AN ABSTRACT OF THE THESIS OF

HERBERT MICHAEL WEHR (Name of student) for the Ph. D. (Degree)

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Title: REACTIONS OF PROTEIN WITH PHENOLS AND QUINONES: EVALUATION OF AMINO ACID MODIFICATION AND PROTEIN DIGESTIBILITY

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Abstract approved: _____
Dr. W. D. Loomis

Plant tissues contain a wide variety of phenolic compounds, frequently in high concentration. Both non-covalent association of intact and oxidized phenols and covalent linkage of oxidized phenols to protein occur. Such interactions can have important biochemical and nutritional significance.

A model system approach was used to identify and quantitate amino acid residue modification in protein exposed to oxidized phenolic material and to identify the probable adduct compound. Digestibility studies were carried out on tanned bovine serum albumin to assess further protein-phenol association and to estimate the extent of covalent and non-covalent binding of oxidized phenolic material to protein.

Preliminary studies indicated that non-enzymatic base-catalyzed air oxidation of pyrocatechol occurred rapidly above pH 7.5. Spectral

analysis of oxidized pyrocatechol showed a continuous absorption throughout the visible region of the spectrum indicating the formation of a wide variety of products. The presence of bovine serum albumin in such a system changed the nature of oxidized products as evidenced by alterations in the visible absorption spectrum. Tanned bovine serum albumin showed a visible absorption spectrum similar to that of oxidized pyrocatechol which, in conjunction with the lack of visible absorption by bovine serum albumin, indicated association of pyrocatechol oxidation products with the protein. The strong nature of these associations was indicated by the failure of solvent stripping agents to completely remove bound material. Spectral analysis of the products of mushroom tyrosinase oxidation of pyrocatechol indicated that o-benzoquinone was the initial oxidation product and that product inhibition of tyrosinase occurred in the systems. Binding of enzymatic pyrocatechol oxidation products to glycine, bovine serum albumin, and poly-L-lysine was indicated.

Amino acid analysis of tanned bovine serum albumin indicated that cystine, lysine, and histidine were the primary residues modified by covalent linkage to oxidized pyrocatechol. A similar pattern of amino acid modification was found in bovine serum albumin tanned by either base-catalyzed or tyrosinase oxidation of pryocatechol, or by incubation with p-benzoquinone. The extent of modification was greatest in the p-benzoquinone system implying that the quinone was

the reactive species. Observed modifications increased with increasing pyrocatechol or p-benzoquinone concentrations and with increasing pH.

Gas-liquid chromatography of N-trifluoroacetyl-L-tryptophan methyl ester and N-trifluoroacetyl-DL-methionine methyl ester exposed to enzymatically oxidized pyrocatechol indicated that these amino acids were also modified, the extent of modification increasing with increasing pyrocatechol level.

Trypsin digestibility of tanned BSA was markedly reduced while pepsin digestibility was less affected. Protein-phenol complex formation as evidenced by decreased digestibility, is a result of covalent and non-covalent binding of phenolic material. Digestibility decreases greater than the observed amino acid modifications resulting from covalent binding of oxidized phenolic material suggest, as one possibility, that non-covalent association of phenolic material is predominant over amino acid modifications.

Modifications occurring to the nutritionally essential amino acids lysine, methionine, tryptophan, and histidine (infants only), and the decrease in digestibility of tanned protein may drastically reduce the nutritional value of a protein. These same modifications may alter the biochemical properties of protein. Procedures to isolate plant proteins must be designed to minimize these effects.

Ultraviolet, infrared, and mass spectral analyses of a

glycine-p-benzoquinone reaction mixture indicated that a phenolic secondary amine (Ar-NH-R) was one of the products. Results suggest a 1, 4-addition reaction as the probable means of amino acid modification in oxidized phenolic systems (literature on amino-acid quinone interaction supports this conclusion).

Reactions of Protein with Phenols and Quinones: Evaluation of Amino Acid Modification and Protein Digestibility

by

Herbert Michael Wehr

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Abbreviations used in the text

BSA, bovine serum albumin

Methyl pyrrolidone, N-methyl-2-pyrrolidone

NATEE, N-acetyl-L-tryptophan ethyl ester

N-TFA-L-tryptophan ME, N-trifluoroacetyl-L-tryptophan methyl ester

N-TFA-DL-methionine ME, N-trifluoroacetyl-DL-methionine methyl ester

TCA, trichloroacetic acid

Tris, tris-(hydroxy methyl) amino methane

$V_o + V_i$, void volume + inner volume

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REACTIONS OF PROTEIN WITH PHENOLS AND QUINONES: EVALUATION OF AMINO ACID MODIFICATION AND PROTEIN DIGESTIBILITY

INTRODUCTION

Phenolic compounds are highly reactive materials and their reactions with proteins have both biochemical and nutritional significance. These interactions are of particular importance in terms of plant biochemistry since plant tissues contain a wide variety of phenolic compounds, frequently in high concentrations. The introductory sections below survey the chemical nature of plant phenolic compounds, modes of phenolic oxidation, and the interactions possible between phenolic material and amino acids, peptides, and proteins.

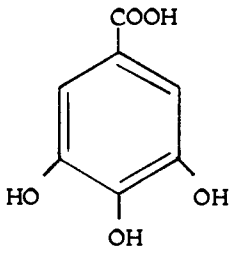
Plant Phenolic Compounds

Chemically, plant phenolic compounds are extremely heterogeneous, ranging from simple monomers to complex polymers. Most of them, however, may be classified into one of two biochemical groupings: the C_6-C_1 and C_6-C_3 compounds, including the hydrolyzable tannins; and the C_6-C_3-C_6 or flavonoid compounds, including the condensed tannins. The term "tannin" itself frequently refers to plant phenolic material in a general sense; it has no exact chemical definition and derives from the ability of many plant phenolic

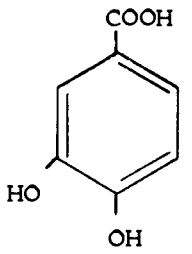
compounds to convert raw hide into leather. The biosynthesis and characterization of plant phenolic compounds is reviewed by Neish (1964), Swain (1965), and Robinson (1967).

The C_6-C_1 and C_6-C_3 phenolic compounds are, according to available data, derived from the shikimic acid pathway and contain hydroxyl and carboxyl groups as reactive centers. Examples of the C_6-C_1 and C_6-C_3 compounds are shown in Figure 1. Hydrolyzable tannins are esters of the C_6-C_1 and C_6-C_3 phenolic compounds, the ester linkage being susceptible to cleavage with boiling dilute acid. The alcoholic portion of the ester is usually a sugar but it may be quinic acid. Complex mixtures of hydrolyzable tannins may arise from esterification at the different positions of the sugar molecule.

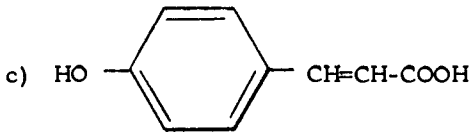
The C_6-C_3-C_6 or flavonoid compounds basically consist of two C_6 aromatic rings linked by a three-carbon chain as shown in Figure 2(a). The predominant flavonoid class has the carbon skeleton shown in Figure 2(b) and tends to have the hydroxylation pattern indicated. The types of flavonoid compounds within this class are based on the chemistry of the heterocyclic ring as indicated in Figure 2 (c-k). Flavonoids most frequently occur as glycosides or methoxy compounds; the methylation and glycosidation steps involving the hydroxyl groups of the flavonoids are generally thought to occur at the end of the biosynthetic sequence. Compounds of the



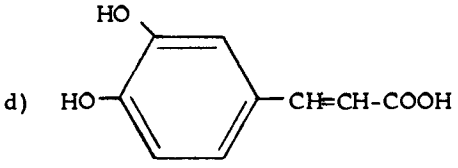
a) gallic acid



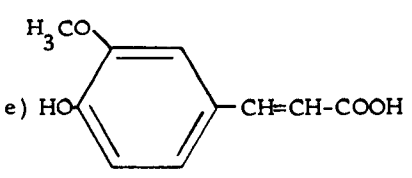
b) protocatechuic acid



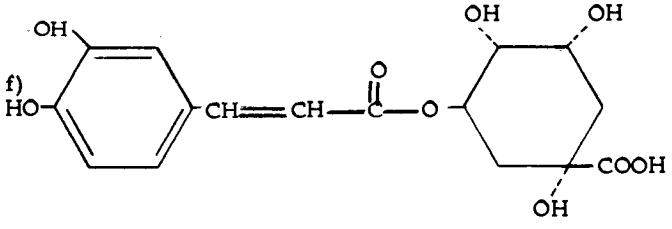
p-coumaric acid



caffeic acid

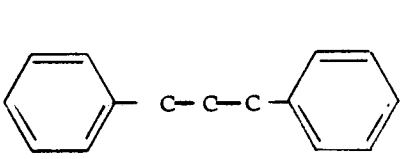


ferulic acid

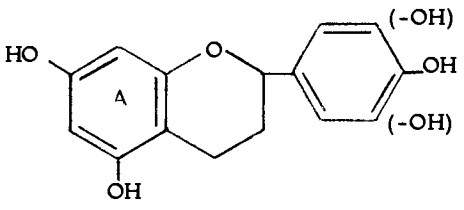


chlorogenic acid

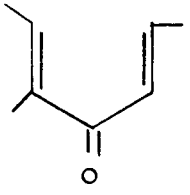
Figure 1. C_6-C_1 (a and b) and C_6-C_3 (c-f) phenolic compounds (from Robinson, 1967).



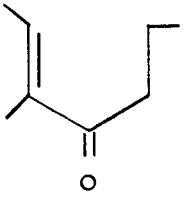
(a)



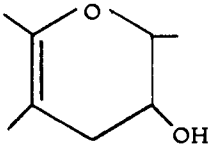
(b)



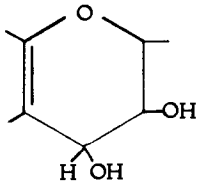
c) chalcones



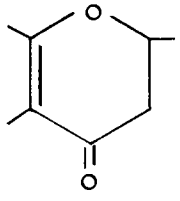
d) dihydrochalcones



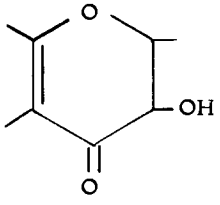
e) catechins



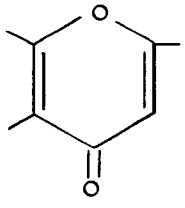
f) leucoanthocyanidins



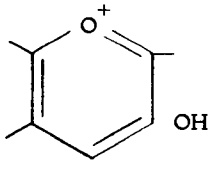
g) flavanones



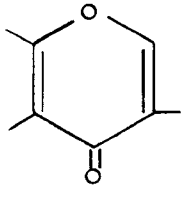
h) flavanonols



i) flavones



j) anthocyanidins



k) isoflavones

Figure 2. Fundamental flavonoid skeletons (from Robinson, 1967).

flavonoid type generally contain only hydroxyl groups as reactive centers. Condensed tannins are complex mixtures of polymeric material generated from oxidative modification and cross-linking of flavonoid monomers.

Oxidative Modification of Plant Phenolic Compounds

Oxidation of phenolic compounds readily occurs by a variety of reagents and usually results in a complex mixture of compounds that are dimeric to polymeric, and quinoid in nature. The following discussion presents a brief overview of phenolic oxidation mechanisms of interest in biochemical systems. Mason (1949 1955a), Kertesz and Zito (1962), Thompson (1964), Musso (1967), Brown (1967) and Mihailovic and Cekovic (1971) review the great amount of information available on non-enzymatic and enzymatic oxidation of phenols.

Phenol oxidation is generally thought to proceed by free radical mechanisms with a one-electron extraction by a suitable reagent resulting in the formation of a phenoxy free radical. One-electron extracting agents of interest in biochemical systems include heavy metal ions, free radicals, molecular oxygen in alkaline solution, and some enzymes (laccases, phenolases peroxidases). Initial formation of the phenoxy radical by such agents is indicated in Figure 3. The generation of the phenoxy radical may occur by the loss of one electron from the corresponding phenoxide anion or by

homolytic cleavage of the O-H bond with the concomitant release of a hydrogen atom. The phenoxy radical that is formed is resonance stabilized as indicated for phenol in Figure 4. As is evident from Figures 3 and 4 a semiquinone radical results from an o- or p-dihydroxy phenolic compound. The phenoxy radical formed from hydroquinone is sufficiently stable that characterization has been possible (Musso, 1967); the ESR spectrum has been determined and characteristics of oxidation-reduction equilibria have been evaluated. Characteristics of phenoxy radicals are reviewed by Musso (1967) and Mihailovic and Cekovic (1971). The phenoxy radical is usually only a transitory species, and subsequent reaction leads to the quinone or, with other radicals, to a variety of coupled dimers, oligomers, and polymers. Further reaction of the phenoxy radical leading to the quinone and to the formation of dimeric compounds from radical condensation involving C-C, C-O, and O-O linkages is illustrated in Figure 5. Usually the initial dimeric compounds tautomerize in protic solvents to yield the more stable aromatic products. At least two coupling mechanisms involving the phenoxy radical and the intact phenol or phenolate anion are possible, as illustrated for phenol in Figure 6. The phenoxy radical may attack a phenol or phenolate anion yielding a dimeric radical which is subsequently modified by either the loss or addition of a hydrogen radical (Figure 6a). This pathway is possible when oxidation is

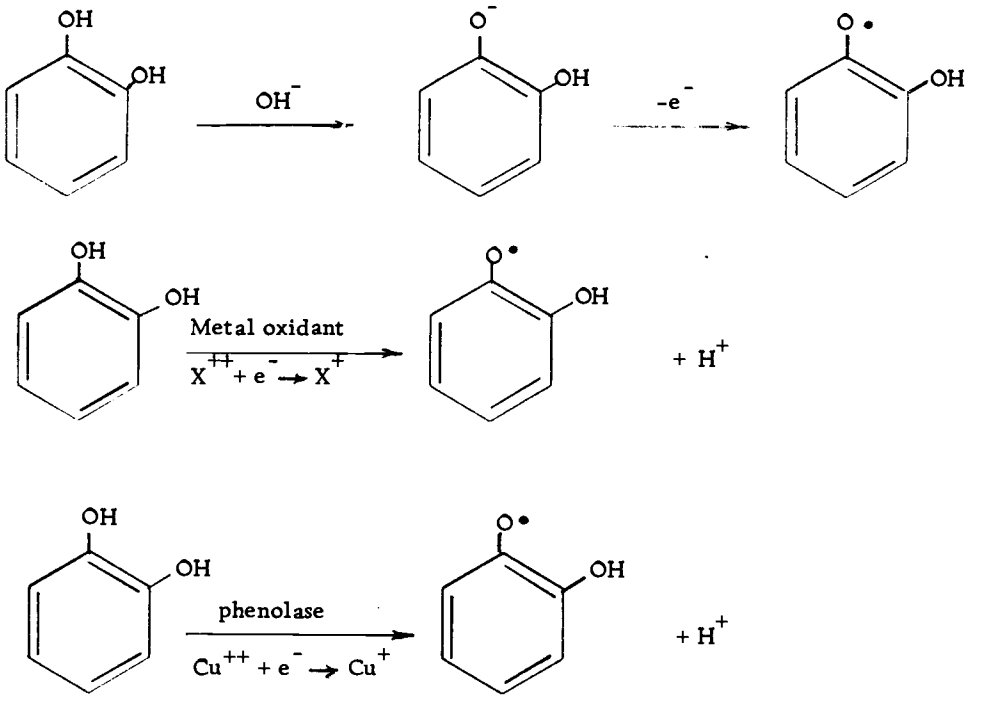


Figure 3. Modes of formation of phenoxy free radicals.

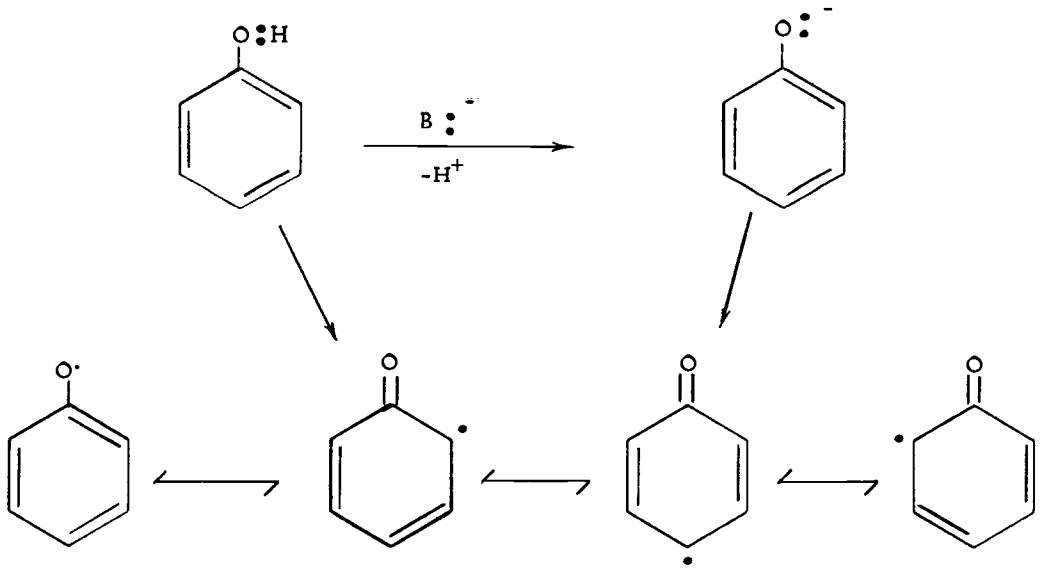


Figure 4. Resonance stabilization of the phenoxy free radical. (From Mihailovic and Cekovic, 1971).

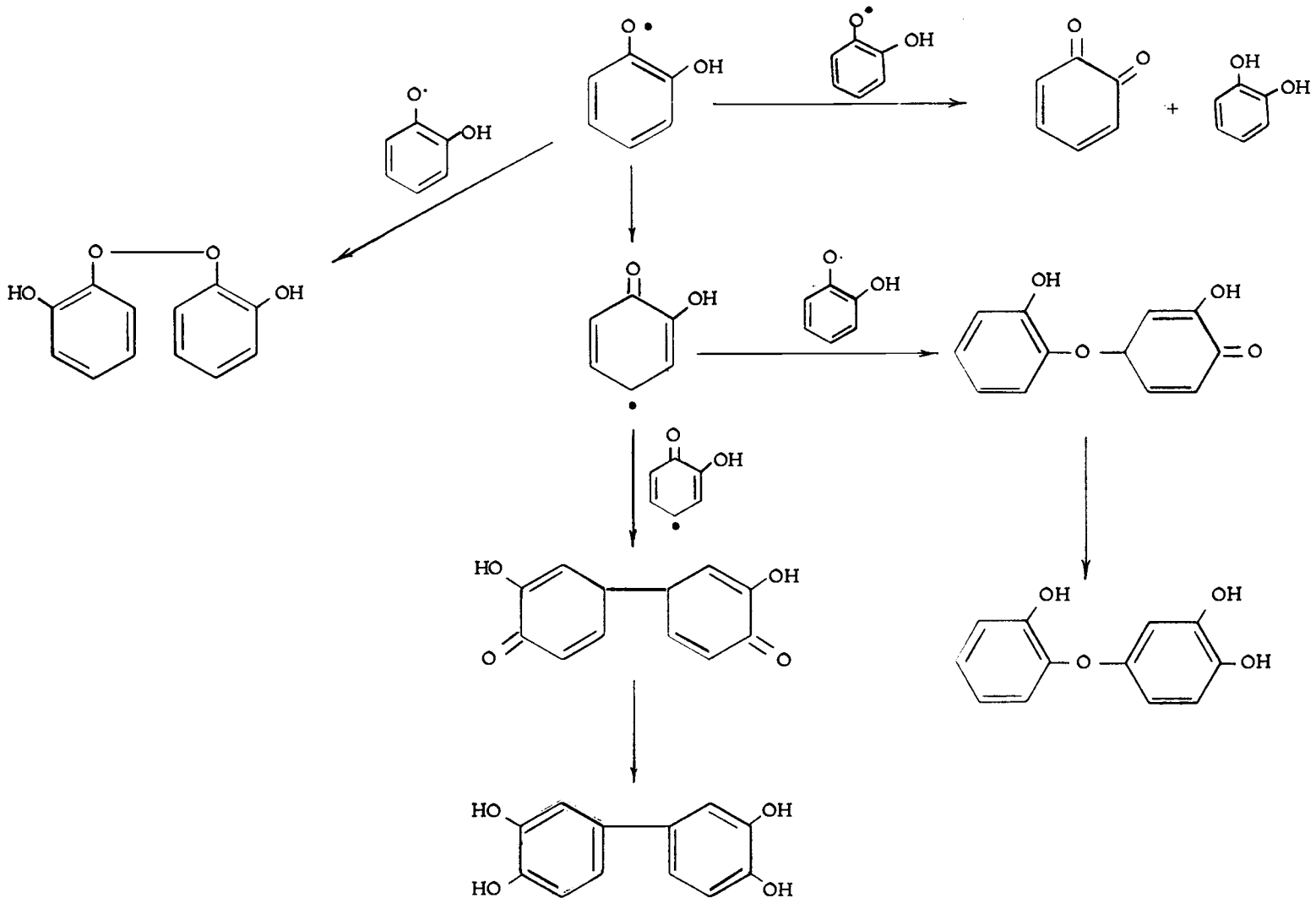


Figure 5. Reactions of the phenoxy radical involving radical condensation and quinone formation.

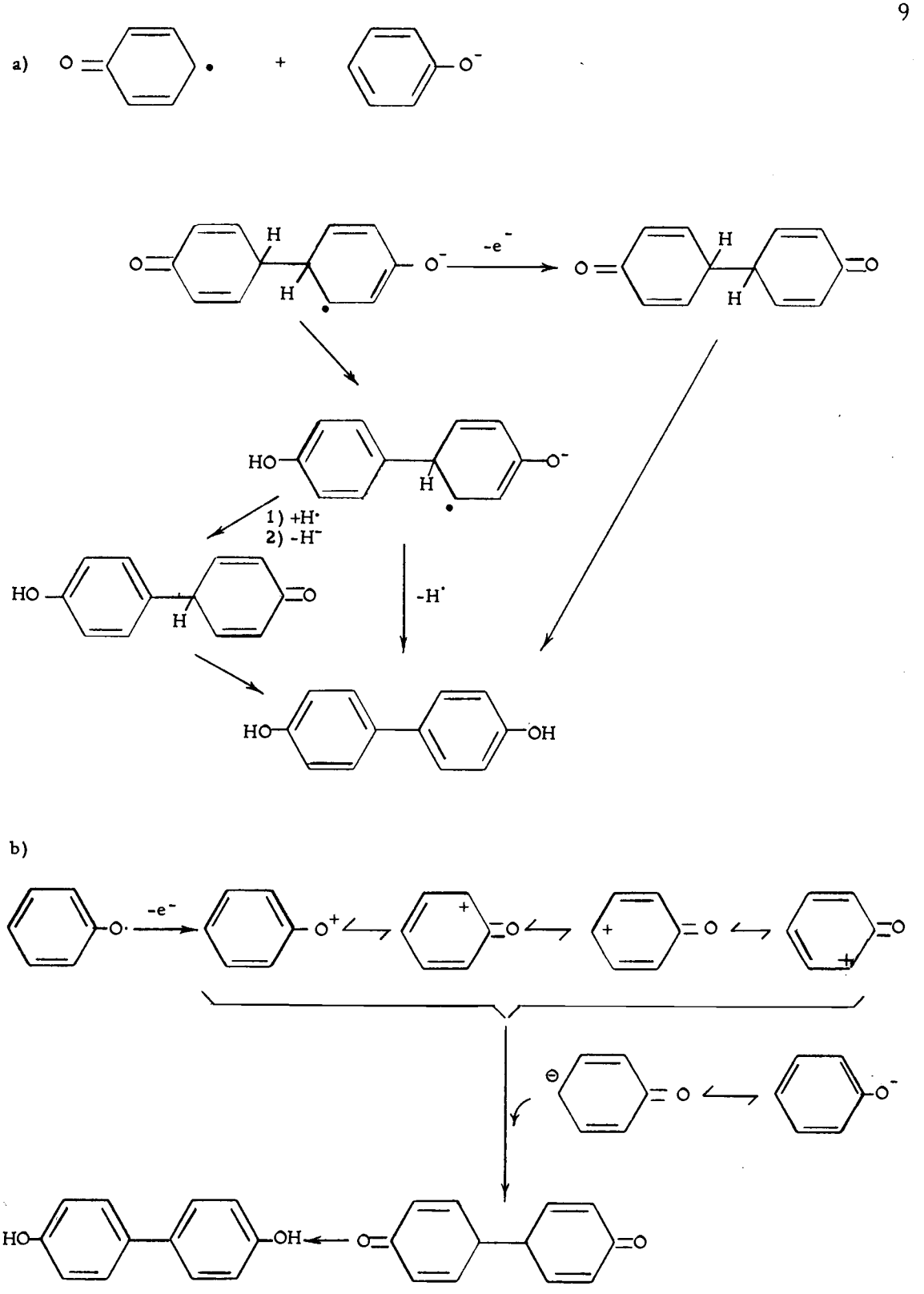


Figure 6. Reaction of the phenoxy radical with intact phenol or phenolate anion (from Musso, 1967).

is slow or an excess of unreacted phenol is present. Additionally (Figure 6b), the phenoxy free radical may be further oxidized to the cation which then rapidly reacts with phenol or its anion. Phenol-quinone addition may occur as illustrated in Figure 7. The formation of oligomeric and polymeric material in oxidizing phenolic systems may be formulated in an analogous manner. The mode of interaction and subsequent modification depends, in part, upon the relative concentration and standard oxidation-reduction potential (E'_o) of the reactants as well as steric factors resulting from ring substitutions.

Enzymatic phenolic oxidation is accomplished primarily by three classes of enzymes, the laccases, tyrosinases, and peroxidases. The peroxidases catalyze the oxidation of various substrates, including phenols, with the reduction of hydrogen peroxide (Brown, 1967). Of greater concern to this study are the laccases and tyrosinases as reviewed by Mason (1955a), Kertesz and Zito (1962), and Brown (1967).

The laccases are copper-containing enzymes of low specificity which catalyze the oxidation of a variety of phenols to quinones and coupled compounds through an aryloxy (phenoxy) free radical (Brown, 1967). The tyrosinases are copper-containing enzymes which catalyze both the ortho-hydroxylation of monophenols and the dehydrogenation of o-diphenols as indicated below.

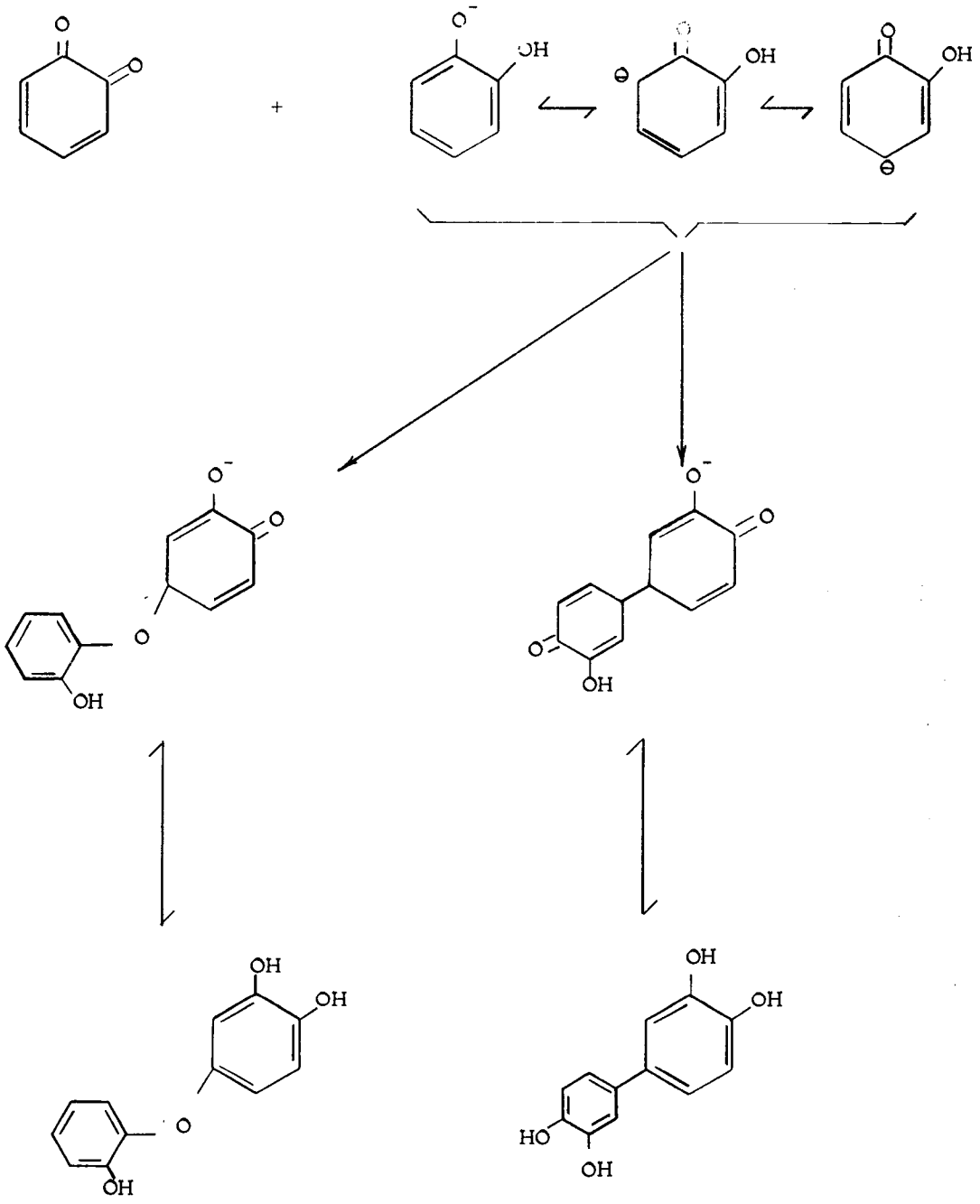


Figure 7. Phenol-quinone coupling.

a) monophenol + O_2 + $2e^-$ → o-diphenol

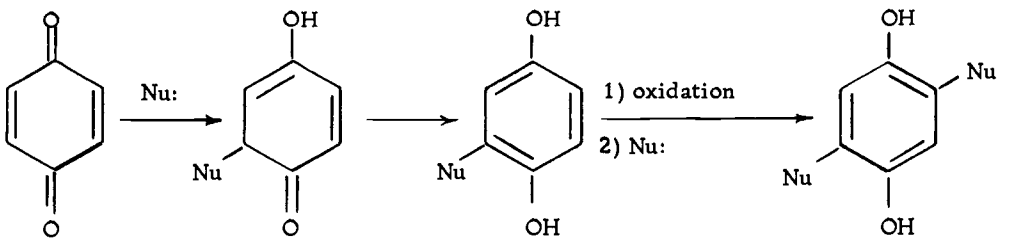
b) o-diphenol + $1/2\,O_2$ → o-quinone + H_2O

Enzymatic reactions of the phenolase complex (Mason, 1955b). Reaction (a) above of the phenolase complex has been termed the cresolase activity, reaction (b) above the catecholase activity (Mason, 1955b). The formation of o-benzoquinone as a product of tyrosinase oxidation of catechol in the presence of oxygen is substantiated by the isolation of o-benzoquinone derivatives from such systems (Pugh and Raper, 1927; Pierpoint, 1966; Janes, 1969), by the appearance of characteristic o-benzoquinone ultraviolet absorption (Mason, 1949) and ESR (Mason et al., 1961) spectra, and by oxygen uptake values that are consistent with the hypothesized mode of action. Phenolases are subject to reaction-inactivation, that is, inhibition by a product of the enzyme-catalyzed phenol oxidation (Richter, 1934; Ludwig and Nelson, 1939; Asimov, 1950; Ingraham, 1954; Ingraham, 1955). Addition of the apparent product, o-benzoquinone, to the enzyme at or near its active site could be responsible for the inactivation. The action of both laccases and phenolases on phenolic compounds may lead, in addition to quinones and quinone-phenolic addition products, to radical condensation products involving C-C, C-O, and O-O bonds (Brown, 1967). Such products are probably due to the formation of an intermediate phenoxy free radical with resultant coupling as indicated in Figure 5. Additional oxidation to

the corresponding quinone with subsequent coupling leading to polymeric material may occur.

