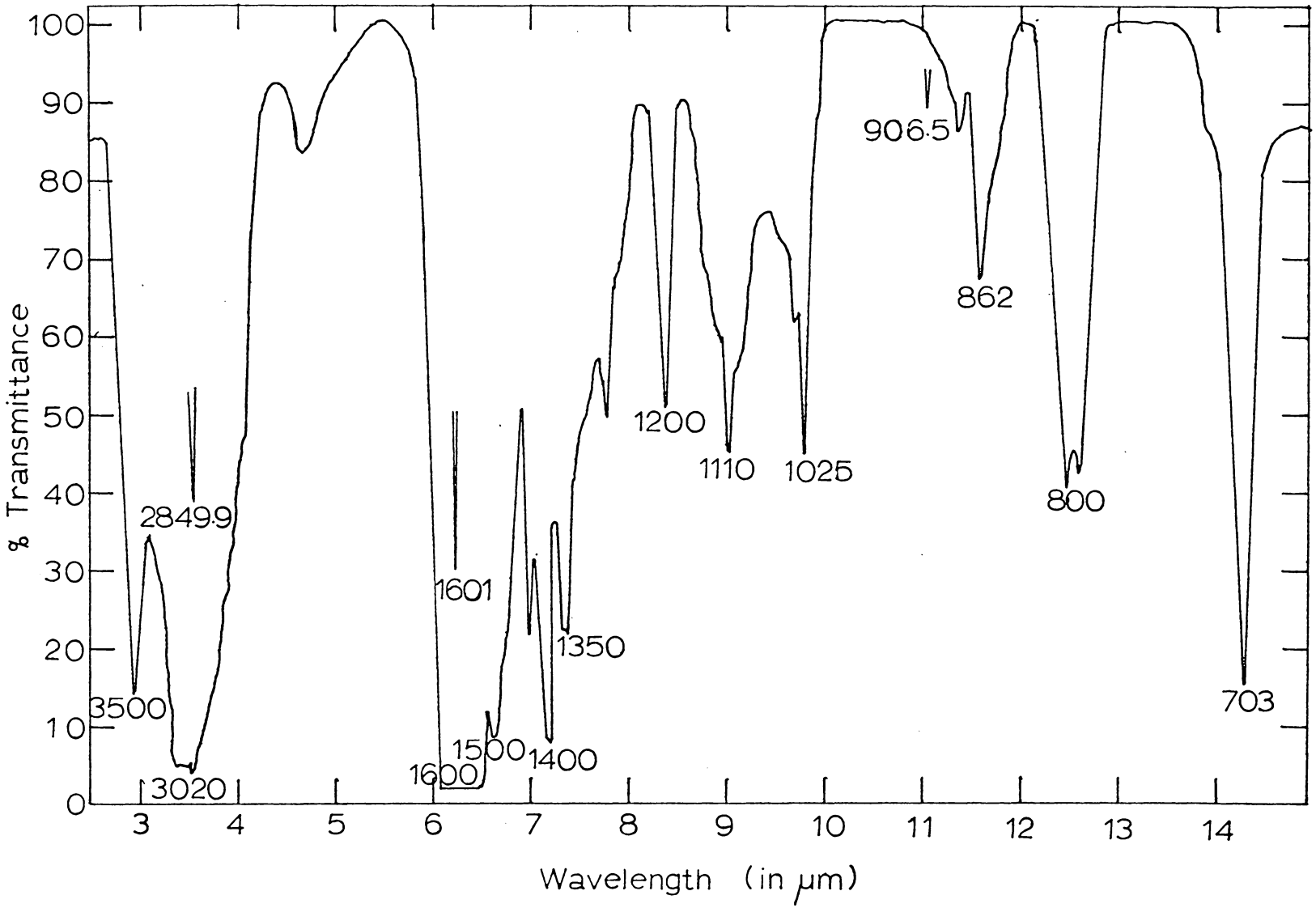
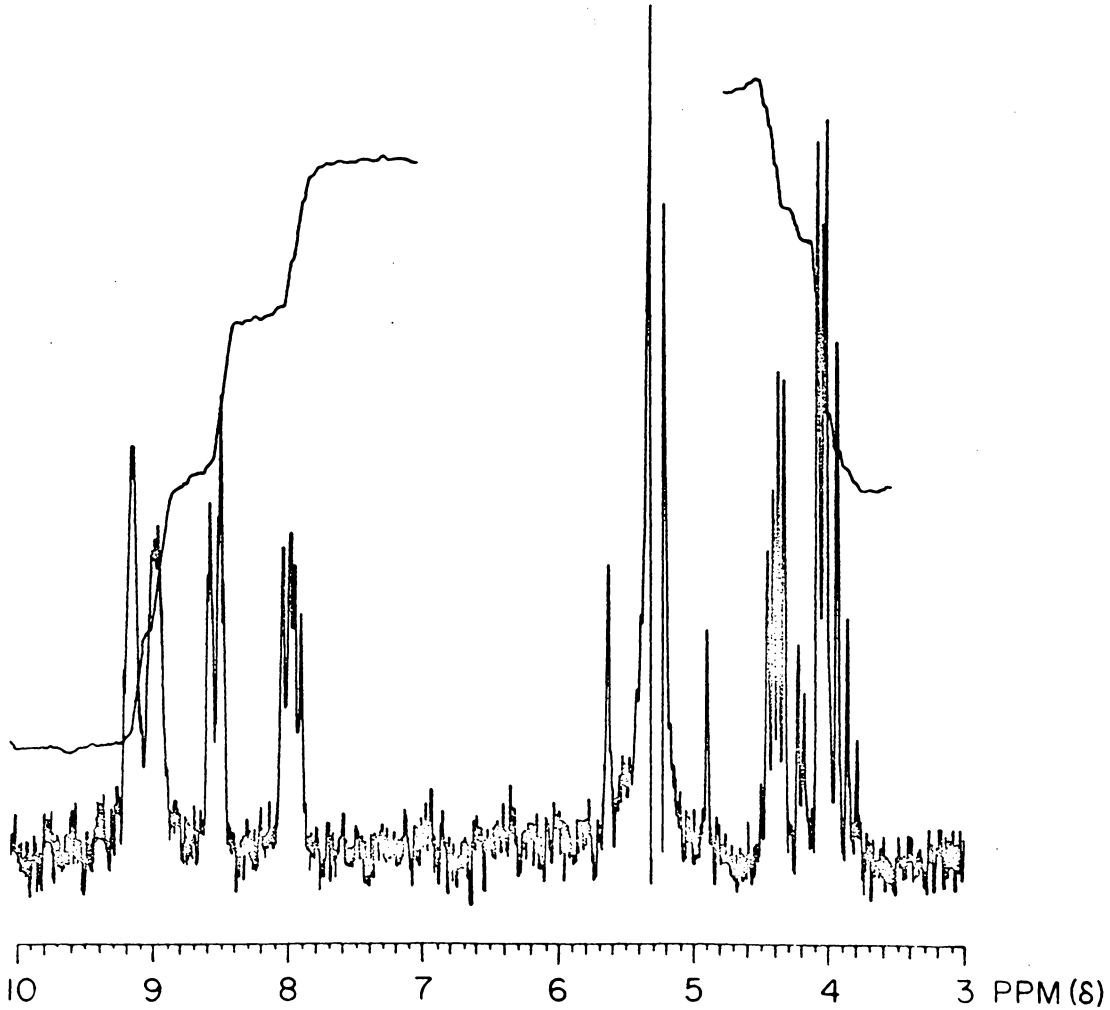

100
90
80
70
60
50
40
30
20
10
0
% Transmittance
3
4
5
6
7
8
9
10
11
12
13
14
Wavelength (in μm)
3500
2849.9
3020
1601
1600
1500
1400
1350
1200
1110
1025
906.5
862
800
703

FIGURE 10.

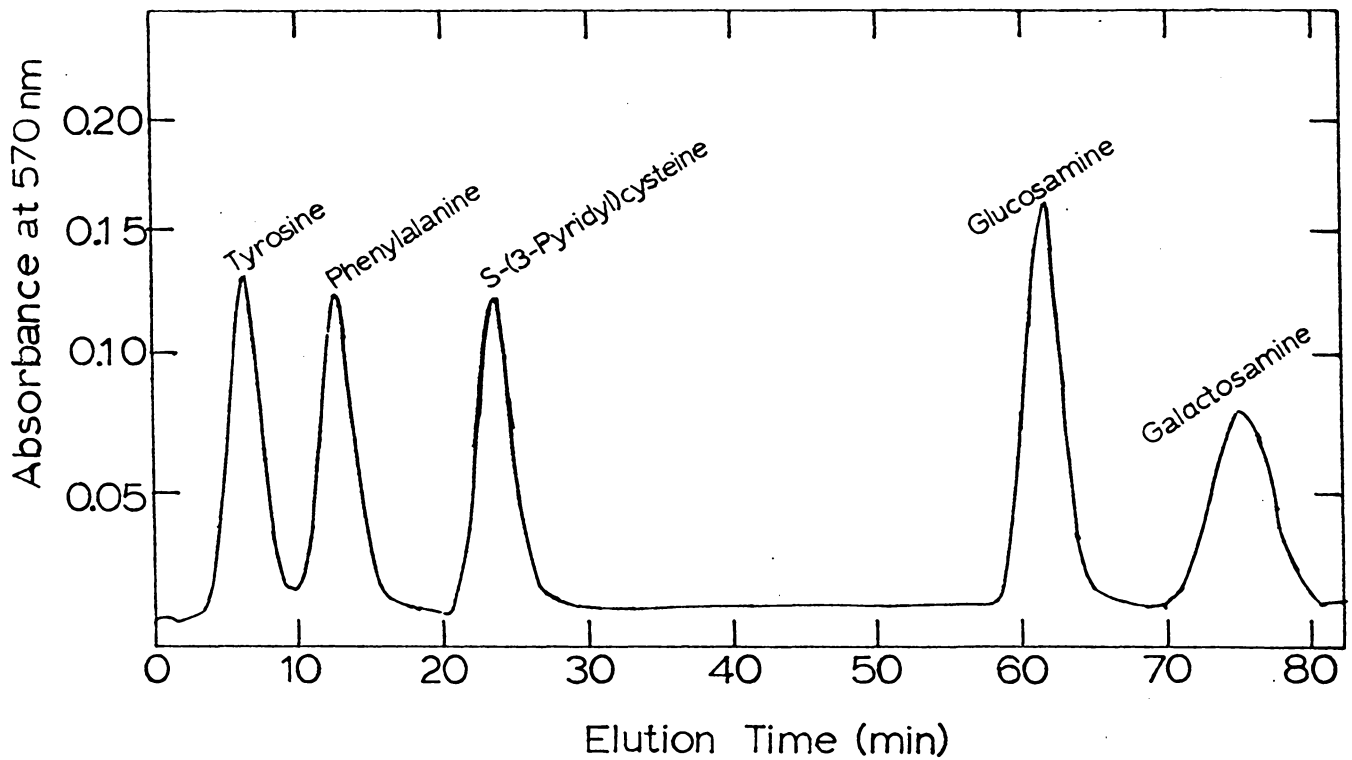
100 MHz nuclear magnetic resonance spectrum of S-(3-pyridyl)-cysteine, 1.5% (w/v) in D_2O, 1080 cycles per second sweep width.


10
9
8
7
6
5
4
3 PPM (δ)

Amino Acid Analysis Studies of S-(3-Pyridyl)cysteine - A solution of the previously synthesized and purified S-(3-pyridyl)-cysteine at 0.02 μmole per ml was prepared. Samples were analyzed by automatic amino acid analysis with the regular amino acid program utilizing both long and short columns according to the method described by Spackman et al. [147]. No discrete identifiable peak was observed. A second program designed for amino sugars [148] and based on the manual procedures of Walborg et al. [149] was then used. This latter program involved elution from the single long column (AA-15 resin) using the short column buffer, 0.38 M sodium citrate, pH 5.28. An amino acid standard containing amino acid calibration mixture Type 1, glucosamine HCl and galactosamine HCl was used. A distinct peak corresponding to S-(3-pyridyl)cysteine was obtained. This peak was clearly distinguishable from the other amino derivatives, tyrosine, phenylalanine, glucosamine and galactosamine which were also identified by this program. The elution times found for these compounds were in the following order, tyrosine, phenylalanine, S-(3-pyridyl)cysteine, glucosamine and galactosamine at 6.4, 12.9, 23.8, 61.3 and 75.4 min, respectively. The color factor K_F determined for S-(3-pyridyl)cysteine was 0.4320. The color factors for tyrosine, phenylalanine, glucosamine and galactosamine were 0.4150, 0.4290, 0.5470 and 0.4310, respectively. The elution pattern of a standard containing all the five compounds as obtained from the amino acid analyzer is shown in Figure 11, page 70.

FIGURE 11.

The amino acid analysis elution pattern of S-(3-pyridyl)cysteine. The sample was eluted from the long column (AA-15 resin) using 0.38 M sodium citrate buffer, pH 5.28. Elution times for tyrosine, phenylalanine, S-(3-pyridyl)cysteine, glucosamine and galactosamine are 6.4, 12.9, 23.8, 61.3, 75.4 min, respectively; K_F values are 0.4150, 0.4290, 0.4320, 0.5470, 0.4310, respectively.


Absorbance at 570 nm
0.20
0.15
0.10
0.05
Tyrosine
Phenylalanine
S-(3-Pyridyl)cysteine
Glucosamine
Galactosamine
0
10
20
30
40
50
60
70
80
Elution Time (min)

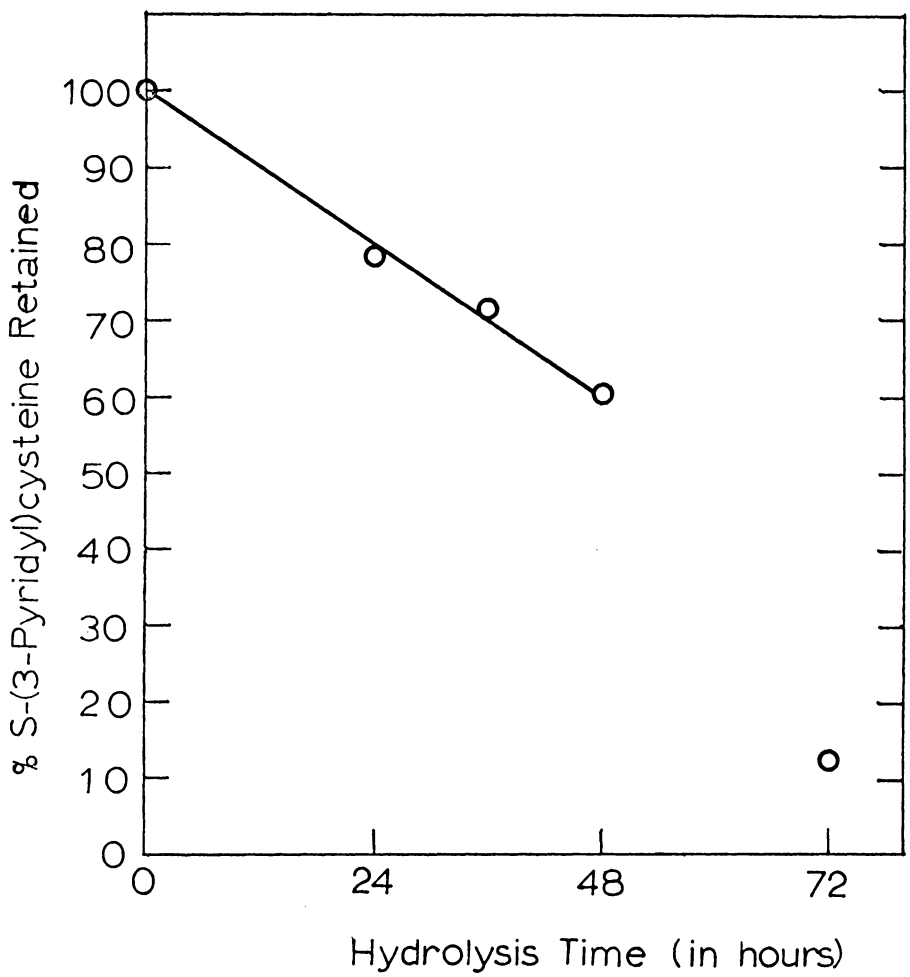
Stability of S-(3-Pyridyl)cysteine in Acid - The stability of S-(3-pyridyl)cysteine during acid hydrolysis was studied. Samples of 0.02 μmole of the compound were treated with 6 N constant boiling glass-distilled HCl in sealed Pyrex glass tubes and kept at 110° for 0, 24, 36, 48 and 72 hours. The samples were then lyophilized, dissolved in 1 ml portions of 0.2 M sodium citrate buffer, pH 2.2 and then analyzed using the amino sugar program. The standard used in this experiment contained amino acid calibration mixture Type 1, glucosamine HCl, galactosamine HCl and synthesized S-(3-pyridyl)-cysteine. The K_F value obtained previously for S-(3-pyridyl)cysteine was used. The decay curve for S-(3-pyridyl)cysteine under acid hydrolysis conditions was plotted for the percentages of residual S-(3-pyridyl)cysteine against hydrolysis time, this is indicated by the graph shown in Figure 12, page 73. Considerable destruction of the compound was noted in the 72-hour sample.

Inactivation Studies of Yeast Alcohol Dehydrogenase - Inactivation of yeast alcohol dehydrogenase by diazotized AAD in the series of enzyme modification experiments was essentially completed in 15 min as shown by the complete loss of enzyme activity. However, when the yeast alcohol dehydrogenase solution was prepared with 0.1% gelatin before treatment with diazotized AAD, activity of the enzyme was maintained as high as the control. After complete inactivation of the enzyme by diazotized AAD, addition of cysteine at 3 μM which amounted to a thousand times that of diazotized AAD in the activity

FIGURE 12.

Stability of S-(3-pyridyl)cysteine during acid hydrolysis.

Samples of 0.02 μmole of the compound were treated with 6 N constant boiling glass-distilled HCl in sealed Pyrex glass tubes and kept at 110° for 0, 24, 36, 48 and 72 hours. The samples were then lyophilized, dissolved in 1 ml portions of 0.2 M sodium citrate buffer, pH 2.2 and then analyzed using the amino sugar program.


% S-(3-Pyridyl)cysteine Retained
100
90
80
70
60
50
40
30
20
10
0
0
24
48
72
Hydrolysis Time (in hours)

assay mixture did not appear to restore any activity. Figure 13, page 76, shows a summary of these results.

Characterization of Modified Yeast Alcohol Dehydrogenase by Spectral Studies - The diazotized AAD for modification of the enzyme in this series of experiments was prepared at 0-4° as follows. To 0.5 ml of 60 mM AAD was added 0.25 ml of 2.0 N HCl and then 0.5 ml of 1.0 M $NaNO_2$. After 10 min, 0.5 ml of 2.0 M ammonium sulfamate was added slowly with stirring to destroy excess nitrous acid. After an additional 10 min, the solution was adjusted to pH 7.0 by adding 0.25 ml of 2.0 N NaOH and 2.0 ml of 0.10 M sodium phosphate buffer, pH 7.0. To the resulting 4.0 ml of 7.5 mM diazotized AAD solution, 0.20 ml of 60 μM yeast alcohol dehydrogenase in 0.10 M sodium phosphate buffer, pH 7.0 was added and the mixture incubated at 0-4°. Inactivation of the enzyme was monitored by assaying periodically for yeast alcohol dehydrogenase activity as detailed under Methods, page 52. Incubation was allowed to proceed for one hour to assure complete inactivation. A control solution, lacking only AAD, was also incubated under the same conditions and its enzyme activity assayed. Both sample and control solutions were dialyzed at 4° against five 1-liter portions of 0.1 M sodium phosphate buffer, pH 7.0, over a two-day period. The ultraviolet spectra of the dialyzed native and modified enzymes as well as their difference spectrum were obtained. The ultraviolet difference spectrum of modified versus native yeast alcohol dehydrogenase after extensive dialysis is given in Figure 14,

FIGURE 13.

Reduction of NAD as a function of time as catalyzed by native yeast alcohol dehydrogenase and by enzyme treated as indicated. Yeast alcohol dehydrogenase activity was measured by the formation of NADH at 340 nm. The assay mixture contained 330 mM ethanol, 8 mM NAD, 16 mM sodium pyrophosphate, pH 8.8, in a total volume of 3 ml. 5 µl of enzyme samples were used in the studies.