Reaction of Quinones with Amino Acids, Peptides, Proteins, and Related Compounds

Characteristic reactions of quinones include the addition of nucleophilic groups to an unsaturated carbon of the quinone. Carbon-carbon double bonds conjugated with electron sinks may serve as substrates for nucleophilic addition reactions. Such is the case with o-, and p-benzoquinones, the nucleophiles of primary interest here being those present in amino acids, peptides, and proteins. The probable nature of the reaction is that of a conjugate addition or 1,4-addition as noted below for p-benzoquinone, yielding a di- or monosubstituted phenolic or quinone derivative, depending upon the relative E_o' of the starting materials and products, concentration of reactants, nature of the nucleophile, and environment of the system.

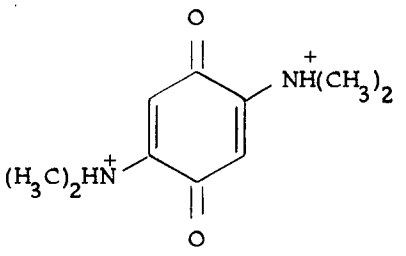


1,4-addition of nucleophile to p-benzoquinone.
(Nu represents any reacting nucleophile)

o-Benzoquinone yields the 4 or 5 monosubstituted or 4-5 disubstituted quinone. Proton shifts leading to the more stable aromatic compound follow nucleophilic addition (in protic solvents) and the phenolic product formed may undergo further oxidation. Mason (1955a) has extensively reviewed reactions of this type as has Pierpoint (1970).

Reactions with Amines

The ability of quinones to react with amines has been known for over a century, and the generality of the reactions has been established for aliphatic and aromatic primary and secondary amines. Hofmann (1863) observed that aniline and p-benzoquinone reacted to yield a dianilino-quinone compound. Mylius (1885) prepared a dialkylamino quinone from dimethylamine and p-benzoquinone and Kehrman (1890) found the structure to be as shown.



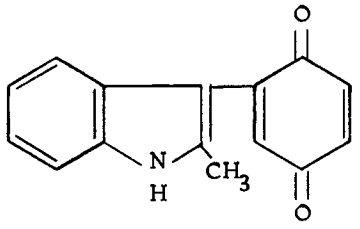
2, 5-bis(dimethylamino)-p-benzoquinone

Harger (1924) indicated the generality of reaction of primary amines with quinones, showing that the bis(dialkylamino)-p-benzoquinone

resulted from oxidation of hydroquinone in the presence of methyl-, ethyl-, isobutyl-, amyl-, allyl-, and benzyl-amine. Reaction products were, in part, characterized by melting point, elemental analysis, and molecular weight estimation. Beevers and James (1948) studied oxygen uptake and spectral changes occurring in enzymatically oxidized pyrocatechol systems containing secondary and tertiary amines. While secondary amines (dimethylamine, proline, nortropine, sarcosine) resulted in the formation of a deep purple color indicative of amine-quinone interaction, tertiary amines (tropine, hyoscyamine) yielded no uniquely colored products and no increase in oxygen consumption over that of the control, indicating the need for at least one unsubstituted position on the amino nitrogen for reaction. More recently Hikosaka (1970) has confirmed the ability of primary and secondary amines to react with quinones. 2, 5-bis(akylamino)-p-benzoquinones were formed from methyl-, ethyl-, n-propyl-, n-butyl-, isopropyl-, s-butyl-, and tert-butyl- amines and p-benzoquinone with the rate of reaction an inverse function of the size of the alkyl group. Secondary amines (dimethyl-, diethyl-, di-n-propyl-, di-n-butyl-amine) yielded mono p-benzoquinone derivatives. Horspool, Smith, and Tedder (1971) substantiated the ability of aniline to react with quinones, the 4, 5-dianilino-1, 2-benzoquinone being obtained from o-benzoquinone in excess of 90 percent yield in hydroxylic solvents and identified by elemental analysis, nuclear

magnetic resonance spectroscopy, and mass spectroscopy.

Heterocylic amines such as indole react readily with both o-, and p-benzoquinones, the mode of reaction in some cases, however, being distinctly different from that of simpler amines. The nitrogen atom in heterocyclic amines may be relatively unreactive, other parts of the molecule showing much greater reactivity. Möhlau and Redlich (1911) obtained the 2-2'-methyl-3'-indoyl-1, 4-benzoquinone from 2'-methylindole and p-benzoquinone as shown.



2-2'-methyl-3'-indoyl-1, 4-benzoquinone

More recent work by Bu'Lock and Harley-Mason (1951) support this mode of reaction, obtaining 4-3'-indolyl-1, 2-benzoquinone and 4-3'-indolyl-5-methyl-1, 2-benzoquinone from indole and o-benzoquinone or 5-methyl-o-benzoquinone. o-Benzoquinone was found to be more reactive than p-benzoquinone toward the indole moiety. Indoles substituted at the 3' position appear to show only a limited reactivity toward quinones and the products are apparently of a different type. Bu'Lock and Harley-Mason (1951) obtained a colorless product upon reacting 3-methyl indole (scatole) with p-benzoquinone which appeared

to involve two molecules of scatole to one of p-benzoquinone. Leopold and Plummer (1961) obtained colored products with indol-3'-ylacetic acid and quinones generated enzymatically from catechol, chlorogenic acid, and caffeic acid. Pierpoint (1966) obtained a compound of the same nature with the quinone of chlorogenic acid, the purple-red pigment having a broad absorption peak between 520 and 530 nm. The exact nature of the 3'-substituted indole derivatives was not determined although Leopold and Plummer (1961) suggested that an addition compound could be formed via the nitrogen of the indole ring or a carbon atom of the furan ring.

Reactions with Amino Acids

In general, amino acids react with quinones in a manner analogous to that of simple amines, reaction usually occurring at the α-amino group (although the ε-amino of lysine and the sulfhydryl of cysteine may also readily react) yielding pigmented products.

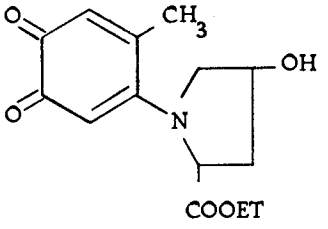
Szent-Györgyi (1925) observed the formation of a reddish-purple pigment during catechol oxidation by crude potato or mushroom extracts and suggested the name "tyrin" for a precursor compound which, with enzyme and catechol, led to the red pigment. He suggested that the compound was an integral constituent of plant systems, serving as a reversibly oxidizable hydrogen carrier. Platt and Wormall (1927), however, concluded that "tyrin" was simply a

mixture of amino acids and that reaction with the enzymatically generated quinone was responsible for the observed pigmented material.

James et al. (1948) observed that several amino acids (alanine, phenylalanine, methionine, valine, leucine, isoleucine, aspartic acid, glutamic acid, histidine, tryptophan) yielded colored complexes and increased oxygen consumption similar to that observed with simpler primary and secondary amines.

Jackson and Kendal (1949) further characterized amino acid-quinone reactions. They observed that the presence of proline in an enzymatically oxidized catechol system yielded a product of the same color as that originally observed by Szent-Györgyi (1925). Furthermore, oxygen uptake in the presence of proline, hydroxyproline, glycine, alanine, glutamic acid, or arginine was found to correspond to the ratio of two oxygen atoms per molecule of catechol, indicating that o-benzoquinone apparently undergoes only one addition reaction under the conditions employed. An oxygen uptake ratio of two atoms oxygen per molecule amino acid was also found to occur with homocatechol (4-methylcatechol) while 4, 5-dimethylcatechol gave no reaction, supporting a 1, 4 addition mechanism as the mode of interaction. Use of 4-methylcatechol allowed isolation of the hydroxyproline ethyl ester- o-benzoquinone adduct, and identification

of the compound showed it to be 4-(4'-hydroxy-2'-carbethoxy-1'-pyrrolidyl)-5-methyl-1, 2-benzoquinone, an imino- o-benzoquinone as indicated below.



4-(4'-hydroxy-2'-carbethoxy-1'-pyrrolidyl)-
5-methyl-1, 2-benzoquinone

Michalik and Szarkowska (1959) and Haider, Frederick, and Flaig (1965) cite evidence for amino acid-quinone reaction, the latter group noting that o-methylated phenolic compounds (e.g., guaiacol, vanillic acid, ferulic acid, syringic acid) show no increase in oxygen uptake in the presence of amino acids.

Mason and Peterson (1965) in studying melanoprotein formation, surveyed the spectral changes occurring in enzymatically oxidized phenolic (catechol, 4-methylcatechol, dihydroxyphenylalanine) systems containing primary and secondary amines, including several amino acids. From a spectroscopic criterion, N-terminal primary amino groups and secondary amino acids (i.e. proline) form condensation products with o-benzoquinone and 4-methyl-o-benzoquinone, yielding compounds with absorption bands at 280-290 nm, and 480-490 nm (N-terminal primary amino groups) or

310-315 nm (secondary amino acids). The absence of these absorption bands with N-acetyl amino acids as well as the lack of more complex spectra with amino acids containing the 3'-substituted indole ring, guanidino, seryl hydroxyl, or 4-substituted imidazole groups indicated that, under the conditions employed, only the α-amino or imino groups were reactive. Work by Pierpoint (1969a) supported and extended these observations. Increased oxygen uptake and formation of distinctive pigmented products indicated that reaction had occurred between the several amino acids studied and quinones generated enzymatically from chlorogenic acid and caffeic acid. With the exception of lysine and cysteine, reaction was shown to occur primarily through the α-amino group as evidenced by the lack of reactivity of N-acetyl amino acid derivatives; lysine and cysteine were shown to react additionally through the ε-amino and thiol groups respectively. Increasing reactivity with increasing nucleophilicity of the attacking species was indicated by a greater "extra oxygen uptake" (oxygen consumption above that of the control) at pH 7.8 as compared with pH 7.0.

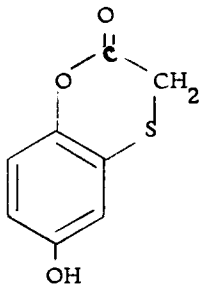
Morrison, Steele, and Danner (1969) employing a p-benzoquinone-glycine system, reported the formation of mono- and di-substituted glycine- p-benzoquinone adducts depending upon the ratio of reactants employed, although no direct evidence was cited for the existence of such compounds.

Mason (1955a) summarized sulfhydryl group reactivity with quinones. Basically, two types of reactions may occur: oxidation of the thiol group to the disulfide with reduction of the quinone to the hydroquinone; and addition of the thiol group to the quinone. Pierpoint (1966) noted the possibility of cysteine conversion to the disulfide in the presence of enzymatically oxidized chlorogenic acid. Clayton (1959) noted in studying tobacco polyphenoloxidase, that cysteine served to keep the phenolic substrate in the reduced form, indicating a possible conversion of the sulfhydryl to the disulfide with the corresponding reduction of the quinone to the phenolic compound.

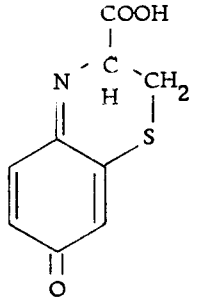
Thiol addition to quinones is extremely facile. Schubert (1947) found that, in excess thiol, a tetra-substituted p-benzoquinone derivative occurs. The reactivity of sulfhydryl groups with quinones is such that they tend to substitute into every available position, in contrast to the selective addition of amines. Furthermore, quinones react preferentially with sulfhydryl groups in the presence of other nucleophiles (Mason, 1955a). Pierpoint(1969a) showed that N-acetyl cysteine reacts in the same manner as does cysteine itself, indicating the preferential reactivity of the thiol group.

In some cases, quinone-thiol compounds may undergo further modification after addition (Mason, 1955a). With p-benzoquinone and excess thioglycolic acid, the monosubstituted derivative may be

isolated as the lactone (A below) while S-1, 4-benzoquinone-2-ylcysteine may undergo an inner condensation to yield a cyclic dihydrothiozine as shown (B below).



(A)



(B)

Non-enzymatic Deamination

Chodat and Schweizer (1913) observed that ammonia and formaldehyde were formed from glycine in the presence of a crude tyrosinase preparation from potatoes and concluded that tyrosinase was an amino acid oxidase. Happold and Raper (1925) however, noted that the presence of p-cresol was necessary to obtain deamination of glycine with the potato phenolase complex and, moreover, that o-benzoquinones obtained from phenol and pyrocatechol with the same phenolase preparation could deaminate glycine. They concluded, therefore, that o-benzoquinone, rather than the enzyme, was the causative agent of glycine deamination and therefore, that the deamination was non-enzymatic in nature. Since that time the deaminating ability of o-benzoquinones has

been well substantiated, not only for glycine, but for other amino acids as well (James et al., 1948; Jackson and Kendal, 1949; Gordon and Paleg, 1961; Haider, Frederick, and Flaig, 1965). James et al. (1948) found that both glycine and alanine were deaminated in the presence of pyrocatechol and the pyrocatechol oxidase of belladonna, yielding glyoxylic acid and pyruvic acid, respectively, and ammonia. Gordon and Paleg (1961) found that deamination of tryptophan occurred in the presence of several o-diphenols and phenoloxidases from several plant sources, yielding indolacetic acid probably via indolpyruvic acid. A probable scheme for deamination (Figure 8) involves Schiffs base formation with subsequent rearrangement, loss of an aldehyde moiety, hydrolysis with concomitant formation of ammonia followed by re-oxidation to the initial quinone condensation product (Mason, 1955a).

Reaction with Peptides and Proteins

Since the functional groups of peptides and proteins are those of the side chains of the constituent amino acids (e.g. amino, imino, heterocyclic amine, thiol, hydroxyl) and the terminal amino acid residues, it would be expected that proteins could react with quinones, and such reactions do occur, although their complexity is greater than that observed for the free amino acid or related compound.

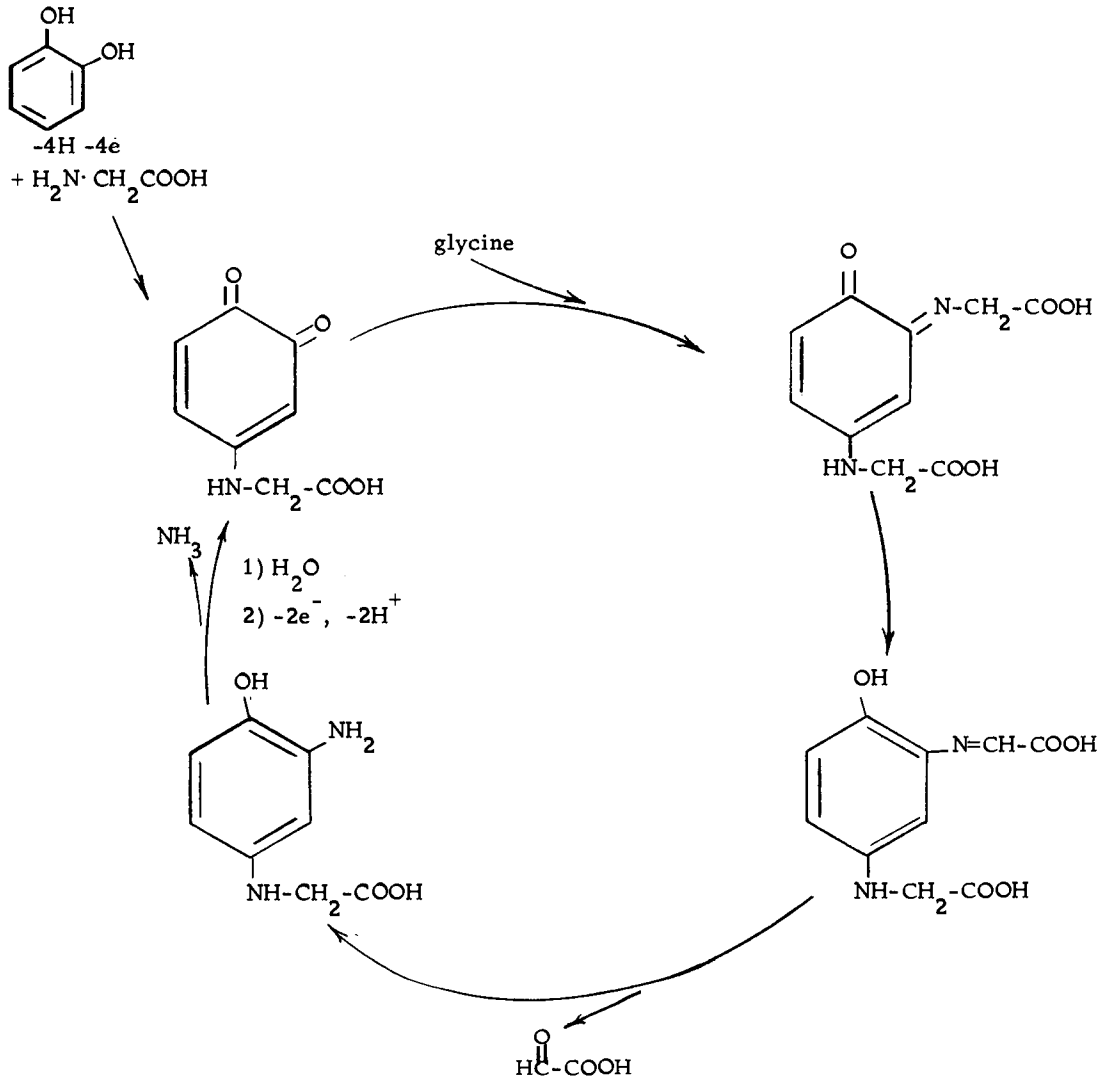


Figure 8. Non-enzymatic deamination of amino acids by mono-substituted o-benzoquinone (from Mason, 1955a).

Early investigations (Wurster, 1889; Raciborski, 1889; Cooper, 1913; Morgan, 1921) determined that colored products were formed in protein-quinone solutions; the nature of the reaction was inferred, in part, through analogy with colored products formed by reactions of quinones with amines and related compounds. Cooper (1913), and Morgan and Cooper (1921) obtained red reaction products when gelatin, egg albumin, horse serum, and casein were incubated with an aqueous benzoquinone solution; prior treatment of the protein with formaldehyde inhibited the color reaction, implying the involvement of amino and imino groups.

Studies on quinone tannage of protein (reviewed by Gustavson, 1956, and Mason, 1955a) have provided much information on protein-quinone interaction. Binding of p-benzoquinone to proteins appears to involve both monomeric and polymeric quinone moieties with fixation generally thought to involve primarily amino, imino, and thiol groups via addition reactions of the type described for free amino acids, although additional quinone seems to be bound by non-covalent means (see below).

Mason (1955b) found that enzyme-generated o-quinones reacted with peptides and proteins to yield N-, and S-catechol derivatives which could not be further oxidized by diphenolases but which could be further oxidized by excess free o-quinone. The coupling of quinones to peptide and protein functional groups appears to occur

more rapidly and, in some cases, more extensively than with the free amino acid. Mason (1955b), utilizing the rate of uptake of the second oxygen molecule as a means of evaluating the effects of substituents on the nucleophile, found an increasing rate of reaction in the order butyl amine, glycine, glycylglycine, glycylglycylglycine, and diethylamine, proline, N-terminal proline(protamine, salmine). The increasing rate of reactivity with increasing peptide chain length has been termed the peptide effect (Mason, 1955a). Mason and Peterson (1955) found that, while proline itself reacts with o-benzoquinone to form only the mono-substituted imino- o-benzoquinone derivative, salmine (in two-fold excess) may yield the di-substituted derivative, indicating a greater reactivity of proline when present as an N-terminal amino acid. Similar results were found by Pierpoint (1969a); however, Haider, Frederick, and Flaig (1965) found that the activity of the amino group of several di- and tri-peptides was not significantly altered. Pierpoint (1969a) found that, although the primary reactions of peptides are likely to involve only the thiol, terminal α-amino, and lysine ε-amino groups, secondary reactions may also be possible; for example, the initial reaction of L-carnosine (β-alanyl-L-histidine) resembled that of β-alanine, while final spectral evidence suggested the participation of the N-bound histidine residues as well.

Wood and Ingraham (1965) found that oxidation of 1-^{14}C phenol

by tyrosinase resulted in the formation of an inactive radioactive enzyme and suggested that an irreversible quinone addition reaction was responsible. Decreased inhibition with 4-methylcatechol and lack of inhibition with 4, 5-dimethylcatechol support this conclusion since with the 4 and 5 positions of the quinone blocked, condensation would not be possible. Byck and Dawson (1968), investigating the allergenic activity of 3-pentadecylcatechol, the active principle of poison ivy, studied the interaction of the corresponding quinone or related compounds with γ-globulin. Binding of the 3-pentadecyl-o-benzoquinone was shown, however 4, 5-dimethyl-, and 4, 5, 6-trimethyl-3-pentadecyl-o-benzoquinone did not appear to react appreciably, supporting a 1, 4-addition mechanism. 4-, 5-, or 6-methyl-3-pentadecyl-o-benzoquinones were intermediate in reactivity, suggesting that ring substituents may sterically hinder the condensation reaction.

Haider, Frederick, and Flaig (1965) found that the N-terminal amino acid of glycylvalylleucine was absent from an acid hydrolysate of an enzymatically oxidized caffeic acid-tripeptide reaction mixture, and, in an additional study of a similar system, found that the α-amino group of the N-terminal aspartate and the ε-amino group of lysine were modified (the extent of modification a function of the phenol level of the system). Pierpoint (1969b) found that the nature of reaction of bovine serum albumin (BSA) with the o-quinones

generated enzymatically from chlorogenic or caffeic acids depended upon the ratio of reactants; with excess protein, oxygen uptake was restricted to one atom of oxygen per molecule of phenolic material whereas with excess phenol, O_2 uptake increased above this value. Pierpoint argued that thiol addition to the quinone in the presence of excess BSA limited oxygen uptake to only that required for quinone formation. With excess phenolic material, the increased oxygen uptake observed is in part, due to an extra oxygen uptake resulting from further oxidation of phenol-BSA adducts. Additionally, Pierpoint found that enzymatic oxidation of chlorogenic and caffeic acids in the presence of BSA yielded a red-pink product that was more strongly absorbed on an anion exchange resin and which exhibited a greater anionic electrophoretic mobility than unreacted BSA; no change in sedimentation coefficient or amino analysis could be observed.

Peptide nitrogen does not apparently react with quinone to form a covalently linked product (Mason and Peterson, 1955). Quinone inhibtion of enzymatic activity is well documented. Quastel (1932) reported the inhibition of urease by several polyhydric phenols, the evidence indicating that the actual inhibitory species was the quinone formed from the corresponding phenol. Quinone inhibition was shown to be exceedingly effective as evidenced by a 57 percent inhibition by p-benzoquinone at a level of one part per 5,000,000. Hellerman and Perkins (1934) and Bahadur and Atreya (1960) have shown that papain is inhibited by p-benzoquinone, the inhibition due to thiol addition to the quinone, or sulfhydryl oxidation. Ophir and Miller

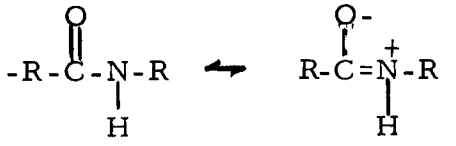
(1969) reported the inhibition of bromelin by p-benzoquinone, again due, in part, to thiol oxidation. Dirr, Fiedler., and Schahbandari (1959) found that aldolase was inhibited by p-benzoquinone, while Lin (1962) reported that catalase was inhibited by several quinones including p-benzoquinone. Grossman (1967) has shown that a C_x-type cellulase isolated from Fusarium oxysporum is inhibited by 10^{-2} M p-benzoquinone. A two to three fold reduction in nitrate reductase activity in the presence of 2×10^{-3} M p-benzoquinone has been reported (Glyanko, Korovin, and Stom, 1970). Blank and Sondheimer (1969) reported the inhibition of potato phosphorylase by a variety of phenolic materials, inhibition being attributed primarily to the quinone formed from the corresponding phenolic material by enzymatic oxidation.

Non-covalent Association of Phenolic Material with Protein

As noted previously plant phenolic materials are extremely heterogeneous, consisting primarily of the C_6-C_1 and C_6-C_3 compounds and their derivatives, with hydroxyl and carboxyl groups as reactive centers, and the flavonoid compounds with hydroxyl groups as the principal reactive centers. Both of these groups of compounds as well as material derived from oxidative polymerization of the two groups may form non-covalent associations with protein, primarily by hydrogen bonding and salt or electrostatic linkages. The

elucidation of the nature of these associations is principally due to investigations associated with the tanning and brewing industries, reviewed by Gustavson (1956, 1966), Shuttleworth (1948, 1968) and Loomis and Battaile (1966).

The primary mode of non-covalent association of unoxidized and oxidized phenolic compounds with protein has been proposed to be hydrogen bonding (Gustavson, 1966). Hydrogen bonding results from proton sharing between two electronegative atoms and requires a proton donor and proton acceptor. In tannin-protein hydrogen bonding the principal proton acceptor is the oxygen of the peptide bond which holds a partial negative charge as the result of resonance of the keto-imide bond.



peptide bond resonance

Such a situation allows for stable hydrogen bonding. The principal donor is the phenolic hydroxyl hydrogen of the tannin complex.

The above situation probably represents the most stable as well as the predominant hydrogen bonding species in tannin-protein complexes; however, other proton-donor, proton-acceptor pairs are possible, e.g., amide hydrogen-tannin carboxyl oxygen. Hydrogen bonding leads to a multi-point attachment of the tannin material in

protein and the formation of stable complexes because of the large numbers of hydrogen bonds involved.

Hydrolyzable and condensed tannins show markedly different pH responses with respect to hydrogen bonding. Hydrolyzable tannins are bound most strongly at pH values below pH 5.0, binding falling off rapidly above this pH, while condensed tannins are bound almost independently of pH below pH 7-8 with binding decreasing rapidly above pH 9.0. The ionization of the carboxyl moiety and the resultant decrease in its ability to hydrogen-bond probably accounts for the pH response of hydrolyzable tannins while the ionization of the phenolic hydroxyl accounts for the behavior of condensed tannins. It should be noted that phenolic compounds with hydroxyl groups ortho to each other may internally hydrogen bond and therefore show a lower affinity for hydrogen bonding with acceptor compounds.

Evidence for the hydrogen bonding of polyphenolic material to protein dates back to the work of Powarin (1914) who showed that polyphenolics could bind with model compounds containing $-\overset{O}{\overset{\|}{C}}-$ and $-\overset{O}{\overset{\|}{C}}-NH-$ linkages by non-covalent means. Freudenberg (1921) re-emphasized such interactions while Pfeiffer (1927) showed that complexes occurred between phenols and a sarcosine derivative containing the grouping $-\overset{O}{\overset{\|}{C}}-\overset{CH_3}{\overset{|}{N}}-$. Such complexes, in conjunction with the then developing concept of the hydrogen bond (Huggins, 1920), indicated the involvement of hydrogen bonding and, furthermore,

emphasized the proton acceptor function of the peptide oxygen. Grassman (1937) found that water soluble urea-formaldehyde condensation products containing the -CONH-moiety as the only functional group could quantitatively precipitate tannins. Such evidence implied the direct involvement of the peptide bond in tannin binding. Subsequent investigations by Batzer (1952) and Batzer and Weissenberger (1952) with hydrated nylon, and by Gustavson (1954) with modified collagen and polyvinylpyrrolidone, all of which have the -CON< group as the sole reactive species, made it clear that the peptide bond was of prime importance in binding tannin material and that the linkage was that of hydrogen bonding. Recently, Santhanam and Nayudamma (1968) and Vijalakshmi and Santhanam (1970) have confirmed the involvement of the peptide bond and hydrogen bonding in the binding of phenolic material to protein. Heidemann and Srinivasan (1967) employing deuterium exchange between collagen and synthetic polypeptides treated with phenolic compounds have found that macro-molecular tannins are capable of breaking, then renewing, hydrogen bonds. Woof and Pierce (1968), utilizing difference spectroscopy, have indicated that flavanoids bind to α-amylase through hydrogen bonds.

Additional support for this mode of binding comes from the use of hydrogen bond breaking agents to strip tanned protein of bound phenolics (Shuttleworth and Cunningham, 1948; Gustavson, 1956;

Gustavson, 1966). Six to eight molar urea at 20° C for seven days removes 50 to 95 percent of the "irreversibly" bound tannin on hide protein. Stripping is due to competition between urea and tannin for the protein hydrogen bonding sites. Similar results are obtained with tanned polyamide and polyvinylpyrrolidone. Solvent stripping of tannin from protein is well known (Gustavson, 1956). Aqueous alcohols, ketones, ethers, and nitriles, as well as reagents of greater polarity approaching that of the peptide bond such as aqueous amides may effectively remove bound phenolics from protein or polyamides. Acetone-water (1:1) will, for example, virtually strip collagen of bound tannic acid; condensed tannins are also removed by this solvent.

Ionic bonding may occur between tannin material and protein. Such binding usually involves the ionized carboxyl of hydrolyzable tannins and the basic groups of protein, particularly arginine and lysine. Reviews of such interactions with respect to tannin-protein complexes are presented by Gustavson (1956) and Shuttleworth (1966).

Asymmetric electron positioning in aromatic systems may result from the presence of electron-donating or withdrawing substituents on the ring. In tannin material, hydroxyl and carboxyl groups act in such a capacity, creating, in highly polymerized material, a large number of weak dipoles. The additive nature of the weak dipole interactions with protein may yield an important

mode of non-covalent binding of tannins. Although these weak dipolar forces extend over only a few ångstroms, the close association of tannin to protein by other means of bonding increases their effectiveness of binding.

Examples of enzyme inactivation by non-covalently associated tannin material are numerous. Often reactivation is achieved with the use of competitive hydrogen bonding agents such as hide powder or polyvinylpyrrolidone (PVP), demonstrating the non-covalent nature of tannin binding. Ehrenberg (1954) reported the inhibition of phosphatase by tannins and noted that lightly chromed hide powder could reactivate the enzyme. Similar reactivation was shown by Friedrich (1956) in the case of β-glucosidase inactivation by tannic acid. Boser (1961), Guerritore et al. (1965), and Firenzuoli et al. (1969) have shown the inhibition of malic dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6PDH), and isocitrate dehydrogenase (IDH) by both hydrolyzable and condensed tannins. Reactivation was possible by the addition of PVP. According to Firenzuoli (1969) MDH, IDH, and G6PDH were 80 to 100 percent inactivated by tannic acid, chlorogenic acid, or potassium pyrogallate while addition of 4.5 mg/ml PVP resulted in the regaining of up to 84 percent of the original activity. Goldstein and Swain (1965) showed that alcohol dehydrogenase, lactic acid dehydrogenase, peroxidase, catalase, and β-glucosidase were partially inhibited by both tannic acid and

wattle tannin (a condensed tannin). Denaturation of the enzymes occurred in the presence of tannins. Partial resolubilization and reactivation of the enzymes could be obtained with a variety of reagents, including borate, polyethylene glycol, polyvinylalcohol, polyvinylpyrrolidone, and non-ionic and cationic detergents. PVP usually showed the greatest degree of reactivation. The inhibition of pectic enzymes by tannin material has been widely reported (Hathway and Seakins, 1958; Porter and Schwarz, 1962; Hall, 1966). Porter and Schwarz (1962) showed that a condensed tannin of a minimum molecular weight of 10,000 almost completely inhibited pectinase, while Hall (1966) showed that tomato pectin esterase was reversibly inhibited by 5×10^{-3}M tannic acid at a pectin substrate concentration of 0.05 percent. Gallic, shikimic, chlorogenic, caffeic, vanillic, and ferulic acids also inhibited the enzyme though to a lesser extent. Gadal and Boudet (1965) and Boudet (1965) showed that β-amylase and peroxidase were inhibited by oak leaf tannins. The inactivation could be reversed by the addition of 0.04 percent PVP (β-amylase) or 2.0 percent PVP (peroxidase) to the previously formed enzyme-tannin complex. Prevention of inhibition could be achieved by incorporating PVP into the enzyme isolation medium. Schneider and Hallier (1970) demonstrated the reactivation of the spinach chloroplast NADP-dependent glyceraldehyde phosphate dehydrogenase-phosphoglycerate kinase system and the pseudocyclic

photophosphorylation system by PVP after prior incubation with tannin material.

Statement of Purpose

Evidence has been cited for the non-covalent association of phenolic material with protein. Additionally, the formation of amino acid-quinone adducts has indicated that covalent association of oxidized phenolic compounds with proteins may modify constituent amino acid moieties. It was of interest therefore from both biochemical and nutritional viewpoints to further characterize the interactions which may occur between protein and oxidized phenolic material. The purpose of this research was to delineate the extent, nature, and mode of amino acid modifications that may occur in protein upon exposure to oxidized phenolic material, and to estimate the relative importance of non-covalent association and covalent binding of oxidized phenolic materials to protein. The oxygen uptake studies, the spectrophotometric evaluation of oxidized pyrocatechol solutions, and the studies on the enzymatic oxidation of pyrocatechol by tyrosinase were preliminary in nature. Major emphasis was directed towards the evaluation of amino acid modifications and alterations in protein digestibility resulting from the reactions of protein with phenols and quinones.

MATERIALS AND METHODS

Materials

Bovine serum albumin (crystalline) was obtained from Nutritional Biochemicals Corporation. L-Amino acid standards, N-acetyl-L-tryptophan ethylester, N-benzoyl-L-arginine ethylester hydrochloride, pepsin (hog stomach, 3x crystallized, 3000 units per mg), trypsin (2x crystallized), and mushroom tyrosinase (1100 units per mg) were obtained from Schwarz-Mann. N-Trifluoroacetyl-L-tryptophan methyl ester, N-trifluoroacetyl-DL-methionine methyl ester, ninhydrin (crystalline), poly-L-lysine, and Trizma base (tris-(hydroxymethyl)amino methane) were obtained from the Sigma Chemical Company. N-Acetyl-L-histidine and glycine were obtained from Calbiochem. N-Nitrosomethylurea was obtained from K and K Laboratories, Inc. Amino acid standard solution was obtained from Pierce Chemical Company. N-Methyl-2-pyrrolidone was kindly supplied by the GAF Corporation. Bio-Gel P-30, Bio-Gel P-2, and DEAE cellulose (Cellex-D) were purchased from Bio-Rad Laboratories. Sephadex G-50 and blue dextran 2000 were obtained from Pharmacia Fine Chemicals, Inc. Carbowax 20M and Chromosorb G (100/120 mesh) were purchased from Varian Aerograph. Quinone (p-benzoquinone) was obtained from Fisher Scientific Company.

Silica gel G was obtained from M. Merck A. G. through Brinkmann Instruments, Inc. Chromagram sheets, cellulose type 6064 without fluorescent indicator, were obtained from the Eastman Kodak Company. Pyrocatechol, hydroquinone, bromocresol green, methyl red, and rhodamine B (practical) were obtained from Eastman Organic Chemicals. Standard 0.1N hydrochloric acid and standard 0.1N sodium hydroxide acculutes were supplied by Hellige, Inc. Potassium phosphate, monobasic, and potassium phosphate, dibasic, were obtained from the J. T. Baker Chemical Company. Acetic acid, ammonium hydroxide, boric acid, citric acid, potassium carbonate, potassium sulfate, sodium hydroxide, and sodium thiosulfate were obtained from Baker and Adamson (Allied Chemicals). Anhydrous diethyl ether, formic acid, mercuric oxide, trichloroacetic acid, and sodium nitrite were purchased from Mallinckrodt Chemical Works. Acetone, benzene, n-butanol, p-dimethylaminobenzaldehyde, isopropanol, methanol, p-nitrophenol, potassium citrate, n-propanol, and 2, 4, 6-trimethylpyridine (s-collidine) were obtained from Matheson, Coleman, and Bell. Hydrochloric acid and sulfuric acid were obtained from Dupont. Ethanol was obtained from Central Solvents and Chemicals. Skellysolve B was purchased from the Skelly Oil Company. Celotate filters (cellulose acetate, 0.5 micrometer pore size) were obtained from the Millipore Corporation.

Other supplies and materials were those commonly found in the laboratory.

Methods

Preliminary Studies

Effect of pH, Time and Bovine Serum Albumin on the Non-enzymatic (Base Catalyzed) Oxidation of Pyrocatechol

A Gilson Medical Electronics Warburg apparatus was used throughout the experimental series. Mannometric calculations were carried out by procedures outlined by Umbreit, Burris, and Stauffer (1957). 0.2 M potassium phosphate of the appropriate pH was employed as noted. It is realized that the buffer capacity of the potassium phosphate system used is low for some of the pH values included in the studies conducted. However, the levels of acid or base produced should not be great in these situations. Pyrocatechol in 0.5 ml glass distilled water was initially placed in the sidearm of a standard Warburg mannometric flask, containing 2.0 ml of the appropriate 0.2 M potassium phosphate solution in the bottom. Samples were temperature equilibrated for 10 minutes prior to mixing.

Effect of pH

Pyrocatechol (55.6 μmoles) was combined with 0.2 M potassium phosphate of the appropriate pH and allowed to air oxidize at 30° C for

two hours.

Effect of Time

Pyrocatechol (148 μmoles) was combined with pH 8.0 buffer and allowed to air oxidize at 30° C for a 12 hour period.

Effect of BSA

Appropriate levels of pyrocatechol were combined with 20 mg BSA in pH 8.0 buffer and allowed to air oxidize for two hours at 30° C. Control samples without BSA were included.

Spectrophotometric Evaluation of Bovine Serum Albumin, Pyrocatechol, Oxidized Pyrocatechol, and Tanned Bovine Serum Albumin

All spectrophotometric evaluations were carried out at room temperature employing a Beckman DB recording spectrophotometer equipped with a 1.0 cm pathlength cuvette. Spectra were read against the appropriate 0.2 M potassium phosphate solution. Dilutions were carried out with the appropriate salt solution. Visible color changes were noted.

Bovine Serum Albumin

The ultraviolet spectrum of BSA was determined using a 1.5 mg/ml solution in 0.1 M potassium phosphate buffer, pH 8.0. The

visible spectrum was determined on a 20 mg/ml solution in the same buffer.

Pyrocatechol

The ultraviolet spectrum of pyrocatechol was determined using a 1:1000 dilution of a solution containing 592 μmoles (65.2 mg) pyrocatechol in 1.0 ml 0.1 M potassium phosphate, pH 5.0. The visible spectrum was determined on the undiluted solution.

Base Catalyzed Air Oxidized Pyrocatechol

Pyrocatechol (296 μmoles) in 0.5 ml distilled water was added to 2.0 ml 0.1 M potassium phosphate buffer, pH 8.0, and allowed to air oxidize at 30° C for 24 hours in a reciprocal shaking water bath (Research Specialties Company). Visible and ultraviolet spectra of the reaction mixture were obtained from 1:25 and 1:1000 dilutions, respectively.

Tanned Bovine Serum Albumin

Pyrocatechol (296 μmoles) in 0.5 ml distilled water was added to 2.0 ml 0.1 M potassium phosphate buffer, pH 8.0, containing 20 mg BSA and allowed to air oxidize at 30° C for 24 hours in a reciprocal shaker. Visible and ultraviolet spectra were obtained from 1:25 and 1:1000 dilutions, respectively. Spectra of the tanned protein

alone were obtained by precipitating replicates of the above solution with four volumes of 10 percent (w/v) trichloroacetic acid (TCA), reagent acetone, or 100 percent ethanol, centrifuging at approximately 27,000 x g for 15 minutes, redissolving the precipitate in 2.5 ml 0.1 M potassium phosphate buffer, pH 8.0, and diluting as above for visible and ultraviolet spectra. An additional TCA-precipitated tanned BSA sample was redissolved in 100 percent methanol, re-precipitated with 10 percent (w/v) TCA, centrifuged at 27,000 x g for 15 minutes, redissolved in pH 8.0 buffer and diluted as above.

Elution Pattern of Tanned Bovine Serum Albumin on Bio-Gel P-30

Column Preparation and Standardization

Bio-Gel P-30 was hydrated overnight in 0.2 M tris-(hydroxymethyl)amino methane (tris) buffer, pH 8.0, at a ratio of one gram dry beads to a minimum of 20 ml buffer. A Pharmacia 1.5 x 30 cm column was packed to a height of 15 cm and the void volume determined using Pharmacia blue dextran 2000.

Preparation and Elution of Tanned BSA

Hydroquinone (91 μmoles) in 0.5 ml glass distilled water was added to 20 mg BSA in 2.0 ml 0.2 M tris buffer, pH 8.0, and air oxidized for eight hours at 30° C in a reciprocal shaker.

The reaction mixture was concentrated to approximately 0.5 ml with Bio-Gel P-2, placed on the Bio-Gel P-30 column, and eluted with 0.2 M tris buffer, pH 8.0. 2.0 ml fractions were collected to 30 ml, then 5.0 ml fractions to 60 ml total eluate. The elution pattern was determined by noting the absorbancy of each fraction at 275 nm. The elution pattern of standard untanned BSA was determined in a similar manner, employing 20 mg BSA concentrated to approximately 0.5 ml with Bio-Gel P-2 prior to column application.

Elution Pattern of Tanned Bovine Serum Albumin Treated with N-methyl-2-pyrrolidone on Sephadex G-50

Pyrocatechol (290 μmoles) in 0.5 ml glass distilled water was added to 20 mg BSA in 2.0 ml 0.2 M potassium phosphate buffer, pH 8.0, and air oxidized for 24 hours at 30°C in a reciprocal shaker water bath. The tanned protein was precipitated with an equal volume of 10 percent (w/v) TCA, centrifuged at 27,000 x g for 15 minutes, and redissolved in 1.0 ml N-methyl-2-pyrrolidone. The tanned mixture was applied to a 1.5 x 13 cm Sephadex G-50 column, the void volume of which had previously been determined with Pharmacia blue dextran 2000. The sample was eluted with 0.2 M phosphate buffer, pH 8.0. Sephadex G-50 was substituted for Bio-Gel P-30 due to the solubility of polyacrylamide in N-methyl-2-pyrrolidone. The elution pattern was noted. The void volume fraction was collected and its

visible and ultraviolet spectra determined. A tanned BSA sample, treated in the same manner except dissolved in 0.2 M potassium phosphate buffer, pH 8.0, was similarly evaluated.

Thin-layer Chromatography of Bovine Serum Albumin Acid Hydrolysates

Two dimensional thin-layer chromatography of BSA acid hydrolysates was carried out on Eastman cellulose chromagram sheets type 6064 without fluorescent indicator. Standard and tanned BSA hydrolysates were prepared as noted below except that the final hydrolysate was taken up in 0.25 ml 0.1 M potassium phosphate buffer, pH 8.0. 2.0 to 3.0 µl of the hydrolysate was spotted in the lower left hand corner of the cellulose sheet, 1.5 cm from either edge. The chromatogram was initially equilibrated for one hour with the first solvent system, butanol:acetic acid:water (60:15:25 v/v), developed to within 1.5 cm of the upper edge (17 cm traveling distance), and air-dried at room temperature overnight. It was then turned 90 degrees, equilibrated for an hour with the second solvent system, ethanol: ammonia:water (90:5:5 v/v), developed to within 1.5 cm of the edge, and air dried. Spots were visualized by spraying the chromatogram with ninhydrin prepared according to von Arx and Neher (1963), followed by heating at approximately 100° C for 10 minutes. Identification of the individual amino acids was carried out by comparison to a composite amino acid standard chromatogram. The standard

chromatogram was prepared by similarly chromatogramming standard amino acid mixtures prepared to yield solutions of 2.0 mg each amino acid per ml in 10 percent reagent isopropanol. Minimal addition of hydrochloric acid was added as needed for complete solution.

Enzymatic Oxidation of Pyrocatechol by Tyrosinase

All spectrophotometric evaluations were carried out employing a Beckman DB recording spectrophotometer equipped with a 1.0 cm pathlength cuvette and a Brinkman Lauda K-2/R constant temperature apparatus. Except where noted the reaction was carried out in the cuvette. Throughout the experimental series 0.2 M potassium phosphate of the appropriate pH and Schwarz-Mann mushroom tyrosinase with a manufacturer-specified activity of 1100 units/mg were used. Absorbancy was read against the appropriate potassium phosphate solution blank.

Spectrophotometric Evaluation of Initial Enzymatic Pyrocatechol Oxidation Products

Pyrocatechol (45.4 μmoles) in 1.0 ml 0.2 M potassium phosphate, pH 5.5, was added to 2.0 ml of the same potassium phosphate solution and the mixture brought to 30°C. Tyrosinase (0.1 ml), 0.1 mg/ml in pH 5.5 phosphate solution) was added and changes in absorbancy of duplicate samples followed at 390 nm and

410 nm. The ultraviolet spectrum of enzymatically oxidized pyrocatechol was determined as described in the section below on the effect of multiple tyrosinase addition on pyrocatechol oxidation.

Effect of pH on Tyrosinase Oxidation of Pyrocatechol

Tyrosinase (0.1 ml of a 0.1 mg/ml solution in the appropriate potassium phosphate solution) was added to 3.0 ml potassium phosphate solution of the same pH and brought to 30° C. Pyrocatechol (45.4 μmoles) in 0.1 ml 0.2 M potassium phosphate was added and the progress of the reaction followed at 390 nm. The rate of tyrosinase activity as a function of pH was determined from the absorbancy change per 30 second period evaluated from the initial straight line portion of the absorbancy versus time curve.

Effect of Pyrocatechol Concentration on
Its Oxidation by Tyrosinase

Pyrocatechol at eight appropriate levels in 1.0 ml pH 5.5, 0.2 M potassium phosphate was added to 2.0 ml of the same potassium phosphate solution and brought to 30° C. Tyrosinase (0.1 ml, 0.1 mg/ml in pH 5.5 phosphate solution) was added and the progress of the reaction followed at 390 nm.

Effect of Tyrosinase Levels on Pyrocatechol Oxidation

Pyrocatechol (45.4 μmoles) in 1.0 ml 0.2 M potassium phosphate, pH 5.5, was added to 2.0 ml of the same pH 5.5 potassium phosphate solution and the mixture brought to 30° C. Tyrosinase

(0.1 ml) at one of four concentrations in pH 5.5 potassium phosphate solution was added, and the progress of the reaction followed at 390 nm.

Effect of Multiple Tyrosinase Addition on Pyrocatechol Oxidation

Pyrocatechol at four levels in 1.0 ml, 0.2 M potassium phosphate, pH 5.5, was added to 2.0 ml of the same potassium phosphate solution, and brought to 30° C. Tyrosinase (0.1 ml, 0.1 mg/ml in pH 5.5 potassium phosphate solution) was added and the progress of the reaction followed at 390 nm until no further change in the absorbancy was observed. An additional 0.1 ml tyrosinase was added at this point and the progress of the reaction again followed at 390 nm. The sequence was repeated a third time.

A second experiment was carried out as follows: To each of three flasks, 0.1 ml tyrosinase (1.0 mg/ml in pH 5.5 potassium phosphate solution) was added to 3.0 ml pH 5.5 potassium phosphate solution containing 9.08 μmoles (1.0 mg) pyrocatechol. Samples were allowed to air oxidize at 30° C for 10 minutes in a reciprocal shaker water bath. At the end of the 10 minute incubation period a 1.0 ml aliquot from the first flask was diluted 1:10 with 0.2 M potassium phosphate, pH 5.5 and the ultraviolet spectrum read, while an additional 0.1 ml tyrosinase was added to the second and third flasks

with continued incubation for a second 10 minute period. The sequence was repeated a third time, obtaining ultraviolet spectra of oxidized pyrocatechol at 10, 20, and 30 minutes incubation time, following the addition of 0.1, 0.2, and 0.3 ml tyrosinase. A control sample without the addition of tyrosinase was included.

Effect of Added Glycine, Bovine Serum Albumin, and Poly-L-lysine on Tyrosinase Oxidation of Pyrocatechol

Pyrocatechol (45.4 μmoles) in 1.0 ml 0.2 M potassium phosphate, pH 5.5, was added to 2.0 ml pH 5.5 potassium phosphate solution containing the appropriate levels of glycine, BSA, and poly-L-lysine and brought to 30° C. 0.1 ml tyrosinase (0.1 mg/ml in pH 5.5 potassium phosphate solution) was added and the progress of the reaction followed at 390 nm. A control sample without glycine, BSA, or poly-L-lysine was included.

Estimation of Tryptophan Modification by Oxidized Pyrocatechol Employing Chemical and Anion Exchange Chromatographic Procedures

Estimation by the Chemical Method of Spies and Chambers (1948)

9.12 μmoles (2.5 mg) N-acetyl-L-tryptophan ethylester (NATEE) in 2.0 ml 0.2 M potassium phosphate buffer, pH 8.0, were added to the appropriate levels of pyrocatechol in 0.5 ml

glass distilled water and allowed to air oxidize at 30° C for 24 hours. 1.0 ml aliquots of triplicate samples were diluted 1:10 with glass distilled water and analyzed for residual NATEE by the p-dimethyl-aminobenzaldehyde condensation method of Spies and Chambers (1948) employing an NATEE standard curve. NATEE was solubilized by taking the material up in anhydrous diethyl ether followed by the addition of buffer with subsequent removal of the ether under nitrogen. Levels of pyrocatechol used yielded pyrocatechol:NATEE mole ratios of 1:10, 1:1, and 10:1. The following control samples were run: A) as above but without pyrocatechol, NATEE equal to 0.365 μmoles; B) as above but without NATEE; C) oxidized pyrocatechol at appropriate levels, with 0.365 μmoles NATEE added just prior to the determination.

Estimation by Separation of N-acetyl-L-tryptophan Ethylester (NATEE) from Reaction Products by Solvent Extraction and Column Chromatography

Column Preparation and Standardization

DEAE cellulose (Bio-Rad Cellex D) was slurried in 100 percent methanol, packed into a 4.2 x 25 cm glass column, washed with 100 percent methanol until the absorbancy at 280 nm was less than 0.05, and then resuspended in 100 percent methanol. A Glenco 1.0 x 30 cm column was packed to a height of 10 cm and the 280 nm absorbancy

rechecked. The elution profile of NATEE was determined.

Analysis of Residual NATEE

Pyrocatechol (9.08 μmoles) in 0.5 ml 0.2 M potassium phosphate solution, pH 5.5, was added to 2.0 ml of the same pH 5.5 potassium phosphate solution containing 9.12 μmoles (2.5 mg) NATEE. Tyrosinase (0.1 ml, 1.0 mg/ml in pH 5.5 potassium phosphate solution) was added and triplicate samples incubated at 30° C for three hours. At the end of the incubation period the samples were quantitatively transferred to round bottom flasks, dried under vacuum, and the residual NATEE extracted with four 1.0 ml portions of 100 percent methanol. The methanol extract was concentrated to approximately 0.5 ml under nitrogen, applied to the 1.0 x 10 cm washed DEAE cellulose column and eluted with 100 percent methanol. The NATEE fraction was collected, its visible spectrum recorded, a 1:50 dilution prepared and the ultraviolet spectrum of this dilution read. Spectrophotometric determinations were carried out at room temperature against a 100 percent methanol blank. Percent residual NATEE was determined by: a) percent of 280 nm absorbancy of the sample as compared to the control without pyrocatechol; b) direct calculation of remaining NATEE from the 280 nm absorbancy of the sample and the determined molar extinction coefficient of NATEE in 100 percent methanol at 280 nm. Correction was made for a 95% recovery of

NATEE in the methanol extraction process.

Principal Studies

Preparation of Tanned Bovine Serum Albumin

Base Catalyzed Air Oxidation of Pyrocatechol

BSA (20 mg) in 2.0 ml 0.2 M potassium phosphate buffer, pH 8.0, or in 2.0 ml 0.2 M potassium carbonate buffer, pH 9.5, was added to the appropriate levels of pyrocatechol in 0.5 ml glass distilled water. Triplicate samples were incubated for 24 hours at 30° C in a reciprocal shaker water bath. At the end of the oxidation period a 1.0 ml aliquot of each sample was removed for acid hydrolysis and amino acid analysis of the tanned BSA as described below. Levels of pyrocatechol used yielded pyrocatechol:BSA amino acid residue mole ratios of 1:10, 1:1, 2:1, and 10:1, holding the BSA level constant. The term BSA-amino acid residue is used to signify a weight of BSA providing a given mole level of amino acid residues and will be used to describe levels of BSA employed in the amino acid modification studies. The amino acid composition of BSA according to Tristram and Smith (1963) was used in these calculations. Control samples were prepared with 0.5 ml glass distilled water replacing the pyrocatechol.

Incubation of BSA with p-benzoquinone

The low solubility of p-benzoquinone in water necessitated an alteration in the protein incubation procedure to the following: 0.60 mg BSA in 2.0 ml 0.2 M potassium phosphate buffer, pH 8.0, was added to the appropriate levels of p-benzoquinone in 0.5 ml glass distilled water and incubated at 30° C for 24 hours. At the end of the incubation period 1.0 ml aliquots of the triplicate samples were removed for acid hydrolysis and amino acid analysis of the tanned BSA. A control sample was prepared substituting 0.5 ml water for the p-benzoquinone solution. Levels of p-benzoquinone used yielded p-benzoquinone:amino acid residue mole ratios of 1:20, 1:10, 1:1, 2:1 and 5:1, holding BSA constant.

Enzymatic Oxidation of Pyrocatechol

Modification of the constituent amino acids of BSA by enzymatically oxidized pyrocatechol was studied with respect to three variables: system pH, amino acid residue: pyrocatechol mole ratio, and enzyme level. 0.2 M potassium phosphate solution of the appropriate pH was used throughout. BSA:pyrocatechol mole ratios were varied by altering pyrocatechol levels, holding BSA constant.

BSA (1.23 mg) in 2.0 ml of the appropriate potassium phosphate solution was added to the appropriate level of pyrocatechol in 1.0 ml

of the same potassium phosphate solution. 0.1 ml tyrosinase in the same phosphate solution at the appropriate level was added and the duplicate or triplicate samples incubated at 30° C for three hours. At the end of the oxidation period a 1.0 aliquot of each sample was taken for amino acid analysis. Amino acid modifications were evaluated at pyrocatechol:amino acid residue mole ratios of 1:1 and 1:10 at pH 5.5 with a tyrosinase level of 110 units (0.1 mg). Amino acid modifications were evaluated at pH values of 5.5, 7.0, 7.5, and 8.0 (pyrocatechol:amino acid residue mole ratio of 1:1, 110 units tyrosinase) and at enzyme levels of 220, 110, 55, and 11 units (pyrocatechol:amino acid residue mole ratio of 1:1, pH 5.5). Control samples treated as above consisted of 1.23 mg BSA in 3.0 ml 0.2 M buffer, pH 7.0, to which was added 0.1 mg or 0.2 mg tyrosinase. Higher levels of pyrocatechol caused rapid reaction-inactivation of the tyrosinase, and were therefore not used.

Acid Hydrolysis and Amino Acid Analysis of Tanned Bovine Serum Albumin

To 1.0 ml aliquots of tanned BSA contained in 5.0 ml drying ampoules (Kontes Glass Company) were added 1.0 ml volumes of concentrated hydrochloric acid. The ampoules were sealed under vacuum after flushing with nitrogen. The samples were hydrolyzed at 110° C for 24 hours in a refluxing toluene bath. At the end of the

24 hour hydrolysis period the samples were opened, quantitatively filtered through a 0.5 micrometer pore size cellulose acetate Millipore filter, dried under vacuum, washed twice with a small volume of glass distilled water, and redried under vacuum. The BSA samples tanned by the base catalyzed procedure were then taken up in 4.0 ml glass distilled water and 0.15 ml aliquots (containing approximately 0.3 mg hydrolyzed protein) were diluted to 2.0 ml with a pH 2.2 sodium citrate buffer. BSA hydrolysate samples tanned by p-benzoquinone were taken up directly in 2.0 ml of citrate buffer following the filtering, washing, and drying procedure. Hydrolysate samples of BSA tanned by the enzymatic oxidation of pyrocatechol were taken up in 3.0 ml citrate buffer. Amino acid analysis of these hydrolysates was carried out according to the procedure of Spackman, Stein, and Moore (1958) employing a Spinco Model 120B automated amino acid analyzer. Amino acid modifications were determined by evaluating changes in the amino acid/glycine ratios. Glycine was chosen as the standard amino acid since an internal glycine residue is most unlikely to react with oxidized phenolics or quinones. The amino acid/glycine ratio value chosen was always the average value obtained from the analyses of triplicate or duplicate samples. Amino acid analysis determines only modifications resulting from covalent bonding of quinone to protein. Statistical evaluation of the observed modifications was carried out employing the Mann-Whitney rank test and Student's

t test.

Treatment of Amino Acid Standard Mixture with Pyrocatechol and p-benzoquinone under Hydrolysis Conditions

To evaluate possible artifacts arising from amino acid modifications by pyrocatechol or p-benzoquinone during the hydrolysis procedure, the following experiment was carried out using an amino acid standard mixture normally employed in amino acid analyzer standardization. The amino acid standard mixture (0.1 ml) was added to 5.0 ml drying ampoules containing the appropriate levels of either pyrocatechol or p-benzoquinone in 0.5 ml glass distilled water. Water (0.4 ml) and 1.0 ml concentrated hydrochloric acid were added; the vials were flushed with nitrogen, sealed under vacuum, and heated at 110° C for 24 hours. The samples were then filtered, washed, and dried as described above and taken up in 2.5 ml sodium citrate buffer, pH 2.2, to yield a theoretical concentration of 0.1 μmole amino acid/ml. Amino acid analysis was carried out as previously described. Levels of pyrocatechol employed yielded a pyrocatechol:amino acid mole ratio of 2:1 while p-benzoquinone was used at levels yielding p-benzoquinone:amino acid mole ratios of 2:1 and 10:1.

Estimation of Tryptophan and Methionine Modification by Enzymatically Oxidized Pyrocatechol Employing Gas-liquid Chromatographic Analysis of N-trifluoroacetyl-L-tryptophan Methyl Ester (N-TFA-L-tryptophan ME) and N-trifluoroacetyl-DL-methionine Methyl Ester (N-TFA-DL-methionine ME)

Chromatography Conditions

Gas-liquid chromatographic analysis of N-TFA-L-tryptophan ME and N-TFA-DL-methionine ME was carried out according to a modified method of Hagen and Black (1965) using a Perkin Elmer Model 990 gas chromatograph equipped with a flame ionization detector. A stainless steel column packed with 0.5 percent carbowax 20 M on chromosorb G (100/120 mesh) and conditioned at 250° C for 24 hours was employed. The injector port temperature was 300° C while the detector temperature was 250° C. Hydrogen and air flows were set to give satisfactory flame response. A retention time of 10 minutes with respect to the solvent front was obtained for the two amino acid derivatives with a nitrogen flow of 30 ml/minute and column temperatures of 185° C for N-TFA-L-tryptophan ME or 95° C for N-TFA-methionine ME.

Sample Preparation and Analysis

N-TFA-L-tryptophan ME or N-TFA-DL-methionine ME (9.08 μmoles) in 0.1 ml reagent grade acetone were added to 2.5 ml 0.2 M

potassium phosphate solution, pH 5.5, containing the appropriate level of pyrocatechol. (An additional 0.1 ml of reagent acetone was needed to bring the N-TFA-L-tryptophan ME into solution; this level of acetone in the reaction system was found not to inhibit tyrosinase oxidation of pyrocatechol.) Tyrosinase (0.2 ml, 1.0 mg/ml in the same pH 5.5 potassium phosphate solution) was added and the samples incubated at 30° C for two hours. Triplicate samples were prepared. After incubation the samples were dried under vacuum and extracted with several volumes of 100 percent methanol to a total volume of 3.0 ml. A 3.0 µl aliquot of each sample was used for gas-liquid chromatographic analysis. Levels of pyrocatechol used yielded pyrocatechol:amino acid derivative mole ratios of 1:1 and 1:10. Control samples containing either N-TFA-L-tryptophan ME or N-TFA-DL-methionine ME treated the same as above but without pyrocatechol were included. Percent modification of the amino acid derivative in the samples subjected to enzymatically oxidized pyrocatechol was determined from measured residual N-TFA-L-tryptophan ME or N-TFA-DL methionine ME.