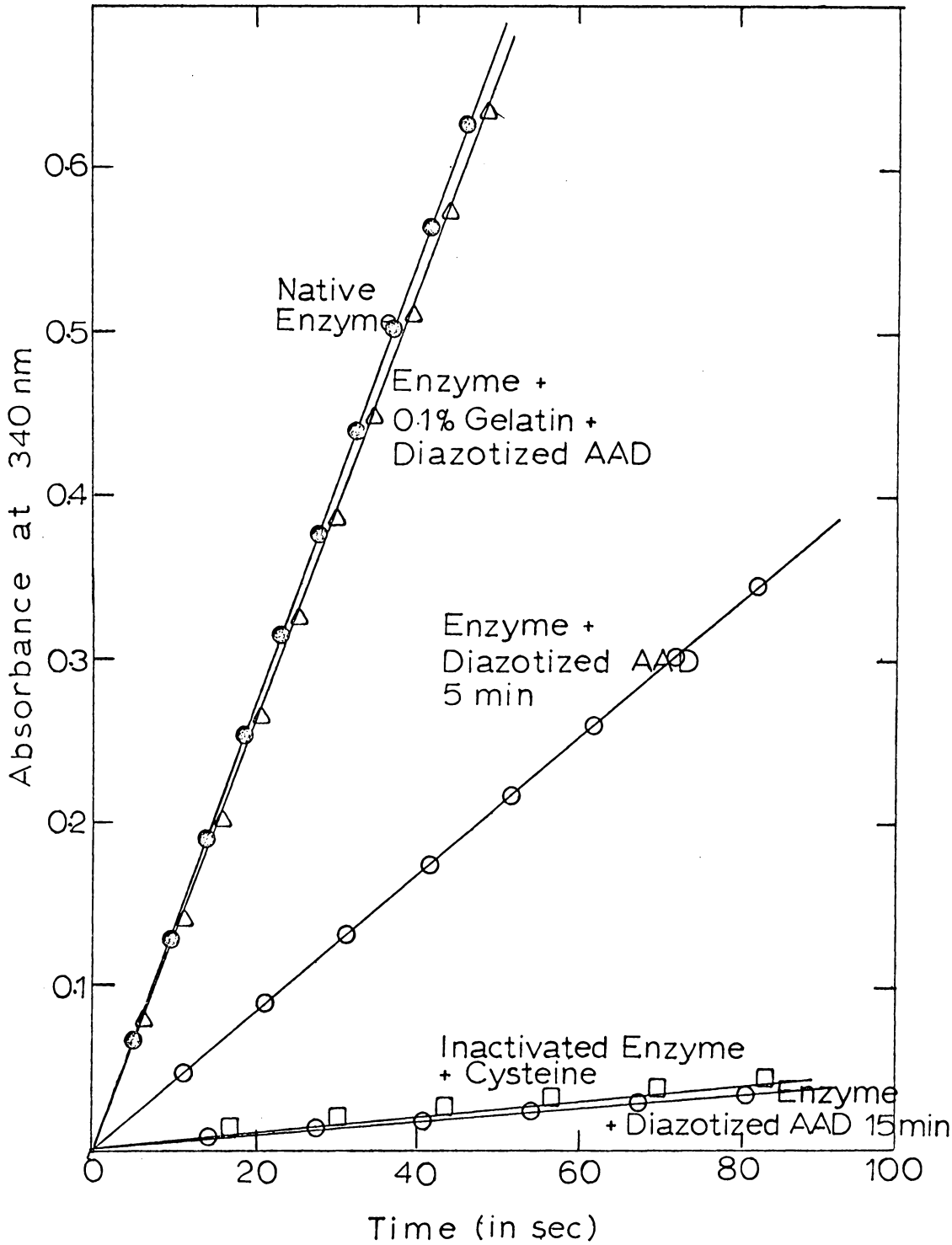
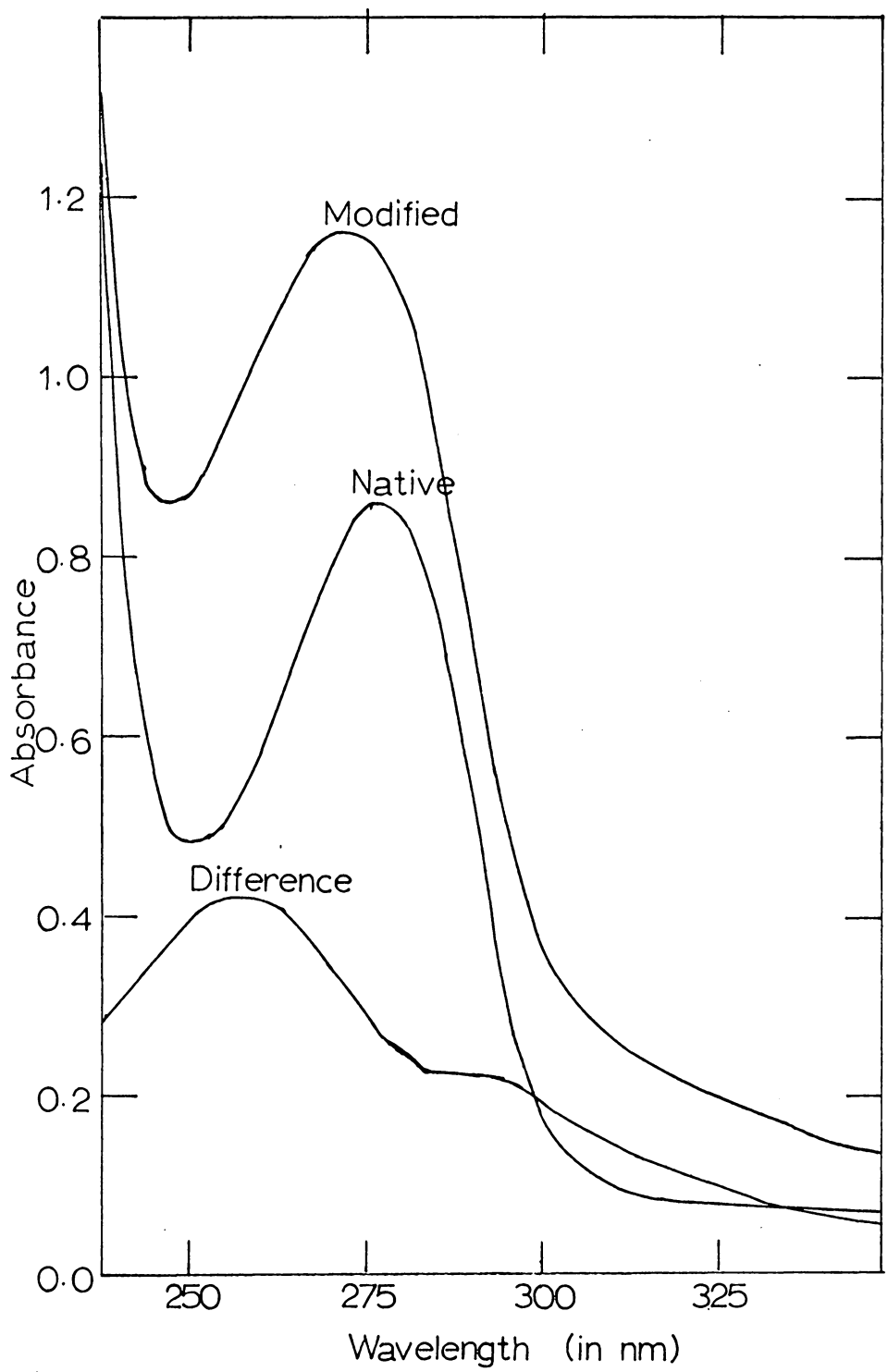

Absorbance at 340 nm
0·6
0·5
0·4
0·3
0·2
0·1
0
20
40
60
80
100
Time (in sec)
Native Enzyme
Enzyme + 0·1% Gelatin + Diazotized AAD
Enzyme + Diazotized AAD 5 min
Inactivated Enzyme + Cysteine
Enzyme + Diazotized AAD 15min

FIGURE 14.

Ultraviolet absorption spectra of native and diazotized AAD inactivated yeast alcohol dehydrogenase, and the corresponding difference spectrum. Both the native and inactivated enzyme were at a concentration of 14 μM in 0.1 M sodium phosphate buffer at pH 7.0, and were extensively dialyzed before the measurements.

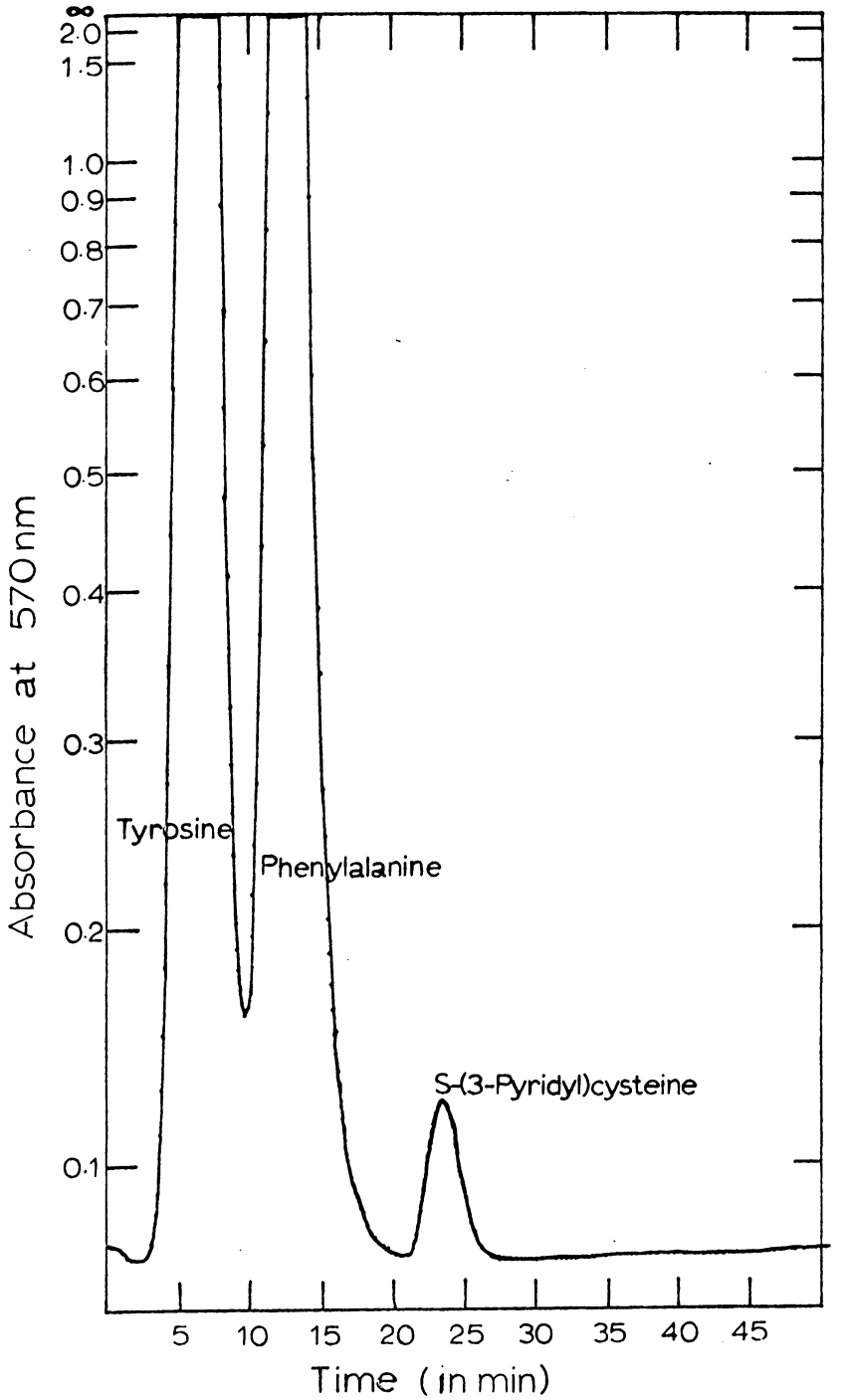

Modified
Native
Difference
Absorbance
1·2
1·0
0·8
0·6
0·4
0·2
0·0
250
275
300
325
Wavelength (in nm)

page 78, and reveals an absorption in the 260-nm region. By assuming the molar extinction coefficient at 260 nm for the AAD residue of the modified enzyme to be the same as diazotized AAD, the amount of the residue was obtained from the 262-nm absorbance value of the difference spectrum. The enzyme concentrations were measured by the absorbances at 280 nm. The number of AAD residues per molecule of tetrameric enzyme was calculated and found to be 4.1-4.4.

Study of Modified Yeast Alcohol Dehydrogenase by Amino Acid Analysis - Samples of the dialyzed modified yeast alcohol dehydrogenase were lyophilized, and hydrolyzed in constant boiling HCl for 24, 36 and 48 hours at 100°. The hydrolyzates were lyophilized and redissolved in 0.2 M sodium citrate buffer, pH 2.2. This was subjected to amino acid analysis using the amino sugar program as detailed under the Results section on amino acid analysis studies of S-(3-pyridyl)cysteine, page 68. Elution peaks with elution times corresponding to tyrosine, phenylalanine and S-(3-pyridyl)cysteine were observed. The elution patterns of acid hydrolyzed modified yeast alcohol dehydrogenase in the amino acid analyzer using the amino sugar program are shown in Figure 15, page 81. The values of S-(3-pyridyl)cysteine released from modified yeast alcohol dehydrogenase through acid hydrolysis were extrapolated to zero time. When the native enzyme used in the control experiments were subjected to similar amino acid analysis procedures, no

FIGURE 15.

The elution pattern in the amino acid analysis of the acid hydrolyzed yeast alcohol dehydrogenase inactivated by diazotized AAD. In this experiment 1 ml of the dialyzed modified enzyme was lyophilized, and hydrolyzed in constant boiling HCl for 24 hours at 110°. The hydrolyzate was lyophilized and dissolved in 1 ml of 0.2 M sodium citrate buffer, pH 2.2. This was subjected to amino acid analysis using the amino sugar program as detailed on page 68.


2·0
1·5
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Absorbance at 570nm
Tyrosine
Phenylalanine
S-(3-Pyridyl)cysteine
5
10
15
20
25
30
35
40
45
Time (in min)

S-(3-pyridyl)cysteine peak occurred in the hydrolyzate, whilst the other amino acids, tyrosine and phenylalanine, did correspond in elution position and quantity.

The number of moles of S-(3-pyridyl)cysteine released from modified yeast alcohol dehydrogenase was obtained from several such experiments, and compared with the number of AAD residues per molecule of modified enzyme as obtained from spectral data in the same series of modification experiments. The values obtained (4.0-4.4 per mole of enzyme) agreed well with the number of AAD residues per molecule of modified enzyme from corresponding difference spectra studies. The results of these experiments are listed in Table VI, page 83.

Reaction Product of Diazotized AAD and Cysteine - Diazotized AAD was also reacted with cysteine, and the product partially purified by Dowex AG 1-X2 chloride form 200-400 mesh, eluted with a LiCl gradient. The 260-nm absorbing fractions were lyophilized and then desalted on a Bio-Gel P2, 200-400 mesh, 5 cm x 200 cm column.

A partially purified product from the reaction of diazotized AAD with cysteine was isolated. However, Bio-Gel P2 column chromatography was not successful in completely removing the inorganic salt present. From the apparent molar extinction coefficient (calculated by assuming a molecular weight of 800) it was indicated that probably 99% of the material by weight was salt. However, when an amount equivalent to 0.02 μmole based on the absorbance at 260 nm was acid hydrolyzed for 24 hours and analyzed for amino acids by the amino sugar program as

TABLE VI

Comparison of spectral analysis and amino acid analysis of yeast alcohol dehydrogenase inactivated by diazotized AAD

Experiment	Moles of adenyl residues per mole of enzyme[a]	Moles of S-(3-pyridyl)cysteine per mole of enzyme[b]
1	4.4	4.4
2	4.4	4.3
3	4.1	4.0
4	4.2	4.0

[a]Calculated from difference spectra of native versus inactivated enzyme.

[b]Amino acid analysis of acid hydrolyzed inactivated enzyme.

for S-(3-pyridyl)cysteine, a peak corresponding to the elution time of S-(3-pyridyl)cysteine was obtained and was comparable to the amount expected.

Discussion

Sulfhydryl Titrations of Native and Modified Yeast Alcohol Dehydrogenase - For the determination of whether sulfhydryl groups are involved in the selective inactivation of yeast alcohol dehydrogenase by diazotized AAD, modified and native yeast alcohol dehydrogenase were titrated and their sulfhydryl contents compared. Although many chemicals react with sulfhydryl groups, they are not specific sulfhydryl reagents. The reagents that are usually considered and used include p-chloromercuribenzoate, iodoacetate, N-ethylmaleimide and 5,5'-dithiobis(2-nitrobenzoic acid). However, p-chloromercuribenzoate titration requires absorbance measurements at 250 nm which is interfered with by diazotized AAD, especially when small values are involved [150]. Iodoacetate has been reported to react with lysyl, histidyl and methionyl residues, and therefore not specific enough for sulfhydryl groups [151]. N-Ethylmaleimide also reacts with lysyl residues, and in addition, the absorbance measurements at 302 nm are too close to the 280-nm absorption of diazotized AAD [152]. Only 5,5'-dithiobis(2-nitrobenzoic acid) has not been shown to react with other amino acid residues. Ellman [146] had demonstrated that 5,5'-dithiobis(2-nitrobenzoic acid) reacted specifically with thiolate ions to form a mixed disulfide with the

release of 5-thio-2-nitrobenzoic acid. Sulfhydryl titrations by absorbance measurements at 412 nm of the 5-thio-2-nitrobenzoic acid released are not interfered with by the other compounds used in the modification of yeast alcohol dehydrogenase. Twu and Wold [142] had used 5,5'-dithiobis(2-nitrobenzoic acid) for sulfhydryl titrations of yeast alcohol dehydrogenase. In the present work this reagent has been successfully applied to the sulfhydryl titration with yeast alcohol dehydrogenase at three different concentrations of both the native and the modified enzyme forms (Figure 6, page 56). The results provided evidence for the loss of titrable sulfhydryl groups after inactivation with diazotized AAD. This conclusion would suggest that sulfhydryl groups are directly involved in reactions with the diazonium ion. Furthermore, as the completely inactivated form has four less sulfhydryl groups per molecule of enzyme it is very possible that the sulfhydryl groups previously known to be present at the active sites of the tetrameric enzyme are the ones that were selectively modified.

Synthesis and Purification of S-(3-Pyridyl)cysteine - To establish the stoichiometry and specificity of the reaction of cysteinyl residues with diazotized AAD in yeast alcohol dehydrogenase, the isolation and identification of the cysteinyl derivative formed would have to be realized. Protein samples were acid hydrolyzed for amino acid analysis. Under such conditions, in addition to releasing free amino acids, the pyridine ribosidic linkage of the attached dinucleotide

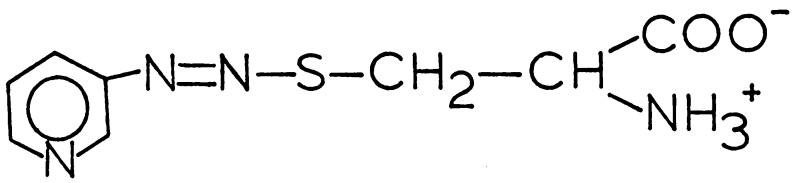
would also be attacked. This in turn would release a cysteinyl derivative that contains the pyridine ring only. Thus experiments to synthesize such a compound by reacting diazotized 3-aminopyridine with cysteine were designed such that an acid stable product may be obtained. This was followed by purification and structural identification. Purification of the synthetic product was first attempted by using various organic solvents. However, as the compound was very soluble in water but not in organic solvents, there was no success in separating it from the other components in the mixture. Finally, Bio-Gel P2 column chromatography was discovered to be an efficient method of purification. The process appeared to be more than gel permeation chromatography, as S-(3-pyridyl)cysteine was retained much longer than expected on the basis of molecular weight. Chromatography of the purified product on cellulose sheets as a single spot in two different solvent systems confirmed that the product was pure, a fact that is further supported by the single elution peak in high pressure liquid chromatography. The positive reaction with ninhydrin revealed that the α-amino group of cysteine was retained in the derivatized compound as a free moiety. Thus the α-amino group was not reacted during the azo-coupling reaction with diazotized 3-aminopyridine. The presence of a minor peak eluted from the Bio-Gel P2 column which contained ultraviolet absorbing and ninhydrin positive material points to the possibility of a second minor component produced during azo-coupling which is also a derivative of cysteine and diazotized 3-aminopyridine.