Preparation, Isolation, and Characterization of a Glycine- p-benzoquinone Adduct Compound

Preparation and Initial Isolation of Adduct Material

p-Benzoquinone (53.4 µmoles) in 1.0 ml glass distilled water

was added to 26.7 μmoles glycine in 4.0 ml 0.2 M potassium phosphate buffer, pH 8.0 and the mixture incubated at 30° C for three hours. At the end of the incubation period the sample was dried under vacuum and taken up in 1.0 ml 10 percent isopropyl alcohol. A control sample consisting of 53.4 μmoles p-benzoquinone in 5.0 ml buffer solution (1.0 ml water plus 4.0 ml pH 8.0 buffer) was similarly incubated, dried, and taken up in 10 percent isopropyl alcohol. Initial separation of the reaction products was carried out by thin layer chromatography on Eastman cellulose chromagram sheets type 6064 without fluorescent indicator employing butanol: glacial acetic acid:water (60:15:25 v/v) as the developing solvent. Isopropyl alcohol:formic acid (88 percent):water (20:1:5 v/v) on cellulose plates, and benzene:acetic acid:water (40:2:2 v/v) on silica gel G plates according to Morrison, Steele, and Danner (1969) failed to give satisfactory separation. Samples to be chromatogramed were spotted 1.5 cm from the bottom edge of the cellulose sheet and at least 1.5 cm from either side, developed to within 1.5 cm of the upper edge (17 cm traveling distance), and air dried. Detection of separated material was carried out by spraying with ninhydrin (von Arx and Neher, 1963) and heating at approximately 100° C for 10 minutes, by viewing the developed chromatogram under ultraviolet light, or by spraying with 0.0005 percent rhodamine B in 95 percent ethyl alcohol and viewing under short ultraviolet light. Preliminary

studies employing the control and glycine- p-benzoquinone samples, as well as a glycine standard (2.0 mg/ml in 10 percent isopropanol) indicated the presence of a band unique to the glycine-p-benzoquinone reaction mixture. Thus, preparative thin-layer chromatography on Eastman cellulose sheets using the butanol:acetic acid:water (60:15:25) solvent system was carried out to obtain sufficient material for further characterization. Several chromatograms were spotted continuously across the plate with the glycine- p-benzoquinone reaction mixture. The sheets were developed and air dried. The band of interest was scraped from the plate and eluted from the cellulose with 10 percent isopropyl alcohol. The extracted material was dried under vacuum, taken up in a minimal volume of 10 percent isopropyl alcohol, rechromatographed, and collected again.

Characterization of the Glycine- p-Benzoquinone Adduct

Determination of the Visible and Ultraviolet Spectra. A portion of the above isolated material was taken up in 3.0 ml distilled water and its visible and ultraviolet (1:10 dilution) spectra determined qualitatively against a water blank. For comparative purposes qualitative visible and ultraviolet spectra of p-benzoquinone, hydroquinone, pyrocatechol, and glycine were determined.

Determination of the Infrared Spectrum. A second portion of the isolated material was slurried in 100 percent methanol and applied

to a sodium chloride disk. The spectrum was recorded on a Beckman IR-5 infrared recording spectrophotometer.

Determination of the Mass Spectrum. Mass spectral analysis of the adduct material was carried out on an Atlas CH7 mass spectrometer using a direct probe technique. Spectra were taken at 70 ev with an accelerating voltage of 3 kv and an ion source temperature of 175° C. Preliminary spectra determined on the above isolated material using probe temperatures of 55° C to 450° C appeared to indicate a lack of volatilization of the material. To improve volatilization of the probable adduct compound, the material isolated from the glycine-p-benzoquinone reaction mixture was methylated employing diazomethane as the methylating agent according to the procedure of Schlenk and Gellerman (1960) with N-nitrosomethylurea serving as the diazomethane generating reagent. Thin-layer chromatography of the methylated material on silica gel G (50 grams silica gel G to 100 ml glass distilled water, plate thickness 250 microns, dried at 100° C and activated at the same temperature just prior to use) using an anhydrous diethyl ether:hexane:absolute methanol (40:10:2 v/v) solvent system yielded four bands in addition to material remaining at the origin. Each of the four bands obtained from the methylated material was scraped from the plate, eluted from the silica gel G by absolute methanol, and dried under vacuum. Material from the origin was eluted with 10 percent isopropyl alcohol and dried under

vacuum. Material from each band as well as the origin was subjected to mass spectral analysis.

Preparation, Isolation, and Characterization of a Possible N-acetyl-L-histidine- p-benzoquinone Adduct Compound

Preparation and characterization of a possible N-acetyl-L-histidine-p-benzoquinone adduct was carried out in a manner similar to that of the glycine-p-benzoquinone compound. p-Benzoquinone (53.4 μmoles) was added to 26.7 μmoles N-acetyl-L-histidine in 4.0 ml 0.2 M potassium phosphate buffer, pH 8.0. The sample was incubated at 30° C for three hours, dried under vacuum, and taken up in 10 percent isopropyl alcohol. Preliminary and preparative thin-layer chromatography on Eastman cellulose plates was carried out as described above employing butanol:glacial acetic acid:water (60:15:25 v/v) as the solvent system. Five distinct bands were observed, but in contrast to the glycine-p-benzoquinone system, no unique band was found for the N-acetyl-L-histidine-p-benzoquinone system when compared to the oxidized p-benzoquinone control. Each of the five bands therefore was scraped from the plate, eluted with 10 percent isopropyl alcohol, dried under vacuum, rechromatographed, and isolated as before. Their infrared spectrum was determined employing a Perkin-Elmer Model 457 grating recording infrared spectrophotometer after application to a salt disk as described above. The

infrared spectrum of unreacted N-acetyl-L-histidine was similarly determined.

Determination of Trypsin Digestibility of Tanned Bovine Serum Albumin

Preparation of Tanned BSA

Base Catalyzed Air Oxidation of Pyrocatechol. BSA (12.5 mg) in 2.0 ml 0.2 M potassium phosphate buffer, pH 8.0 was added to the appropriate levels of pyrocatechol in 0.5 ml glass distilled water and allowed to air oxidize at 30° C for 24 hours. At the end of the incubation period the triplicate samples were quantitatively transferred to round bottom flasks, lyophilized (Virtis Research Equipment, Model 10-140), and taken up in 0.5 ml 0.001 M potassium phosphate buffer, the pH of which had been adjusted such that the final sample pH was approximately 7.5. The samples were eluted with the same 0.001 M buffer through a Bio-Gel P-30 column (1.0 x 12.0 cm), the void volume of which had previously been determined with Pharmacia blue dextran 2000. The void volume fraction containing the tanned protein was collected and made to 5.0 ml with the 0.001 M buffer. Three 1.0 ml aliquots of each sample were subjected to trypsin hydrolysis as described below while a 1.0 ml aliquot of each sample was reserved for Kjeldahl analysis according to the procedure of Steyermark (1961). BSA control samples prepared

by adding 5.0 ml 0.001 M buffer to 12.5 mg lyophilized BSA were included. Levels of pyrocatechol employed yielded pyrocatechol: amino acid residue mole ratios of 1:10 and 1:1.

Enzymatic Oxidation of Pyrocatechol. As a result of the requirement of the titrator for adequate protein substrate in the trypsin digestion procedure, the levels of pyrocatechol needed to provide pyrocatechol:amino acid residue mole ratios approaching 1:1 resulted in rapid inactivation of tyrosinase. Sample preparation was therefore restricted to a pyrocatechol:amino acid residue mole ratio of 1:10.

Preparation of tanned BSA was as follows: 12.50 mg BSA in 2.0 ml 0.2 M potassium phosphate solution, were added to 9.26 μmoles pyrocatechol in 0.5 ml of the same pH 5.5 solution. Tyrosinase (0.1 ml, 1 mg/ml in pH 5.5 phosphate solution) was added. Triplicate samples were incubated at 30° C for three hours. The samples were eluted through a Bio-Gel P-30 column (1.7 x 12 cm) with 0.001 M potassium phosphate buffer, the pH of which had been adjusted such that the final sample pH was 7.5. The void volume was collected, and made to 5.0 ml with the same 0.001 M buffer. Three 1.0 ml aliquots were subjected to trypsin digestion, while a 1.0 ml aliquot was reserved for Kjeldahl analysis. BSA control samples prepared by adding 5.0 ml 0.001 M buffer to 12.5 mg BSA plus 0.1 mg tyrosinase were employed.

Trypsin Digestion of Tanned BSA

Trypsin digestion of control and tanned BSA samples was measured by the determination of the μmoles base uptake required to maintain a constant pH employing a Radiometer TTTlc automatic titrator, type SBR2c equipped with a type SBU1a syringe burette. A 1.0 ml aliquot of the sample to be tested was placed in the titrator sample cup and brought to 30° C. The pH was adjusted to the endpoint value of 7.5 with standard 0.01 N sodium hydroxide. Trypsin solution (0.1 ml, 2.5 mg/ml in 0.001 M potassium phosphate buffer, final pH 7.5) was added and the reaction was followed for a 10 minute period; the volume of 0.01 N base required to maintain pH 7.5 was recorded with time. Triplicate samples were corrected for base uptake due to the sample alone, due to auto-hydrolysis of the trypsin solution, and due to the addition of trypsin. Protein content of the control and tanned BSA samples was determined by Kjeldahl analysis on a 1.0 ml aliquot identical to that employed in trypsin digestion. Changes in trypsin digestibility of the tanned samples were determined from comparison of base uptake values per mg BSA per 10 minute period in control and tanned samples. A synthetic substrate, N-benzoyl-L-arginine ethylester was added at the end of the 10 minute period to ascertain that enzymatic activity had been maintained in the presence of the tanned substrate. The extent of base uptake upon

addition of the synthetic substrate was determined and compared to a control employing only N-benzoyl-L-arginine ethylester at the same level.

Determination of Pepsin Digestibility of Tanned Bovine Serum Albumin

Preparation of Tanned BSA

For BSA tanned by base-catalyzed air oxidation of pyrocatechol, 12.5 mg BSA in 2.0 ml potassium phosphate buffer, pH 8.0, were added to appropriate levels of pyrocatechol in 0.5 ml glass distilled water to give pyrocatechol:amino acid residue mole ratios of 1:10 and 1:1. Triplicate samples were air oxidized for 24 hours at 30° C. The samples were then quantitatively transferred to round bottom flasks, lyophilized, taken up in 0.5 ml 0.001 M potassium citrate buffer, pH 3.45, and eluted through a Bio-Gel P-30 column (1 x 12 cm) with the same 0.001 M buffer to remove unassociated phenolics. The tanned protein samples were collected and made to 5.0 ml with the pH 3.45 buffer. One ml aliquots were subjected to pepsin digestion as described below while separate 1.0 ml aliquots were analyzed for total protein by the Kjeldahl procedure. BSA control samples were prepared by adding 5.0 ml 0.001 M citrate buffer, pH 3.45, to 12.5 mg lyophilized BSA. In the case of BSA tanned by enzymatically oxidized pyrocatechol, only a 1:10 pyrocatechol:amino acid

residue mole ratio sample was prepared, due primarily to tyrosinase reaction-inactivation as noted in the trypsin digestibility experiment above. Pyrocatechol (9.26 μmoles) in 0.5 ml 0.2 M potassium phosphate solution, pH 5.5 and 0.1 ml tyrosinase (1 mg/ml in pH 5.5 phosphate solution) were added to 12.5 mg BSA in 2.0 ml of the same pH 5.5 phosphate solution and triplicate samples incubated at 30° C for three hours. The samples were eluted through a Bio-Gel P-30 column (1.7 x 12 cm) with 0.001 M potassium citrate buffer, pH 3.45. The tanned protein sample was collected and made to 5.0 ml with the same 0.001 M buffer. One ml aliquots were subjected to pepsin digestion or Kjeldahl analysis. Samples prepared by adding 5.0 ml 0.001 M citrate buffer to 12.5 mg BSA plus 0.1 mg tyrosinase were employed as controls.

Pepsin Digestion of Tanned BSA

Pepsin digestion of control and tanned BSA samples was determined by measuring the μmoles acid uptake required to maintain a constant pH value, employing thesame Radiometer titrator described in the trypsin digestion experiments above. A 1.0 ml aliquot was placed in the titrator cup and brought to 30° C. The pH was adjusted to the endpoint value of pH 2.5 with standard 0.1 N and 0.01 N hydrochloric acid. Pepsin (0.1 ml, 2.5 mg/ml pepsin in 0.001 M potassium citrate buffer adjusted to pH 2.5) was added, and the reaction

was followed for a 5 minute period. The volume of 0.01 N hydrochloric acid required to maintain the pH 2.5 endpoint value was recorded with time. Triplicate analyses were corrected for acid uptake by the sample alone, for acid uptake by enzyme alone due to autolysis and acid hydrolysis, and for acid uptake occurring upon addition of pepsin. Protein content was determined by Kjeldahl analysis of 1.0 ml aliquots of control and tanned BSA samples identical to those employed in the pepsin digestion. Changes in pepsin digestibility of the tanned samples were determined from comparison of acid uptake values per mg BSA per 5 minute period in control and tanned samples. Results were expressed as percentage of the control value.

RESULTS AND DISCUSSION

Preliminary Studies

Effect of pH, Time, and Bovine Serum Albumin on the Non-enzymatic Air Oxidation of Pyrocatechol

Non-enzymatic air oxidation of pyrocatechol, determined by oxygen uptake measurements, occurred readily at pH 7.5 or above (Figure 8), with an increasing rate of oxidation occurring with higher pH values (Figure 9). No oxygen uptake was observed at pH 7.0 or below. Air oxidation of pyrocatechol involves a one-electron extraction from the phenolic compound, initially yielding the phenoxy radical. Although the phenoxy radical may be generated directly from the intact phenol, the rate of formation from the phenolate anion is greater. The greater concentration of the anion formed with increasing pH probably accounts for the several-fold change in the rate of oxygen uptake as shown in Figures 9a and 9b. Data therefore substantiate that the air oxidation of pyrocatechol is base catalyzed.

Oxygen uptake measured over a 12 hour period in the base-catalyzed oxidation of pyrocatechol is shown in Figure 10. The total measured oxygen uptake at the end of the 12 hour period corresponded to slightly more than 0.5 atoms of oxygen consumed per molecule of pyrocatechol initially present. This indicates incomplete

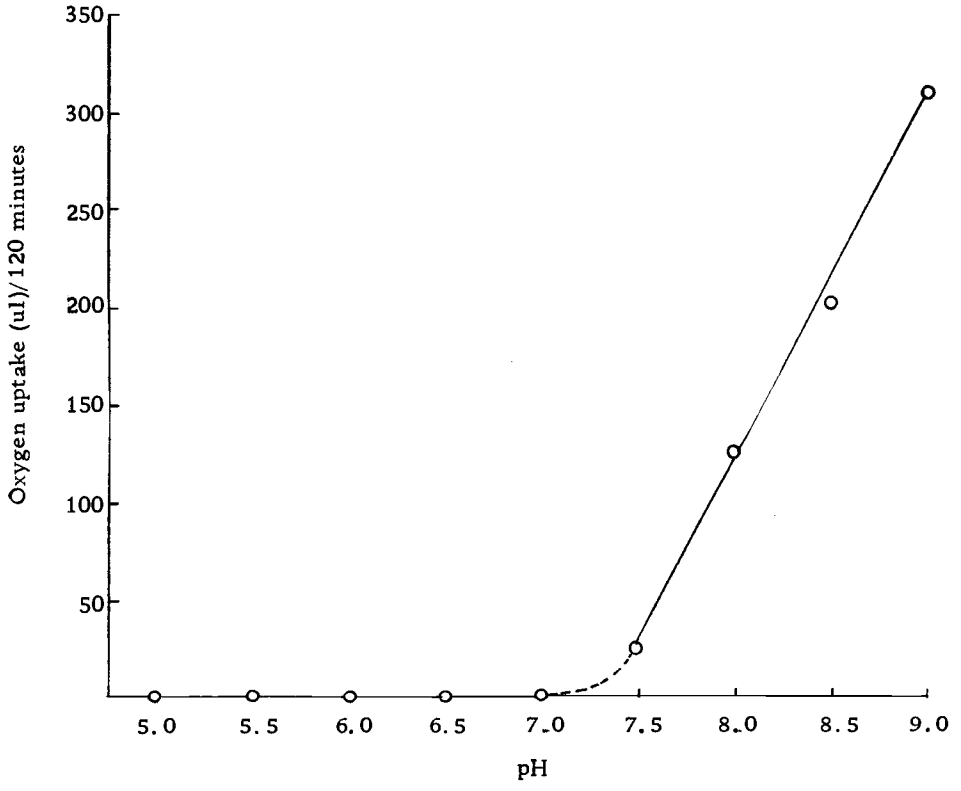


Figure 9. Effect of pH on the non-enzymatic oxidation of pyrocatechol. Reaction mixture contained: potassium phosphate of appropriate pH, 400 μmoles; pyrocatechol, 55.6 μmoles. Total volume, 2.5 ml. Reaction time, 2 hours. Temperature, 30°C.

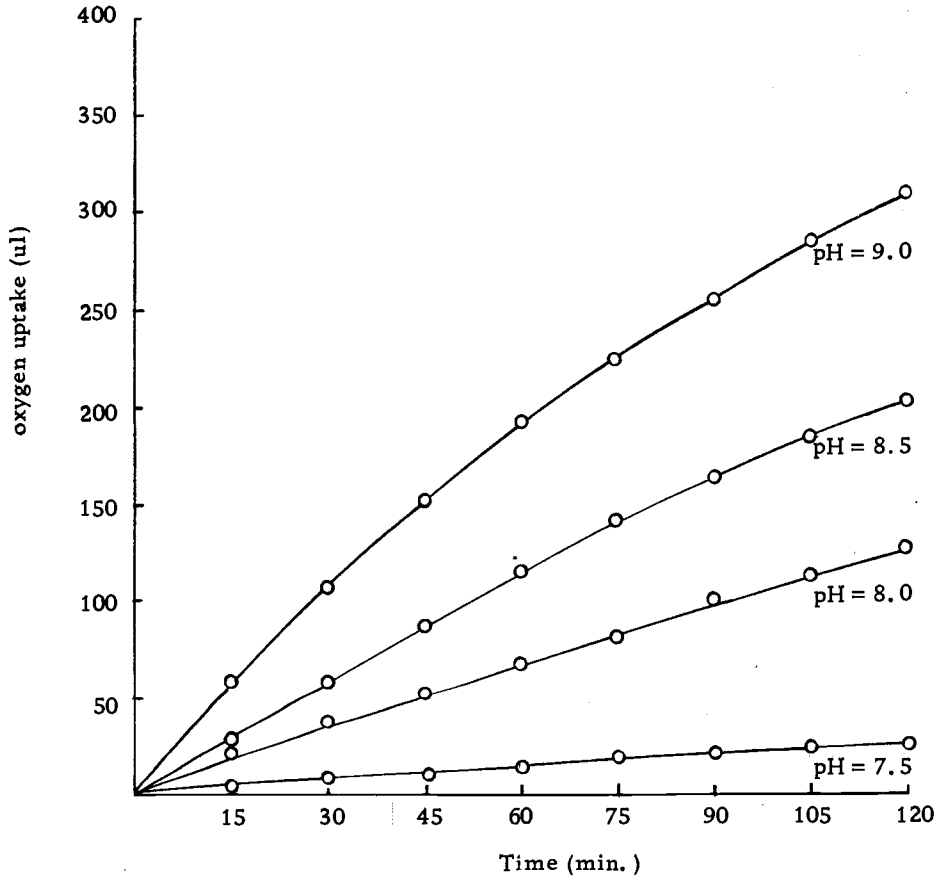


Figure 9b. Time course of non-enzymatic oxidation of pyrocatechol at varied pH. See Figure 9a for reaction conditions.

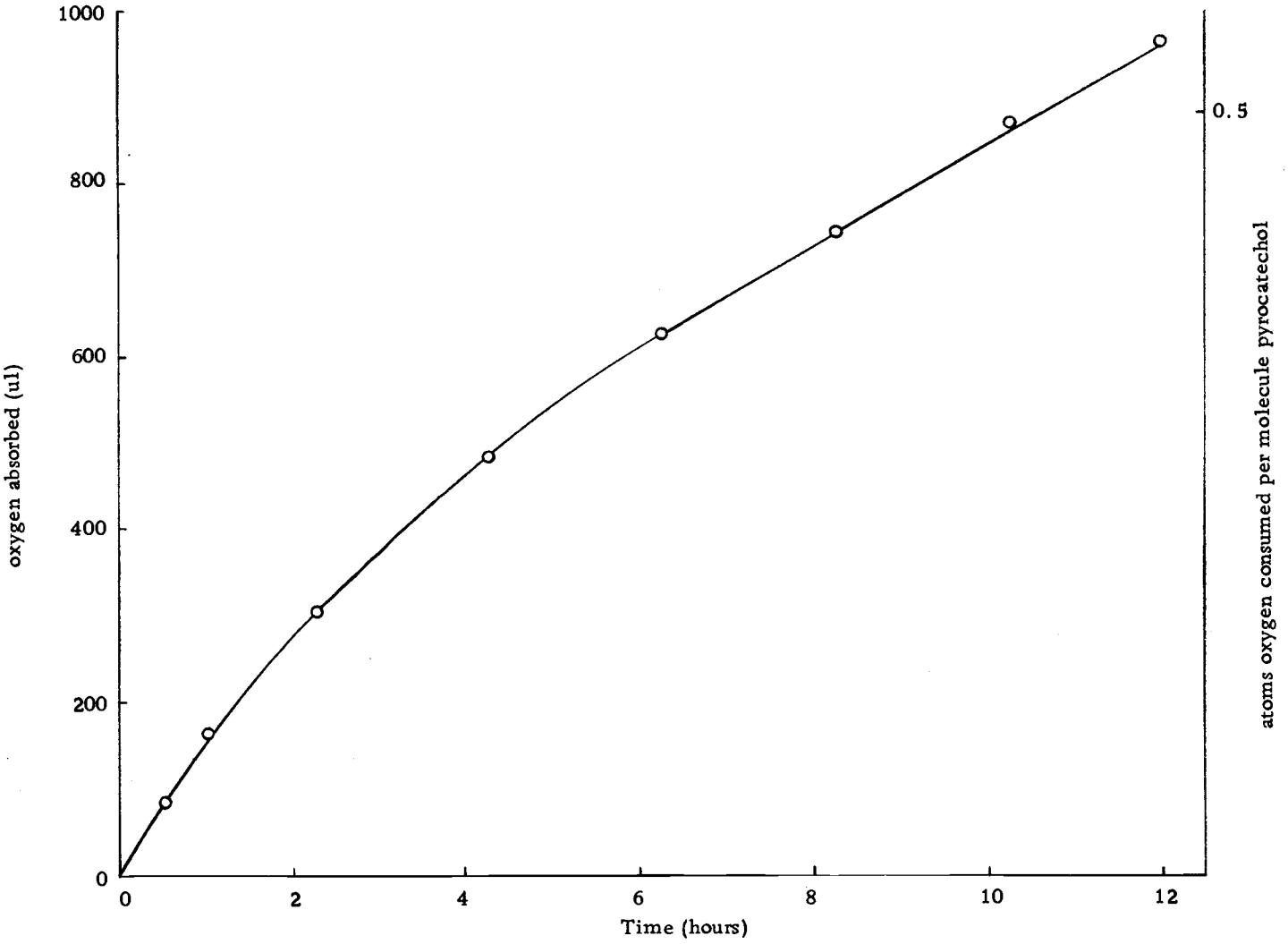


Figure 10. Oxygen uptake with time for the base-catalyzed air oxidation of pyrocatechol. Reaction mixture contained: buffer, potassium phosphate, pH 8.0, 400 μmoles; pyrocatechol, 148 μmoles. Total volume, 2.5 ml. Temperature, 30°C.

oxidation of the pyrocatechol present (complete conversion of pyrocatechol to o-benzoquinone would result in 1.0 atom of oxygen consumed per molecule of pyrocatechol). The system is certainly more complex than this, however. (Literature on phenolic oxidation reviewed emphasizes this complexity.) Secondary reactions between initial oxidation products and pyrocatechol would alter both the rate and the extent of oxygen uptake.

The presence of BSA during the non-enzymatic base catalyzed air oxidation of pyrocatechol resulted in a lag period before the onset of oxygen uptake as shown in Figure 11. After the lag period, however, the rate of oxygen uptake was similar to that of samples containing no BSA. The lag in oxygen uptake in the presence of BSA and the absence of color development during this lag period suggests that BSA delays autocatalytic pyrocatechol oxidation.

Spectrophotometric Evaluation of Bovine Serum Albumin, Pyrocatechol, Oxidized Pyrocatechol, and Tanned BSA

The ultraviolet spectrum of pyrocatechol shows a maximum at 274 nm and a minimum at 244 nm, while that of BSA shows a maximum at 276 nm and a minimum at 250-254 nm. Both pyrocatechol and BSA show no absorption in the visible range. The molar extinction coefficient of pyrocatechol was determined experimentally to be 2470 l $mole^{-1}$ cm^{-1} at 274 nm, room temperature, in 0.2 M potassium

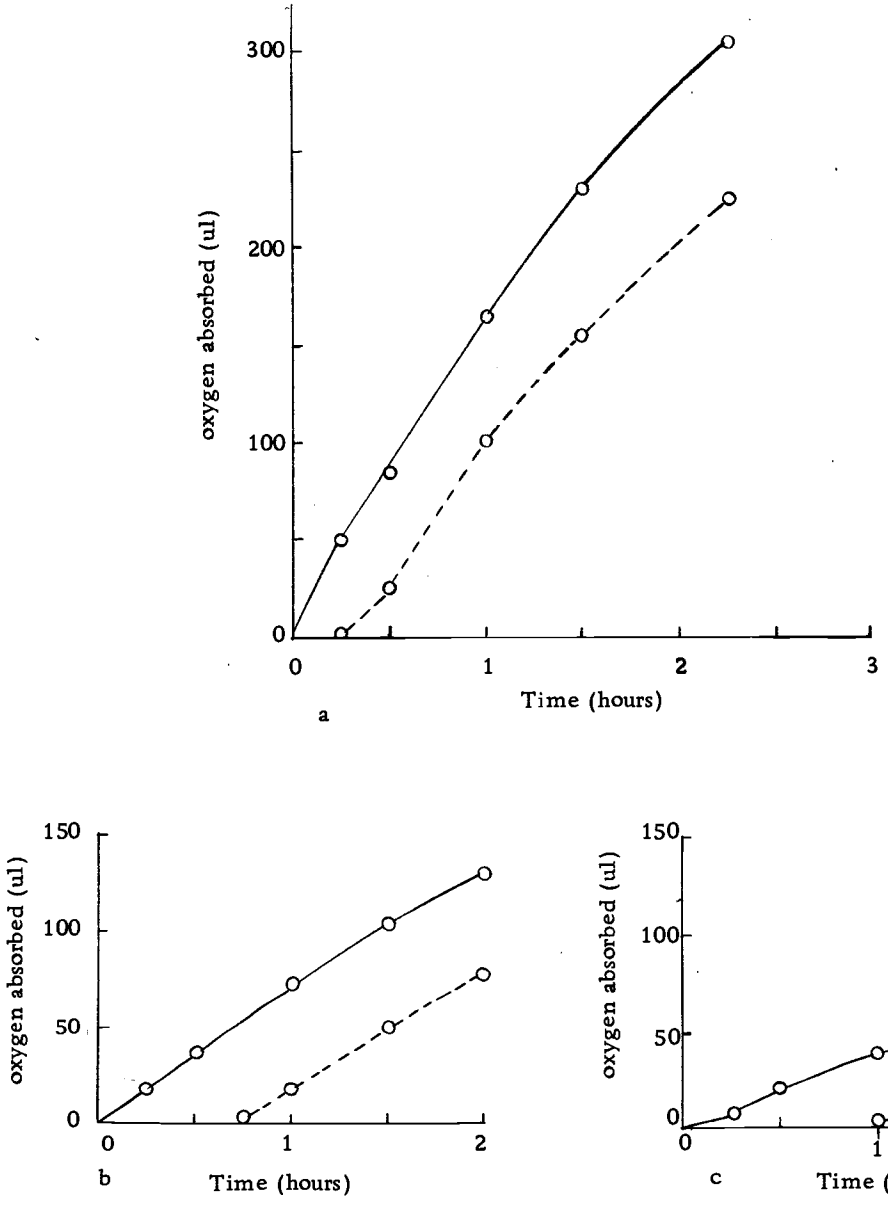


Figure 11. Effect of bovine serum albumin (BSA) on the non-enzymatic oxidation of pyrocatechol. Pyrocatechol only (____) and in the presence of BSA (-----). Reaction mixture contained: buffer, potassium phosphate, pH 8.0, 400 µmoles; pyrocatechol, 148 µmoles a), 55.6 µmoles b), 27.8 µmoles c); BSA, 20 mg. Total volume, 2.5 ml. Temperature, 30°C.

phosphate, pH 5.0.

Base catalyzed air oxidation products of pyrocatechol showed moderate to strong absorption throughout the visible region of the spectrum while the ultraviolet absorption appeared similar to unmodified pyrocatechol with some broadening of the absorption band probably the result of other ultraviolet absorbing species formed during the oxidative process. The base catalyzed pyrocatechol oxidation system changed from colorless to green-black at the end of the 24 hour period, passing through red-brown, brown, and green-brown stages. The continuous absorption of oxidized pyrocatechol throughout the visible region of the spectrum, contrasting with the lack of visible absorption by unmodified pyrocatechol, indicates the formation of a wide variety of products of varying electronic character during the oxidative process. Literature on phenol oxidation indicating the formation of a wide variety of products support this conclusion. The presence of BSA during base catalyzed oxidation of pyrocatechol appears to alter the nature of the oxidation products (Figure 12); the spectrum of an oxidized pyrocatechol-BSA reaction mixture shows lower absorption in the visible region with greater absorption in the ultraviolet. BSA appears to decrease the formation of those polymerized oxidation products that are more highly conjugated. This is probably due, in part, to the formation of adduct compounds between protein and oxidation products of

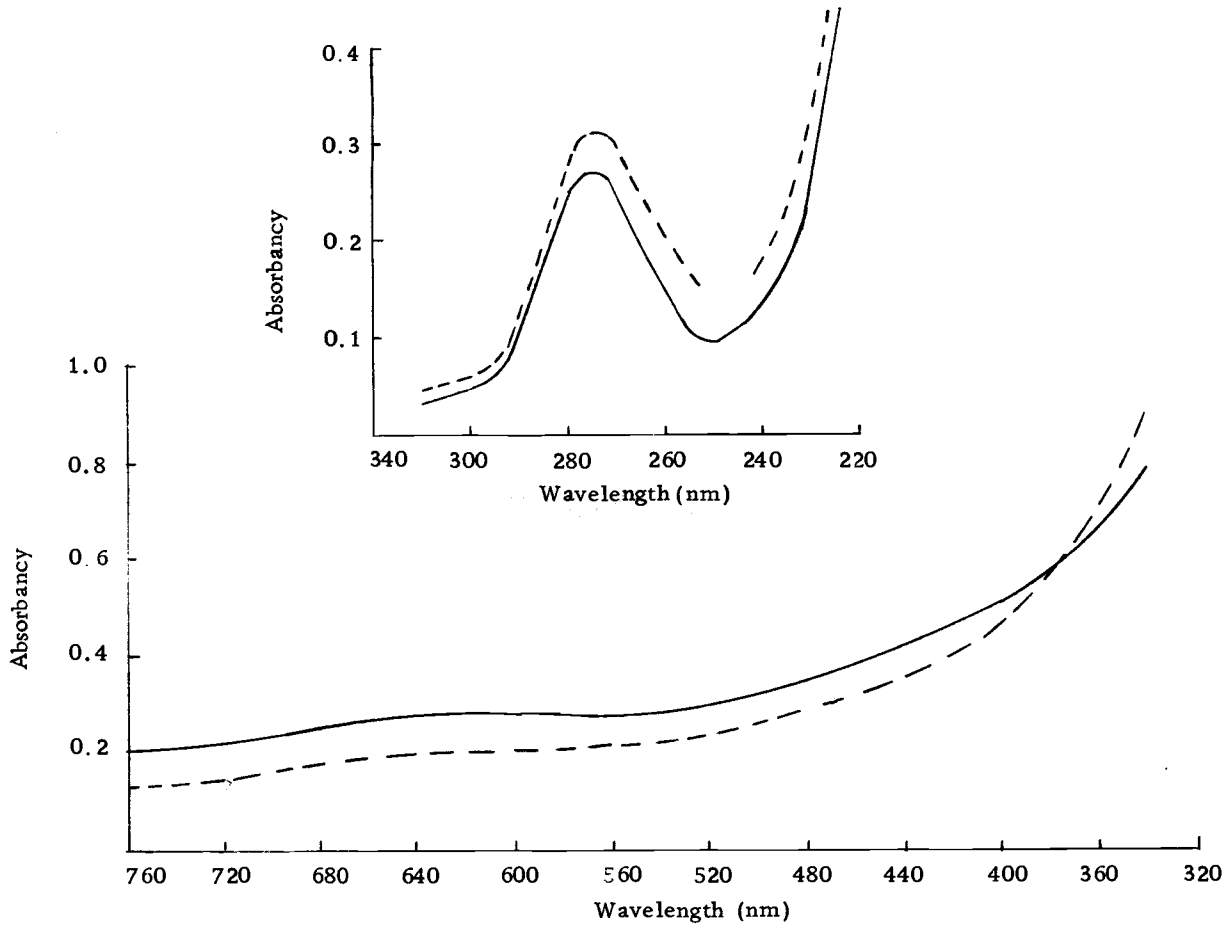


Figure 12. Ultraviolet and visible spectra of oxidized pyrocatechol (——) and oxidized pyrocatechol with bovine serum albumin (----). Reaction systems. oxidized pyrocatechol; buffer, potassium phosphate, pH 8.0, 400 µmoles; pyrocatechol, 296 µmoles. Total volume, 2.5 ml. Temperature, 30°C. Air oxidized, 24 hours. Diluted 1:1000 prior to reading. Oxidized pyrocatechol with bovine serum albumin (BSA): same as oxidized pyrocatechol-only system but with 20 mg BSA. Diluted 1:25 prior to reading.

pyrocatechol (copolymerization with protein), reducing the availability of reactants for polymer formation. The final color of the oxidized pyrocatechol-BSA mixture reflects this difference, the reaction mixture appearing green-brown rather than the green-black of oxidized pyrocatechol alone.