Structural Elucidation of S-(3-Pyridyl)cysteine - Structural elucidation of S-(3-pyridyl)cysteine was based upon the data obtained from elemental analysis, ultraviolet, infrared and nuclear magnetic resonance spectral studies. If the compound is a cysteinyl derivative with the sulfhydryl group modified in the azo-coupling reaction with diazotized 3-aminopyridine, two structures may be proposed based upon the present knowledge of the mechanism of diazonium reactions [2]. The two structures, S-(3-pyridyldiazo)-cysteine and S-(3-pyridyl)cysteine are indicated in Figure 16, page 89. The formula of the diazomercaptide would be $C_8H_{10}N_4O_2S$, that of the thioether, $C_8H_{10}N_2O_2S$. The percentages of carbon, hydrogen and nitrogen obtained experimentally were in good agreement with the calculated values for the thioether, S-(3-pyridyl)cysteine (page 61). The close correspondence of the nitrogen value is especially significant since the presence of one, two, three or four atoms of nitrogen per molecule for all the possibilities in the reaction products would yield great differences in this value. Here, the evidence of only two nitrogen atoms per molecule suggests that one would be the pyridinium ring nitrogen and the other the cysteinyl α-amino nitrogen. S-(3-Pyridyl)cysteine was found to decompose at its melting point. This behavior points to a similarity with other thioethers such as methionine which also decompose on melting.

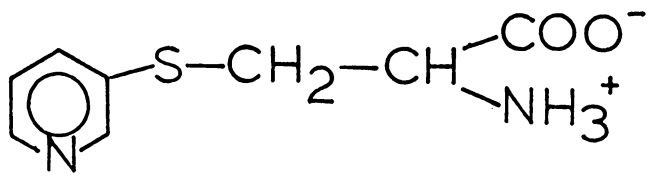
The characteristic absorption maxima of pyridine in water were reported to be at 257 and 270 nm, with molar extinction coefficients of 27.5 and 4.5 x 10^2 cm^{-1} M^{-1}, respectively [153]. S-(3-Pyridyl)-

FIGURE 16.

Molecular structures for S-(3-pyridyldiazo)cysteine and S-(3-pyridyl)cysteine.



S-(3-Pyridyldiazo)cysteine



S-(3-Pyridyl)cysteine

cysteine was found to show similarly the B bands of a $\pi \rightarrow \pi^*$ transition characteristic of aromatic and heteroaromatic molecules, with absorption maxima at 250 and 280 nm (Figure 8, page 63). The much greater molar extinction coefficients, 11.2 and 7.6 x 10^3 cm^{-1} M^{-1} probably reflect substitution effects of the S-cysteinyl group at the 3-position of the pyridine ring. However, the shorter wavelength absorption is still the one of greater absorptivity like that of pyridine. In the infrared spectrum, the 3020 (s) and 1600 (s) cm^{-1} absorptions reflected N^+-H_n and CO_2^- stretching vibrations, respectively, suggesting an amino acid type of zwitterionic structure $-CH<^{COO^-}_{NH_3^+}$ for S-(3-pyridyl)cysteine (Figure 9, page 65).

The proton nuclear magnetic resonance spectrum (Figure 10, page 67) provided strong evidence both for the presence of the pyridine ring system as well as that of the cysteinyl residue. The proton resonances of pyridine have been reported [153] to occur as three groups, α, β and γ, with chemical shifts at τ 1.5, τ 2.6 and τ 3.0, respectively. In the case of S-(3-pyridyl)cysteine, the down field absorptions at τ 1.0, τ 1.5 and τ 2.0 with a proton ratio of 2:1:1 reflect the presence of the α, α', β and γ protons of a 3-substituted pyridine ring system. The splitting of the absorption at τ 1.0 into two peaks of almost equal magnitude indicated the non-equivalence of the α and α' protons which is expected from an inspection of the proposed structure for S-(3-pyridyl)cysteine. The proton absorption at τ 5.6 is equivalent to a single proton and indicates the presence of the methine proton. The proton absorption at τ 6.0 at the high field

region of the spectrum is equivalent to two protons and indicates the presence of the methylene protons. The complex spin-spin coupling revealed in the spectrum suggested an ABX system of protons. This can be explained if the S-(3-pyridyl)cysteine structure is realized to be related with L-cysteine, and the protons near the asymmetric carbon atom are not chemically equivalent. The methylene protons are capable of coupling with each other and each can have a different coupling to a vicinal proton. Analysis of proton magnetic resonance spectra of cysteine and other amino acids by Martin and Mathur [154] also indicated such spin multiplicity characteristics. The three protons of the quaternary ammonium nitrogen in S-(3-pyridyl)cysteine were not observed in the spectrum due to rapid exchange with the D_2O solvent.

Thus elemental analysis, ultraviolet, infrared and proton magnetic resonance spectra all pointed to the structure for the synthetic product as S-(3-pyridyl)cysteine. The evidence presented confirmed the thioether structure with the heterocyclic ring resembling the pyridine ring substituted at the 3-position through the sulfur atom. The aliphatic substituent, in addition to a sulfur atom also involved a methylene and a methine group, the methine carbon being asymmetric. The α-amino acid group exists in the zwitterionic form as the carboxylate and the protonated quaternary ammonium ion.

Analytical Methodology for S-(3-Pyridyl)cysteine - The regular amino acid program was not useful for the detection of S-(3-pyridyl)-

cysteine. However, upon consideration of the suggested structure of this derivative as containing the pyridine ring system, it was argued that there should be a close similarity to tyrosine and phenylalanine, each possessing an aromatic nucleus. As expected, in the amino sugar program, S-(3-pyridyl)cysteine was eluted at a position immediately after tyrosine and phenylalanine (Figure 11, page 70), probably due to the slightly higher basicity. This is a convenient identification because no other amino acids were eluted at this position. The color factor of 0.4320 is in the same order as other amino acids indicating a similarity in the reaction with ninhydrin reagent. Stability studies showed that S-(3-pyridyl)cysteine decomposed under acid hydrolysis conditions (Figure 12, page 73). However, the decay curve is linear and its decay rate is low enough for the assessment at zero time provided that values from 24, 36 and 48 hours of hydrolysis are obtained. The decay curve was very similar to that of methionine under similar hydrolysis conditions, probably due to the fact that both are thioethers.

Characterization of Modified Yeast Alcohol Dehydrogenase - In the inactivation studies of yeast alcohol dehydrogenase by diazotized AAD, gelatin was found to protect the enzyme from inactivation (Figure 13, page 76). This may be due to one or both of two factors. The enzyme may be stabilized by gelatin structurally such that the active site becomes less acessible to diazotized AAD. It is also possible that the presence of gelatin may contribute to a large number of sulfhydryl

groups which reacted with the reagent, appreciably reducing the amount of diazotized AAD available for enzyme inactivation. Gelatin was known to maintain high activity for yeast alcohol dehydrogenase. Excess cysteine did not appear to be capable of reactivating the enzyme. This suggested that the covalent linkage formed by the diazonium-sulfhydryl inactivation is not reversed by cysteinyl sulfhydryl groups. However, it may also be due to the inability of cysteine molecules to approach the vicinity of these covalent linkages.

The ultraviolet difference spectra obtained for the modified versus native enzyme (Figure 14, page 78) confirmed the data obtained from previous studies [1]. The covalent binding of the AAD residues with the enzyme has a stoichiometry of one AAD residue per active site. The modified yeast alcohol dehydrogenase was hydrolyzed and analyzed by amino acid analysis. The detection of a peak recognized as S-(3-pyridyl)cysteine indicated that some cysteinyl residues have been derivatized by reacting with diazotized AAD. The derivatization occurs as a diazonium-sulfhydryl reaction. This is a very interesting parallel with the reaction of diazotized 3-aminopyridine and cysteine. It is not demonstrated whether a diazomercaptide or a thioether was formed by the enzyme cysteinyl residue with diazotized AAD. However, acid hydrolysis of the reaction product obtained from azo-coupling of cysteine with diazotized AAD did yield upon amino acid analysis an elution peak identical with S-(3-pyridyl)cysteine. It can be interpreted that during complete acid hydrolysis, the pyridine ribosidic linkage of the attached dinucleotide was actually hydrolyzed

to produce a cysteinyl derivative containing the pyridine ring only. Thus although the nature of the covalent bonding of the AAD residue with cysteinyl residues on the enzyme has not been ascertained, acid hydrolysis of the inactivated enzyme should be expected to yield S-(3-pyridyl)cysteine. This may involve a simple release of the thioether or a conversion of the diazomercaptide to the thioether, depending on the actual covalent linkage formed during the inactivation.

When the results of the amino acid analysis of modified and native yeast alcohol dehydrogenase are considered, it can be concluded that no other amino acid residues have been modified. This is demonstrated by the absence of peaks corresponding to other amino acid derivatives, and the fact that the amount of tyrosine and phenylalanine from both the modified and native enzyme hydrolyzates remained unchanged.

The results of characterization experiments on yeast alcohol dehydrogenase indicate that close to four molecules of S-(3-pyridyl)-cysteine were released per molecule of modified enzyme. This result is in close agreement with the difference spectra studies where four AAD residues have been found to covalently bind to each molecule of the modified enzyme (Table VI, page 83).

General Observations - The experiments reported in this chapter are related to the inactivation of yeast alcohol dehydrogenase by diazotized AAD. The nature of the reaction has been studied in detail,

and the derivative formed during the enzyme inactivation process has been investigated. It was concluded that the cysteinyl residue of the active site is derivatized by diazotized AAD. Although whether the derivative is a thioether or a diazomercaptide was not demonstrated, it was shown that upon acid hydrolysis it yielded the thioether S-(3-pyridyl)cysteine.

The application of diazotized AAD as a selective reagent for modification of functional groups in dehydrogenases has been demonstrated. If the usefulness of a reagent in site labeling experiments is assessed in terms of the criteria proposed by Friedman [30], diazotized AAD appears to offer promising possibilities. Under conditions of neutral pH and 0-4° where most proteins are stable, reaction with sulfhydryl groups of the active site is rapid and specific. Although the diazotized AAD-cysteine derivative is hydrolyzed during acid treatment, it has given rise to another fairly stable cysteinyl derivative. This derivative, S-(3-pyridyl)cysteine, can be well characterized and was found to elute in a convenient position as a well-resolved peak using the amino sugar program. The ninhydrin color yield of S-(3-pyridyl)cysteine was proportional to concentration and in the same magnitude as other amino acids. S-(3-Pyridyl)cysteine also contains a chromophore with high molar extinction coefficient which permits independent determination by ultraviolet spectroscopy. Since AAD has been shown to be a coenzyme analog capable of inhibiting competitively several NAD-requiring

enzymes, it is likely that diazotized AAD can be bound specifically to the active sites of some of these enzymes and react with sulfhydryl groups where these are present. Thus there is a great potential for diazotized AAD to be used as an active site specific sulfhydryl reagent which forms identifiable cysteinyl derivatives that can be quantitated. The procedures developed above for the determination of S-(3-pyridyl)cysteine by amino acid analysis can be readily applied to other enzymes that are irreversibly inactivated by diazotized AAD. Recently, glycerophosphate dehydrogenase was demonstrated to be inactivated by diazotized AAD[2], and experiments are in progress for characterization of the modified enzyme by the procedures outlined in this study.

In attempts to bind diazotized AAD to insolubilized L-cysteine attached to carboxymethyl cellulose for preparation of affinity column materials, the reaction product was subjected to hydrolysis and then analyzed for S-(3-pyridyl)cysteine. The appearance of a peak at the position of S-(3-pyridyl)cysteine in amino acid analysis seemed to suggest that azo-coupling of the diazotized AAD with the cysteinyl sulfhydryl groups was achieved[2].

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[2]Anderson, B. M., personal communications.

DIAZONIUM-SULFHYDRYL REACTIONS OF SOME DIAZONIUM DERIVATIVES WITH SIMPLE SULFHYDRYL COMPOUNDS

In contrast to the fairly large amount of protein modification experiments utilizing aromatic diazonium compounds, studies designed to investigate the nature of sulfhydryl-diazonium reactions under comparable conditions are virtually non-existent. In fact, any diazonium coupling reaction was almost immediately ascribed to an involvement with tyrosyl, histidyl or lysyl residues. However, in the previous chapter, experimental data have been presented to indicate that in the inactivation of yeast alcohol dehydrogenase by diazotized AAD, the diazonium derivative functioned as an active-site directed sulfhydryl reagent. There is a definite need to gain a greater understanding of the nature of the chemistry of diazonium-sulfhydryl reactions, as this could contribute significantly to future possibilities of protein sulfhydryl group diazonium reactions. Furthermore, since preliminary experiments seemed to indicate that in certain cases the sulfhydryl group is more reactive than the other amino acid functions aforementioned, it is likely that some previous experiments of protein modification with diazonium compounds may have to be re-interpreted in the light of diazonium-sulfhydryl reactions. To avoid the complex nature of protein reactions, detailed studies of the reaction in simpler systems were used. Diazonium derivatives of aniline, p-aminobenzoic acid, sulfanilic acid, arsanilic acid, 3-aminopyridine, 1-methyl-3-aminopyridinium chloride, AAD and AADP

were used to react with simple thiols such as cysteine, homocysteine, glutathione and mercaptoethanol. Experimental approaches involved an analysis of spectral changes that occurred during the reactions, product studies by thin layer chromatography, determination of the kinetics of some of the reactions, and the preparation and purification of the reaction products for structural analysis and chemical studies.

Experimental Procedure

Materials - NAD glycohydrolase (E. C. 3.2.2.5) from pig brain as purified acetone-dried powder, NAD, NADP, mercaptoethanol, L-cysteine, glutathione (reduced form), N-acetyltyrosine, sulfanilic acid, aniline (twice distilled), 5,5'-dithiobis(2-nitrobenzoic acid) and ninhydrin aerosol spray were obtained from Sigma Chemical Company. DL-Homocysteine was obtained from ICN K & K Laboratories. p-Aminobenzoic acid was obtained from General Biochemicals, Inc. 3-Aminopyridine was obtained from Eastman Kodak Company and sublimed before use. Chromagram cellulose sheets with fluorescent indicator were also obtained from Eastman Kodak Company. Dowex AG 1-X8 formate form, 200-400 mesh and Bio-Gel P2, 200-400 mesh were obtained from BioRad Laboratories.

1-Methyl-3-aminopyridinium chloride was synthesized by the Menschutkin reaction according to the procedure of Fisher et al. [1]. The compound AAD was prepared by means of the Hofmann hypobromite reaction according to the procedure detailed under Materials in the

previous chapter (page 50). The compound AADP was prepared according to the procedure of Anderson et al. [59]. The NAD glycohydrolase source, pig brain purified acetone-dried powder (8 g) was stirred with NADP (857 mg) and 3-aminopyridine (3.764 g) in 400 ml of 0.3 M Tris-HCl buffer, pH 8.1, at room temperature. Samples of 0.1 ml volumes were withdrawn at 30 min intervals, added to 2.9 ml of 1 M KCN and the progress of the reaction assessed by measuring the absorbance at 327 nm. The reaction was completed after three hours when there was no further decrease in absorbance. The mixture was centrifuged for 30 min at 5,000 rpm, and the supernatant poured slowly into four liters of ice-cold acetone with stirring, and then allowed to settle overnight at 4°. The precipitate was obtained by decanting the acetone, and blown with dust-free air until no acetone was detectable. The precipitate was extracted several times with small amounts of water and the mixture was centrifuged at 5,000 rpm for 30 min. The supernatant was applied to a Dowex AG 1-X8 formate column, 3.5 cm x 37 cm. The column was eluted with a quasilinear gradient (mixer: 2 liters of H_2O; reservoir: 2 liters of 2.0 M ammonium formate). The fractions corresponding to the AADP peak were lyophilized, dissolved in a minimum amount of water and applied to a Bio-Gel P2 column, 4.5 cm x 200 cm. Fractions with absorbance > 1 at 260 nm were pooled and lyophilized. The white powder was dried under vacuum over phosphorus pentoxide.

Methods -

1. Ultraviolet spectral studies of diazonium-sulfhydryl reactions. Stock solutions of amines and thiols were freshly made before the experiments. Aniline, p-aminobenzoic acid, sulfanilic acid, arsanilic acid, 3-aminopyridine and 1-methyl-3-aminopyridinium chloride were prepared at 3×10^{-3} M. The compounds AAD and AADP were prepared at 1.5×10^{-3} M. Cysteine, homocysteine, glutathione and mercaptoethanol were prepared at 3×10^{-3} M. The ultraviolet spectra of the amines, thiols, diazotized compounds and diazonium-thiol mixtures were obtained in 0.1 M sodium phosphate buffer, pH 7.0 with a Beckman ACTA III spectrophotometer in the range 350 to 200 nm, against buffer as a blank.

2. Sulfhydryl titrations. The solutions of the diazonium-thiol reactions to be assayed were removed as 0.5 ml aliquots at timed intervals, and 2.5 ml of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M sodium phosphate buffer, pH 8.0 were then added. The absorbance at 412 nm was measured at room temperature, using a Zeiss PMQ II spectrophotometer against a blank containing the control.

3. Thin layer chromatographic analysis. The compounds were analyzed by thin layer chromatography using cellulose sheets in two solvent systems, 0.1 M acetic acid-95% ethanol (1:1 by volume) and butanol-1 M acetic acid (82:18 by volume). Both ultraviolet and ninhydrin detection methods were used.

4. Elemental analysis. Compounds were dried overnight at the temperature of refluxing toluene under vacuum over phosphorus

pentoxide immediately before analysis. Elemental analysis for carbon, hydrogen and nitrogen was performed by the Chemistry Department, Virginia Polytechnic Institute and State University.

5. Melting point determinations. Melting points were determined by a Thermodyne hot stage microscope melting point apparatus without temperature correction.

Results

Ultraviolet Spectral Studies of Diazonium-Sulfhydryl Reactions - In the ultraviolet spectral studies, aniline, p-aminobenzoic acid, sulfanilic acid, arsanilic acid, 3-aminopyridine and 1-methyl-3-aminopyridinium chloride at 10^{-4} M, and AAD and AADP at 5×10^{-5} M were used. The concentrations of the thiols, cysteine, homocysteine, glutathione and mercaptoethanol used were at 10^{-3} M.

The amines were diazotized immediately prior to use. Stock solutions of amines at 1 ml volumes were mixed with 1 ml of 0.2 M HCl and then 1 ml of 0.1 M $NaNO_2$ at 0-4°. After 10 min, 1 ml of 0.2 M ammonium sulfamate was added slowly with stirring to destroy excess nitrous acid. After an additional 10 min, the solutions were neutralized with 1 ml of 0.2 N NaOH. Ultraviolet spectra of the diazonium derivatives were obtained in 0.1 M sodium phosphate buffer, pH 7.0. For diazotized aniline, diazotized p-aminobenzoic acid, diazotized sulfanilic acid, diazotized arsanilic acid, diazotized 3-aminopyridine and diazotized 1-methyl-3-aminopyridinium chloride, concentrations of 10^{-4} M were used. For diazotized AAD and

diazotized AADP, concentrations of 5×10^{-5} M were used.

Solutions containing one of the above diazonium derivatives and one of the thiols in 0.1 M sodium phosphate buffer, pH 7.0 were prepared for all the 32 possible combinations. The concentrations of the diazonium derivatives used corresponded to their concentrations indicated in the previous paragraph. The concentrations of thiols were at 10^{-3} M. Ultraviolet spectra of these solutions were obtained at 5, 15 and 30 min after mixing.

The spectral characteristics of the above amines, thiols, diazonium derivatives and the diazonium-thiol mixtures were then compared. A summary of the major spectral changes in the 32 diazonium-sulfhydryl reactions studied is given in Table VII, page 103.

Two groups of diazonium-sulfhydryl reactions giving different spectral changes can be distinguished. The first group included diazotized aniline, diazotized p-aminobenzoic acid, diazotized sulfanilic acid, diazotized arsanilic acid and diazotized 3-aminopyridine. The spectra of aniline, diazotized aniline and the reaction mixture of diazotized aniline and mercaptoethanol are given as an example in Figure 17, page 105. In this first group, the spectral changes are characterized by the loss of the diazonium absorption maximum at around 265 nm and the appearance of a new absorption maximum at around 325 nm. The change was rapid, and already came to completion in 5 min. The spectra for the diazotized p-aminobenzoic acid-glutathione reaction are shown in Figure 18, page 107 and the diazotized 3-aminopyridine-cysteine reaction in Figure 19, page 109.

TABLE VII

Ultraviolet spectral characteristics of diazonium-sulfhydryl reactions[a]

Amine	Diazonium Derivative	Diazonium-Sulfhydryl Reaction Mixture			
		Cysteine	Homocysteine	Glutathione	Mercaptoethanol
Aniline	265	320	325	320	322
p-Aminobenzoic acid	265	325	325	327	327
Sulfanilic acid	265	322	315	322	315
Arsanilic acid	265	322	315	320	315
3-Aminopyridine	235	315	315	320	315
MAP[b]	282	282[c]	282[c]	282[c]	282[c]
AAD	262	262[c]	262[c]	262[c]	262[c]
AADP	260	260[c]	260[c]	260[c]	260[c]

[a]Showing major absorption above 230 nm in nm.

[b]MAP, 1-methyl-3-aminopyridinium chloride.

[c]In these cases no new absorption maximum appeared, however, a time dependent decrease in the original absorption maximum was observed.

FIGURE 17.

Ultraviolet spectra of 10^{-4} M aniline, 10^{-4} M diazotized aniline, and a mixture of 10^{-4} M diazotized aniline and 10^{-3} M mercaptoethanol. Measurements were made in 0.1 M sodium phosphate buffer at pH 7.0 and at room temperature.

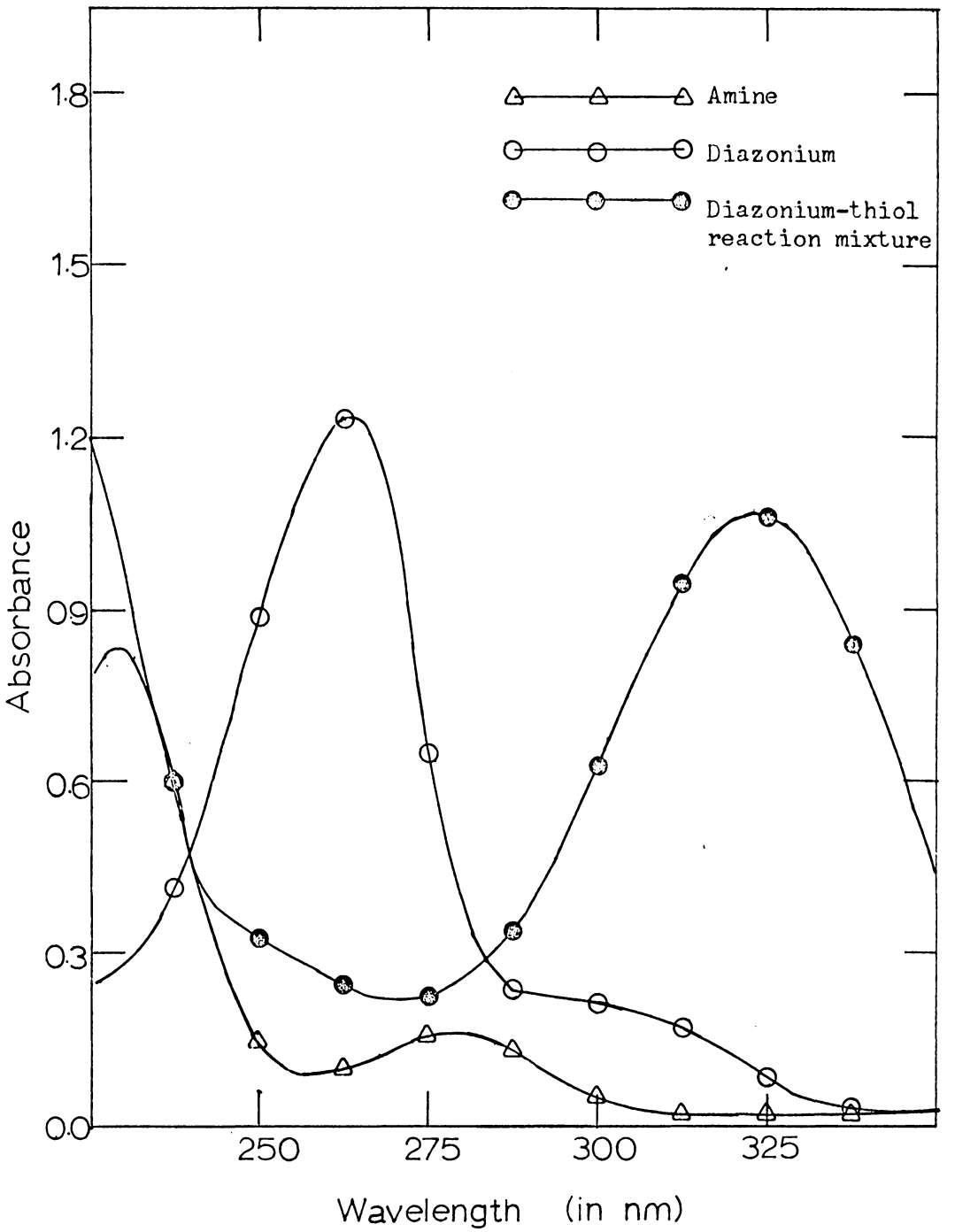

Amine
Diazonium
Diazonium-thiol reaction mixture
Absorbance
1.8
1.5
1.2
0.9
0.6
0.3
0.0
250
275
300
325
Wavelength (in nm)

FIGURE 18.

Ultraviolet spectra of 10^{-4} M p-aminobenzoic acid, 10^{-4} M diazotized p-aminobenzoic acid, and a mixture of 10^{-4} diazotized p-aminobenzoic acid and 10^{-3} M glutathione. Measurements were made in 0.1 M sodium phosphate buffer at pH 7.0 and at room temperature.

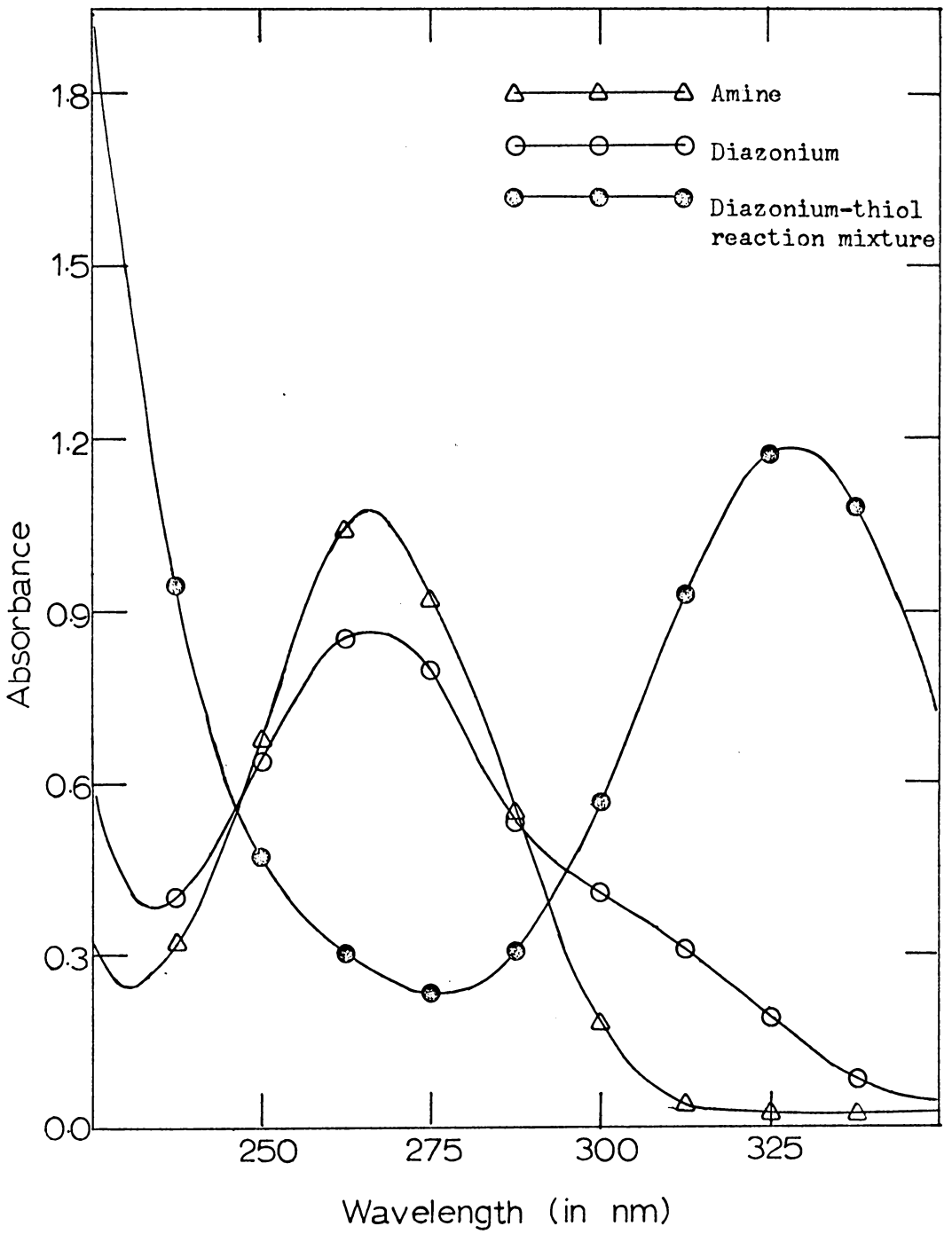
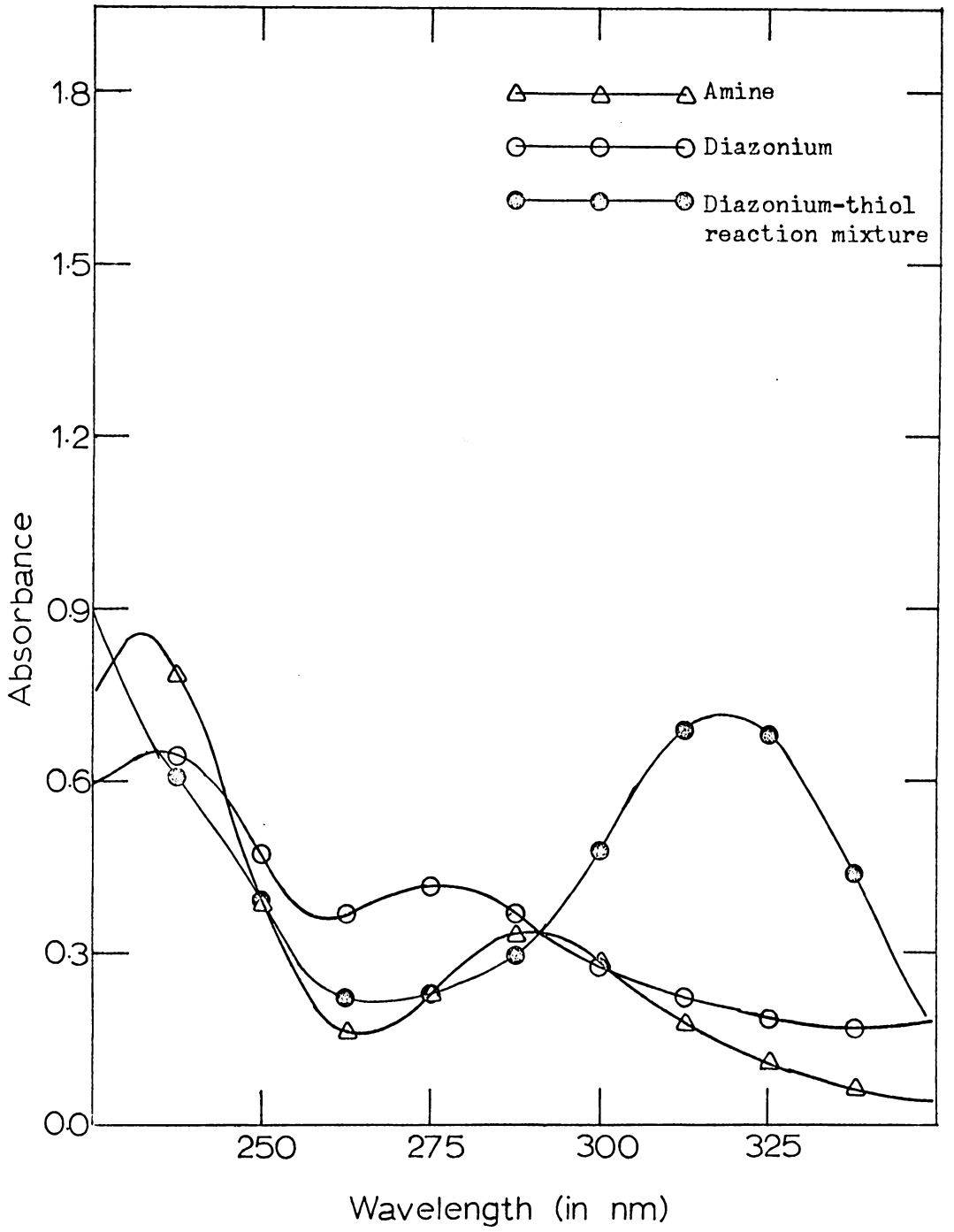

Amine
Diazonium
Diazonium-thiol reaction mixture
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Absorbance
250
275
300
325
Wavelength (in nm)

FIGURE 19.

Ultraviolet spectra of 10^{-4} M 3-aminopyridine, 10^{-4} M diazotized 3-aminopyridine, and a mixture of 10^{-4} M diazotized 3-aminopyridine and 10^{-3} M cysteine. Measurements were made in 0.1 M sodium phosphate buffer at pH 7.0 and at room temperature.


Amine
Diazonium
Diazonium-thiol reaction mixture
Absorbance
1·8
1·5
1·2
0·9
0·6
0·3
0·0
250
275
300
325
Wavelength (in nm)

The four thiols, cysteine, homocysteine, glutathione and mercaptoethanol all reacted with these diazonium derivatives to give very similar spectral changes. The second group included diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP. The spectra of 1-methyl-3-aminopyridinium chloride, diazotized 1-methyl-3-aminopyridinium chloride and the reaction mixture of diazotized 1-methyl-3-aminopyridinium chloride and homocysteine are given in Figure 20, page 112. In this second group, the spectral changes are characterized only by the loss of the diazonium absorption maximum, without any other discernible changes between 350 and 200 nm. Furthermore, the spectral changes were much slower, allowing the time dependency to be observed. Again, the four thiols gave essentially the same results in all these experiments. The spectra for the diazotized AAD-cysteine reaction are shown in Figure 21, page 114.

Reactivities of Cysteine and Tyrosine with Diazotized p-Aminobenzoic Acid - Diazotized p-aminobenzoic acid was prepared similarly as above. Visible and ultraviolet spectra from 600 to 200 nm were obtained at 10^{-4} M concentrations in 0.1 M sodium phosphate buffer for pH values 6.0, 6.5, 7.0, 7.5 and 8.0. The spectra of N-acetyltyrosine at concentrations of 10^{-3} M in 0.1 M sodium phosphate buffer at these different pH's were also obtained. Solutions containing 10^{-4} M diazotized p-aminobenzoic acid and 10^{-3} M N-acetyltyrosine in the five different pH's from 6.0 to 8.0 were prepared and their spectra obtained at 5, 15 and 30 min after mixing.

FIGURE 20.

Ultraviolet spectra of 10^{-4} M 1-methyl-3-aminopyridinium chloride, 10^{-4} M diazotized 1-methyl-3-aminopyridinium chloride, and a mixture of 10^{-4} M diazotized 1-methyl-3-aminopyridinium chloride and 10^{-3} M homocysteine. Measurements were made in 0.1 M sodium phosphate buffer at pH 7.0 and at room temperature. In the case of the diazonium-thiol mixture, the spectra were obtained at 1, 15 and 30 min after mixing.

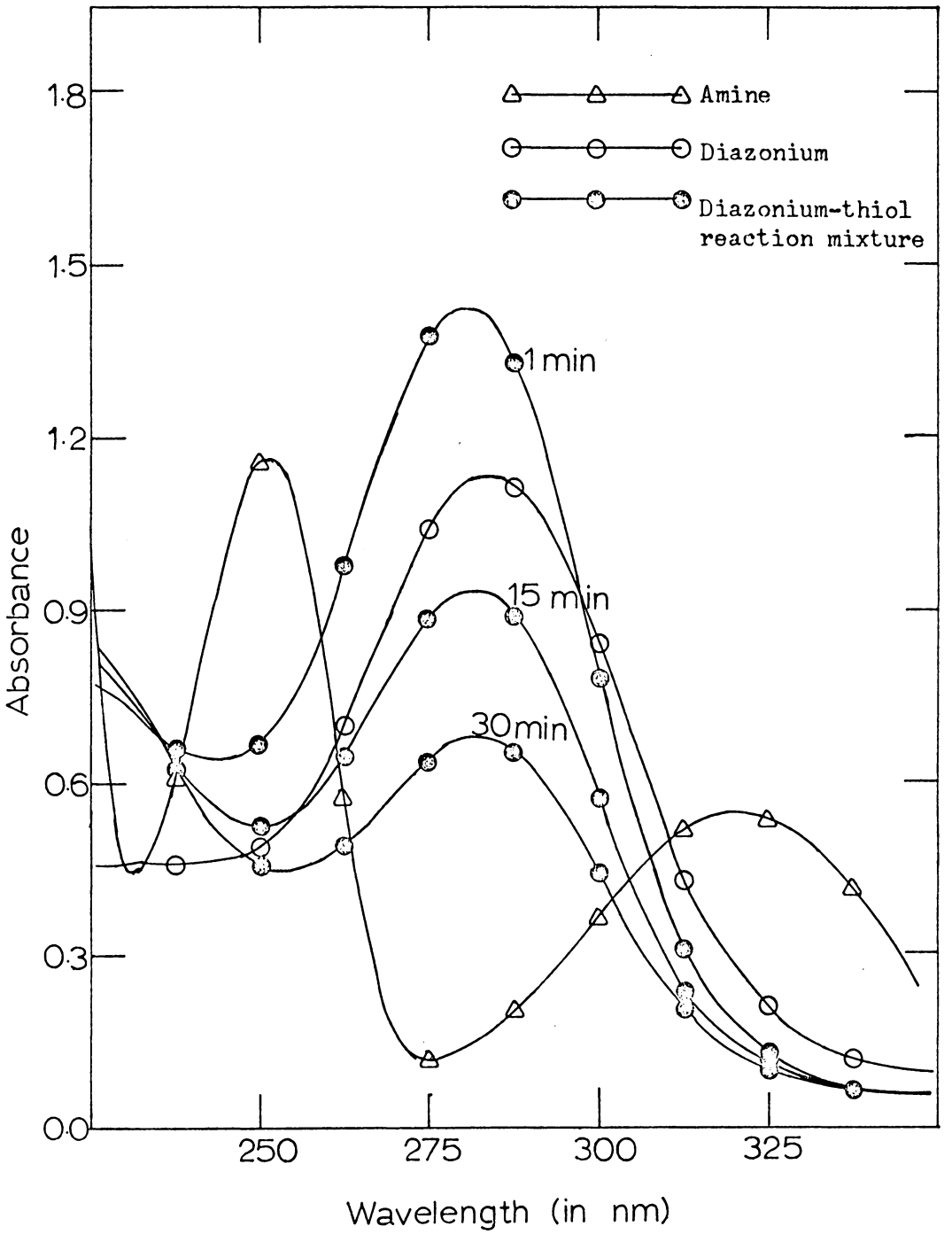
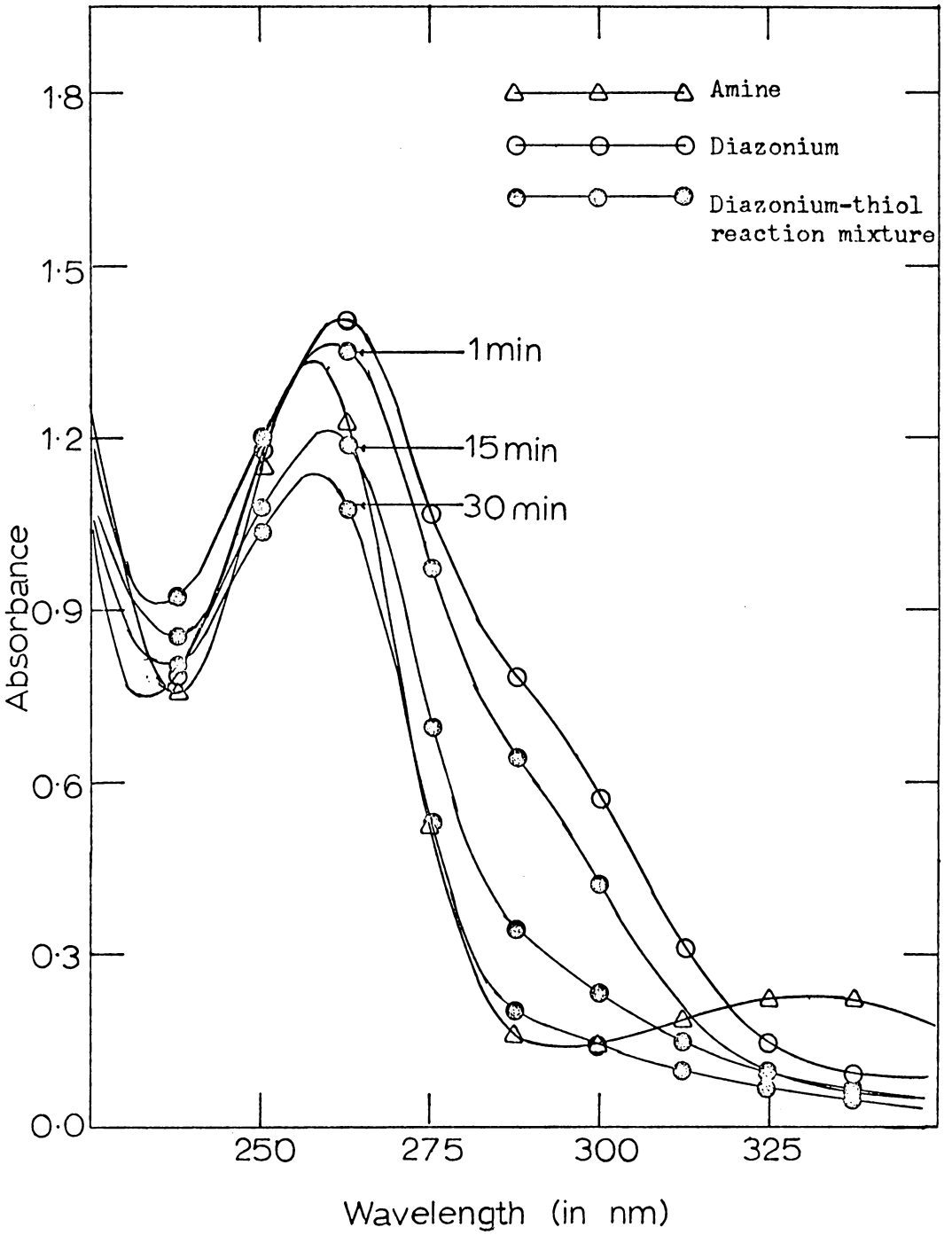

Amine
Diazonium
Diazonium-thiol reaction mixture
1 min
15 min
30 min
Absorbance
Wavelength (in nm)
1·8
1·5
1·2
0·9
0·6
0·3
0·0
250
275
300
325

FIGURE 21.