Tanned BSA precipitated by trichloroacetic acid or acetone from an oxidized pyrocatechol-BSA reaction mixture shows a visible absorption spectrum similar to that of oxidized pyrocatechol (Figure 13) which, in conjunction with the lack of visible absorption by BSA, indicates the association of pyrocatechol oxidation products with BSA. Treatment of oxidized phenolic material with TCA yielded no visible precipitate and only a slight precipitate after centrifugation. Such association is well documented, as noted in the introductory sections, and is reported to involve both covalent and non-covalent bonding of tannin material to protein. Acetone or 100 percent ethyl alcohol precipitation of tanned BSA, or methanol washing of TCA precipitated tanned BSA results in a lower visible absorption than that of TCA precipitated tanned BSA. The ability of such solvents to remove pyrocatechol oxidation products would appear to be connected with their ability to form hydrogen bonds as noted by Gustavson (1956), Shuttleworth and Cunningham (1948) and Loomis and Battaile (1966). Additionally, the fact that only a portion of the absorbed material is solvent stripped from BSA indicates the relatively strong nature of

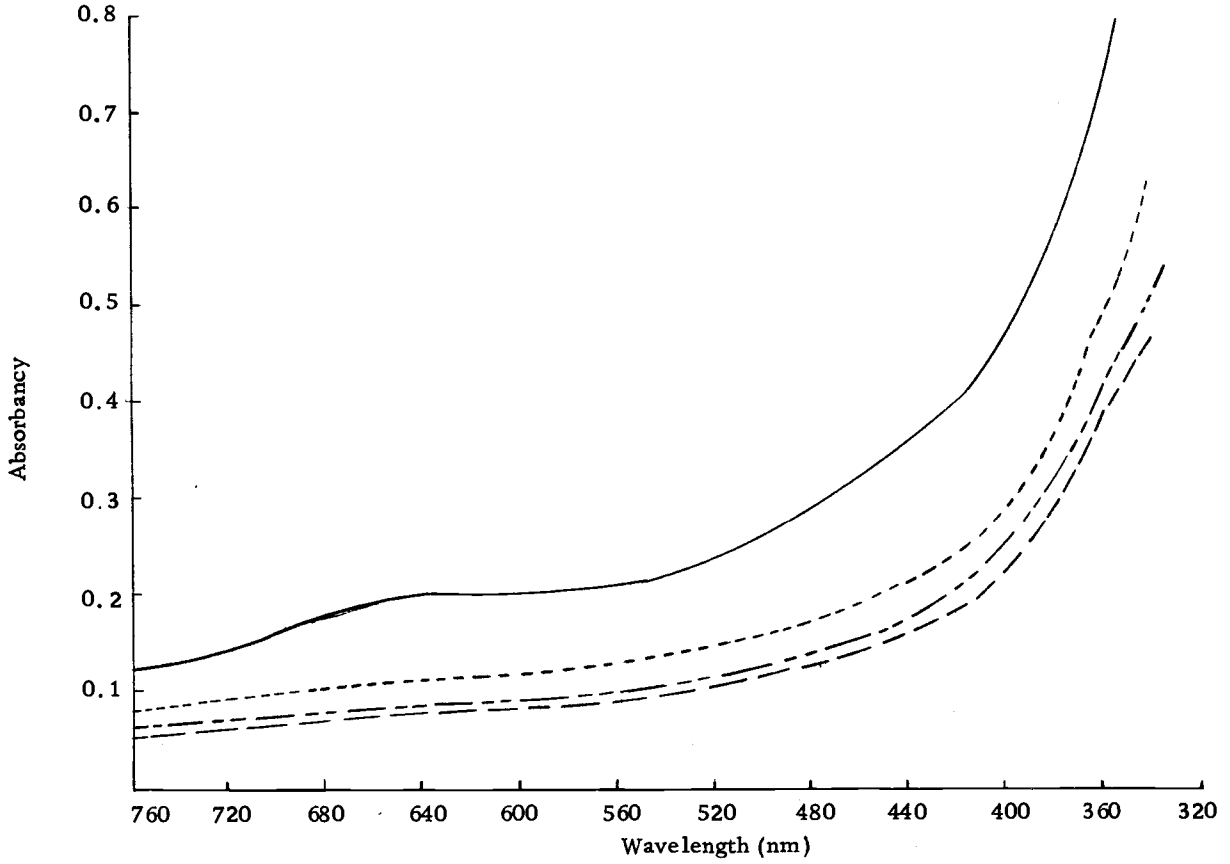


Figure 13. Visible spectra of: oxidized pyrocatechol containing bovine serum albumin (BSA) (——); tanned BSA, trichloroacetic acid precipitated (- - - -); tanned BSA, acetone precipitation (— · — · —); tanned BSA, trichloroacetic acid precipitated and methanol washed (— — —). Reaction system: buffer, potassium phosphate, pH 8.0, 400 μmoles; pyrocatechol, 296 μmoles; BSA, 20 mg. Total volume, 2.5 ml. Temperature, 30°C. Air oxidized, 24 hours. Tanned protein obtained by precipitating with four volumes of stated solvent, centrifuging at 27,000 x g, 15 minutes, with redissolving in 2.5 ml buffer, samples diluted 1:25 prior to reading.

the association of some pyrocatechol oxidation products with protein. Covalent bonding between protein and catechol oxidation products is probably, in part, the reason for this strong assocation.

Elution Pattern of Tanned Bovine Serum Albumin on Bio-Gel P-30, and on Sephadex G-50 after Treatment with N-methyl-2-pyrrolidone

As indicated in Figure 14 both tanned BSA and unmodified BSA elute at the same position on Bio-Gel P-30, approximately at the void volume of the column. A similar pattern was obtained for tanned BSA on Sephadex G-50. The elution of oxidized hydroquinone-BSA reaction mixture (base catalyzed) from Bio-Gel P-30 with 0.2 M tris-(hydroxymethyl)amino methane buffer, pH 8.0, resulted in the initial separation of the mixture into two bands; the leading band, tan in color, eluted at the column void volume and a second dark brown band, more diffuse than the leading band, eluted near the column V_o+V_i volume (Figure 14). Considering the exclusion size of Bio-Gel P-30 (MW 30,000 for globular proteins) and the elution patterns of unmodified BSA and unoxidized hydroquinone, the leading tan colored band was probably tanned BSA and the second brown band probably hydroquinone and oxidized hydroquinone products of a varied nature. The occurrence of a brown protein band upon elution of an oxidized hydroquinone-BSA mixture through Bio-Gel P-30 is consistent with the ability of oxidized phenolic material to strongly

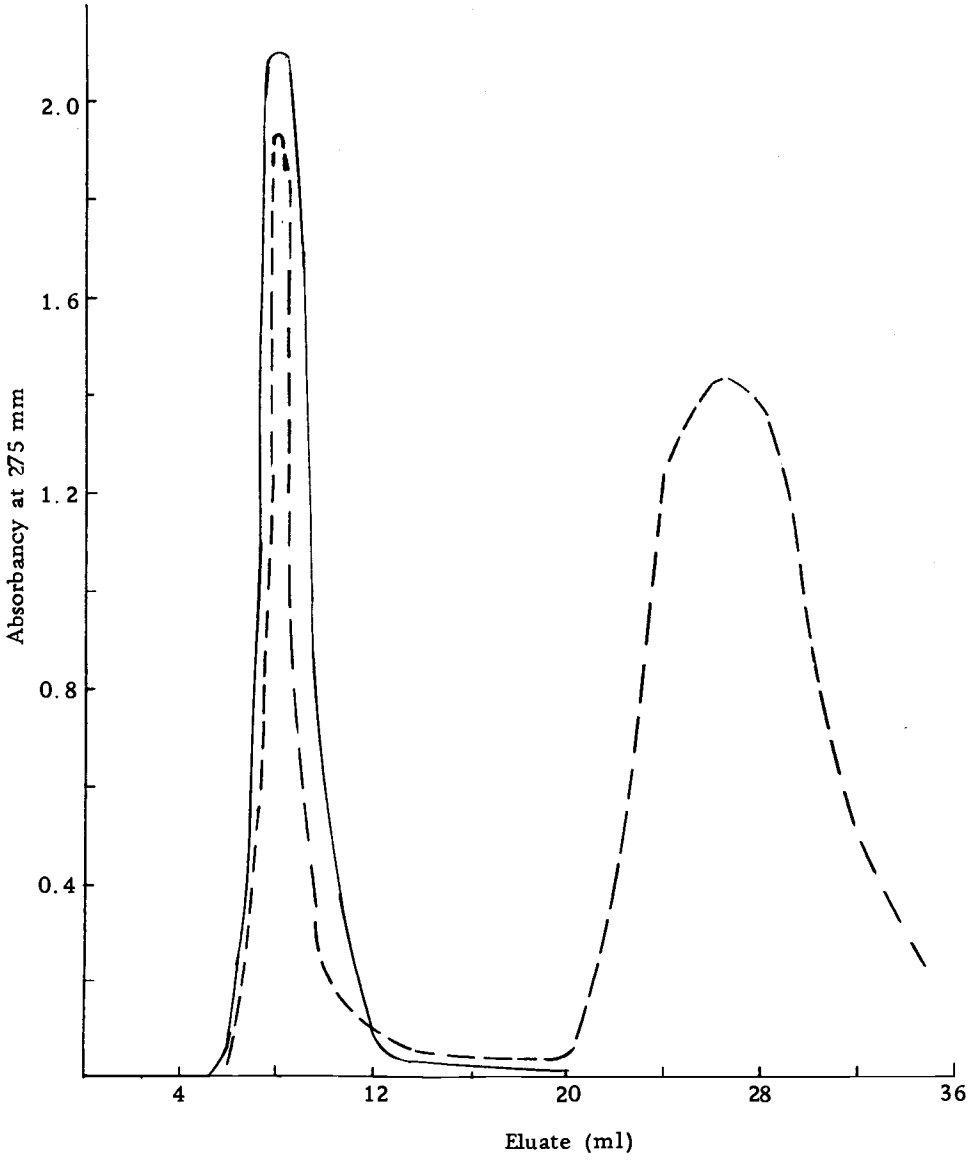
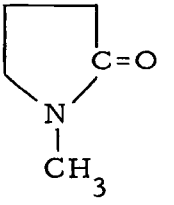


Figure 14. Elution pattern of bovine serum albumin (BSA) (——) and a BSA-hydroquinone reaction mixture (----) on Bio-Gel P-30. Column size, 1.5 cm x 15 cm; column developed with 0.2 M Tris buffer, pH 8.0. Room temperature. Reaction mixture contained: buffer, Tris, pH 8.0, 400 µmoles; hydroquinone, 91 µmoles; BSA, 20 mg. Total volume, 2.5 ml. Oxidized 8 hours at 30°C. Concentrated to approximately 0.5 ml with Bio-Gel P-2 prior to column application. BSA standard; 10 mg/ml in 0.2 M Tris buffer, pH 8.0, concentrated in the same manner as above to provide 20 mg/0.5 ml volume.

associate with protein.

A trichloroacetic acid precipitated tanned BSA sample taken up in 1.0 ml N-methyl-2-pyrrolidone (methyl pyrrolidone) and passed through Sephadex G-50 with 0.2 M potassium phosphate buffer, pH 8.0, gave two distinct bands, a brown band eluting at the colum void volume and a dark green-brown band which appeared strongly associated with the column material and was removable only with extensive washing with 0.2 M phosphate buffer, pH 8.0. In contrast, a similar TCA precipitated sample taken up instead in pH 8.0 buffer, yielded only one band upon passage through Sephadex G-50, the brown material eluting at the column void volume. Since methyl pyrrolidone contains an amide bond analogous to the protein peptide bond and can hydrogen-bond in a similar fashion, it may competitively remove phenolic material bound to protein.



N-methyl-2-pyrrolidone

McFarlane (1961) has described the competitive removal of similarly bound phenolic material by methyl pyrrolidone. Phenolic material is strongly bound to Sephadex dextran gel (Gelotte, 1960; Determann and Walter, 1968; Brook and Housley, 1969; Brook and Munday,

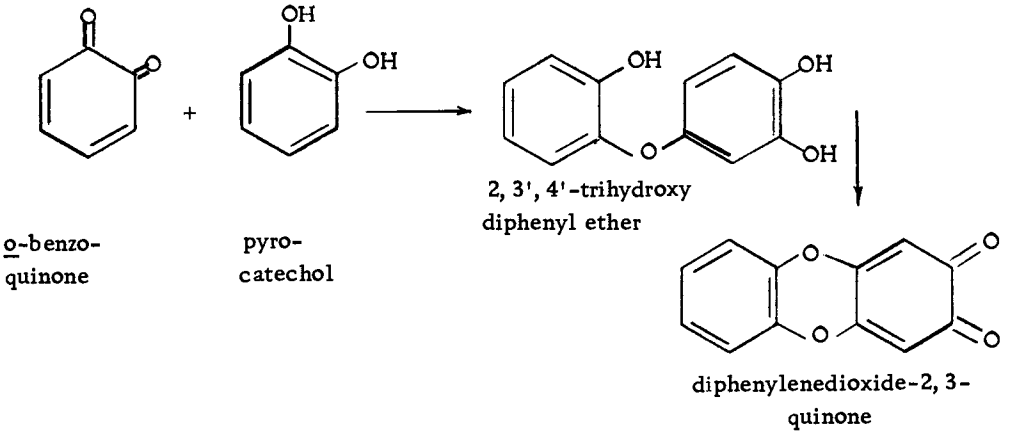
1970). Thus the competitive removal by methyl pyrrolidone of phenolic material originally bound to BSA and subsequent attachment of this material to Sephadex may account for the strongly absorbed green-brown band in the methyl pyrrolidone treated sample. Tanned protein might account for part of this band if the affinity of the BSA-bound phenolics for Sephadex is sufficiently high. However, this possibility is argued against by the elution of tanned BSA without methyl pyrrolidone treatment as a single band at the column void volume. (It should be noted that Sephadex G-50 was substituted for Bio-Gel P-30 in this experiment due to the solubility of polyacrylamide in methyl pyrrolidone.)

Enzymatic Oxidation of Pyrocatechol by Tyrosinase

Initial studies of BSA tanned by tyrosinase oxidized pyrocatechol failed to yield significant amino acid residue modifications. These results were questioned in light of the modifications observed in BSA tanned by base catalyzed oxidized pyrocatechol or by p-benzoquinone (see below). The absence of amino acid modifications was believed to be caused by a pyrocatechol level sufficiently high to result in reaction-inactivation of tyrosinase. To define the characteristics of tyrosinase in this reaction system, a series of experiments was carried out involving spectrophotometric evaluation of initial pyrocatechol oxidation products, variation of enzyme and substrate levels

on pyrocatechol oxidation, and the effect of added polypeptides and amino acids on enzymatic pyrocatechol oxidation.

Enzymatic oxidation of phenols leads to a complex variety of products. o-Benzoquinone, with an absorption maximum at 390 nm, is the probable initial reaction product of tyrosinase oxidation of pyrocatechol (Mason, 1949; Mason et al., 1961; Pierpoint, 1966). Subsequent products of the reaction system depend on a number of factors including pyrocatechol concentration and pH (Mason, 1949; Dawson and Tarpley, 1963). Figure 15 shows the change in absorbancy with time at 390 nm and 410 nm during tyrosinase oxidation of pyrocatechol at pH 5.5, 30° C. The initial increase in absorption at 390 nm is consistent with the formation of o-benzoquinone in the system, the reactants showing no absorption above 300 nm. In the presence of excess pyrocatechol, a quinone chromogen (diphenylenedioxide-2,3-quinone) absorbing maximally at 410 nm may be formed as shown (Dawson and Tarpley, 1963).



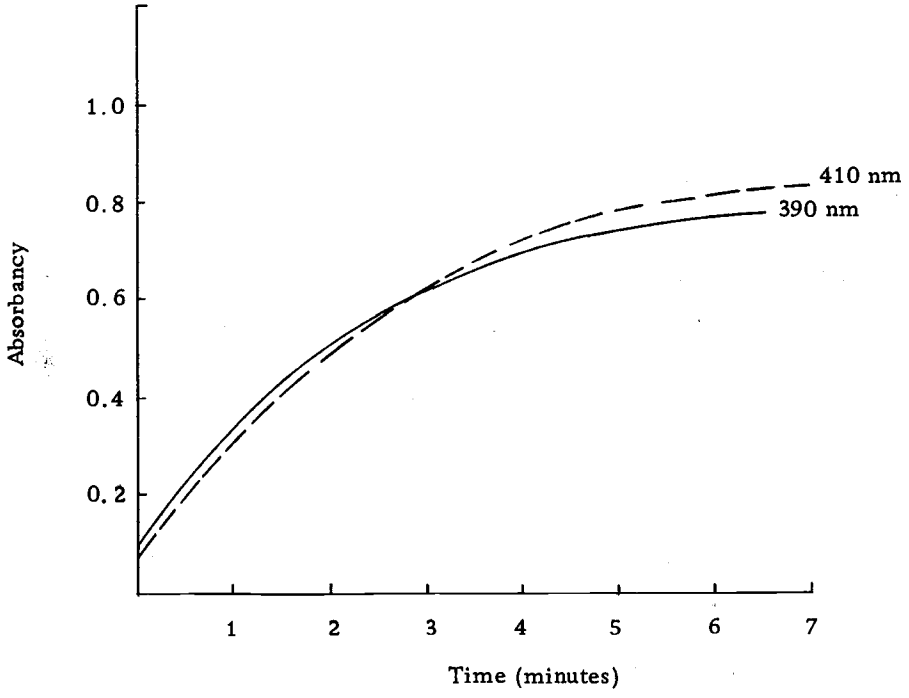


Figure 15. Spectrophotometric evaluation of initial enzymatic pyrocatechol oxidation products. Reaction system: potassium phosphate solution, pH 5.5, 620 μmoles; pyrocatechol 45.4 μmoles (5.0 mg); tyrosinase, 10 ug. Total volume, 3.1 ml. Temperature, 30°C.

The increase in 410 nm over 390 nm absorption after three minutes incubation is consistent with the formation of the 410 nm quinone chromogen in the tyrosinase oxidation system.

The ultraviolet absorption spectrum of an enzymatically oxidized pyrocatechol reaction mixture changed, with time, from the characteristic absorption of pyrocatechol centered at 274 nm to a general absorption throughout the ultraviolet region. The absorbancy of oxidized pyrocatechol products was always greater than that of pyrocatechol itself.

The effect of pH on the initial rate of tyrosinase oxidation of pyrocatechol is shown in Figure 16. An increase in the initial oxidation rate occurred from pH 5.0 through pH 7.0. The effect of pH on pyrocatechol oxidation by tyrosinase is more complex than is suggested by the initial reaction rate data, however, as is indicated in Figure 17. Color formation increases through pH 6.0, while at pH 7.0 and pH 7.5 the color intensity is less than at pH 6.0 even though the initial rate of pyrocatechol oxidation is greater at these higher pH values. Additionally, maximal color formation occurs earlier at the higher pH values. Such results suggest that tyrosinase is inactivated, with more rapid inactivation occurring with increasing pH.

To test for possible inhibition of tyrosinase, the effect of multiple enzyme addition in the tyrosinase-pyrocatechol system

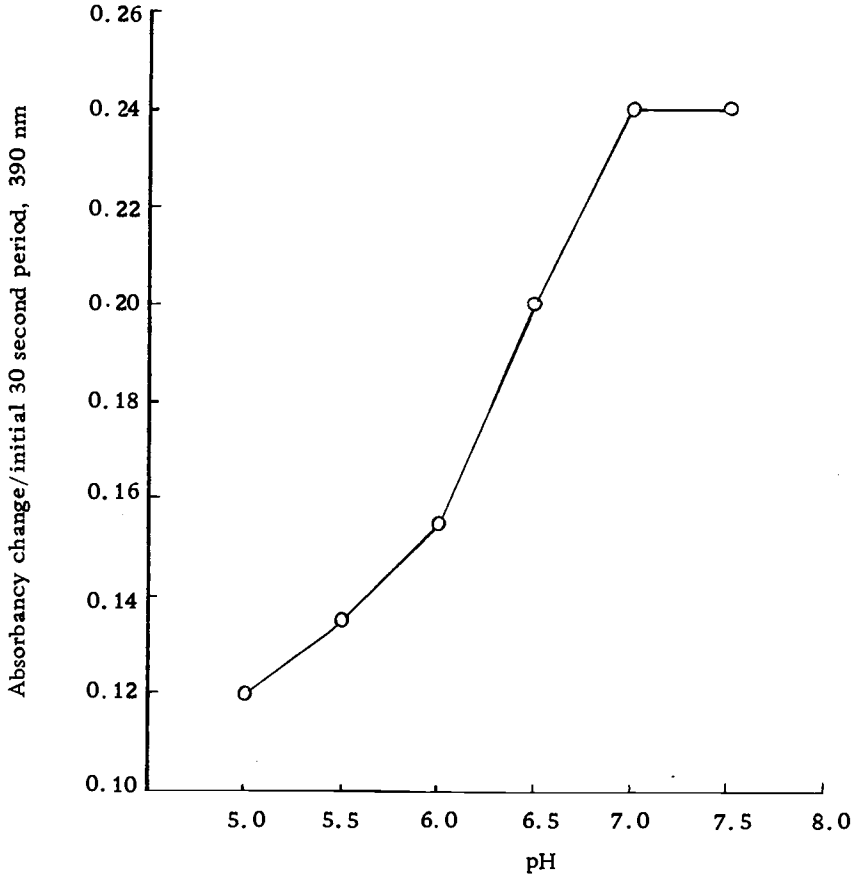


Figure 16. Effect of pH on initial tyrosinase activity. Reaction mixture contains: potassium phosphate solution, 640 μmoles, of appropriate pH; pyrocatechol, 45.4 μmoles (5.0 mg); tyrosinase, 10 μg. Total volume, 3.2 ml. Temperature, 30°C.

was studied as shown in Figure 18. The addition of a second or third identical aliquot of enzyme, following the initial oxidation of pyrocatechol, resulted in further color formation. This additional color formation suggests that tyrosinase rather than pyrocatechol is the limiting factor in the system. Nearly equal initial rates for the two substrate levels would argue against substrate inhibition at these substrate concentrations. Thus, the causative agent in limiting the enzyme under these conditions is probably the product. Inactivation of tyrosinase is well documented (Richter, 1934; Ludwig and Nelson, 1939; Asimov and Dawson, 1950; Ingraham, 1955). Kinetic studies by Ingraham (1954) indicate that the agent responsible for inactivation must be formed after modification of the phenolic substrate and is probably the semiquinone or quinone.

Decreasing color formation with increasing pH is consistent with the conclusion that reaction-inactivation of tyrosinase by the quinone has occurred. Increasing basicity would enhance quinone-protein coupling through a 1, 4-addition reaction, which could result in critical enzyme modification and loss of activity. Figure 17 also indicates that formation of complex reaction products may occur more rapidly at higher pH values. Absorbancy at 390 nm reaches a maximum, then decreases, at pH 7. 5, consistent with the conversion of the initial o-benzoquinone to further reaction products. Similar decreases in 390 nm absorbancy were also observed at lower pH

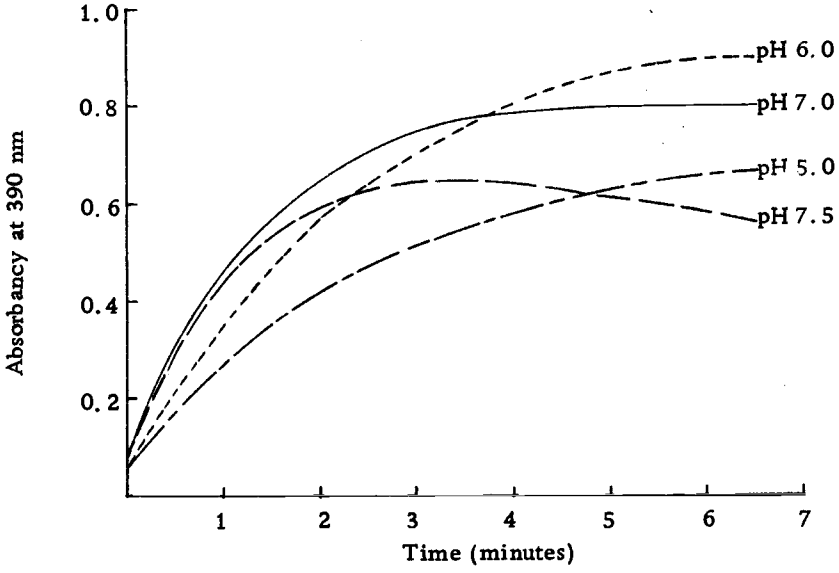


Figure 17. Tyrosinase oxidation of pyrocatechol with time as a function of pH. Reaction system: potassium phosphate solution, 640 μmoles, of appropriate pH; pyrocatechol, 45.4 μmoles (5.0 mg); tyrosinase, 10 μg. Total volume, 3.2 ml. Temperature, 30°C.

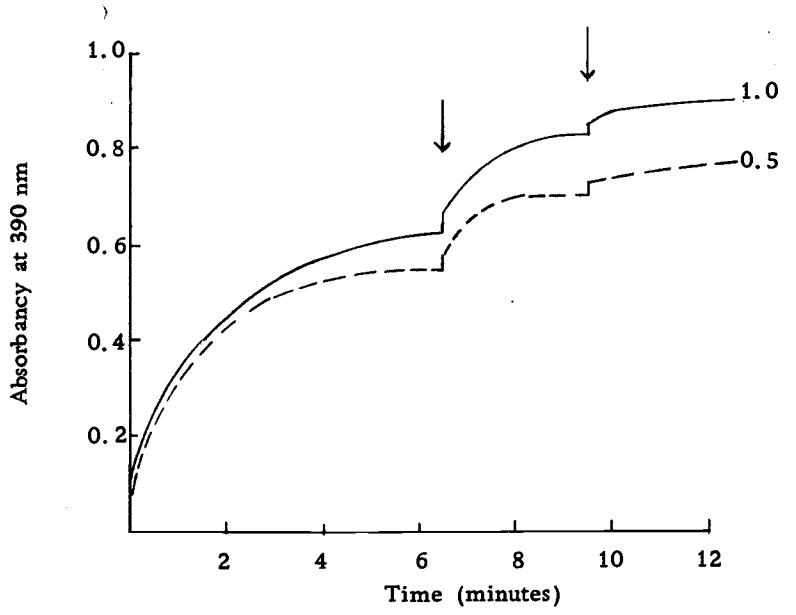


Figure 18. Effect of multiple enzyme addition on tyrosinase oxidation of pyrocatechol. Reaction system: potassium phosphate solution, pH 5.5, 620 μmoles; pyrocatechol 4.54 μmoles (0.5 mg), or 9.08 μmoles (1.0 mg); tyrosinase, 10 μg/aliquot. Volume, 3.1 ml. Temperature, 30°C. Enzyme added initially and at times designated by arrows.

values but only after a longer time.

The effects of various enzyme and substrate levels on tyrosinase oxidation are shown in Figures 19 and 20. The initial rate of pyrocatechol oxidation increases with increasing enzyme level (Figure 19). However, it appears that equivalent amounts of product per unit of enzyme are not formed. Increasing substrate levels above 10 mg/3.1 ml decrease both the initial rate of color formation and the total amount of color formed (Figure 20). Substrate inhibition occurring at high levels of substrate could, in part, account for these observations. It is probable that such substrate inhibition is not unique but rather an example of the general ability of phenolic material to inhibit a wide variety of enzymes. Thus, tyrosinase may be inhibited both by high levels of substrate and by reaction products.

Figure 21 shows the effect of added bovine serum albumin (BSA), glycine, and poly-L-lysine on color formation during the enzymatic oxidation of pyrocatechol. In the presence of any of these compounds the color formed was always less than in their absence (Figure 21-A). As shown for BSA (Figure 21-B), this effect was a function of the level of the compound added. Similar results were obtained for glycine. Product binding by these added compounds, rather than lowered enzymatic activity, may be the reason for the observed decrease in colored product. Unavailability of pyrocatechol as a result of hydrogen bonding might also be a cause of decreased

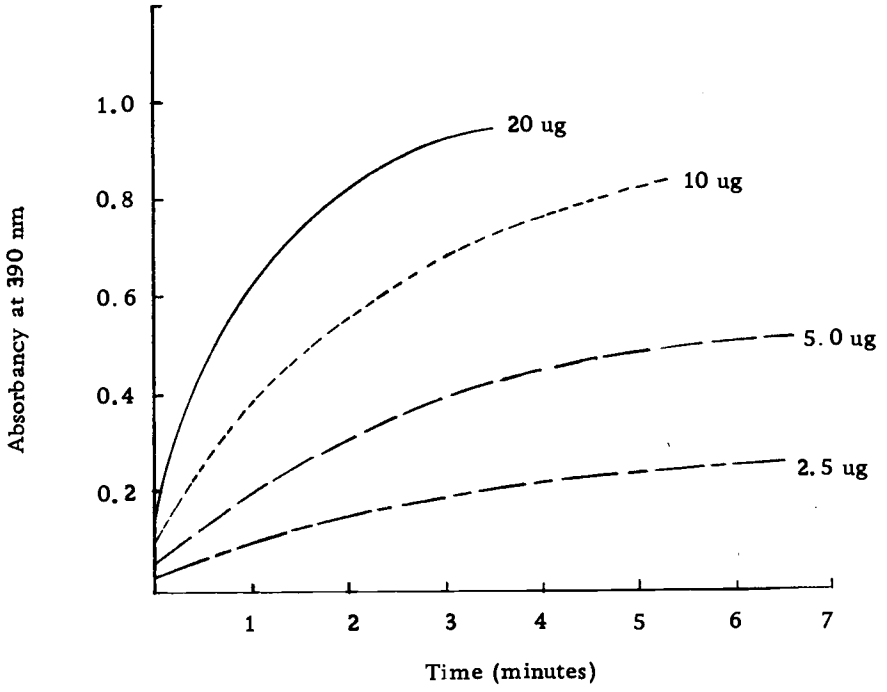


Figure 19. Effect of varying tyrosinase levels on pyrocatechol oxidation. Reaction system: potassium phosphate solution, pH 5.5, 620 µmoles; pyrocatechol 45.4 µmoles (5.0 mg). Tyrosinase at stated levels. Total volume, 3.1 ml. Temperature, 30°C.