Ultraviolet spectra of 5×10^{-5} M AAD, 5×10^{-5} M diazotized AAD, and a mixture of 5×10^{-5} M diazotized AAD and 10^{-3} M cysteine. All measurements were made in 0.1 M sodium phosphate buffer at pH 7.0 and at room temperature. In the case of the diazotized AAD-cysteine mixture, the spectra were obtained at 1, 15 and 30 min after mixing.

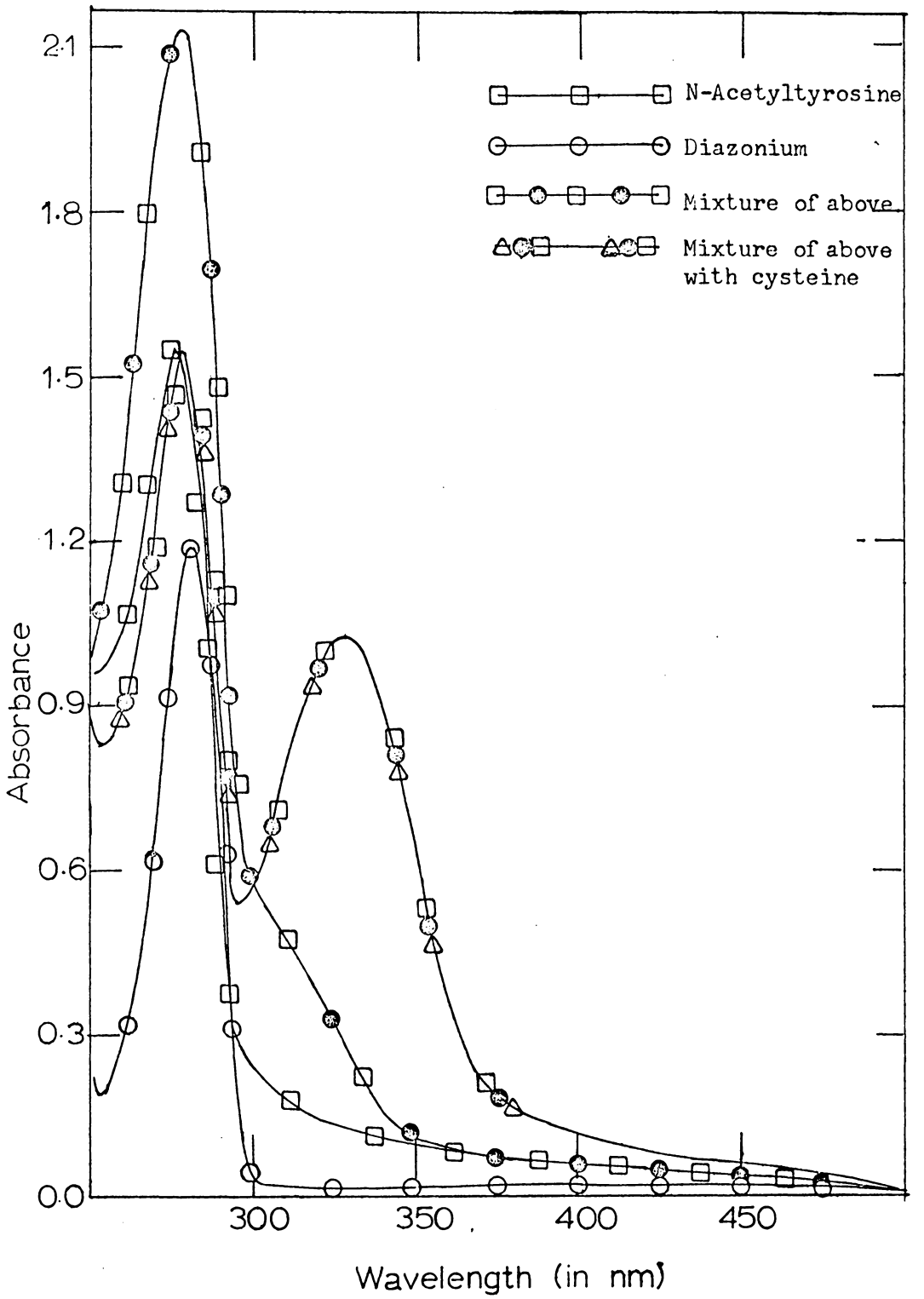

Amine
Diazonium
Diazonium-thiol reaction mixture
1min
15min
30 min
Absorbance
1·8
1·5
1·2
0·9
0·6
0·3
0·0
250
275
300
325
Wavelength (in nm)

It was found that under the given conditions when N-acetyltyrosine was mixed with diazotized p-aminobenzoic acid, the spectrum obtained was essentially equal to the sum of their individual spectra. No other spectral changes were observed between the pH range 6.0 to 8.0 even under prolonged periods of up to 1 hour. However, when cysteine was used it reacted with diazotized p-aminobenzoic acid rapidly with the loss of 265-nm absorption and appearance of 325-nm absorption at all pH values from 6.0 to 8.0. Furthermore, when cysteine was added to solutions of diazotized p-aminobenzoic acid that had been kept over a period of 24 hours, the reaction to give the 325-nm absorption was still instantaneous. Finally, solutions containing diazotized p-aminobenzoic acid and N-acetyltyrosine were first prepared, their spectra obtained, and then a few crystals of cysteine were added into the cuvettes. It was observed that an instantaneous appearance of the 325-nm absorption and a decrease of the 265-nm absorption occurred to the extent that the remaining absorbance may be completely accounted for by the N-acetyltyrosine absorption at this wavelength. This last experiment is illustrated in Figure 22, page 117.

Kinetics of Diazonium-Sulfhydryl Reactions - The kinetics of diazonium-sulfhydryl reactions were studied through sulfhydryl titration experiments. Diazonium derivatives of aniline, p-aminobenzoic acid, sulfanilic acid, arsanilic acid, 3-aminopyridine, 1-methyl-3-aminopyridinium chloride, AAD and AADP were prepared as

FIGURE 22.

Ultraviolet spectra of 10^{-3} M N-acetyltyrosine, 10^{-4} M diazotized p-aminobenzoic acid, a mixture of 10^{-3} M N-acetyltyrosine and 10^{-4} M diazotized p-aminobenzoic acid, and the same mixture with addition of a few crystals of cysteine. All measurements were made in 0.1 M sodium phosphate buffer at pH 7.0 and at room temperature.


N-Acetyltyrosine
Diazonium
Mixture of above
Mixture of above with cysteine
Absorbance
2·1
1·8
1·5
1·2
0·9
0·6
0·3
0·0
300
350
400
450
Wavelength (in nm)

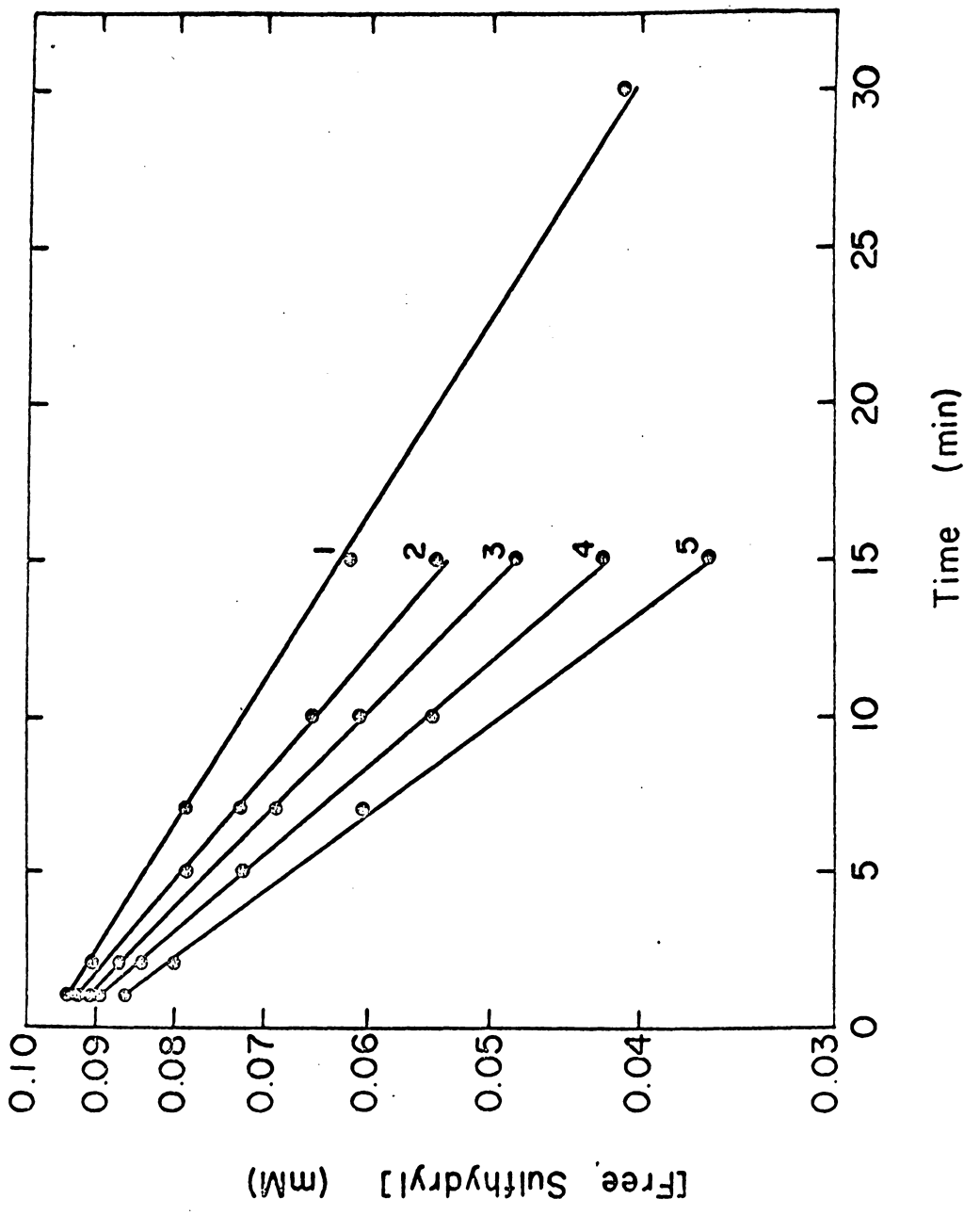
mentioned in the previous section on ultraviolet spectral studies. These compounds were then mixed with sodium phosphate buffer, a thiol solution, and incubated at 0-4°. The resulting reaction mixtures each contained 0.1 M sodium phosphate buffer, pH 7.0, 10^{-4} M thiol and the diazonium derivative in a total volume of 6 ml. Aliquots of 0.5 ml were removed at timed intervals and assayed for sulfhydryl content by 5,5'-dithiobis(2-nitrobenzoic acid).

In the reactions of the diazonium derivatives of aniline, p-aminobenzoic acid, sulfanilic acid and arsanilic acid, as well as diazotized 3-aminopyridine with cysteine, homocysteine, glutathione and mercaptoethanol, sulfhydryl titration indicated the loss of sulfhydryl groups to be instantaneous. Kinetic constants for these reactions were therefore not obtainable.

In the case of reactions involving diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP with cysteine, homocysteine, glutathione and mercaptoethanol, this disappearance of sulfhydryl groups was not instantaneous and therefore the rates of these reactions could be measured. The rates of disappearance of sulfhydryl groups in cysteine, homocysteine, glutathione and mercaptoethanol with five different concentrations (1 to 2.33 mM) of diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP were obtained. The results were analyzed graphically. Pseudo first order kinetics were observed as exemplified by the reactions of diazotized 1-methyl-3-aminopyridinium chloride with cysteine as shown in Figure 23, page 120. Pseudo first order rate

FIGURE 23.

Pseudo first order rate plots of the reaction of diazotized 1-methyl-3-aminopyridinium chloride with cysteine. Reactions were carried out at 0-4°, in 0.1 M sodium phosphate buffer, pH 7.0, with a fixed cysteine concentration (100 μM) and varying diazonium concentrations. Aliquots were taken from reaction mixtures at timed intervals and assayed for sulfhydryl content using 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M sodium phosphate buffer, pH 8.0. Concentrations of diazotized 1-methyl-3-amino-pyridinium chloride used were: line 1, 1mM; line 2, 1.33 mM; line 3, 1.66 mM; line 4, 2.0 mM; line 5, 2.33 mM.


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
[Free Sulfhydryl] (mM)
0
5
10
15
20
25
30
Time (min)
1
2
3
4
5

constants k_{obs} were calculated using the equation $k_{obs} = 0.693/t_{\frac{1}{2}}$, where $t_{\frac{1}{2}}$ is the time for 50% completion of the reaction. When the pseudo first order rate constants were plotted against the concentration of the diazonium derivative a graph showing linear relationship was obtained. This is given in Figure 24, page 123. From the slope of the graph, a second order rate constant of 30 M^{-1} min^{-1} was determined for this reaction. In the same manner, second order rate constants were determined for the 12 sets of reactions of diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP with cysteine, homocysteine, glutathione and mercaptoethanol, and are listed in Table VIII, page 124.

Thin Layer Chromatographic Studies of Diazonium-Cysteine Reaction Products - Calculated amounts of aniline, p-aminobenzoic acid, sulfanilic acid and 3-aminopyridine in mg quantities were diazotized and reacted with cysteine at pH 7.0 and 0-4°. Two sets of such experiments were carried out, one having 10% equivalent excess in the diazonium derivative and the other having 10% equivalent excess in cysteine. The products were obtained by lyophilization of the reaction mixtures. These products were then dissolved in a minimum amount of water and applied on to Eastman Chromagram cellulose sheets containing fluorescent indicator. A series of solvent mixtures containing 0, 1, 5, 10 and 18% (by volume) of 1 M acetic acid to butanol were used for chromatographic analysis to obtain the best

FIGURE 24.

Second order rate plot of the reaction of diazotized 1-methyl-3-aminopyridinium chloride with cysteine.

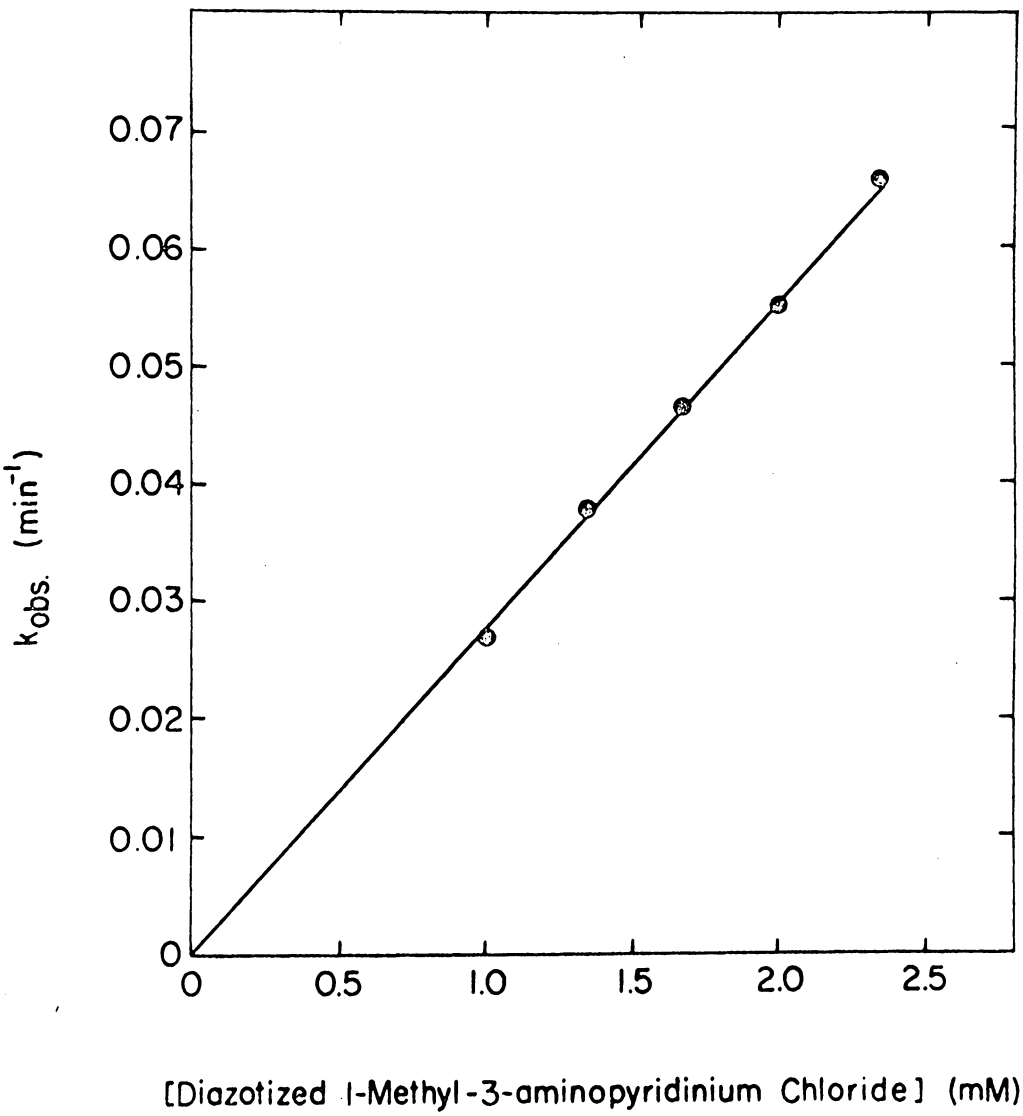

0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
$k_{obs.}$ (min^{-1})
0
0.5
1.0
1.5
2.0
2.5
[Diazotized 1-Methyl-3-aminopyridinium Chloride] (mM)

TABLE VIII

Second order rate constants for a number of diazonium-sulfhydryl reactions[a] (in M^{-1} min^{-1})

Diazonium Compounds	Sulfhydryl Compounds			
	Cysteine	Homocysteine	Glutathione	Mercaptoethanol
Diazotized MAP[b]	30	18	9	9
Diazotized AAD	14.4	9	4.2	4.8
Diazotized AADP	24	16.2	12	6.6

[a]The reactions were carried out in 0.1 M sodium phosphate buffer pH 7.0 at 0-4°, using a fixed sulfhydryl concentration of 0.1 mM, and varying diazonium concentrations 1 to 2.33 mM. Sulfhydryl contents of aliquots were assayed by 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M sodium phosphate, pH 8.0.

[b]MAP, 1-methyl-3-aminopyridinium chloride.