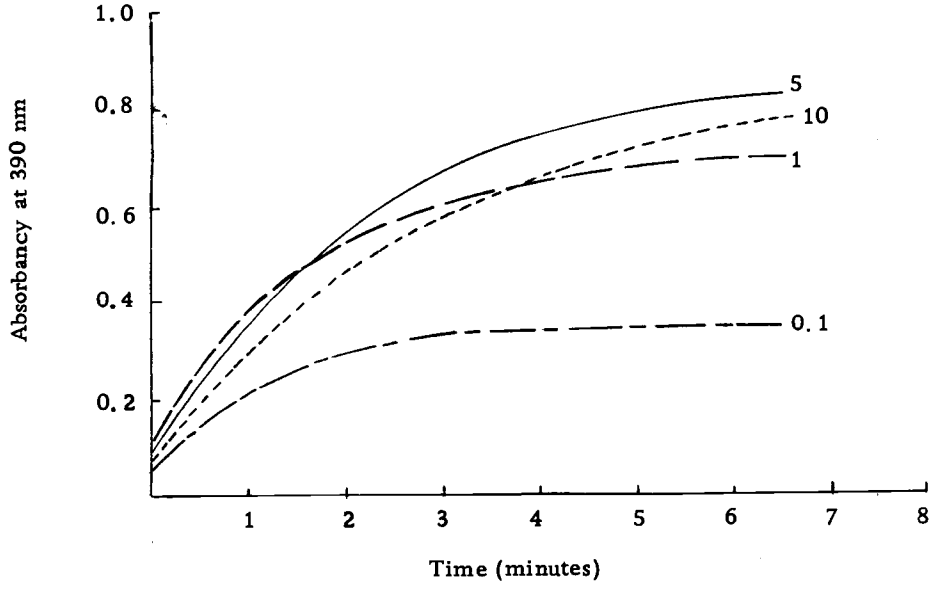
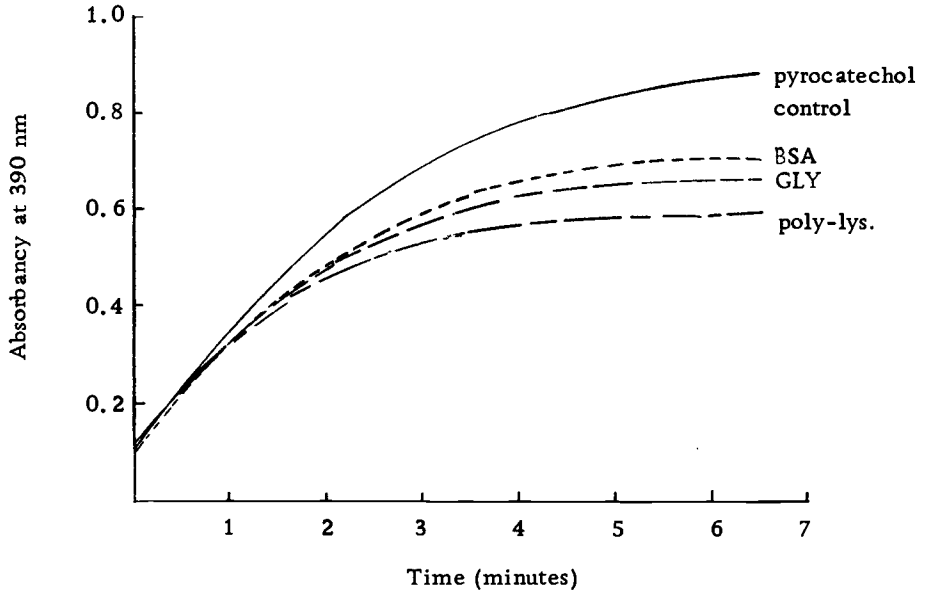
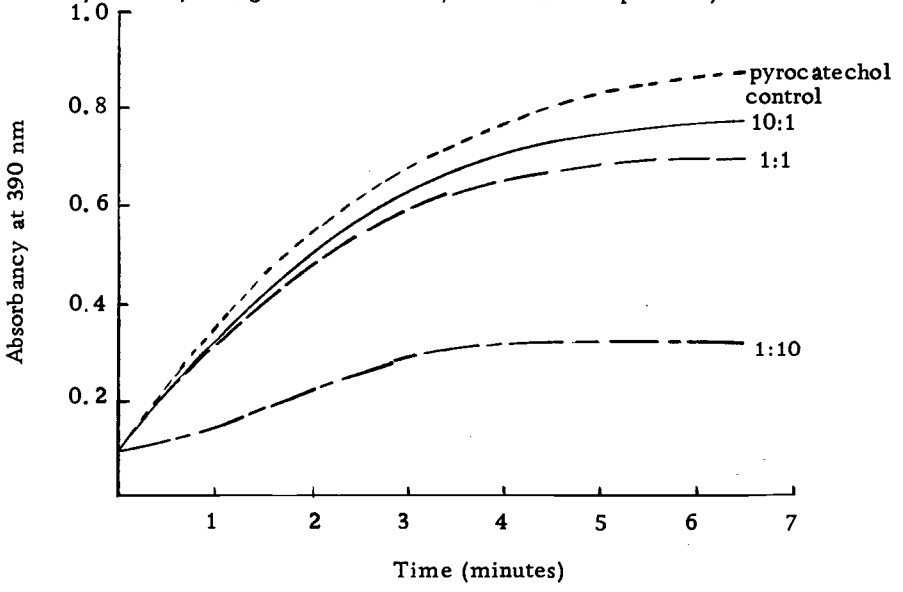


Figure 20. Effect of pyrocatechol level (mg) on its oxidation by tyrosinase. Reaction system: potassium phosphate solution, pH 5.5, 620 µmoles. Pyrocatechol at the following levels; 10 mg (90.8 µmoles), 5 mg (45.4 µmoles), 1 mg (9.08 µmoles), 0.1 mg (.91 µmoles). Tyrosinase, 10 µg. Total volume, 3.1 ml. Temperature, 30°C.



A) Effect of bovine serum albumin (BSA), glycine (GLY), and poly-L-lysine (poly-lys) on tyrosinase oxidation of polycatechol. Reaction system: potassium phosphate solution, pH 5.5, 620 μmoles. Pyrocatechol, 45.4 μmoles (5.0 mg). BSA, GLY, and Poly-lys at a level to provide a pyrocatechol:BSA amino acid residue mole ratio of 1:1. Tyrosinase, 10 μg. Total volume, 3.1 ml. Temperature, 30°C.



B) Effect of bovine serum albumin on tyrosinase oxidation of pyrocatechol at pyrocatechol: BSA-amino acid residue mole ratios of 10:1, 1:1, and 1:10. Reaction system: potassium phosphate solution, pH 5.5, 620 μmoles; pyrocatechol, 45.4 μmoles (5.0 mg); BSA at levels to provide stated mole ratios; tyrosinase, 10 μg. Total volume, 3.1 ml. Temperature, 30°C.

Figure 21. Effect of bovine serum albumin, glycine, and poly-L-lysine on tyrosinase oxidation of pyrocatechol.

color formation in the case of added BSA or poly-L-lysine. Such associations might account for the lag in color formation as well as the low level of color formed at the highest BSA level (Figure 21-B).

Principal Studies

Evaluation of Amino Acid Modifications in Tanned Protein and Amino Acid Systems

Quantitative evaluation of amino acid modifications in tanned bovine serum albumin was carried out by amino acid analysis of acid hydrolysates of protein. Tryptophan is modified during acid hydrolysis and cannot be determined by this procedure. Additionally, methionine was not evaluated due to its apparent modification during the hydrolysis procedure in the presence of p-benzoquinone or pyrocatechol. Treatment of a standard amino acid mixture with p-benzoquinone or pyrocatechol under acid hydrolysis conditions at p-benzoquinone:amino acid mole ratios of 1:1 and 10:1 followed by amino acid analysis indicated that only methionine was apparently modified by phenolic or quinoid compounds and could not be evaluated accurately (refer to Appendix II). Estimation of tryptophan and methionine modification by enzymatically oxidized pyrocatechol was carried out by gas-liquid chromatography of their N-trifluoroacetyl methyl ester derivatives. Initial analysis of amino acid modifications in

the base catalyzed system by thin-layer chromatography of protein hydrolysates did not prove satisfactory due to the lack of adequate quantitative results. Subsequent review of later results indicated that the low level of amino acid modification in this particular system might have been responsible for these results. Compilations of the actual amino acid analysis data are presented in the appendix section of the thesis; the data presented in this section have been expressed as percents of control values. Amino acid modifications have been determined from amino acid/glycine ratios assuming that glycine modification does not occur. The unreactive side chain of glycine, a single hydrogen, and the internal position of glycine in BSA justifies this assumption. Any error in glycine analysis would, however, be incorporated into the overall experimental error. Under normal hydrolysis conditions cysteine is converted to cysteic acid. The low level of cysteine in BSA (less than one free thiol group per peptide chain (Pierpoint, 1967b)) prohibits an accurate estimation of cysteine modification. Cystine is reported as half-cystine in the hydrolysis procedure. Cystine modification was estimated from a cystine/glycine peak height ratio rather than a peak area ratio due to the, at times, poor alanine-cystine chromatographic separation. It should also be noted that an amino acid analyzer buffer change occurring close to the valine elution point made estimation of this amino acid difficult. Many of the amino acid modifications were

carried out at pH 5.5 or 8.0. It is recognized that buffer capacity of the potassium phosphate system is low at these values. The phosphate system was chosen at pH 8.0 to minimize interfering reactions that might be caused by quinone reaction with amino functions of buffers normally used in this range. The use of pH 5.5 in many of the tyrosinase studies was done to minimize reaction-inactivation of tyrosinase.

The results of amino acid analysis of BSA tanned by the base catalyzed, air oxidation of pyrocatechol are presented in Tables 1 and 2. Amino acid modifications, determined as a function of pyrocatechol concentration (Table 1) and pH (Table 2), indicate that modifications are restricted to cystine and lysine. Cystine is the only amino acid modified at pH 8.0 (Table 1) (α = 0.05 according to a one-tailed Mann-Whitney rank test at 2:1 and 10:1 pyrocatechol: amino acid residue mole ratios, where α is termed the significance level). In the discussion below α determined from the Mann-Whitney test will be designated α_{mw}; that determined from the t-test will be designated α_t. Amino acid modifications result in a decrease from the control value of the particular amino acid; therefore the use of a one-tailed test is applicable here. Using these same statistics, lysine and histidine modification appear to occur at the higher pyrocatechol concentrations (Table 1). However, noting that amino acid analysis values vary within approximately ±10% of

Table 1. Amino acid analysis of bovine serum albumin tanned by the base catalyzed oxidation of pyrocatechol, pH 8.0. Effect of pyrocatechol:BSA-amino acid residue mole ratio on amino acid modification. Data expressed as percent of control values.

Amino acid	Mole ratios - pyrocatechol:BSA-amino acid residue 1:10	1:1	2:1	10:1
Aspartic acid	101	102	98	93
Threonine	103	103	101	97
Serine	105	108	103	99
Glutamic acid	104	100	99	96
Proline	105	100	101	96
Glycine	100	100	100	100
Alanine	106	109	101	99
Half cystine	88	96	84	54
Valine	110	89	106	104
Isoleucine	107	95	98	94
Leucine	104	106	101	97
Tyrosine	103	108	104	103
Phenylalanine	100	108	97	92
Lysine	94	95	88	89
Histidine	98	95	93	88
Arginine	106	101	98	98

Reaction mixture contained: buffer, potassium phosphate, pH 8.0, 400 μmoles; BSA, 20 mg; pyrocatechol, 14.8, 148, 296, 1480 μmoles to provide pyrocatechol:BSA-amino acid residue mole ratios noted above. Total volume, 2.5 ml. Temperature, 30°C. Air oxidized 24 hours. 1.0 ml aliquot used for amino acid analysis.

Table 2. Amino acid analysis of bovine serum albumin tanned by the base catalyzed oxidation of pyrocatechol. Effect of pH on amino acid modification. Data expressed as percent of control values.

Amino acid	pH 8.0	pH 9.5
Aspartic acid	98	97
Threonine	101	98
Serine	103	103
Glutamic acid	99	101
Proline	101	87
Glycine	100	100
Alanine	101	104
Half-cystine	84	82
Valine	106	103
Isoleucine	98	107
Leucine	101	104
Tyrosine	104	96
Phenylalanine	97	101
Lysine	88	69
Histidine	93	94
Arginine	98	82

Reaction mixture contained: buffer, 400 μmoles, potassium phosphate, pH 8.0, or, potassium carbonate, pH 9.5; BSA, 20 mg; pyrocatechol, 296 μmoles, to provide a pyrocatechol:BSA-amino acid residue mole ratio of 2:1. Total volume, 2.5 ml. Temperature, 30°C. Air oxidized, 24 hours. 1.0 ml aliquot used for amino acid analysis.

control values, there is reason to question whether these amino acids are modified.

Lysine modification increases with increasing pH (Table 2) suggesting that the ε-amino group is more reactive in the non-ionized form or that catechol is more reactive in the ionized form. An interesting application of these results is that, since lysine is an essential dietary amino acid, a food product based on a plant source may yield a nutritionally inferior product if subjected to alkaline treatment during processing. At pH 9.5, modification of arginine (α_{mw}=0.05) occurs in addition to that of cystine and lysine.

The effect of p-benzoquinone on amino acid modification in BSA at pH 8.0 is shown in Table 3. The pattern of amino acid modification is similar to that observed with BSA tanned by base-catalyzed, air-oxidized pyrocatechol; however, modifications of cystine, lysine, and histidine, are considerably greater in the p-benzoquinone incubation system. Modifications of these amino acids increase with increasing p-benzoquinone level, losses reaching 52, 58, and 76 percent for cystine, lysine, and histidine respectively at a p-benzoquinone:amino acid residue mole ratio of 5:1. These values contrast with those of 46, 11, and 12 for the same three respective amino acids in the base catalyzed system at pH 8.0 and a pyrocatechol:amino acid mole ratio of 10:1. α_{mw} equals 0.05 for these modifications in the p-benzoquinone system. The similar,

Table 3. Amino acid analysis of bovine serum albumin tanned by p-benzoquinone, pH 8.0. Effect of p-benzoquinone:BSA-amino acid residue mole ratio on amino acid modification. Data expressed as percent of control values.

Amino acid	Mole ratios-p-benzoquinone:BSA-amino acid residue 1:10	1:1	2:1	5:1
Aspartic acid	92	100	92	96
Threonine	96	100	94	98
Serine	105	108	111	106
Glutamic acid	94	99	96	98
Proline	86	95	89	104
Glycine	100	100	100	100
Alanine	96	102	97	100
Half-cystine	66	66	58	48
Valine	95	96	97	108
Isoleucine	96	95	98	94
Leucine	97	99	97	99
Tyrosine	92	101	91	94
Phenylalanine	96	99	95	98
Lysine	87	56	47	42
Histidine	85	51	42	24
Arginine	94	104	93	98

Reaction mixture contains buffer, potassium phosphate, pH 8.0, 400 μmoles; BSA, 600 μg; p-benzoquinone, 0.44, 4.43, 8.86, 22.2 μmoles to provide p-benzoquinone:amino acid residue mole ratios stated above. Total volume 2.5 ml. Temperature, 30°C. Air oxidized 24 hours. 1.0 ml aliquot used for amino acid analysis.

though more marked, amino acid modifications in the p-benzoquinone system as contrasted with the base catalyzed pyrocatechol oxidation procedure imply that the quinone is the probable reactive species and, furthermore, that effective quinone levels are low in the base catalyzed system. In p-benzoquinone treated samples, ammonia levels greater than controls found during amino acid analysis suggest that some non-enzymatic deamination may have occurred. Reactions of this type have been reviewed by Mason (1955a).

Results of amino acid analysis of BSA exposed to tyrosinase-oxidized pyrocatechol are shown in Tables 4, 5, and 6. Amino acid modifications were studied as a function of tyrosinase level, pH, and pyrocatechol concentration. In general, the pattern of amino acid modification was similar to that previously observed; cystine, lysine, and histidine were the principal amino acids modified.

Table 4 shows the effect of varying tyrosinase levels on observed amino acid modifications at pH 5.5 and a pyrocatechol:amino acid residue mole ratio of 1:1. Cystine modifications were observed at tyrosinase levels of 55 units or more with levels of cystine remaining essentially constant at approximately 70 percent of controls (α=0.025). Modifications of lysine and histidine increased with increasing enzyme level. Modifications observed reached 30 and 34 percent for lysine and histidine at a tyrosinase level of 220 units (α_t for all modifications at 110 or 220 units of tyrosinase was 0.025 or lower). Although

Table 4. Amino acid analysis of bovine serum albumin tanned by the tyrosinase oxidation of pyrocatechol, pH 5.5. Effect of enzyme level on amino acid modification. Data expressed as percent of control values.

Amino acid	Tyrosinase levels, units of enzyme 11	55	110	220
Aspartic acid	105	106	104	98
Threonine	104	106	105	100
Serine	104	105	104	97
Glutamic acid	108	109	107	100
Proline	110	115	110	106
Glycine	100	100	100	100
Alanine	107	108	104	98
Half-cystine	93	69	71	69
Valine	117	111	105	92
Isoleucine	94	91	94	91
Leucine	102	102	106	96
Tyrosine	107	104	98	88
Phenylalanine	108	108	106	97
Lysine	95	85	82	70
Histidine	95	84	77	66
Arginine	98	96	101	93

Reaction mixture contained: potassium phosphate solution, pH 5.5, 620 μmoles. BSA, 1.23 mg; pyrocatechol, 9.08 μmoles, to provide a pyrocatechol:BSA-amino acid residue mole ratio of 1:1; tyrosinase, ‡100 units/mg stated activity, used at the levels noted above. Total volume, 3.1 ml. Temperature, 30°C. Incubation time, 3 hours. 1.0 ml aliquot used for amino acid analysis.

amino acid analysis values vary up to ±15 percent of controls, modification of lysine and histidine may occur at a tyrosinase level of 55 units considering that modifications do increase at higher tyrosinase levels. Greater modifications of cystine at lower enzyme levels would suggest that this amino acid residue is more reactive to pyrocatechol oxidation products than are histidine and lysine. This is also supported by the observed greater modifications for cystine than for other amino acids at lower pyrocatechol concentrations on the base catalyzed system.

The free thiol content of BSA is, at most, one free thiol group per peptide chain (Pierpoint, 1967b). The literature reports only the reactivity of the cysteine residue. The modifications of cystine observed throughout these studies may result from the reduction of disulfides to thiol groupings by phenols. The E_o' values of -0.340 for cystine/cysteine and -0.350 for hydroquinone/o-benzoquinone (Sober, 1968) support this conclusion. The preferential reactivity of sulfhydryls with one of the oxidation products of phenol, quinone, has been reported (Mason, 1955a; Pierpoint, 1969a).

Table 5 shows the effect of pH on observed amino acid modifications at a 1:1 amino acid residue:pyrocatechol mole ratio and 110 units of tyrosinase. Cystine, lysine, and histidine were observed to be the amino acids modified. Cystine modification remained constant at approximately 30 percent throughout the pH range of

5.5 to 8.0 (α_t no greater than 0.005). Lysine modification increased with increasing pH, reaching a level of 34 percent modification at pH 8.0 (α_t=0.005 in all cases). Histidine levels range between 77 percent and 88 percent of controls with no apparent pattern of increased modification with increasing pH (α_t no greater than 0.025 in any case).

The effect of pyrocatechol concentration on amino acid modification at pH 5.5 using 110 units of tyrosinase is shown in Table 6. No apparent modification occurs at a pyrocatechol:amino acid residue mole ratio of 1:10. (Although the isoleucine value is 83 percent of the control at this mole ratio, the α_t values in most cases indicate non-rejection of the null hypothesis at α_t = 0.10, casting doubt on its actual modification in this case.) Cystine, lysine, and histidine were modified at a 1:1 mole ratio (α_{mw}=0.05, α_t=0.005 in all cases).

Modifications of a similar nature occur in all systems studied. Cystine, lysine, and histidine are the principal amino acids modified. At similar reactant mole ratios, greater modifications occur at pH 5.5 in the tyrosinase system than occur at pH 8.0 in the base catalyzed system. Additionally; modifications occur at lower pyrocatechol concentrations in the tyrosinase system as compared to the base catalyzed system. These modifications probably reflect, in part, a greater effective quinone level in the enzymatic systems. The even greater modifications observed in the p-benzoquinone system

Table 5. Amino acid analysis of bovine serum albumin tanned by the tyrosinase oxidation of pyrocatechol. Effect of pH on amino acid modification. Data expressed as percent of control values.

Amino acid	pH 5.5	7.0	7.5	8.0
Aspartic acid	104	104	100	102
Threonine	105	106	101	100
Serine	104	106	104	101
Glutamic acid	107	107	104	104
Proline	110	112	111	102
Glycine	100	100	100	100
Alanine	104	104	102	104
Half-cystine	71	69	71	67
Valine	105	109	110	109
Isoleucine	94	96	94	91
Leucine	106	104	99	100
Tyrosine	98	103	107	103
Phenylalanine	106	107	105	105
Lysine	82	74	65	66
Histidine	77	84	78	88
Arginine	101	102	91	93

Reaction mixture contains: potassium phosphate solution, 620 μmoles, of stated pH; BSA, 1.23 mg; pyrocatechol, 9.08 μmoles, to provide pyrocatechol:BSA-amino acid residue mole ratio of 1:1; Tyrosinase, 110 units (100 μg). Total volume, 3.1 ml. Temperature, 30°C. Incubation time, 3 hours. 1.0 ml aliquot used for amino acid analysis.

Table 6. Amino acid analysis of bovine serum albumin tanned by the tyrosinase oxidation of pyrocatechol. Effect of pyrocatechol:BSA-amino acid residue mole ratio on amino acid modification. Data expressed as percent of control values.

Amino acid	1:10	1:1
Aspartic acid	110	104
Threonine	108	105
Serine	114	104
Glutamic acid	106	107
Proline	100	110
Glycine	100	100
Alanine	106	104
Half-cystine	104	71
Valine	92	105
Isoleucine	83	94
Leucine	102	106
Tyrosine	99	98
Phenylalanine	106	106
Lysine	95	82
Histidine	88	77
Arginine	95	101

Reaction mixture contains: potassium phosphate solution, pH 5.5, 620 μmoles; BSA, 1.23 mg; pyrocatechol, 9.08 and 0.91 μmoles to provide pyrocatechol:BSA-amino acid residue mole ratios stated above; tyrosinase, 110 units (100 μg). Total volume, 3.1 ml. Temperature, 30°C. Incubation time, 3 hours. 1.0 ml aliquot used for amino acid analysis.

support this viewpoint. It is interesting to note that serine, threonine, and tyrosine, all with potentially nucleophilic hydroxyl groups in their side chains, show no apparent modification.

These amino acid modifications are consistent with observations of Pierpoint (1969b), Haider, Frederick, and Flaig (1965), and others (Mason, 1955a, Wood and Ingraham, 1965) on the reactivity of protein functional groups with quinones. Evidence was presented by these investigators for the primary involvement of α- and ε-amino groups and sulfhydryl groups in the quinone reaction with protein.

As noted above, estimation of tryptophan and methionine modification in tanned BSA by amino acid analysis of protein acid hydrolysates was not possible. Estimation of tryptophan modification, in particular, presented unique analytical problems due to the presence of phenolic material and the probable nature of oxidized phenolic-amino acid interaction. As noted previously, tryptophan is modified during acid hydrolysis of protein and cannot be determined by this procedure. Estimation of tryptophan modification by base hydrolysis of protein was not possible, however, due to the increase in quinone-amino acid interaction in basic systems. Additionally, estimation by microbial means was not possible because of the presence of growth inhibiting phenolic material. Estimation of modification by the spectrophotometric p-dimethylaminobenzaldehyde condensation method of Spies and Chambers (1948) proved to be unsatisfactory;

the presence of phenolic material decreased absorbancy as shown by an N-acetyl-L-tryptophan ethyl ester standard. Estimation of tryptophan modification employing methanol extraction of the reaction system followed by column chromatography on DEAE cellulose also proved unsatisfactory. Preliminary studies indicated that elution of N-acetyl-L-tryptophan ethyl ester (NATEE) from DEAE cellulose employing methanol as the eluting solvent was rapid and quantitative, and that pyrocatechol oxidation products remained bound to the anion exchange cellulose column. Additionally, employing methanol solvent extraction, added NATEE was quantitatively recoverable from enzymatic oxidation reaction products. Analysis of NATEE exposed to enzymatically oxidized pyrocatechol indicated, however, that an apparent modified NATEE eluted along with unreacted NATEE, preventing estimation of tryptophan modification by difference. NATEE and tryptophan have no absorption in the visible region of the spectrum, but have similar absoprtion spectra in the ultraviolet. The ultraviolet absorption spectrum for NATEE is shown in Figure 22. The eluted fraction from the reacted NATEE system was pink in color and gave absorption spectra shown in Figure 23. Although these results do not permit quantitation of tryptophan modification, they do, however, suggest that tryptophan was modified by enzymatically oxidized pyrocatechol, producing a pink chromogen with a visible absorption spectrum as

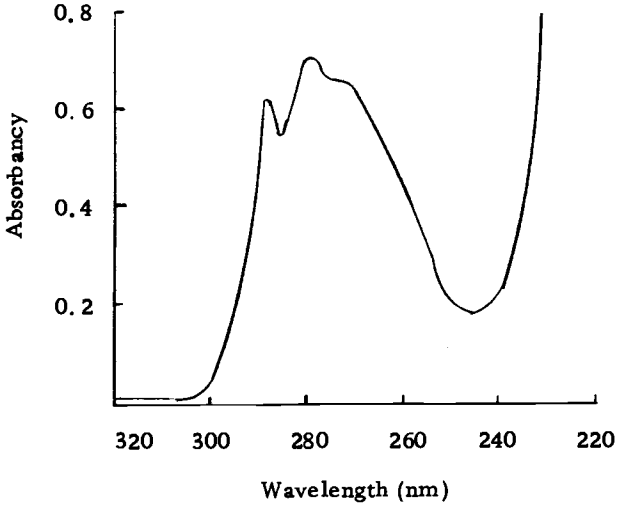


Figure 22. Ultraviolet spectrum of N-acetyl-L-tryptophan ethyl ester. 3.13 μmoles (0.858 mg)/ml in 100 percent methanol; diluted 1:25 with the same solvent. Final concentration, 0.125 μmoles/ml. Room temperature.

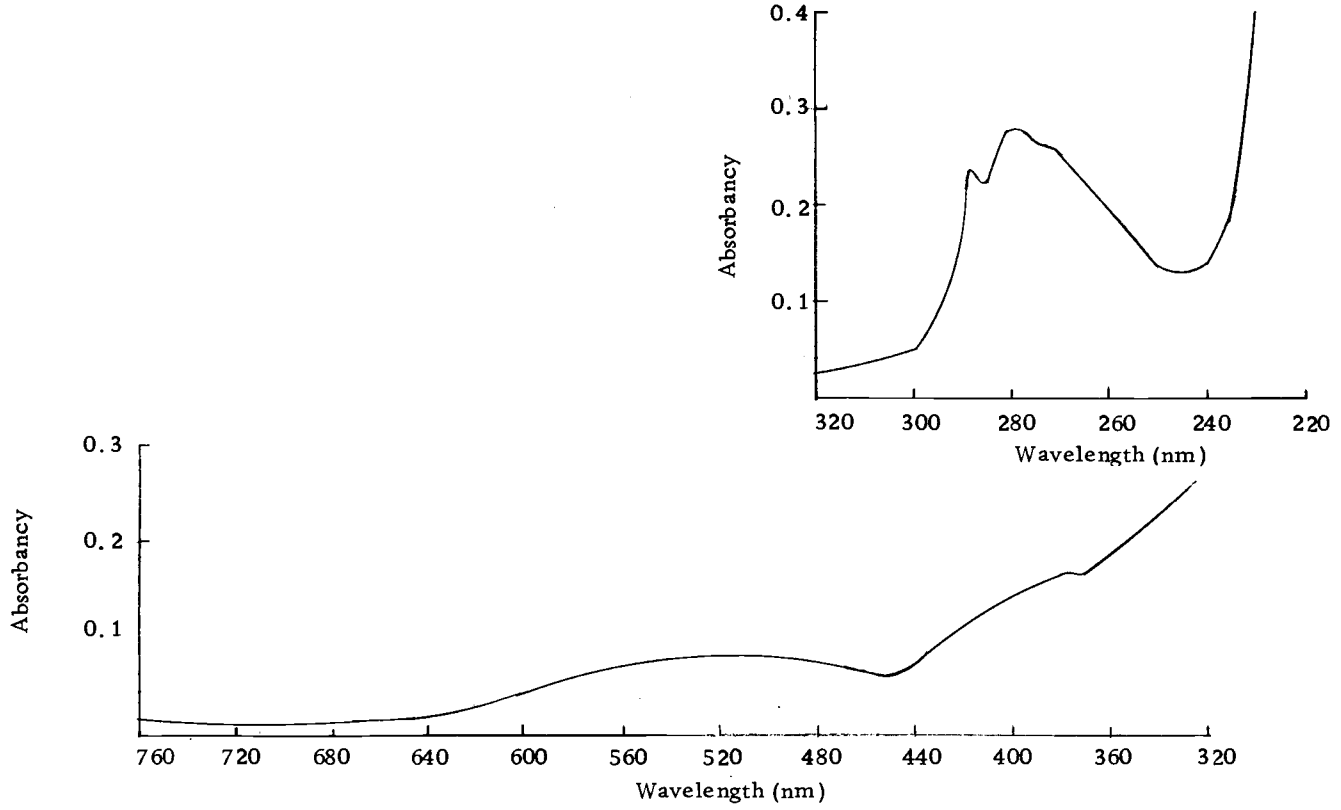


Figure 23. Visible and ultraviolet absorption of N-acetyl-L-tryptophan ethyl ester (NATEE) exposed to enzymatically (tyrosinase) oxidized pyrocatechol. Reaction mixture contained: potassium phosphate solution, pH 5.5, 520 μmoles; pyrocatechol, 9.08 μmoles (1.0 mg); NATEE, 9.12 μmoles (2.5 mg); tyrosinase, 110 units (0.1 mg). Total volume, 2.6 ml. Temperature, 30°C. Incubation time, 3 hours. Material from vacuum dried reaction mixture methanol extracted, concentrated to 0.5 ml, and placed on 1.0 x 10 cm DEAE cellulose column. NATEE fraction collected. Solvent employed, 100% methanol. Ultraviolet spectrum determined on a 1:50 dilution of the NATEE fraction.

shown. Pierpoint (1966) observed a similar chromogen with a broad absorption peak centered between 520 and 530 nm when indole was exposed to enzymatically oxidized chlorogenic acid.