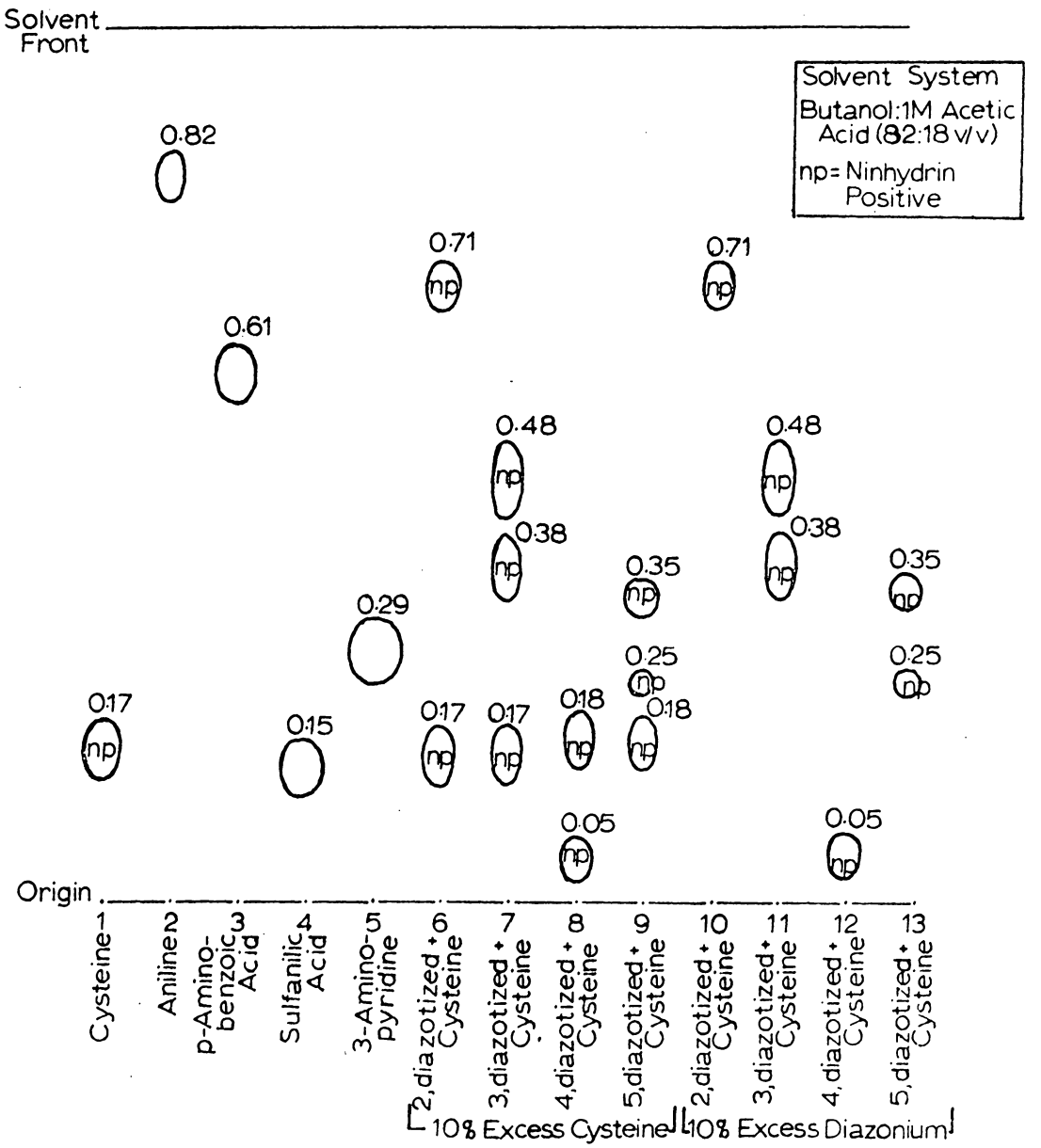
possible separation of the components in the mixtures. Cysteine, aniline, p-aminobenzoic acid, sulfanilic acid and 3-aminopyridine were also used to locate their corresponding positions on the chromatogram. The chromatograms were air-dried, observed under ultraviolet light, then sprayed with ninhydrin aerosol spray and observed after developing for 1 hour at room temperature.

The best solvent system in which the reaction products of cysteine with the diazonium derivatives of aniline, p-aminobenzoic acid, sulfanilic acid and 3-aminopyridine were distinguishable on the same chromatogram was found to be butanol-1 M acetic acid (82:18 by volume). The positions of these products as well as their R_f values are indicated in Figure 25, page 127. In the diazotized aniline-cysteine and the diazotized sulfanilic acid-cysteine reactions the products each gave only one ninhydrin positive spot which was also ultraviolet light quenching. For the diazotized p-aminobenzoic acid- and diazotized 3-aminopyridine-cysteine reactions, the products each gave two spots that were ninhydrin positive and ultraviolet light quenching. It was also observed that when 10% equivalent excess of cysteine was used, free cysteine was also found in the reaction products, which was not the case when the diazonium derivatives were in 10% equivalent excess.

Preparation of Reaction Product of Diazotized p-Aminobenzoic Acid with Cysteine - Synthesis of the product from a reaction in neutral pH was carried out as follows. Sodium nitrite (200 mg) and

FIGURE 25.

Thin layer chromatographic studies of diazonium-cysteine reaction products.


Solvent Front
Solvent System
Butanol:1M Acetic Acid (82:18 v/v)
np= Ninhydrin Positive
0.82
0.71
0.71
0.61
0.48
0.48
0.38
0.38
0.35
0.35
0.29
0.25
0.25
0.17
0.17
0.17
0.18
0.18
0.15
0.05
0.05
np
Origin
1 Cysteine
2 Aniline
3 p-Amino-benzoic Acid
4 Sulfanilic Acid
5 3-Amino-pyridine
6 2,diazotized + Cysteine
7 3,diazotized + Cysteine
8 4,diazotized + Cysteine
9 5,diazotized + Cysteine
10 2,diazotized + Cysteine
11 3,diazotized + Cysteine
12 4,diazotized + Cysteine
13 5,diazotized + Cysteine
10% Excess Cysteine
10% Excess Diazonium

p-aminobenzoic acid (270 mg) were stirred in 50 ml of N HCl at 0-4° until a clear solution was formed. Ammonium sulfamate (330 mg) was then added with vigorous stirring to remove excess nitrous acid. Pellets of NaOH were added to bring the solution to pH 7.0. A saturated solution containing cysteine (240 mg) was slowly added to the reaction mixture. A yellowish orange product was immediately precipitated. The precipitate obtained was filtered, wash thrice with distilled water, then ethanol, and dried. The product was only slightly soluble in water, yielding an ultraviolet spectrum with absorption maximum at 325 nm. The product was found to be more soluble in dilute acid. A portion of the crude product was dissolved in a minimum volume of 1 N HCl, chromatographed on a Bio-Gel P2 column, 5 cm x 200 cm and eluted with distilled water by gravity. Fractions of 20 ml volumes were collected after an initial 750 ml were eluted. A peak containing material with an absorption maximum at 325 nm was eluted at a volume of approximately 3 liters. At this point small orange yellow needle-like crystals appeared in the more concentrated fractions. The crystals were collected by centrifugation. The fractions were scanned from 350 to 200 nm, and those that showed an absorption maximum at 325 nm were pooled and lyophilized. All the samples obtained were dried under vacuum over phosphorus pentoxide.

Characterization of S-(4-Carboxyphenyldiazo)cysteine - Elemental analysis of the crystals obtained from experiments in the previous section indicated a formula corresponding to the diazomercaptide

$C_{10}H_{11}N_3O_4S$.

Calculated: C 44.60, H 4.12, N 15.60

Found: C 44.53, H 4.18, N 15.20

When the compound was heated, it was observed to decompose at above 210° with evolution of gas. Thin layer chromatographic studies of the compound gave an ultraviolet quenching spot which was also ninhydrin positive. For the solvent system 0.1 M acetic acid-95% ethanol (1:1 by volume), R_f = 0.63; for the system butanol-1 M acetic acid (82:18 by volume), R_f = 0.5. The infrared spectrum of S-(4-carboxyphenyldiazo)cysteine in KBr was found to exhibit prominent absorption bands at 690 (w), 740 (m), 775 (m), 860 (m), 1010 (w), 1130 (m), 1155 (w), 1270 (s), 1400 (s), 1500 (s), 1630 (s), 1750 (s), 2000 (w), 3050 (s) and 3450 (s) cm^{-1}. The ultraviolet spectrum of the compound in 0.1 M sodium phosphate buffer at pH 7.0 has absorption maxima at 225 and 325 nm with molar extinction coefficients of 7.2 and 9.9 x 10^3 cm^{-1} M^{-1}, respectively.

When the crude product was dissolved in 6 N HCl and heated, effervescence occurred. When the reaction was studied spectroscopically it was found that there was a loss of the 325-nm absorption and appearance of a new absorption at 265 nm. At room temperature, this reaction proceeded more slowly, with the 325-nm absorption gradually disappearing, and progressive appearance of the 265-nm absorption (Figure 26, page 131). When the logarithm of the absorbance at 325 nm was plotted with respect to time, a linear relationship was obtained (Figure 27, page 133). The pseudo first

FIGURE 26.

Spectral changes that occur upon the formation of S-(4-carboxyphenyl)cysteine (λ_{max} 265 nm) from S-(4-carboxyphenyldiazo)cysteine (λ_{max} 325 nm). Reaction was carried out in 6N HCl at room temperature. The decrease in absorbance at 325 nm corresponds to a concomitant increase at 265 nm.

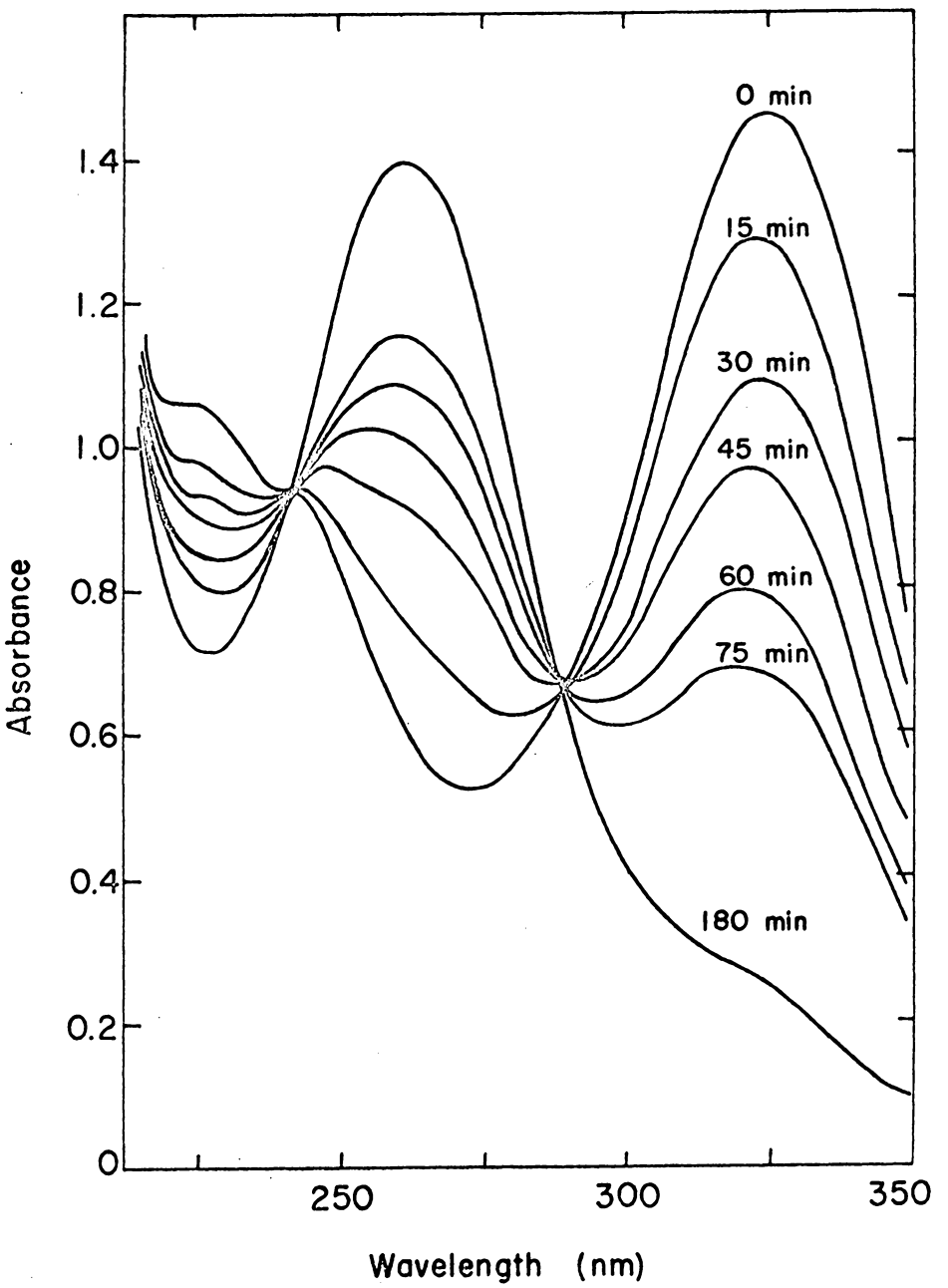
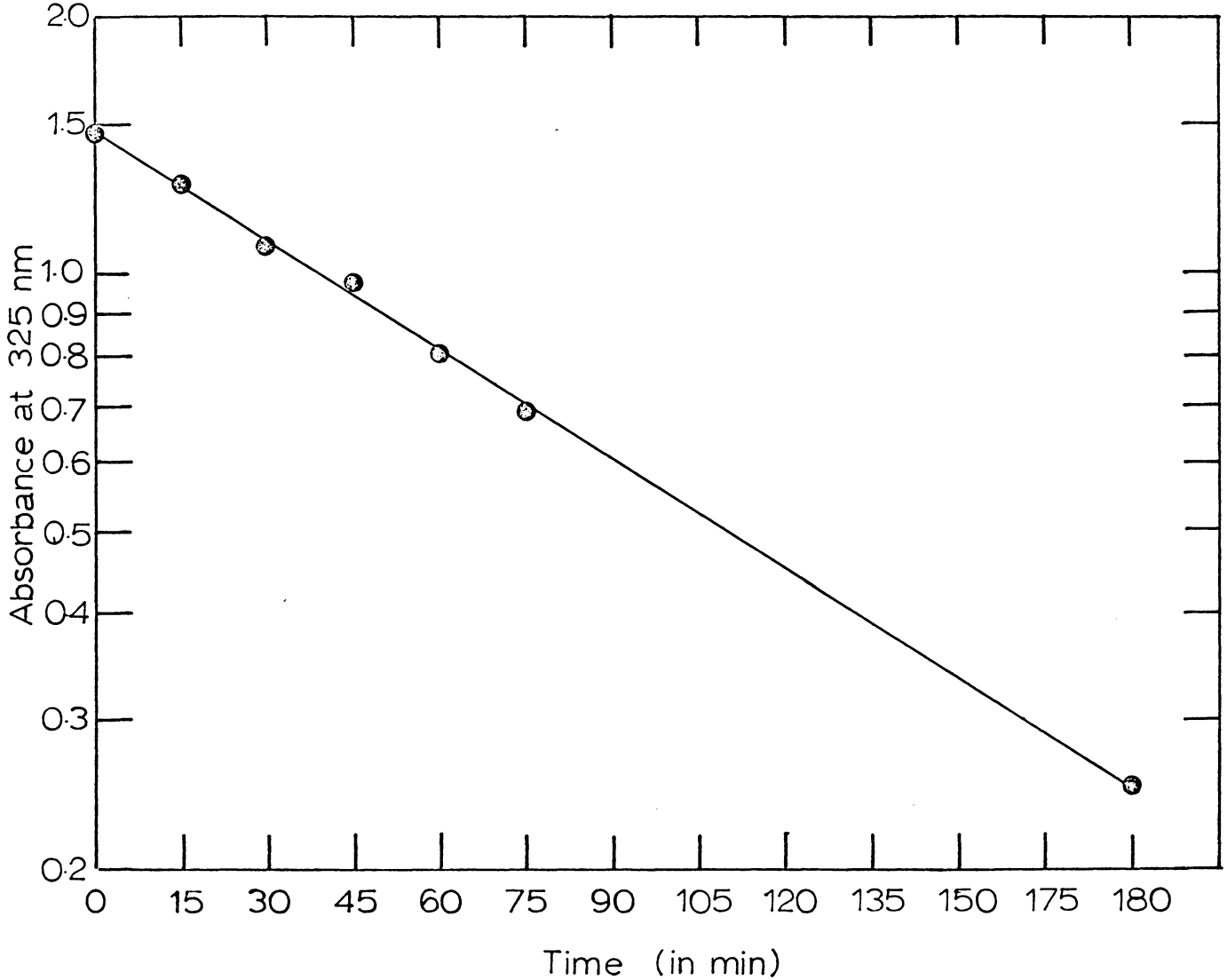

0 min
15 min
30 min
45 min
60 min
75 min
180 min
Absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
250
300
350
Wavelength (nm)

FIGURE 27.

Pseudo first order rate plot of the conversion of S-(4-carboxyphenyldiazo)cysteine to S-(4-carboxyphenyl)cysteine.


Absorbance at 325 nm
2·0
1·5
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0
15
30
45
60
75
90
105
120
135
150
175
180
Time (in min)

order rate constant was calculated as $k_{obs} = 1.02 \times 10^{-2}$ min^{-1}.

Preparation of Decomposition Product from S-(4-Carboxyphenyldiazo)cysteine - A 200-ml saturated solution of the crude product in 2 N HCl was stirred overnight at room temperature, and monitored spectrophotometrically. After 72 hours, the solution was neutralized with NaOH pellets and lyophilized. This product was dissolved in a minimum amount of water and chromatographed on a Bio-Gel P2 column, 5 cm x 200 cm. The eluent was monitored at 280 nm and collected as 20 ml fractions. A major peak containing ultraviolet absorbing materials appeared at an elution volume of about 2.5 liters. The fractions within this peak showed an absorption maximum at 270 nm. These were pooled and lyophilized, yielding a white solid.

Characterization of S-(4-Carboxyphenyl)cysteine - Thin layer chromatography of the product from the previous experiment gave an ultraviolet quenching spot which was also ninhydrin positive. For the solvent system 0.1 M acetic acid-95% ethanol (1:1 by volume), $R_f = 0.58$; for the system butanol-1 M acetic acid (82:18 by volume), $R_f = 0.38$. The compound was dried and elemental analysis performed. The results indicated a formula corresponding to the thioether, $C_{10}H_{11}NO_4S$.

Calculated:	C 49.78,	H 4.60,	N 5.81
Found:	C 48.69,	H 5.39,	N 5.50

The compound was found to melt at 201-3° (uncorrected) with decomposition.

Preparation and Studies of the Diazomercaptide Derivative of 3-Aminopyridine - Synthesis of the diazomercaptide was carried out using the same amounts of sodium nitrite, 3-aminopyridine, HCl, ammonium sulfamate and cysteine under the same conditions as mentioned in the previous chapter for the synthesis of S-(3-pyridyl)cysteine, page 57. The main difference was that the acid solution containing the diazotized 3-aminopyridine was neutralized with NaOH pellets and the reaction with cysteine was carried out at pH 7.0. The reaction mixture was immediately chromatographed on a jacketed Bio-Gel P2 column, 3.5 cm x 40 cm. Fractions were eluted by distilled water and collected in 5 ml portions in a Buchler refrigerated fraction collector. The whole unit including the column was maintained at 4°. Fractions that showed maximum absorption at 315 nm were eluted at about 200 ml elution volume. The fractions were pooled, lyophilized and dried, and a yellowish brown solid was obtained. The product was readily soluble in water and ethanol, giving an absorption maximum at 315 nm. Thin layer chromatography of the product revealed a major ultraviolet quenching and ninhydrin positive spot with a minor spot which was also ultraviolet quenching and ninhydrin positive. R_f values for the solvent system 0.1 M acetic acid-95% ethanol (1:1 by volume) were 0.75 and 0.65, respectively.

Elemental analysis for carbon, hydrogen and nitrogen gave the following composition:

Calculated for diazomercaptide:

C 42.47, H 4.46, N 24.76

Calculated for thioether:

C 48.47, H 5.08, N 14.13

Found: C 45.06, H 4.48, N 18.74

The product was found to melt in the range 195-206° (uncorrected) with decomposition.

When the product was dissolved in 6 N HCl and ultraviolet spectra recorded by repetitive scanning from 350 to 200 nm at 15 min intervals, the 325 nm absorption was observed to decrease with time.

Discussion

Ultraviolet Spectral Studies of Diazonium-Sulfhydryl Reactions - In the experiments of the diazonium derivatives of aniline, p-aminobenzoic acid, sulfanilic acid and arsanilic acid with the thiols cysteine, homocysteine, glutathione and mercaptoethanol, reactions were instantaneous, all yielding a similar characteristic absorption in the 325-nm region (Table VII, page 103). This similarity, together with apparently comparable extinction coefficients suggested that a similar new chromophoric group was formed. Considering the structure of the aromatic diazonium derivatives, and the possibility of reaction with the sulfhydryl groups, it is suggested that the new chromophore is an aromatic diazomercaptide. The bond formation of the electronegative sulfur atom with the aromatic diazonium β nitrogen could well help to facilitate the delocalization of π electrons of

the diazo linkage with the aromatic nucleus, thus lowering the resonance energy and causing a shift of the $\pi \rightarrow \pi^*$ absorption maximum to a longer wavelength. The fact that diazotized 3-aminopyridine behaved similarly in reactions with thiols indicated that the chemistry of the pyridyl diazonium ion is similar to that of the other aromatic derivatives. Since the α-amino acids cysteine and homocysteine reacted in the same way as mercaptoethanol, the α-amino and carboxylic groups are shown not to be involved in the reactions, nor do they affect the reactivity or spectral characteristics significantly. Thus the functional group that would be involved in all these cases would be the sulfhydryl group. The significant reactivity of glutathione demonstrated that sulfhydryl groups in peptides are capable of reacting with diazonium compounds under mild conditions of pH and temperature, providing a basis for correlating the results of the diazotized AAD reaction with yeast alcohol dehydrogenase. The concomitant disappearance of the diazonium absorption in the 265-nm region also further supported the interpretation that diazonium ions were involved in these reactions.

In the case of diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP, the progressive disappearance of their characteristic diazonium absorption in presence of thiol compounds would suggest that reactions are involved with the sulfhydryl groups (Figure 20, page 112 and Figure 21, page 114). The products formed in these reactions appeared not to have distinctive spectral characteristics, which may be partly due to the masking of

new absorption maxima by the relatively high absorbance of the adenyl and pyridyl residues in these compounds. If the extent of reaction is assessed from the disappearance of diazonium ions, the reaction rate, although significant, is appreciably slower than those of the diazotized aromatic derivatives and diazotized 3-aminopyridine. The slower reactions observed with the diazotized pyridinium derivatives may reflect a more favorable equilibrium of diazotate formation due to a greater electron withdrawing capacity of the positively charged ring system. Thus the lower effective concentration of diazonium ion would result in a slower reaction with thiols.

The observation that diazotized p-aminobenzoic acid reacted with cysteine in all pH values between pH 6.0 and 8.0 suggests that the reaction is not sensitive to the ionization states of the sulfhydryl group within this range. It is interesting to find that N-acetyltyrosine does not appear to react with diazonium ions appreciably at this concentration. An inspection of previous reports indicated that reactions were carried out in more concentrated solutions at more alkaline pH values. Thus cysteine is obviously much more reactive than N-acetyltyrosine at pH 6-8. This is confirmed by the experiments where cysteine was added to a reaction mixture containing diazotized p-aminobenzoic acid and N-acetyltyrosine, and reacted to form the characteristic chromophore of 325 nm with the disappearance of the diazonium derivative (Figure 22, page 117). The fact that cysteine could still react with diazotized p-aminobenzoic acid kept overnight at room temperature seems to indicate that the

sulfhydryl group can react with the diazohydroxide form. The reactivity of the sulfhydryl is not surprising, since this function has been known to be among the strongest of biological nucleophiles.

Kinetics of Diazonium-Sulfhydryl Reactions - For the aromatic diazonium derivatives as well as diazotized 3-aminopyridine, no detailed kinetic picture can be drawn as the reaction is too fast for rate measurements by ordinary spectroscopic techniques. However, the immediate complete disappearance of the sulfhydryl groups in the reaction mixtures as indicated by 5,5'-dithiobis(2-nitrobenzoic acid) titration confirmed the interpretations in the previous section that the sulfhydryl group is involved in these reactions. In the cases of diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP, a similar loss of sulfhydryl groups provided essentially the same conclusion. However, the slower rate of reaction permitted the study of the kinetics in these cases. The rates of reactions measured in the presence of excess diazonium derivative with all four thiols were first order with respect to both the diazonium and thiol concentration. This suggests a relatively simple reaction mechanism such as nucleophilic attack on the β nitrogen of the diazonium cation by the sulfhydryl group, or heterolytic dediazoniation with the nucleophile. In reactions with cysteine, second order rate constants indicated that diazotized 1-methyl-3-aminopyridinium chloride is twice as reactive as diazotized AAD. However, this can be compared with the inactivation of yeast

alcohol dehydrogenase as reported by Fisher et al. [1] where the N-methyl derivative was shown to be only one tenth as reactive as diazotized AAD. This latter observation would reflect that the specificity of binding of diazotized AAD to the enzyme active site is an important factor in the enzyme inactivation. The higher reactivity of diazotized 1-methyl-3-aminopyridinium chloride over that of the diazotized pyridine dinucleotides in the simpler systems may be due to the unfavorable structural contributions of the adenyl portions of the dinucleotides in the reaction. The second order rate values obtained for N-ethylmaleimide with cysteine and glutathione as reported by Heitz et al.[155] at pH 5.0 are 1210 and 1545 M^{-1} min^{-1}, respectively. A comparison of these values would suggest that the pyridinium diazonium derivatives are less reactive; however, the difference in reaction conditions of pH prevented an actual quantitative comparison.

Amongst the thiols, cysteine is the more reactive, being followed closely by homocyteine. The α-amino acid zwitterionic structure may be the controlling factor here, since mercaptoethanol and glutathione appear to have essentially similar reactivities.

The sulfhydryl titration is the first experimental demonstration that diazotized AADP can also react with sulfhydryl groups. Thus it would be expected that in certain biochemical systems diazotized AADP would be able to serve as an active-site directing sulfhydryl reagent.

Chromatographic Studies of Diazonium-Sulfhydryl Reaction Products -Thin layer chromatography revealed that the reactions of cysteine with diazotized aniline, diazotized p-aminobenzoic acid, diazotized sulfanilic acid and diazotized 3-aminopyridine all give rise to products that are ninhydrin positive and ultraviolet absorbing (Figure 25, page 127). This indicates that the α-amino group of the amino acid is retained in all cases, and that the aromatic and pyridyl residues of the diazonium derivatives are now linked with the amino acid residues. This is added evidence for the confirmation of diazonium-sulfhydryl reactions. In the case of diazotized aniline and diazotized sulfanilic acid only single spots were detected. The R_f of the diazotized aniline derivative is very large, suggesting that in the solvent system of butanol-1 M acetic acid, nonpolar compounds will migrate faster towards the solvent front. The low R_f value for the diazotized sulfanilic acid derivative is due to a strong polar nature of the p-sulfonic acid substituent which remains ionized in acetic acid. In the case of diazotized p-aminobenzoic acid and diazotized 3-aminopyridine reactions with cysteine, two spots were detected in each case, both being ninhydrin positive and ultraviolet absorbing with fairly close R_f values. In each case, it is suggestive of the formation of two products from the diazonium-sulfhydryl reaction. Mechanistic considerations of the reactions as well as information from the latter sections of the Discussion indicate that one is likely to be the diazomercaptide amd the other the thioether. The R_f values for the diazotized-p-aminobenzoic acid

derivatives are related to the non-ionization of the p-carboxylic acid substituent to the benzene ring in acetic acid, rendering the compound to be relatively non-polar. The R_f values for the diazotized 3-aminopyridine derivatives are somewhat lower, this is probably due to the protonation of the pyridine ring nitrogen in acid medium providing a more polar property. Evidence from thin layer chromatography for two products from the reaction of diazotized 3-aminopyridine with cysteine can be correlated with the results of the synthesis of S-(3-pyridyl)cysteine in acid medium presented previously (page 57) where purification by Bio-Gel P2 chromatography revealed a major component. The minor component had an absorption maximum at 325 nm which seems to relate well to the results from ultraviolet spectral studies of diazotized aromatic and diazotized 3-aminopyridine reactions with thiols presented in a previous section of this study (page 102). Such observations led to the decision to proceed with experiments for the isolation and purification of products from reactions of diazotized p-aminobenzoic acid and diazotized 3-aminopyridine with cysteine.

The fact that in all the above diazonium-sulfhydryl reactions, the same spots were identified with either diazonium or cysteine in slight excess, indicated that the same products are formed independent of the proportions of reactants. Furthermore, in the case of slight excess of cysteine, an additional ninhydrin positive spot identical in position to cysteine was present in every reaction of the four diazonium derivatives used. This suggests immediately that the

stoichiometry of the reactions must be approaching a molar ratio of one to one. This is supported by the fact that in slight excess of diazonium derivatives, no cysteine spot was identified in all the cases.

Reaction Products of Diazotized p-Aminobenzoic Acid-Cysteine Reaction - The reaction of diazotized p-aminobenzoic acid and cysteine in neutral medium yielded a product only slightly soluble in water. To obtain effective separation from Bio-Gel P2 column chromatography, the material has to be applied as a narrow band and this was effected using a solution of the precipitated product in the minimum amount of 1 N HCl. Upon elution, the concentrated band undergoes a process of crystallization because the amount of water was insufficient to dissolve the eluted material. This process of chromatography and crystallization yielded very pure crystals and accounted for the excellent elemental analysis data obtained. The spectrum of this product with an absorption maximum at 325 nm indicated that it is the same compound obtained instaneously during the ultraviolet spectral experiments of diazonium-sulfhydryl reactions for diazotized p-aminobenzoic acid and cysteine.

Elemental analysis indicated that the compound has a formula identical to S-(4-carboxyphenyldiazo)cysteine (page 129). Thin layer chromatographic analysis showed that this compound corresponded with the faster moving spot of $R_f = 0.48$ in the chromatographic experiments on the diazonium-cysteine reaction products with reference

to diazotized p-aminobenzoic acid (Figure 25, page 127). The infrared spectrum is characterized by major bands at 1400 (s), 1500 (s) and 3050 (s) cm^{-1}. Very recently [156], Carroll et al. in their studies involving ligand abstraction in the reaction of aryldiazonium ions with some iron complexes containing co-ordinated cysteine reported that S-(arenediazo)cysteine and related compounds were obtained. Subsequently, they synthesized a series of S-(arenediazo)cysteines directly. All were pale yellow compounds, quite insoluble in all the common solvents while solutions in mineral acids slowly evolve gas. Vibrational spectroscopy shows unambiguously that binding of the diazo group is actually to sulfur. Raman bands at 2567 cm^{-1} that reflected the S-H stretching in cysteine are absent in these derivatives. Infrared spectra of the products show absorptions in the 1600 cm^{-1} region including bands of the NH_3^+ and CO_2^- groups of the zwitterionic form of cysteine. The bands in the region 1350-1500 cm^{-1} were attributed to N=N vibrations. All these data are consistent with the results obtained in the present study, confirming that the compounds synthesized have the diazomercaptide structure.

The compound S-(4-carboxyphenyldiazo)cysteine was unstable in acid, converting to S-(4-carboxyphenyl)cysteine as the product which was isolated and identified from elemental analysis. Although it was not unambiguously demonstrated that the thioether is the immediate decomposition product, the spectral data for the decomposition reaction exhibited by the loss of 325-nm absorption of S-(4-carboxyphenyldiazo)cysteine and gain in 265-nm absorption

of S-(4-carboxyphenyl)cysteine seemed to suggest this (Figure 26, page 131). The reaction apparently takes place by homolytic dediazoniation with the evolution of nitrogen gas. The thioether was found to correspond with the lower spot of R_f = 0.38, in the chromatographic experiments on diazonium-cysteine reaction products as reported in a previous section (Figure 25, page 127). This explains why chromatographic analysis indicated two compounds to be formed in the reaction. The diazomercaptide was likely the first reaction product, followed by partial decomposition to the thioether so that a mixture of two compounds was derived.

Diazomercaptide Derivative of 3-Aminopyridine - Since in the diazotized p-aminobenzoic acid-cysteine reaction, the diazomercaptide was initially formed, this led to the question whether diazotized 3-aminopyridine would give a similar result. This is because from other studies such as spectral, kinetic and chromatographic, there seem to be a close similarity between the two diazonium derivatives and their reactions. As the preparation of S-(3-pyridyl)cysteine reported previously (page 57) was performed in acid medium, there is no information for the situation in neutral medium. Thus the same synthetic procedures for S-(3-pyridyl)cysteine were repeated except that the diazonium-cysteine reaction was carried out at pH 7.0. The product obtained did show absorption characteristics at 315 nm, resembling that of the diazomercaptide. However, elemental analysis did not conform to the formula of this compound. Instead, the

nitrogen composition was somewhat intermediate between the diazomercaptide and the thioether. It was suggested that the product may be a mixture of the two compounds. Thin layer chromatography revealed that this was indeed a mixture. Thus although the diazomercaptide has not been obtained as a pure product and identified unequivocally, the data do strongly suggest that it is formed initially in the diazonium-cysteine reaction, but its stability is so low that the thioether can be formed in significant proportions even at neutral pH.

CONCLUSION

In the present work, experiments have been conducted along two lines- namely, inactivation studies of yeast alcohol dehydrogenase with diazotized AAD and the investigation of diazonium-sulfhydryl reactions of certain diazonium derivatives with simple sulfhydryl compounds. The recent successful synthesis of the NAD coenzyme analog, AAD and the finding that diazotized AAD can cause specific inactivation of yeast alcohol dehydrogenase [1] generated the work of the inactivation studies. Preliminary results of the inactivation studies suggesting the involvement of sulfhydryl groups necessitated detailed experimental work to provide a sound basis for interpreting diazonium-sulfhydryl reactions. Thus these two lines of investigation are intimately related.

Active Site Sulfhydryl Groups in Yeast Alcohol Dehydrogenase

The inactivation of yeast alcohol dehydrogenase by diazotized AAD has been identified as resulting from a selective reaction between sulfhydryl groups of the enzyme and the diazonium compound. After complete inactivation, four sulfhydryl groups per tetrameric form of enzyme were derivatized. The evidence for stoichiometric modification was provided by comparative studies of the modified and native enzymes through 5,5'-dithiobis(2-nitrobenzoic acid) titration, ultraviolet spectra and amino acid analysis. The isolation and identification of the modified cysteinyl residue of the

inactivated enzyme as S-(3-pyridyl)cysteine established the occurrence of a diazonium-sulfhydryl reaction. According to our knowledge, this would constitute the first documentation of a diazonium-sulfhydryl reaction with protein sulfhydryl groups. Previous studies of yeast alcohol dehydrogenase have shown that the presence of free sulfhydryl groups is necessary for catalytic activity [27, 130]. Several sulfhydryl reagents have been used to inactivate this enzyme. These include iodoacetic acid [157], iodoacetamide [135], N-ethylmaleimide [136], fluorescein mercuric acetate [158], p-hydroxymercuribenzoate [133] and butyl isocyanate [142]. At low concentrations of iodoacetic acid, four sulfhydryl groups per molecule are attacked. At higher concentrations and longer reaction time, as many as eight sulfhydryl groups are attacked. N-Ethyl-maleimide and the mercurials react with eight sulfhydryl groups during inactivation of the enzyme. More recently, Twu and Wold [142] used butyl isocyanate to study the sensitive sulfhydryl groups of yeast alcohol dehydrogenase. They reported that three sulfhydryl groups per molecule of enzyme were attacked during inactivation. From peptide analysis, the modified sulfhydryl groups were shown to be different from those derivatized by iodoacetamide [143]. Twu *et al.* [143] proposed that there are two distinct "essential" sulfhydryl groups (denoted x and y) per active site necessary for enzyme activity. They have demonstrated that iodoacetate, iodoacetamide and butyl isocyanate react with the 'x' and 'y' sulfhydryl groups in a proportion dependent on the pH of the reaction [159].

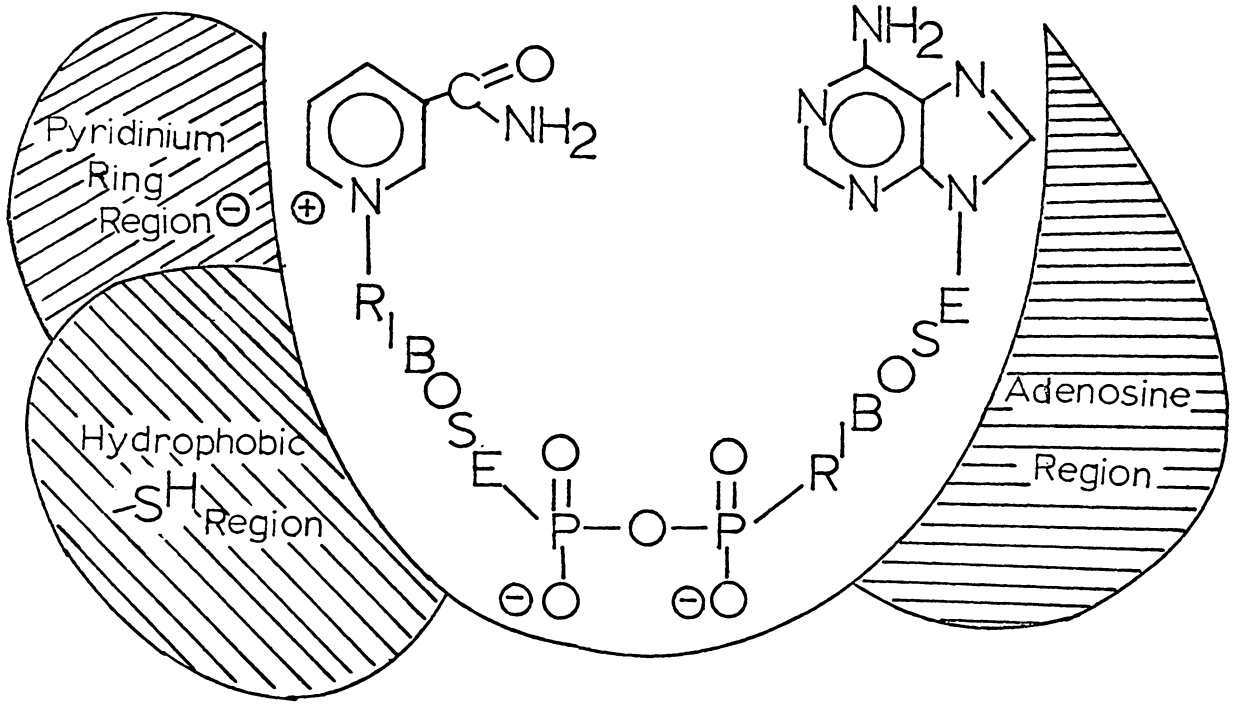
The above reagents are not necessarily selective for sulfhydryl groups. Thus the haloacetates are known to be able to react with lysyl, histidyl and methionyl residues, and N-ethylmaleimide can react with lysyl residues. Even if some of these reagents under given experimental conditions can be selective for sulfhydryl groups, they are not necessarily site-directed reagents. However, since diazotized AAD is a structural analog of NAD, it can be preferentially bound at the active site of the enzyme. The parent compound AAD has also been found to be a coenzyme-competitive inhibitor of yeast alcohol dehydrogenase [1]. Thus diazotized AAD is both active-site directed and sulfhydryl group specific.

The results from the studies of the interaction of yeast alcohol dehydrogenase by diazotized AAD can be interpreted with reference to earlier studies of the catalytic processes of this enzyme. The reversible binding of inhibitors at the coenzyme binding site of this enzyme indicated that three distinct regions exist where selective interactions can occur. A model depicting these regions [160] is shown in Figure 28, page 151. Inhibition by different types of compounds, mainly adenosine derivatives [161, 162] and N^1-alkylnicotinamide chlorides [139, 163] revealed the existence of an adenosine region, a pyridinium ring region and a hydrophobic region at the coenzyme binding site. These regions were found to be distinct from one another on the basis of multiple inhibitor analysis as described by Yonetani and Theorell [164].

N^1-Alkylnicotinamide chlorides which use the pyridinium ring

FIGURE 28.

Schematic representation of the yeast alcohol dehydrogenase coenzyme binding site (modified from Heitz [160]).



SCHEMATIC REPRESENTATION OF THE
YEAST ALCOHOL DEHYDROGENASE
COENZYME BINDING SITE

region in binding were observed not to exclude the binding of NADH, indicating that the 1,4-dihydronicotinamide ring of NADH is bound differently than the oxidized nicotinamide ring of NAD . The alkyl side chains of the N^1-alkylnicotinamide chlorides which interact with the hydrophobic region of the site were shown to enhance the binding of NADH suggesting the involvement of the hydrophobic region in the binding of the reduced coenzyme.