In light of the above difficulties in estimating tryptophan modification by oxidized pyrocatechol, a gas-liquid chromatographic analysis of N-trifluoroacetyl-L-tryptophan methyl ester exposed to enzymatically oxidized pyrocatechol was attempted. Such a derivative would be related to the form in which tryptophan occurs within a polypeptide chain and would permit some estimation of tryptophan residue reactivity with oxidized phenolics. Additionally, because methionine appeared to be modified during the acid hydrolysis procedure when quinone or phenolic material was present, a similar gas chromatographic procedure was carried out using N-trifluoroacetyl-DL-methionine methyl ester which had been exposed to enzymatically oxidized pyrocatechol. Table 7 presents the observed modifications of N-TFA-L-tryptophan ME and N-TFA-DL-methionine ME as a function of pyrocatechol:amino acid derivative mole ratio. The actual analytical data are presented in Appendix III. Modification of both derivatives occurred at pyrocatechol:amino acid mole ratios of 1:10 and 1:1. N-TFA-L-tryptophan ME levels averaged 88 percent of controls (α_t equal to 0.05 or less except in one instance where α_t = 0.20) for a 1:10 mole ratio and 73 percent of controls (α_t=0.005) for a 1:1 mole ratio. N-TFA-DL-methionine ME levels

Table 7. Modification of N-trifluoroacetyl-L-tryptophan methyl ester and N-trifluoroacetyl-DL-methionine methyl ester by enzymatically oxidized pyrocatechol. Data expressed as percent of control value.

Pyrocatechol: amino acid mole ratio	N-TFA-L-tryptophan methyl ester	N-TFA-DL-methionine methyl ester
1:10	88	92
	(86-89)	(90-95)
1:1	73	79
	(72-74)	(76-82)

Reaction mixture contained: potassium phosphate solution, pH 5.5, 540 μmoles; N-TFA-L-tryptophan ME or N-TFA-DL-methionine ME, 9.08 μmoles; pyrocatechol, 9.08 or 0.91 μmoles; tyrosinase, 220 units (0.2 mg); reagent acetone, 0.1 ml. Total volume, 2.8 ml. Temperature, 30°C. Incubation time, 2 hours. Degree of modification was determined by gas-liquid chromatography of methanol extracts of vacuum dried reaction mixtures, as compared to controls.

averaged 92 percent of controls (α_t=0.10 or less) at a 1:10 mole ratio and 79 percent of controls (α_t=0.005) at a 1:1 mole ratio.

Digestibility of Tanned Bovine Serum Albumin

Formation of complexes between proteins and phenolic material may readily take place by both covalent and non-covalent bonding as noted in these studies and in the introduction. It would be expected that such complexes would modify the susceptibility of the protein to digestion by proteases. Digestibility represents, therefore, an additional technique for assessing the extent of interaction between protein and phenolic material.

Bovine serum albumin tanned in the same manner as that employed in the amino acid modification study was subjected to pepsin and trypsin digestion, using the acid or base uptake required to maintain a constant pH to evaluate proteolytic activity. Table 8 presents the results of the tanned BSA digestibility study with data expressed as percents of control values; compilation of the actual analytical data is presented in Appendix 3. Trypsin digestibility of BSA tanned by base catalyzed air oxidized pyrocatechol (pH 8.0) was reduced by approximately 85 percent. BSA tanned by enzymatically oxidized pyrocatechol was essentially indigestible by trypsin according to this procedure. A synthetic substrate, N-benzoyl-L-arginine-ethyl ester was used to insure that trypsin inactivation by oxidized

Table 8. Proteolytic digestibility of tanned BSA. Data expressed as percent of control values.

	Trypsin	Pepsin
Base catalyzed air oxidation of pyrocatechol, pH 8.0		
1:10*	13	70
1:1*	14	64
Enzymatic oxidation of pyrocatechol, pH 5.5		
1:10*	1	53

*Pyrocatechol:BSA-amino acid residue mole ratios

Reaction systems: base catalyzed air oxidation of pyrocatechol: buffer, potassium phosphate, pH 8.0, 400 μmoles; BSA, 12.5 mg; pyrocatechol, 92.6 or 9.26 μmoles to yield pyrocatechol: BSA-amino acid residue mole ratios of 1:10 and 1:1. Total volume, 2.5 ml. Temperature, 30°C. Time, 24 hours. Enzymatic oxidation of pyrocatechol: potassium phosphate solution, pH 5.5, 520 μmoles; BSA, 12.5 mg; pyrocatechol, 9.26 μmoles; tyrosinase, 110 units (0.1 mg). Total volume, 2.6 ml. Temperature, 30°C. Incubation time 3 hours. Digestibility determined by evaluating enzymatic activity using an automated pH stat method employing a radiometer type TTTlc automatic titrator. Pepsin or trypsin levels used, 0.1 mg/1.0 mg tanned BSA. pH of digestion: trypsin, pH 7.5; pepsin, pH 2.5.

phenolic material did not occur. Similar base uptakes by a synthetic substrate control and by tanned BSA-trypsin samples to which synthetic substrate was added after completion of the digestion study indicated that inactivation of trypsin did not occur. Pepsin digestibility of tanned BSA was also markedly reduced but to a lesser extent than was trypsin digestibility. Pepsin digestion of BSA tanned by the base catalyzed system was reduced by approximately 30-35 percent while digestion of BSA tanned enzymatically was reduced by 47 percent. The low level of oxidized phenolic material required to reduce greatly proteolytic activity, particularly in the case of trypsin digestion, is noteworthy; a phenolic level only one-tenth that of the amino acid level present in the system was sufficient to markedly reduce protease action. The greater pepsin digestibility of tanned BSA may be due to at least three factors. Since the pepsin digestion was carried out at pH 2.5, it is probable that the BSA was denatured to some extent, resulting in a greater exposure of peptide bonds susceptible to pepsin cleavage with a corresponding increase in measured hydrolysis. Secondly, pepsin is less specific than trypsin, cleavage occurring most rapidly at those peptide bonds formed by the amino or carboxyl groups of the aromatic amino acids, phenylalanine and tyrosine, although peptide bonds involving leucine, alanine, glutamic acid, cystine, or cysteine residues may also be involved (Smyth, 1967). Tryptic activity, on the other hand,

is directed towards cleavage of the peptide bond between the carboxyl group of lysine or arginine and the amino group of the adjacent amino acid. Thirdly, sites of trypsin cleavage can be reduced by modification of the lysine side chains. No amino acids that are specifically adjacent to peptide bonds cleaved by pepsin are greatly modified whereas trypsin digestion would be reduced by the demonstrated lysine modification in tanned BSA. The marked reduction in digestibility, particularly in the case of trypsin, would suggest that complex formation had taken place between oxidized pyrocatechol and BSA. Similar decreases in digestibility of tanned protein have been noted by Feeny (1969); casein incubated at pH 7.6 with oak leaf tannins was found to be almost completely indigestible by trypsin. The lower digestibility of the tyrosinase treated samples is of interest since enzymatic oxidation of phenolic material is probably the important mode of phenolic modification in biological systems.

The decrease in digestibility of tanned protein would be a function of the additive effect of covalent and non-covalent binding of phenolic material to protein. The extent of amino acid modification in the base catalyzed or enzymatically oxidized pyrocatechol system is seldom as great as the observed decreases in digestibility. This would suggest therefore that, as one possibility, non-covalent association of phenolic material may be more extensive than amino acid modifications. This point of view is perhaps most evident in

the tyrosinase oxidized system where the greatest amino acid residue modification observed was 35 percent for lysine (pH 7.5, 1:1 pyrocatechol:amino acid residue mole ratio, 110 units tyrosinase), yet a similarly tanned BSA was found to be almost completely indigestible by trypsin. It may also be possible, however, that structural changes in the protein brought about by tanning may result in digestibility decreases.

The nutritional and biochemical aspects of the observed amino acid modifications and digestibility decreases should be emphasized. The complexing of unoxidized and oxidized phenolic material with protein through covalent or non-covalent bonding results in altered physical and chemical properties reflected in modified biochemical and nutritional behavior.

The above studies indicated that lysine, histidine, methionine, tryptophan and cystine were modified by pyrocatechol oxidation products. All of these amino acids except cystine are nutritionally essential (amino acid requirements for man have recently been reviewed by Irwin and Hegsted (1971)). Additionally, cystine may spare methionine if methionine is present in suboptimal amounts (Irwin and Hegsted, 1971). Modifications to nutritionally essential amino acids would be critical, reducing the biological value of a protein to its least limiting amino acid.

The decrease in digestibility of tanned protein demonstrated

in this study and by others (Feeny, 1969; Horigome and Kandatsu, 1964, 1966a, 1966b) can lead to proteins of lower nutritional value. Growth depression resulting from the formation of an indigestible nitrogen fraction due to phenolic-protein complexes has been demonstrated (Tamir and Alumot, 1970).

Such modifications in the nutritional value of protein resulting from amino acid modification and a reduction in digestibility would be especially critical in such items as plant protein concentrates designed as dietary protein supplements. It is imperative that food processing operations be designed so as to minimize the formation of phenolic-protein complexes. The nutritional aspects of protein phenol complexes are reviewed more fully in Appendix I.

The complexing of unoxidized and oxidized phenolic material with protein often results in damaging effects on the biochemical aspects of a system. Such complexes may distort the native information of a protein resulting in altered solubility properties (Caldron et al., 1968; Van Buren and Robinson, 1969) and decreased or eliminated enzymatic activity. Cysteine and histidine are often involved in enzyme active sites; modifications occurring to these amino acids during isolation procedures could result in inactive enzymes. The formation of such phenol-protein complexes has been reduced through the use of competitive phenol binding agents and by inhibiting phenol oxidation (Loomis and Battaile, 1966; Anderson, 1968).

Methionine has been reported to be low in plant proteins (Swaminathan, 1967). The low values found may, in part, be attributed to methionine modification by quinones or phenolics during the acid hydrolysis portion of the analytical procedure as was found in the studies discussed above. In some situations phenolic compounds may interfere with and prevent accurate protein determination in biochemical systems. The biuret, Lowry, and ultraviolet spectrophotometric procedures are susceptible to interference by phenolics (Solecka et al., 1968; Potty, 1969). A more complete review of the biochemical aspects of protein-phenol complexes is presented in Appendix I.

Isolation and Characterization of a Glycine-p-benzoquinone Adduct

To more clearly define the nature of the probable amino acid-quinone adduct formed in the base catalyzed and enzymatic phenolic oxidation-protein systems employed, thin-layer chromatography of a glycine- p-benzoquinone reaction mixture was carried out followed by ultraviolet, infrared, and mass spectral analyses of reaction products. Additionally, an N-acetyl-L-histidine reaction mixture was subjected to a similar, though more limited, analysis.

Figure 24-c indicates the thin-layer chromatographic separation of the glycine- p-benzoquinone reaction system on cellulose using a butanol:acetic acid:water (60:15:25 v/v) solvent system.

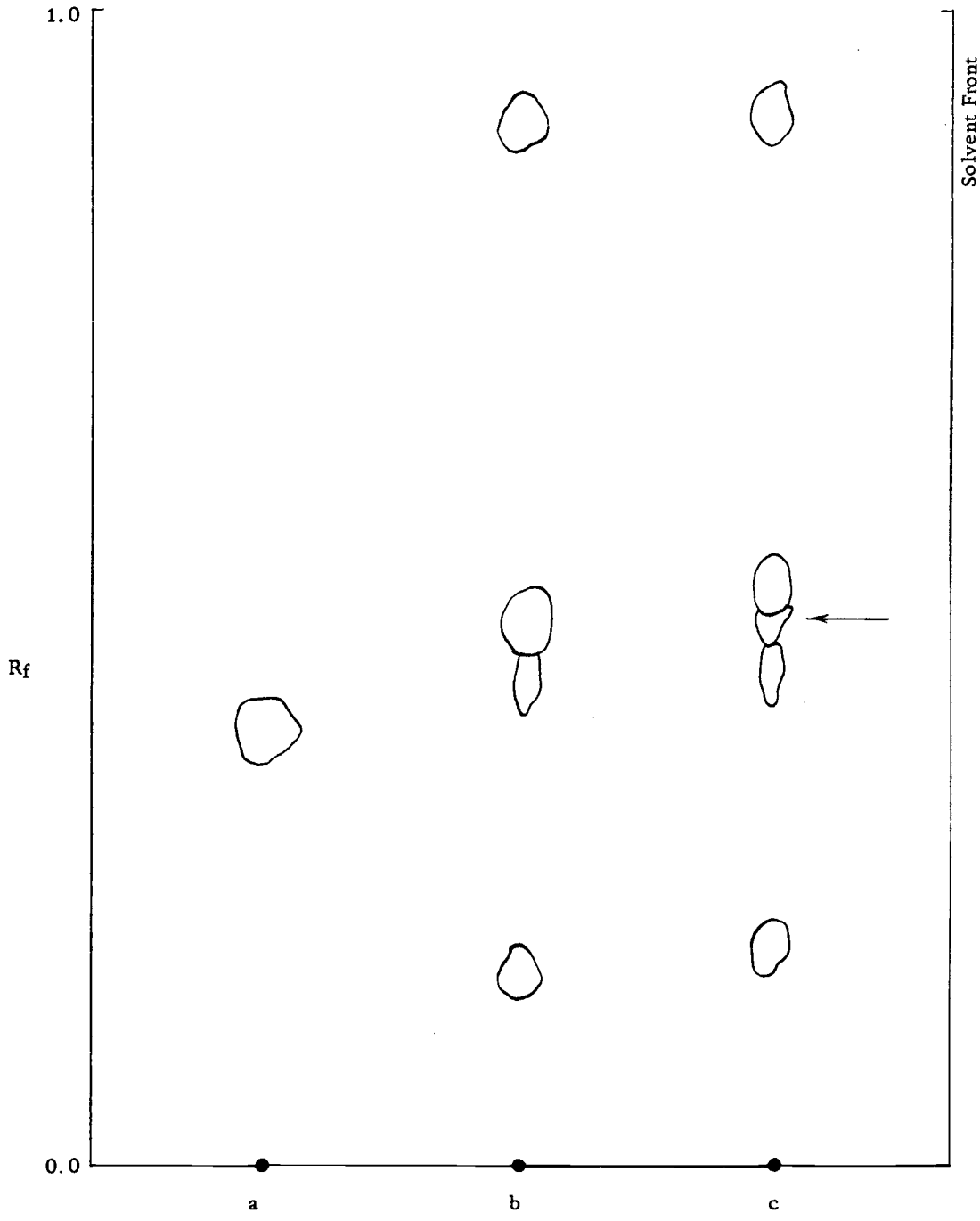


Figure 24. Thin layer chromatography of a) glycine, b) p-benzoquinone reaction mixture; c) glycine-p-benzoquinone reaction mixture. Systems chromatographed: a) glycine, 2.0 mg/ml in 10% isopropyl alcohol. b) p-benzoquinone mixture; p-benzoquinone, 53.4 μmoles: c) p-benzoquinone-glycine reaction mixture; p-benzoquinone, 53.4 μmoles, glycine 26.7 μmoles (2.0 mg). For systems b and c: Buffer, potassium phosphate, pH 8.0, 800 μmoles. Total volume, 5.0 ml. Temperature, 30°C. Incubation time, 3 hours. Systems b and c vacuum dried and taken up in 1.0 ml isopropyl alcohol. TLC carried out on Eastman cellulose chromagram sheets. Solvent system; butanol:Acetic acid:water (60:15:25 v/v). Unique orange-brown material isolated from glycine-p-benzoquinone system designated by arrow.

Chromatographic analysis of a p-benzoquinone reaction mixture (Figure 24-b), and glycine (Figure 24-a) are also shown. Glycine gave a single ninhydrin positive spot with an R_f of 0.38. The p-benzoquinone reaction mixture yielded several spots, as noted, plus material that tailed between all spots, indicating the presence of a wide variety of reaction products. All bands in the p-benzoquinone reaction mixture were ninhydrin negative but were visible under ultraviolet light before or after spraying with Rhodamine B. In addition, the lower two spots were colored and visible without the aid of a detecting reagent. Thin-layer chromatography of the incubated glycine- p-benzoquinone reaction mixture yielded bands and tailing material identical in character to those of the p-benzoquinone sample with the addition, however, of an orange-brown ninhydrin-negative band with an R_f value of 0.47. This band (noted with an arrow in Figure 24-c) was the only material unique to the glycine- p-benzoquinone reaction system and, as such, it was selected as a possible adduct compound and examined in more detail. Preparative thin-layer chromatography was carried out to obtain sufficient sample for ultraviolet, infrared, and mass spectral analyses.

Qualitative ultraviolet spectral analysis of the orange-brown band unique to the glycine- p-benzoquinone sample suggested that the reaction product was probably phenolic in nature; the ultraviolet spectrum (Figure 25) resembled that of hydroquinone or pyrocatechol

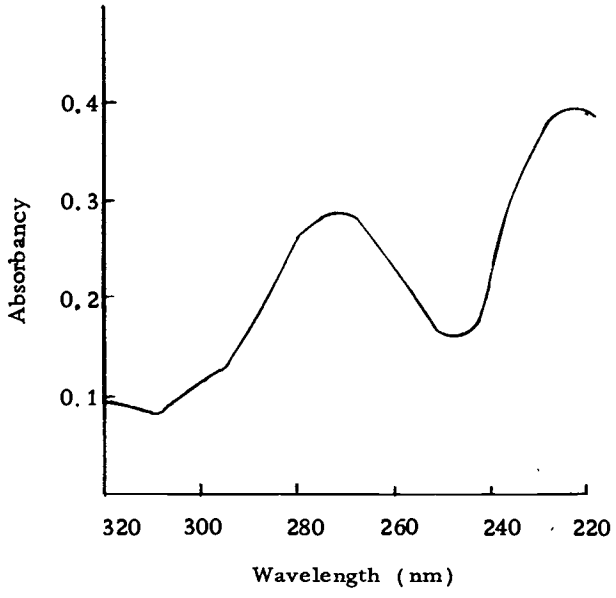


Figure 25. Ultraviolet spectrum of the probable glycine-p-benzoquinone adduct material. Isolated by TLC from the following reaction mixture: buffer, potassium phosphate, pH 8.0, 800 μmoles; glycine, 26.7 μmoles (2.0 mg); p-benzoquinone, 53.4 μmoles. Total volume, 5.0 ml. Temperature, 30°C. Incubation time, 3 hours. TLC carried out on Eastman cellulose sheets; solvent system; butanol:acetic acid:water (60:15:25 v/v).

rather than the starting materials, glycine and p-benzoquinone, although the spectrum was not identical to either of these model phenolic compounds. The ultraviolet spectrum of the isolated adduct material (Figure 25) shows an absorption maximum in the 272 nm region with a trough at 248 nm. Pyrocatechol shows an absorption maximum at 273 nm and a minimum at 244 nm. Hydroquinone shows an absorption maximum at 286 nm and a minimum at 250 nm. p-Benzoquinone, on the other hand, shows a strong maximum at 245 nm, a weak maximum centering at approximately 295 nm and a minimum at 275 nm. Glycine absorption in the ultraviolet occurs below 240 nm.

Figure 26 shows the infrared spectrum of the isolated thin-layer band corresponding to the probable glycine- p-benzoquinone adduct. Bands characteristic of the stretching (3100-2600 cm^{-1}) and bending (2200-2000 cm^{-1}) modes of the free amino group are absent. Additionally, the C=O stretch at 1690-1655 cm^{-1} characteristic of quinones is absent. Absorption bands corresponding to the -OH stretch in the 3600-3000 cm^{-1} region and the asymmetric and symmetric stretch of the C-O grouping characteristic of a free carboxyl group are present. Furthermore, a weak band occurs at approximately 3300 cm^{-1} which probably corresponds to the N-H stretch of an Ar-NH-R type secondary amine. Additional bands occur in the area of 1350 cm^{-1} to 1200 cm^{-1} (1310, 1240, 1195 cm^{-1})

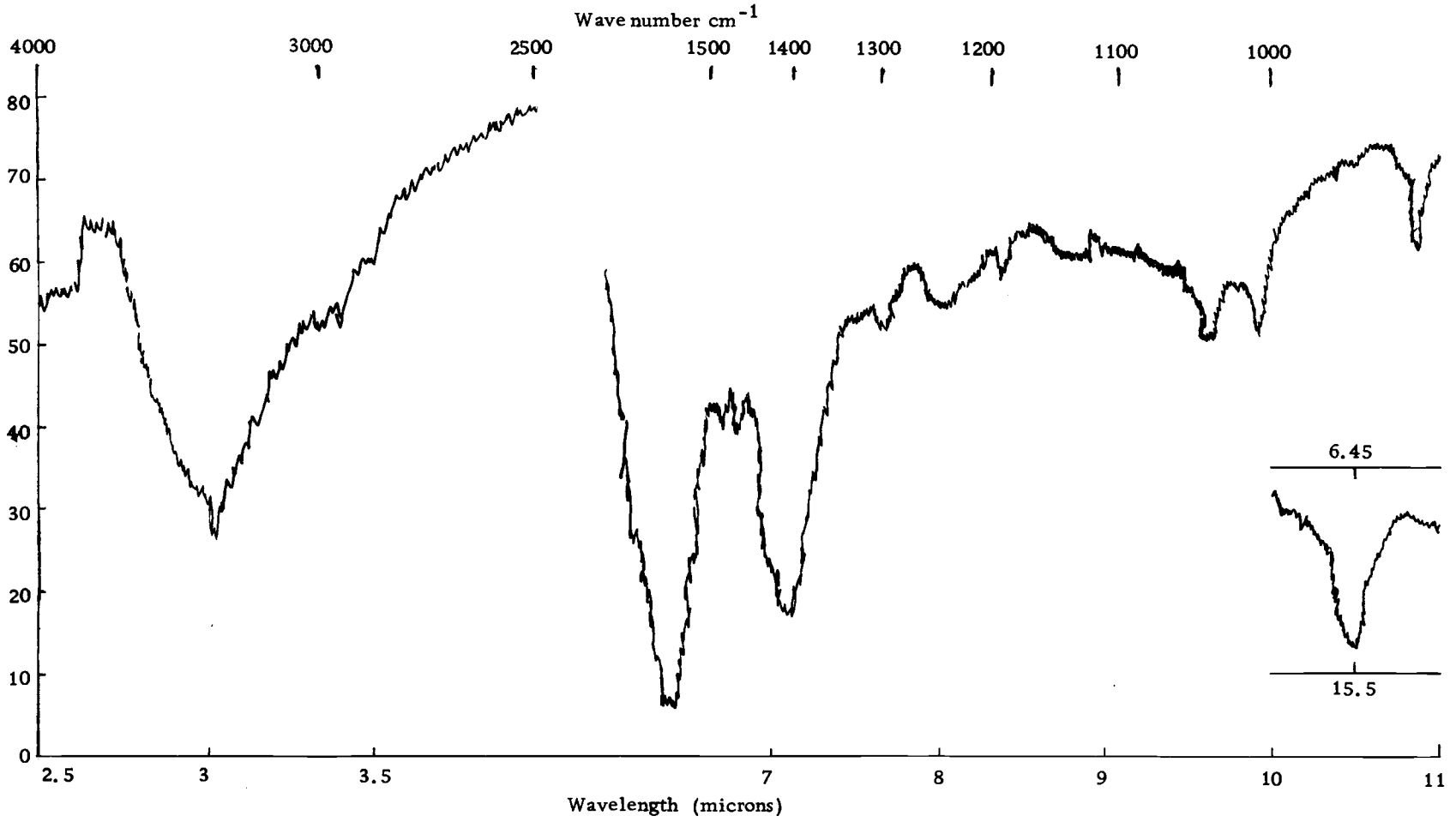


Figure 26. Infrared spectrum of probable glycine-p-benzoquinone adduct material. Isolated by TLC from the following reaction system: buffer, potassium phosphate, pH 8.0, 800 µmoles; glycine, 26.7 µmoles (2.0 mg); p-benzoquinone, 53.4 µmoles. Total volume, 5.0 ml. Temperature, 30°C. Incubation time, 3 hours. TLC carried out on Eastman cellulose sheets; solvent system; butanol:acetic acid: water (60:15:25 v/v). Infrared spectrum determined with isolated material applied to salt disk, using a Beckman IR-5 infrared recording spectrophotometer.

and probably correspond to the =C-O stretch (1260-1180 cm^{-1}) and =C-N stretch (1340-1320 cm^{-1} and 1315-1250 cm^{-1}). The band observed at 645 cm^{-1} is probably the result of a ring bend. An Ar-NH-R group amine stretch may account for one of the bands observed in the 1100-1000 cm^{-1} region while the other could be the result of a C-O stretch of a CR_2O group due to the presence of contaminating polymerized phenolic material. The infrared spectrum indicates that, a) neither of the initial starting compounds, glycine, or p-benzoquinone is present in the isolated material and, b) that an Ar-NH-R type secondary amine that could be phenolic in nature is formed.

Preliminary mass spectral analysis of the material corresponding to the probable glycine- p-benzoquinone adduct failed to yield a molecular ion or a fragmentation pattern indicative of a compound formed from either one or two moles of glycine and one mole of p-benzoquinone. Insufficient volatility was thought to be responsible for this result. To increase volatility, the isolated material was methylated. Thin-layer chromatography of the methylated material on silica gel G yielded four bands (with R_f values of 0.21, 0.30, 0.39, and 0.62) plus material remaining at the origin. Material from each band as well as the origin was isolated and subjected to mass spectral analysis using a direct probe technique. Tabulated data from band one material (that closest to the origin, R_f = 0.21)

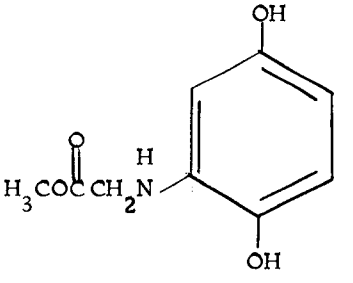
are shown in Table 9. An apparent molecular ion was observed at m/e = 197, corresponding to the proposed structure shown in Figure 27, 2-N-glycyl-1, 4-dihydroxybenzene methyl ester. An observed m -2 peak (m/e=195) probably results from the conversion of this compound to the corresponding p-benzoquinone; such m-2 conversions of phenolic compounds to the quinone have been reported (Aplin and Pike, 1966). The observed m/e value of 167 probably represents an m-28 peak corresponding to a CO loss from the quinone, while the m-31 peaks at m/e = 166 and m/e = 164 probably represent the loss of the methoxy group from the parent phenolic compound and the quinone, respectively. Ions observed at m/e = 141 and m/e = 54 probably correspond to ring cleavage fragments resulting from quinone cleavage between carbons 3 and 4 and 1 and 6. Ions observed at m/e = 138 and m/e = 136 probably represent an m-59 loss of an H_3COCO grouping from the hydroxybenzene and quinone compounds respectively while peaks at m/e = 109 and m/e = 107 probably represent an m-88 loss of the entire amino acid methyl ester moiety ($H_3COCOCH_2NH$) from the same respective parent phenolic and quinone compounds. It should be noted that band one (from silica gel G TLC plates, $R_f = 0.21$) was not "clean"; many additional peaks were present other than those noted. Additionally, only band one material presented m/e bands corresponding to a fragmentation pattern representative of an initial glycine- p-benzoquinone adduct compound; it is

Table 9. Mass spectral analysis of probable glycine-p-benzoquinone adduct material.

Relative abundance (R. A.) values for selected mass (m/e) peaks of band one material

m/e	R. A.	m/e	R. A.	m/e	R. A.
197	100	164	18	109	97
195	34	141	51	107	96
167	72	138	41	54	67
166	30	136	67		

Isolated by TLC on Eastman cellulose plates (solvent system; butanol: acetic acid:water (60:15:25 v/v)) followed by methylation and re-chromatogramming on silica gel G (solvent system; Anhydrous diethyl ether:hexane:absolute methanol (40:10:2 v/v) from the following reaction mixture: buffer, potassium phosphate, pH 8. 0, 800 µmoles; glycine, 26. 7 µmoles (2. 0 mg); p-benzoquinone, 53. 4 µmoles. Total volume, 5. 0 ml. Temperature 30° C. Incubation time, 3 hours. Mass spectral analysis carried out by a direct probe technique using an Atlas CH7 mass spectrometer.



2-N-glycyl-1, 4-dihydroxybenzene methyl ester

Figure 27. Proposed structure of the glycine-p-benzoquinone adduct.

possible that the material occurring in other bands may, in part, be polymeric material which includes glycine as a substituent.

The information obtained from ultraviolet, infrared, and mass spectral analyses of the glycine- p-benzoquinone reaction system indicates, as expected from the literature, that an adduct is formed from glycine and p-benzoquinone which is phenolic in nature and in which the the amino acid is linked to the benzene ring of the phenolic compound through its α-amino group forming an Ar-NH-R type secondary amine. The proposed structure of such a compound is indicated in Figure 27. The reaction mixture also contains, however, a wide variety of other products derived from p-benzoquinone modifications, some of which may include glycine as a substituent.

An effort was made to determine the nature of reaction products resulting from an N-acetyl-L-histidine- p-benzoquinone reaction system. Thin-layer chromatography of the reaction material on cellulose sheets employing butanol:acetic acid:water (60:15:25 v/v) yielded five principal bands (A through E with A nearest the origin) with tailing material between each band as determined by direct visual observation or under ultraviolet light before or after spraying with Rhodamine B. Each band was isolated and subjected to infrared spectral analysis. None of the five bands yielded a spectrum similar to that of standard N-acetyl-L-histidine, suggesting that some modification had occurred. Two samples, band B and bands C and D combined,

contained infrared bands corresponding to the C-O symmetric and asymmetric stretches indicative of a free carboxyl grouping. Additionally, these samples absorbed strongly in the area of 3600-3000 cm^{-1} indicating the probable presence of hydroxyl groups in the isolated material. (Band E gave a weak absorption in this area with no other definitive infrared bands while band F gave no definitive bands.) Indications of histidine in the two samples from C-O stretches characteristic of a free carboxy group suggest the formation of one or more initial histidine- p-benzoquinone adducts. However, the lack of clearly definitive infrared bands other than those mentioned prohibited an evaluation of the possible nature of the adduct compound(s).