Positive chainlength effects in the inactivation of yeast alcohol dehydrogenase by N-alkylmaleimides were interpreted to indicate that one of the essential sulfhydryl groups of the enzyme exists in a nonpolar environment [136], presumably in the hydrophobic region of the coenzyme binding site. NAD, ADPR and N^1-alkylnicotinamide chlorides were observed not to protect the enzyme against the maleimide inactivation [139-141]. NADH, which utilizes the hydrophobic region in binding, was shown to protect the enzyme against N-alkylmaleimide inactivation. The lack of protection by NAD and N^1-alkylnicotinamide chlorides indicates that the sulfhydryl group involved is not the negatively-charged group that interacts with pyridinium derivatives. Since diazotized AAD can selectively inactivate the enzyme by modifying one sulfhydryl group per active site, it can be suggested that the same sulfhydryl group is involved. The position of this sulfhydryl group in the hydrophobic region would be within a distance approachable by the diazonium group of diazotized AAD. Sloan and Mildvan [165], from magnetic resonance studies of the geometry of bound NAD and isobutyramide on spin-labeled yeast alcohol

dehydrogenase, have also indicated that the spin label attached to cysteine is close to the dihydropyridine ring of bound NADH.

The direct transfer of hydrogen from substrate to coenzyme has been demonstrated by the absence of equilibration with solvent during the catalytic reaction. This transfer of hydrogen would be best accomplished in a hydrophobic region where the solvent is excluded. Since the sulfhydryl group appears to be located in the hydrophobic region, it is likely that there is a catalytic role for this group.

Diazotized AAD as Site-directed Sulfhydryl Reagent

The evidence for the specificity for NAD binding sites as well as the reactivity with sulfhydryl groups have been discussed above for diazotized AAD. Since this diazonium-sulfhydryl reaction can occur with diazotized AAD under mild conditions of temperature and neutral pH, and appears specific for sulfhydryl groups under these conditions, diazotized AAD may be used as an active site-directed sulfhydryl reagent for studies of other dehydrogenases. The established ultraviolet spectral data as well as amino acid analysis procedures are immediately applicable once modified dehydrogenases are obtained. The AAD residue can also serve as an ultraviolet absorbing label in peptide analysis studies for the identification of peptides containing the modified sulfhydryl group. Such site-directed studies can be extended to include NADP-requiring dehydrogenases since AADP, recently prepared and characterized [59], exhibits the same reactivity with sulfhydryl groups after

diazotization.

The studies of diazotized AAD inactivation of yeast alcohol dehydrogenase and other dehydrogenases are especially facilitated by the availability of S-(3-pyridyl)cysteine synthesized as a major product from the reaction of diazotized 3-aminopyridine and cysteine under acid conditions. The compound has been characterized in detail. The ninhydrin positive reactions indicated that the free amino group of cysteine is retained in the product. The ultraviolet spectrum indicated that the compound possessed a chromophore $\pi \rightarrow \pi^*$ transitions related to the pyridine nucleus. The infrared data supported the zwitterionic character of the α-amino and carboxyl functions. The nuclear magnetic resonance data indicated four pyridyl protons, a methine proton and two methylene protons. These, together with elemental analysis, confirmed the compound to be the thioether, S-(3-pyridyl)cysteine. S-(3-Pyridyl)cysteine was very suitable for analysis of diazotized AAD site labeling inactivations of enzymes. It is readily derived by hydrolysis from diazotized AAD modified cysteinyl residues under acid conditions. It is fairly stable and can survive acid hydrolysis to the extent as some of the amino acids such as methionine. A convenient amino acid analysis program is available for automatic quantitative analysis as it is eluted in a convenient position as a well resolved peak and with the proper ninhydrin color yield from the amino acid analyzer. It also possesses an ultraviolet absorbing chromophore that permits its quantitative determination by an independent ultraviolet

spectroscopic method since its absorption maxima and molar extinction coefficients have been determined.

Chemistry of Diazonium-Sulfhydryl Reactions

In the modification of proteins by aromatic diazonium compounds it has long been established that tyrosyl, histidyl and lysyl residues are involved [6-10]. Azo derivatives formed from such reactions have been identified and characterized [7]. However, detailed analysis of the conditions under which such reactions can occur has not been presented. More significantly, the reactivity of sulfhydryl groups under comparable situations has neither been experimentally investigated nor suggested to occur. In view of the general lack of data concerning diazonium sulfhydryl reactions, more detailed studies of this reaction in systems other than proteins were carried out. Reactions with diazonium compounds were demonstrated with cysteine, homocysteine and glutathione, indicating that sulfhydryl-containing amino acids and small peptides likewise react. It was also demonstrated that under comparable conditions, N-acetyltyrosine did not react significantly. The essentially equivalent reactivity of mercaptoethanol compared to the other thiols confirms that the critical functional group for this reaction is the sulfhydryl group. The diazotized p-aminobenzoic acid-cysteine reaction was shown to occur instantaneously in the pH range 6.0 to 8.0 at room temperature.

The rates of reactions involving excess diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP with

simple sulfhydryl compounds were first order with respect to both the diazonium derivatives and the sulfhydryl compounds. This suggests a relatively simple reaction mechanism such as nucleophilic attack on the β nitrogen of diazonium cation by the sulfhydryl group, or heterolytic dediazoniation with the nucleophile [2].

To understand the chemistry of diazonium-sulfhydryl reactions, isolation and characterization of products are necessary. In one reaction, diazotized p-aminobenzoic acid was reacted with cysteine at neutral pH. A single product was obtained which upon elemental analysis gave a formula corresponding to the diazomercaptide, S-(4-carboxyphenyldiazo)cysteine. Under acid conditions there was progressive loss of the S-(4-carboxyphenyldiazo)cysteine 325-nm characteristic absorption with concomitant appearance of a new 265-nm peak. The reaction revealed a first order pattern suggestive of homolytic dediazoniation [2]. The decomposition product was isolated and purified, yielding from elemental analysis a compound corresponding to the thioether, S-(4-carboxyphenyl)cysteine. In the above series of reactions, it is likely that the diazomercaptide is formed by nucleophilic attack on the diazonium cation at the β nitrogen, after which the product can decompose to the thioether by loss of nitrogen. Such a reaction is also likely for the reaction of diazotized 3-aminopyridine with cysteine, since ultraviolet data of the reaction at pH 7.0 indicated an initial rapid formation of a 325-nm absorption very similar to that of diazotized p-aminobenzoic acid reaction with cysteine. A product which is only partially

purified indicated an intermediate nitrogen percentage between diazomercaptide and thioether, with its absorption maximum at 325 nm decreasing in presence of acid similar to the case of S-(4-carboxyphenyldiazo)cysteine.

The exact nature of the reaction of diazotized AAD with active site sulfhydryl groups of yeast alcohol dehydrogenase is as yet unclear. The concomitant appearance of the 300-nm absorption, together with the 260-nm adenine absorption in the difference spectrum of modified versus native enzyme [1], seemed to suggest possible diazomercaptide formation. Whether the initial cysteinyl derivative formed during the inactivation process is a diazomercaptide or a thioether remains to be established; however, acid hydrolysis of either of these derivatives would release S-(3-pyridyl)cysteine. Therefore, the application of diazotized AAD or diazotized AADP in active site sulfhydryl studies should be unaffected by the actual intermediate initially formed.

Biochemical Implications of Diazonium-Sulfhydryl Reactions

This investigation has demonstrated that diazonium-sulfhydryl reactions occurred at a significant rate under mild conditions of temperature and pH. The high reactivity of sulfhydryl groups with the diazonium ion must give caution to all interpretations of biochemical experiments with diazonium compounds where the possible involvement of the cysteinyl residue has not been considered. Thus in the diazo coupling reactions with native and acetylated bovine

serum albumin by diazotized arsanilic acid at pH 8.0 as reported by Tabachnick and Sobotka [31], only about 50% of protein bound arsenic that was quantitated could be attributed to the azotyrosine and azohistidine that were assayed spectrophotometrically. The authors suggested that other amino acids such as tryptophan and arginine were involved. However, it is even more likely that the cysteinyl residues should be involved in the light of results from the present study. In the affinity labeling study of the electroplax acetylcholine receptor [39], it was reported that the diazonium irreversible inactivator would become a reversible activator if the electroplax membrane was exposed to dithiothreitol before the experiment. For the reaction with the enzyme acetylcholinesterase, the diazonium derivative became a reversible inhibitor if the enzyme was previously exposed to dithiothreitol. The authors only suggested that the diazonium had reacted with tyrosyl, histidyl or lysyl residues. But in the present analysis the possibilities of dithiothreitol reacting with the diazonium derivatives must be considered, in addition to the possibility of reaction with the free protein sulfhydryl groups generated by such a treatment. The report that in bovine acetylcholinesterase [40], 2,500-fold or more of free histidine was unable to prevent the irreversible inhibition by the diazonium derivative at 10^{-7} to 10^{-5} M should reveal the fact that reactivity of histidine towards diazonium ion was probably not significant at all under those conditions. In the experiments of triose phosphate isomerase [49] with diazotized sulfanilic acid, the absence of

absorption above 320 nm in the modified enzyme was assumed to credit the involvement of lysyl residues (since histidyl or tyrosyl residues were discredited). Again the important possibilities with cysteinyl residues were overlooked. In deoxyribose-5-phosphate aldolase [50], the inactivation by diazotized p-nitroaniline was ascribed to histidyl or tyrosyl residues without even an attempt to investigate experimentally the involvement of any amino acid residue. In this case, cysteinyl residues could just as likely be involved in the inactivation. Thus many previous diazonium modification experiments may have to be reinterpreted or new experiments performed to investigate the possibiliites of the diazonium-sulfhydryl reactions established in the present work.

Possibilities for Future Work

In the inactivation studies of yeast alcohol dehydrogenase this investigation has opened up many future possibilities in understanding the active site characteristics of this enzyme. Thus the peptide containing the diazotized AAD modified cysteinyl residue may be identified by peptide mapping using the absorption characteristics of the AAD residue as an ultraviolet spectral label. The isolation of such a peptide and its amino acid analysis will be useful for comparison with the results obtained by Harris [157] and Twu et al. [143] and to evaluate the consequences of inactivation by diazotized AAD, iodoacetate, iodoacetamide and butyl isocyanate. Information about the approximate distance and region of localization of the

diazotized AAD modified sulfhydryl group can also be obtained. As in the case of diazotized arsanilic acid inactivated carboxypeptidase A, the diazotized AAD inactivated enzyme may be studied for its optical rotatory dispersion characteristics. This is because the asymmetric carbon atom of the L-cysteinyl residue is attached to the AAD chromophore, and theoretically should be optically sensitive to polarized light. The microenvironment of the active site around this modified sulfhydryl can be studied from the multiple extrinsic Cotton effects exhibited in the circular dichroic spectrum. The results of the above-mentioned experiments may also contribute to a clearer understanding of the nature of covalent bonding of diazotized AAD with the sulfhydryl group whether it is a diazomercaptide or a thioether. The tertiary and quaternary structure of the modified enzyme may also be investigated with polyacrylamide gel electrophoresis, Sephadex gel permeation chromatography and ultracentrifugation studies. Application of these techniques to both the native and modified enzymes will indicate in addition to other molecular information, whether there is still tetrameric molecular association after modification. Although a preliminary study of the stability and reversibility of the diazotized AAD sulfhydryl linkage was undertaken using cysteine, a more detail analysis is also needed.

Future studies using diazotized AAD will probably be very significant biochemically. If other NAD-dependent enzymes are found that are inactivated by diazotized AAD, the established methodology in the present study of yeast alcohol dehydrogenase can be readily

applied and information about their active sites especially in terms of sulfhydryl groups can be obtained. The current work started on glycerophosphate dehydrogenase is the first such example. It should also be noted that enzymes that are reversibly inhibited but not inactivated by diazotized AAD could be an indication of non-involvement of sulfhydryl groups in their activity or a significant difference in the localization of sulfhydryl groups. It should be noted that diazotized AADP will have similar applicability for NADP-dependent enzymes. The possibilities for applying diazotized 3-aminopyridine as a specific sulfhydryl quantitation reagent should should be investigated and developed. The establishment of a much faster and just as sensitive analytical technique for S-(3-pyridyl)-cysteine by high pressure liquid chromatography, rather than the routine amino acid analysis presented in this work, will be useful for certain aspects of investigation. The chromatographic procedure can be readily developed from the introductory work reported in this study.

In the area of chemical studies, isolation and purification of the reaction products of the diazonium-sulfhydryl reactions demonstrated in this work should be carried out. Detailed structural elucidation can provide a better understanding of many areas of diazonium chemistry. The especially promising examples would be the products from the reaction of cysteine with diazotized aniline and diazotized sulfanilic acid, since thin layer chromatography studies have indicated that a single product appeared to be formed in each

case. The further purification and studies of the possible S-(3-pyridyldiazo)cysteine should be pursued. Products derived from glutathione and mercaptoethanol with the aromatic diazonium derivatives would also be very informative. The question of cis, trans isomers in diazomercaptide should be analyzed. Although the isolation of products from the reactions of cysteine with diazotized 1-methyl-3-aminopyridinium chloride, diazotized AAD and diazotized AADP are expected to be more difficult, this should be attempted. The successful purification and identification of these products would be instrumental in providing detailed chemical information of the diazotized AAD inactivation process with yeast alcohol dehydrogenase. The availability of all the above diazonium-sulfhydryl reaction products will open a host of possibilities in ultraviolet, infrared and nuclear magnetic resonance spectral studies.

Finally, more general possibilities that are suggested by this work would include design and synthesis of various suitable diazonium reagents estimated to be able to serve as site labeling reagents for various sulfhydryl enzymes other than dehydrogenases. The availability of a large number of diazotizable aromatic and hetero-aromatic amines can give numerous opportunities. However, it should not be assumed that diazonium compounds react only with sulfhydryl groups, in all cases, each must be investigated individually. The recent interest in affinity chromatography also points to the possibility of utilizing the diazonium-sulfhydryl reaction in the binding of inhibitors, coenzyme analogs, etc., such as AAD on to the

chromatographic solid matrix materials.

SUMMARY

The present study was initiated as a consequence of the preliminary findings of Fisher et al. [1] that diazotized AAD inactivated yeast alcohol dehydrogenase specifically. The aim was to delineate the possibilities and to develop the potentialities of diazotized AAD as an active site specific labeling reagent. It was also necessary to provide a basic understanding of the chemical processes involved in the inactivation.

Experiments in the first part of these studies mainly involved enzyme inactivation and associated studies. 5,5'-Dithiobis-(2-nitrobenzoic acid) titration of native and diazotized AAD modified enzyme revealed that sulfhydryl groups were attacked. The complete inactivation of yeast alcohol dehydrogenase was associated with the loss of four sulfhydryl groups per tetramer of enzyme. In considering diazotized AAD as an analytical reagent, the presumed acid hydrolysis product of diazotized AAD derivatized cysteine, S-(3-pyridyl)cysteine was synthesized and studied. S-(3-Pyridyl)cysteine was purified by Bio-Gel P2 column chromatography. Elemental analysis indicated a formula of $C_8H_{10}N_2O_2S$. The ultraviolet spectrum exhibited absorption maxima at 250 and 280 nm of molar extinction coefficients 11.2 and 7.6 x 10^3 cm^{-1} M^{-1}, respectively. Infrared spectrum showed absorption bands at 1600 and 3020 cm^{-1} indicative of a zwitterionic structure. Nuclear magnetic resonance spectrum contained groups of protons of α pyridyl: β pyridyl: γ pyridyl: methine: methylene in a ratio of

2:1:1:1:2 and with spin-spin coupling characteristic of the presence of an asymmetric center. All these physical and chemical data established that the compound was a thioether. The amino sugar program for amino acid analysis was conveniently used to elute S-(3-pyridyl)cysteine at 23.8 min with a K_F = 0.4320. The compound was sufficiently stable under protein hydrolysis procedures. Amino acid analysis of the acid hydrolyzate of diazotized AAD modified yeast alcohol dehydrogenase revealed a peak corresponding to S-(3-pyridyl)cysteine. Four S-(3-pyridyl)cysteine residues were obtained per molecule of modified enzyme, and this agreed with the four AAD residues per molecule of modified enzyme calculated on basis of difference spectra studies.

These results were interpreted with reference to earlier studies of yeast alcohol dehydrogenase catalytic processes, and a model of the active site with the catalytic sulfhydryl group located in the hydrophobic region was found to best fit the data. Diazotized AAD was evaluated as an active site reagent with selectivity under mild conditions, yielding a stable modified derivative with characteristic elution pattern, proper ninhydrin color yield, and alternative quantitation procedure by spectroscopy. Thus diazotized AAD was shown to be valuable for investigating problems concerning active site sulfhydryls in dehydrogenases.

Experiments in the second part of these studies utilized the diazonium derivatives of aniline, p-aminobenzoic acid, sulfanilic acid, arsanilic acid, 3-aminopyridine, 1-methyl-3-aminopyridinium chloride, AAD and AADP in reactions with the simple thiols cysteine,

homocysteine, glutathione and mercaptoethanol. Ultraviolet spectral studies revealed that the diazotized aromatic compounds and diazotized 3-aminopyridine reacted to form diazomercaptides with absorption maxima in the 325-nm region instantaneously. The reactions with the diazotized pyridinium derivatives were slower and no new characteristic absorption maxima were observed. It was also demonstrated that diazotized p-aminobenzoic acid reacted readily with cysteine in the pH range 6.0 to 8.0, but N-acetyltyrosine did not seem to react under similar experimental conditions. Kinetic studies of the disappearance of sulfhydryl groups during diazonium-sulfhydryl reactions again reflected the instantaneous nature of the reactions with aromatic and 3-aminopyridine diazonium derivatives. In the case of the diazotized pyridinium derivatives, reactions showed pseudo first order rate characteristics. The second order rate constants varied within a range of 4 to 30 M^{-1} min^{-1}. Thin layer chromatographic analysis of the reaction products of diazotized aniline and diazotized sulfanilic acid with cysteine showed that only a single product which was ninhydrin positive was obtained in each case. The R_f values in butanol:1 M acetic acid (82:18 by volume) were 0.71 for the diazotized aniline-cysteine product, and 0.05 for the diazotized sulfanilic acid-cysteine product. Diazotized p-aminobenzoic acid-cysteine and diazotized 3-aminopyridine-cysteine reactions each gave two ninhydrin positive spots for their products on chromatographic analysis, R_f values being 0.38 and 0.48 for the former and 0.25 and 0.35 for the latter. The stoichiometry of the diazonium-cysteine reactions was

estimated to be in a ratio of one to one in all cases. S-(4-Carboxyphenyldiazo)cysteine was prepared as the initial reaction product from diazotized p-aminobenzoic acid and cysteine. Its structure was confirmed from elemental analysis, ultraviolet and infrared spectral studies. In acid medium, S-(4-carboxyphenyldiazo)cysteine decomposed to S-(4-carboxyphenyl)cysteine. The diazomercaptide of the diazonium-cysteine reaction of diazotized 3-aminopyridine was also prepared in crude form.

These studies indicated that diazonium-sulfhydryl reactions are very general, and they take place readily. It is likely that the diazomercaptide is the initial product formed in those cases considered, but the thioether may be derived by decomposition. The significance of these experiments is that in all diazonium modifications of proteins, diazonium-sulfhydryl reactions must be considered as a possibility.

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A NOVEL DIAZONIUM-SULFHYDRYL REACTION IN THE INACTIVATION OF YEAST ALCOHOL DEHYDROGENASE BY DIAZOTIZED 3-AMINOPYRIDINE ADENINE DINUCLEOTIDE

by

Jack Cham-Kit Chan

(ABSTRACT)

Diazotized 3-aminopyridine adenine dinucleotide has been found to modify four sulfhydryl groups per molecule of enzyme during the complete inactivation of yeast alcohol dehydrogenase. The reaction of sulfhydryl groups was indicated by titration studies with 5,5'-dithiobis(2-nitrobenzoic acid) as well as isolation and quantitation of the cysteinyl derivative released by acid hydrolysis of the modified enzyme. The cysteinyl derivative was identified as S-(3-pyridyl)cysteine. Authentic S-(3-pyridyl)cysteine was synthesized and structurally characterized for these studies.

Diazonium-sulfhydryl reactions were demonstrated for a number of diazonium derivatives with cysteine, homocysteine, glutathione and mercaptoethanol at 0-4° and neutral pH. Second order rate constants were determined in reactions of these sulfhydryl compounds with diazotized 1-methyl-3-aminopyridinium chloride, diazotized 3-aminopyridine adenine dinucleotide and diazotized 3-aminopyridine adenine dinucleotide phosphate. Chemical studies of the diazonium-sulfhydryl reaction of diazotized p-aminobenzoic acid with cysteine indicated the initial formation of a diazomercaptide which can then decompose to yield the thioether.