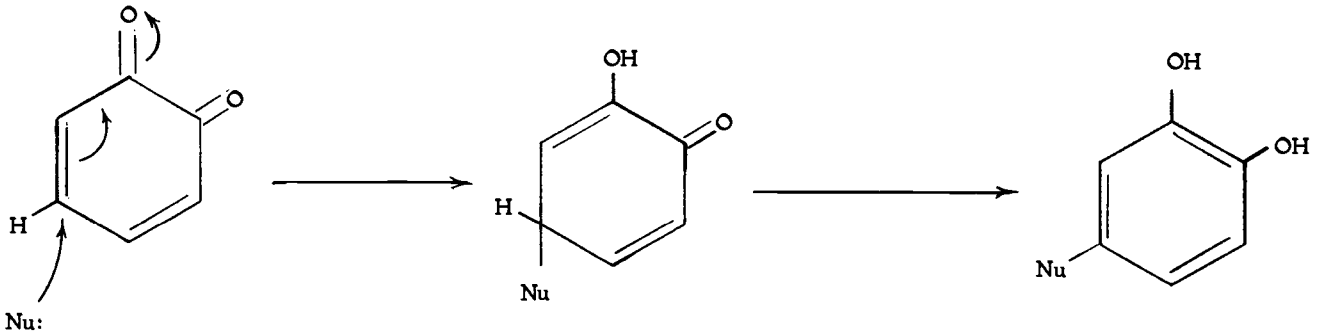
It is clear from the amino acid analysis data that histidine residues in protein are modified by p-benzoquinone. Additionally, infrared analysis of the reaction mixture suggests that modification has occurred. However, no definitive information on the nature of the adduct compound formed was obtained from this experiment. Further work should be done to determine the nature of the compound(s) formed between histidine and quinone.

A Possible Reaction Mechanism for Amino Acid Modification by Quinones

Amino acid analysis of tanned bovine serum albumin indicates that the reaction occurring between constituent amino acids and

oxidized phenolic material is both nucleophilic in nature and quinone-requiring. Amino acid modifications in tanned BSA are observed only with those amino acids which have nucleophilic functions in their side chains, i.e., sulfhydryl, amino, imidazole, and guanidino groups. Spectrophotometric and manometric techniques have indicated similar results as reported in the literature. Additionally, the extent of amino acid modifications, considering both quantitative modification in certain circumstances and the variety of amino acids modified, increases with increasing pH. Observed modifications are greater in BSA tanned by incubation with p-benzoquinone than in that tanned by either base catalyzed or enzymatically oxidized pyrocatechol under equivalent conditions, reflecting the quinone requirement.

Carbon to carbon double bonds conjugated with electron sinks can serve as good substrates in nucleophilic addition reactions. Such a situation exists with both o- and p-benzoquinones. As reported in the literature, these compounds may undergo nucleophilic attack to yield 1, 4-addition products as indicated in Figure 28. Amino acid modifications that are observed in tanned BSA probably result from such 1, 4-addition reactions. As indicated in Figure 28, the nucleophile would attack at the partially positive site resulting from the electron withdrawing character of the carbonyl center. The initial product would be the semiquinone, which, in protic solvents, may undergo proton rearrangement to yield the more stable aromatic



e.g. Nu: = -SH, $-NH_2$,

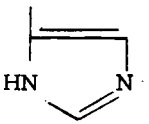


Figure 28. 1, 4 addition reaction mechanism.

compound. Under proper conditions such a substituted dihydroxybenzene may be reoxidized to the quinone derivative and undergo further substitution if suitable centers for nucleophilic attack are available (Mason, 1955a). Analysis of the glycine- p-benzoquinone reaction system supports this mode of interaction. The probable adduct compound obtained from this system, 2-N-glycyl-1,4-dihydroxybenzene methyl ester is shown in Figure 27. This compound would, in its non-methylated form, result from the attack of glycine through its α-amino group on p-benzoquinone via a 1, 4-addition reaction.

Proposed structures of initial products of some amino acids moieties modified by oxidized pyrocatechol are shown in Figure 29. The structures shown are based upon information obtained from amino acid modifications in tanned BSA, the glycine-p-benzoquinone reaction system, and known reactions that are similar in nature (Mason, 1955a; Gurd, 1967). The mode of tryptophan modification is not clear. 3-Substituted indoles such as tryptophan have been reported to show only limited reactivity towards o- and p-benzoquinones although indole derivatives with the 3 position free are highly reactive (Mason, 1955a); the nitrogen atom evidently remains relatively inert. Leopold and Plummer (1961) reported the formation of red pigments when indolacetic acid was incubated with the quinone generated enzymatically from chlorogenic acid. Pierpoint (1966) described the formation of a similar purple-red chromogen

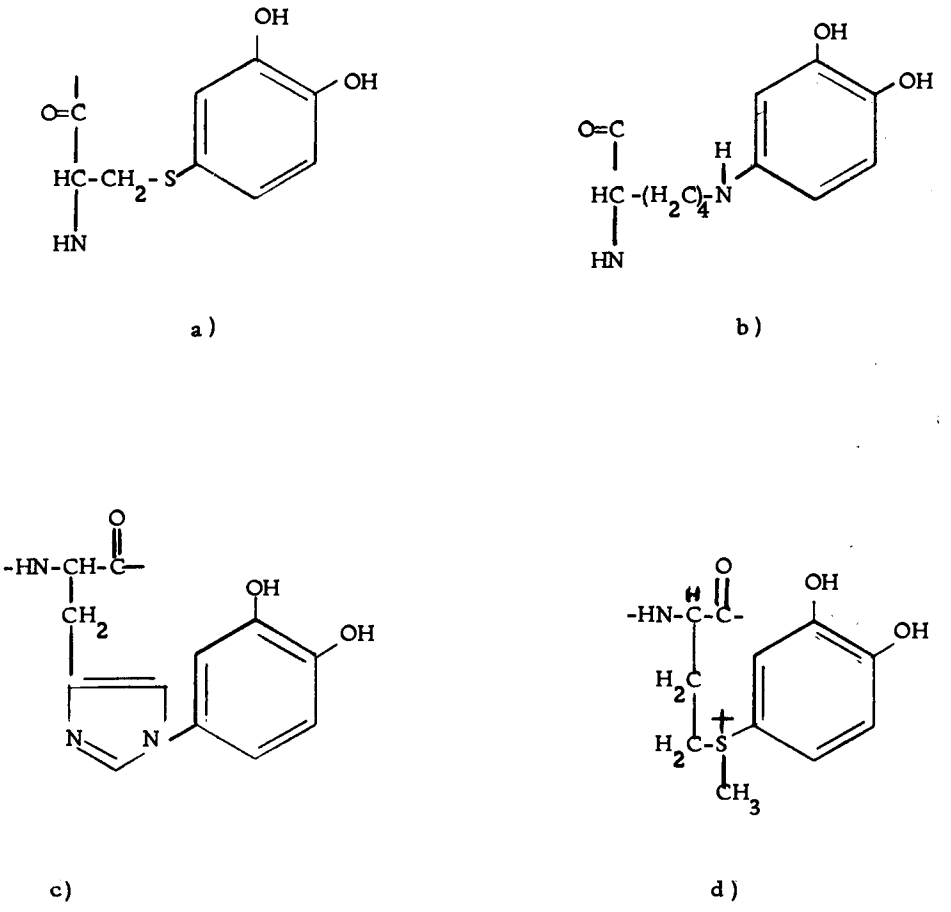


Figure 29. Proposed initial reaction products from o-benzoquinone and a) cysteine, b) lysine, c) histidine and d) methionine.

with indol-3-ylacetic acid. A tryptophan derivative of a similar nature is probably formed in the tyrosinase oxidized pyrocatechol system, although the chemical nature of the derivative is not known.

SUMMARY

Plant tissues contain a wide variety of phenolic compounds, frequently in high concentrations. Earlier work has shown that both non-covalent association of phenols with protein and covalent binding of oxidized phenolic compounds to amino acids can occur; additional studies have indicated that modification of amino acid residues in protein may occur. It was of interest, therefore, from both a biochemical and nutritional point of view to determine the types of amino acid modifications that can occur in protein upon exposure to oxidized phenolic material.

A model system approach was used to identify and quantitate amino acid residue modification in protein by oxidized phenolic material and to identify the probable adduct compound. Digestibility studies were carried out on tanned bovine serum albumin to assess further protein-phenol association and to estimate the extent of covalent and non-covalent binding of oxidized phenolic material to protein.

Preliminary oxygen uptake studies indicated that the non-enzymatic base-catalyzed air oxidation of pyrocatechol occurred rapidly above pH 7.5. This reaction mixture showed moderate to strong absorption throughout the visible region of the spectrum, indicating the formation of a wide variety of products. The presence of bovine serum albumin (BSA) in such a system changed the nature

of oxidized products, as evidenced by alterations in the visible absorption spectrum. Isolated tanned BSA showed a visible absorption spectrum similar to that of oxidized pyrocatechol. This observation, in conjunction with the lack of visible absorption by BSA, indicated the association of pyrocatechol oxidation products with protein. The failure of solvent stripping agents (acetone, methanol, N-methyl-2-pyrrolidone) to completely remove oxidized pyrocatechol material bound to BSA indicates the strong nature of such associations. Elution of an oxidized pyrocatechol or hydroquinone-BSA reaction mixture through either Bio-Gel P-30 or Sephadex G-50 yielded at the void volume a brown band corresponding to tanned BSA; residual oxidized phenolic material remained quite firmly bound to the column. Spectral analysis of the products of mushroom tyrosinase oxidation of pyrocatechol was consistent with the formation of o-benzoquinone as the initial oxidation product which rapidly was converted to more complex products, particularly at pH 7.0-7.5 and above. Product inhibition (reaction-inactivation) of tyrosinase was indicated from multiple enzyme addition studies and from studies involving the effect of pH and enzyme level on pyrocatechol oxidation. Decreased color formation during enzymatic pyrocatechol oxidation in the presence of glycine, BSA, and poly-L-lysine suggested that product binding by these compounds had occurred.

Amino acid analysis of tanned BSA indicated that modifications

were restricted primarily to lysine, histidine, and cystine. Cystine modification was probably the result of its reduction to cysteine by pyrocatechol followed by modification of this thiol. Modifications were a function of initial mole ratio of reactants, mode of pyrocatechol oxidation, and pH.

Little modification was observed in BSA tanned by air oxidized (base catalyzed) pyrocatechol at pH 8.0; cystine was the only amino acid residue clearly modified, this modification requiring a large excess of pyrocatechol. At pH 9.5 lysine modification was observed in addition to that of cystine, suggesting, in part, a greater reactivity of the ε-amino group in the non-ionized form. Arginine modification was also observed at this pH.

Amino acid residue modifications in BSA tanned by tyrosinase-oxidized pyrocatechol were similar in nature but more extensive than those observed in the base-catalyzed system. Modifications were generally found to be a function of enzyme level, pH, and pyrocatechol concentration. Lysine and histidine modification increased with increasing enzyme levels, while cystine modification remained constant, although generally greater than lysine or histidine modifications. Greater cystine modifications at lower pH levels indicate that the probable reduction product, cysteine is more reactive to oxidized phenolics than are histidine and lysine, an observation that has been previously reported. Lysine modification increased with

increasing pH in the enzymatically oxidized pyrocatechol system, while cystine modification remained constant throughout the pH range studied. Histidine modification was observed but with no apparent pattern of increased modification with increasing pH. Modifications of cystine, lysine, and histidine increased with increasing pyrocatechol concentration.

The pattern of amino acid residue modification in BSA exposed to p-benzoquinone was similar to that in the above studies, with cystine, lysine, and histidine modifications observed. Modifications were usually much greater with p-benzoquinone, suggesting that the quinone was the principal reactive phenol oxidation product.

Modifications of a similar nature occurred in all systems studied. Cystine, lysine, and histidine were the principal amino acids modified in tanned BSA under the conditions employed. Modifications occurred at lower pH values and lower pyrocatechol concentrations in the tyrosinase system as compared with the base-catalyzed system. These modifications probably reflect, in part, a greater effective quinone level in the enzymatic system. The greater modifications observed with p-benzoquinone support this viewpoint.

Gas-liquid chromatography of N-trifluoroacetyl-L-tryptophan methyl ester and N-trifluoroacetyl-DL-methionine methyl ester subjected to enzymatically oxidized pyrocatechol indicated that these

amino acids were modified by oxidized phenolic material; these modifications were found to increase with increasing pyrocatechol concentration.

Proteolytic digestibility of tanned BSA was greatly reduced. Digestion by trypsin was reduced approximately 85 percent in BSA tanned by the base catalyzed system; BSA tanned by tyrosinase oxidized pyrocatechol was almost completely indigestible. Pepsin digestion was also impaired although to a lesser degree than that of trypsin. This decrease in digestibility by proteases reflects extensive complex formation between BSA and oxidized phenolic material. Complex formation is a result of covalent and non-covalent binding of phenolic material to protein. The extent of amino acid residue modification resulting from the covalent binding of oxidized phenolic material seldom is as great as the observed decreases in proteolytic digestibility. This suggests, as one possibility, that non-covalent association of phenolic material would be predominant over amino acid modifications.

The nutritional and biochemical aspects of amino acid residue modifications are important. Lysine, methionine, tryptophan, and histidine (infants only) are nutritionally essential amino acids. Additionally, cystine may spare methionine if methionine is present in suboptimal amounts. Modifications occurring to these amino acids within a protein would reduce its nutritional value to the least limiting

essential amino acid. The impaired digestibility of tanned protein also results in a decrease in its nutritional value. Cysteine and histidine can be involved in enzyme active sites. Modifications occurring to these amino acids could result in inactive enzymes. Methionine modifications were found to occur during the acid hydrolysis procedure in the presence of phenols and quinones. The low levels of methionine reported for many plant proteins may be the result of methionine modification during the acid hydrolysis portion of the analytical procedure.

Ultraviolet, infrared, and mass spectral analyses of a glycine-p-benzoquinone reaction mixture were carried out to define the nature of the amino acid-quinone adduct formed in the reaction systems studied. Ultraviolet spectral analyses of a chromatographic band specific to the glycine-p-benzoquinone reaction mixture suggested that the reaction product was phenolic in nature, while infrared analysis of the same material indicated that an aromatic Ar-NH-R type secondary amine, possibly phenolic in nature, was formed. Mass spectral analysis of material isolated after methylation of the specific chromatographic band supported the results obtained from ultraviolet and infrared analyses, indicating the formation of 2-N-glycyl-1, 4-dihydroxybenzene, a phenolic Ar-NH-R type secondary amine, as one product of the reaction system.

The spectral data obtained from the glycine-p-benzoquinone

system, in conjunction with the nucleophilic nature of the amino acid modifications and the greater modifications in the presence of p-benzoquinone, suggest a 1, 4-addition reaction between the quinone and the amino acid residues. Literature on amino acid-quinone interactions support this conclusion.

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APPENDICES

APPENDIX I

SOME BIOCHEMICAL AND NUTRITIONAL ASPECTS OF THE REACTIONS OF PHENOLS AND QUINONES WITH PROTEIN

The complexing of phenolic material with protein often results in damaging effects. Thus, tanned protein exhibits markedly altered physical and chemical properties reflected in modified biochemical and nutritional behavior. Phenolic material bound to protein by non-covalent forces may distort the native conformation of the protein resulting in altered solubility properties (Caldron et al., 1968; Van Buren and Robinson, 1969) and decreased or eliminated enzymatic activity. In addition, as indicated by quinone-amino acid model systems and studies discussed in the text, covalent association of oxidized phenolic material to protein contributes to these changes while also modifying the chemical nature of the protein. It is interesting to note that some glycoproteins are apparently resistant to the damaging effect of tannin material. Strumeyer and Malin (1970) reported an extracellular yeast invertase, a glycoprotein, which showed no apparent decrease in activity in the presence of a hundred fold excess of condensed tannin. Other glycoproteins (e.g. tannase) also show resistance to tannins. It is probable that the carbohydrate portion of the glycoprotein serves as a protective barrier, allowing formation of a reversible tannin complex with the

sugar moieties rather than with protein.

The formation of protein complexes with phenolic material may be reduced by the addition of competitive phenol binding agents and by inhibiting phenol oxidation. Often a combination of both methods is necessary to insure against damaging complex formation. Competitive phenol binding agents such as hide powder, nylon, and polyvinylpyrrolidone have been used successfully to reduce non-covalent association of phenols to protein. Polyvinylpyrrolidone has proven to be especially effective in the isolation of active enzyme systems from plant materials high in phenol content (Loomis and Battaile, 1966). Anderson (1968) has reviewed the use of thiols and other reducing agents to decrease complex formation in plant enzyme isolation. Decrease in complex formation may also result from phenol oxidase inhibition by copper chelating agents and certain reducing agents (Pierpoint, 1966).

Additional techniques for inhibiting protein-phenol complex formation, which may be applicable under certain circumstances, include the use of anion exchange resins for the removal of phenolics from plant extracts (Lam and Shaw, 1970) and the use of aliphatic acids to lower pH, thus retarding phenol oxidation (Arora, 1969). Enzymatic conversion of phenolic material to lesser or non-reactive species with respect to protein complex formation may have potential

application. A ring cleaving catechol oxidase, protocatechuate-3, 4 dioxygenase, from Pseudomas aeruginosa has been used to prevent browning of apple juice (Kelly and Finkle, 1969). Neujahr and Varga (1970) evaluated phenolic degradation by a Trichosporon cutaneum enzyme preparation with broad substrate specificity; the pathway of degradation involved hydroxylation followed by ring cleavage. Gibson (1968) has reviewed the microbial degradation of aromatic compounds.

Analytical use has been made of protein and amino acid complexes with phenols and quinones. Columns packed with tanned gelatin have been employed for protein separation (Dennsion, 1970). Lorentz and Flatter (1970) reported the use of p-benzoquinone as a staining reagent for the detection of amino acids.

Phenolic compounds may interfere with accurate protein determination. The biuret, Lowry, and ultraviolet spectrophotometric procedures are susceptible to interference by phenolics (Solecka et al., 1968; Potty, 1969; Leovey and Loomis, unpublished). The similar nature of phenolic compounds to tyrosine, tryptophan, and phenylalanine gives rise to spurious results with the ultraviolet spectrophotometric procedure. The ability of phenolic material to complex copper ion and Folin's reagent may yield artifactual results with the biuret and Lowry techniques. Prior precipitation of the protein from solution will reduce but not eliminate the interference. Caution must therefore be exercised in evaluating protein levels in

systems containing phenolic compounds, particularly plant material.

Decreased nutritional value of protein can result from its complexing with phenolic material. Possible alteration of amino acid content as well as impairment of digestibility and absorption may occur.

Covalent coupling of nucleophilic functions present in protein to quinones would result in the nutritional unavailability of these amino acid residues, altering overall nitrogen metabolism. Especially important are modifications to the essential amino acids, lysine, methionine, and tryptophan, and to cystine which may spare methionine (Irwin and Hegsted, 1971). Possible modification of histidine may also be critical, especially with respect to human infant requirements.

Non-covalent association of phenolic material to protein may decrease nutritional availability of the protein through lowered digestibility. Feeny (1969) found that trypsin hydrolysis of casein was virtually inhibited after prior incubation of the protein with oak leaf tannins at a tannin:casein ratio of 2:1. Casein digestibility decreased with increasing tannin level, with condensed tannins a more effective inhibitor of casein digestibility than hydrolyzable tannins. In a continuing series of studies on the nutritive value of grass proteins, Horigome and Kandatsu (1964, 1966a, 1966b) reported that interaction of proteins with o-diphenols in the presence of o-diphenoloxidase caused

lowered digestibility; pepsin and trypsin digestibility of pasture grass acetone powders decreased with increasing levels of o-diphenols. Incubation of grass leaf protein with p-coumaric, caffeic, and chlorogenic acids and polyphenoloxidase decreased pepsin digestibility.

Ingestion of tannins may cause significant growth depression due to at least three factors: their astringent taste resulting in decreased feed intake; the formation of an indigestible nitrogen fraction resulting from phenolic-protein complexes; and the inactivation of digestive enzymes by tanning processes. Tamir and Alumot (1970) studied the growth depression and insoluble nitrogen levels in the rat digestive tract resulting from carob tannin ingestion. Results indicated that both a decreased feed intake as a result of carob tannin astringency and the formation of an insoluble nitrogen fraction in the digestive tract were the cause of growth depression. Mitjavila et al. (1970) showed that tannic acid can alter intestinal absorption. Perfusion of tannic acid at a one gram per liter level into mouse intestine markedly reduced glucose and methionine absorption while also lowering butyric acid uptake. Tannic acid denaturation of the proteins of the protective barrier of the intestine, resulting in a lesion of the outer cellular layer of the mucous membrane, was thought to be responsible for the impaired absorption. Kratzer and Williams (1951) found that significant growth depression resulted from the

incorporation of carob material at a five percent level or greater in chick diets; high mortality resulted when the dietary carob level was 40 percent. Bornstein et al. (1963, 1965) obtained similar results and indicated that growth depression was due, in part, to the presence of a hot water extractable appetite depressing factor, probably phenolic in nature. Joslyn and Glick (1969) studied the comparative effects of a variety of phenolic compounds on the growth of rats. Tannic acid, gallic acid, D-catechin, and condensed tannins from quebracho, grape seed, chestnut oak bark, and wattle bark were all found to be growth depressants. The growth depressing effect of tannins has also been reported by Chang and Fuller (1964), Vohra (1966), and Rayudu et al. (1970). Tamir and Alumot (1969) found condensed tannins to be strongly inhibitory to trypsin, α-amylase, and lipase. Prior incubation with up to 1.2 mg carob tannins gave from 70-90 percent inhibition of these enzymes. Histological and histochemical effects of tannin toxicity have been investigated by Horvath et al., 1960, Camp et al., 1967, Booth and Bell, 1968, and Tamir and Alumot, 1970.

Provision of adequate high quality dietary protein is a critical aspect of the world food supply problem. Protein concentrates are needed as dietary supplements to provide adequate protein levels (Pirie, 1968). Plant protein concentrates, especially leaf protein concentrates, comprise an important protein source which is at

present utilized to only a limited extent for direct consumption. Green plants are the ultimate source of virtually all food, including proteins, and the direct use of plant protein concentrates as dietary supplements avoids efficiency losses which occur in the utilization of other protein concentrates. Inherent problems are associated with plant protein isolation however. Certain seeds have a relatively high protein content but the amino acid balance is poor. Leaf protein on the other hand is of better nutritional quality, comparing favorably with animal protein (Chibnall, 1939; Gerloff, 1965; Akeson and Stahmann, 1965; Swaminathan, 1967); however leaf protein levels are commonly only one to two percent as contrasted with animal tissue protein levels of approximately 20 percent. Arkcoll and Festenstein have partially characterized some of the agronomic factors affecting the yield of extractable leaf protein. Lexander et al. (1970) delineated factors governing plant type selection and extractability parameters in relation to quantities and qualities of leaf protein concentrates produced under controlled conditions. Buchanan (1969) reviewed in-vivo and in-vitro methods of assessing the nutritive value of leaf protein preparations. Plant protein concentrate production involves tissue pulping with subsequent protein removal through heat or acid coagulation (Pirie, 1961) or solvent extraction (Jennings, 1968). In reviews on leaf and plant proteins Pirie (1959) and Stahmann (1963) noted factors which interfere with

plant protein isolation. Vacuole acids, carbohydrates, proteolytic enzymes, and tannins may interact detrimentally with protein upon tissue maceration. Tannin-protein complexing may be particularly troublesome, potentially leading to amino acid modification and decreased digestibility and absorption. Jennings et al. (1968) found that leaf protein concentrates prepared in air were heavily contaminated with aromatic compounds throught to be derived from plant phenolics while Lexander et al. (1970) and Arkcoll and Festenstein (1970) noted phenolic interference in the preparation of plant protein concentrates. In a discussion of amino acid composition and in-vitro digestibility of leaf protein fractions as related to leaf protein concentrates, Byers (1971) noted the apparent unavailability of certain amino acids in chloroplastic and unfractionated leaf protein following phenolase modification of plant phenols. It is imperative that plant protein isolation procedures be evaluated critically in terms of minimizing the reactions of phenols and quinones with protein.

APPENDIX II

Amino acid analysis: control and tanned bovine serum albumin samples

Throughout the section bovine serum albumin is abbreviated BSA.

For amino acid analysis of BSA acid hydrolysates:

A. Methionine was not evaluated due to apparent modification by pyrocatechol or its oxidized products, or by p-benzoquinone during the acid hydrolysis procedure.

B. Half-cystine modification was determined as percent differences from control values of half-cystine/glycine peak height ratios. This means of evaluation was utilized due to the at times inadequate separation of half-cystine and alanine.

Amino Acid Analysis

BSA control (base catalyzed oxidation of pyrocatechol and benzoquinone systems)

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.081	3.24	0.080	3.12	0.088	3.26	3.21
Threonine	0.046	1.84	0.047	1.81	0.051	1.89	1.85
Serine	0.035	1.40	0.037	1.42	0.039	1.44	1.42
Glutamic acid	0.118	4.72	0.116	4.46	0.135	5.00	4.73
Proline	0.046	1.84	0.047	1.81	0.054	2.00	1.88
Glycine	0.025	1.00	0.026	1.00	0.027	1.00	1.00
Alanine	0.064	2.56	0.066	2.54	0.071	2.63	2.58
Half-cystine		0.51		0.49		0.50	0.50
Valine	0.053	2.12	0.057	2.19	0.061	2.26	2.19
Isoleucine	0.020	0.80	0.023	0.88	0.022	0.81	0.83
Leucine	0.087	3.48	0.092	3.53	0.098	3.62	3.54
Tyrosine	0.026	1.04	0.030	1.15	0.030	1.11	1.10
Phenylalanine	0.038	1.52	0.043	1.65	0.042	1.56	1.58
Lysine	0.088	3.52	0.088	3.39	0.098	3.62	3.51
Histidine	0.026	1.04	0.024	0.92	0.028	1.04	1.00
Ammonia	0.074	2.96	0.072	2.77	0.082	3.04	2.92
Arginine	0.033	1.32	0.034	1.31	0.039	1.44	1.36

Amino Acid Analysis

Tanned BSA: Base catalyzed oxidation of pyrocatechol, pH 8.0

Pyrocatechol:BSA-amino acid residue mole ratio of 1:10

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.093	3.32	0.091	3.13	0.097	3.23	3.23
Threonine	0.054	1.93	0.056	1.93	0.056	1.87	1.91
Serine	0.041	1.46	0.043	1.48	0.046	1.53	1.49
Glutamic acid	0.136	4.85	0.144	4.98	0.149	4.97	4.93
Proline	0.055	1.96	0.057	1.96	0.060	2.00	1.97
Glycine	0.028	1.00	0.029	1.00	0.030	1.00	1.00
Alanine	0.074	2.64	0.081	2.79	0.084	2.80	2.74
Half-cystine		0.41		0.47		0.44	0.44
Valine	0.069	2.46	0.069	2.38	0.073	2.43	2.42
Isoleucine	0.023	0.82	0.027	0.93	0.028	0.93	0.89
Leucine	0.102	3.65	0.110	3.79	0.109	3.63	3.69
Tyrosine	0.032	1.14	--	--*	0.034	1.13	1.13
Phenylalanine	0.043	1.54	--	--*	0.049	1.63	1.58
Lysine	0.094	3.36	0.094	3.25	0.099	3.30	3.30
Histidine	0.028	1.00	0.028	0.96	0.029	0.97	0.98
Ammonia	0.080	2.86	0.080	2.76	0.086	2.87	2.83
Arginine	0.040	1.43	0.040	1.38	0.045	1.50	1.44

*Undetermined due to amino acid analyzer malfunction

Amino Acid Analysis

Tanned BSA: Base catalyzed oxidation of BSA, pH 8.0

Pyrocatechol:BSA-amino acid residue mole ratio of 1:1

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.121	3.27	0.112	3.11	0.116	3.41	3.26
Threonine	0.072	1.94	0.066	1.83	0.067	1.97	1.91
Serine	0.056	1.51	0.053	1.47	0.055	1.62	1.53
Glutamic acid	0.176	4.76	0.166	4.61	0.165	4.85	4.74
Proline	0.073	1.97	0.066	1.83	0.063	1.85	1.88
Glycine	0.037	1.00	0.036	1.00	0.034	1.00	1.00
Alanine	0.106	2.86	0.098	2.72	0.097	2.85	2.81
Half-cystine		0.46		0.49		0.49	0.48
Valine	0.068	1.84	0.070	1.94	0.071	2.09	1.96
Isoleucine	0.030	0.81	0.026	0.72	0.029	0.85	0.79
Leucine	0.139	3.76	0.129	3.58	0.133	3.91	3.75
Tyrosine	0.043	1.16	0.042	1.17	0.042	1.24	1.19
Phenylalanine	0.063	1.70	0.060	1.67	0.060	1.76	1.71
Lysine	0.122	3.30	0.119	3.31	0.115	3.38	3.33
Histidine	0.035	0.94	0.034	0.94	0.033	0.97	.95
Ammonia	0.089	2.41	0.083	2.31	0.085	2.50	2.41
Arginine	0.050	1.35	0.051	1.42	0.047	1.38	1.38

Amino Acid Analysis

Tanned BSA: Base catalyzed oxidation of pyrocatechol, pH 8.0

Pyrocatechol:BSA-amino acid residue mole ratio of 2:1

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg.Value AA/Gly Ratio
Aspartic acid	0.078	3.25	0.087	2.90	0.094	3.25	3.13
Threonine	0.045	1.88	0.054	1.80	0.056	1.93	1.87
Serine	0.035	1.46	0.042	1.40	0.044	1.52	1.46
Glutamic acid	0.114	4.76	0.135	4.50	0.140	4.83	4.70
Proline	0.045	1.88	0.055	1.83	0.057	1.96	1.89
Glycine	0.024	1.00	0.030	1.00	0.029	1.00	1.00
Alanine	0.061	2.54	0.075	2.50	0.080	2.76	2.60
Half-cystine		0.43		0.40		0.42	0.42
Valine	0.053	2.21	0.069	2.30	0.072	2.48	2.33
Isoleucine	0.018	0.75	0.022	0.73	0.028	0.96	0.81
Leucine	0.085	3.55	0.105	3.50	0.105	3.62	3.56
Tyrosine	0.028	1.17	0.034	1.13	0.033	1.14	1.15
Phenylalanine	0.039	1.63	0.044	1.47	0.044	1.52	1.54
Lysine	0.076	3.16	0.089	2.97	0.092	3.17	3.10
Histidine	0.023	0.96	0.027	0.90	0.027	0.93	0.93
Ammonia	0.077	3.22	0.083	2.77	0.086	2.97	2.99
Arginine	0.033	1.38	0.038	1.27	0.040	1.38	1.34

Amino Acid Analysis

Tanned BSA: Base catalyzed oxidation of pyrocatechol, pH 8.0

Pyrocatechol:BSA-amino acid residue mole ratio of 10:1

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg.Value AA/Gly Ratio
Aspartic acid	0.136	3.09	0.098	2.88	0.149	3.04	3.00
Threonine	0.080	1.82	0.060	1.76	0.088	1.80	1.79
Serine	0.062	1.41	0.048	1.41	0.068	1.39	1.40
Glutamic acid	0.203	4.61	0.154	4.52	0.223	4.54	4.56
Proline	0.082	1.86	0.062	1.82	0.086	1.75	1.81
Glycine	0.044	1.00	0.034	1.00	0.049	1.00	1.00
Alanine	0.113	2.57	0.089	2.62	0.122	2.49	2.56
Half-cystine		0.29		0.25		0.27	0.27
Valine	0.103	2.34	0.076	2.24	0.110	2.25	2.28
Isoleucine	0.035	0.80	0.026	0.76	0.038	0.78	0.78
Leucine	0.152	3.46	0.118	3.47	0.166	3.39	3.44
Tyrosine	0.051	1.16	0.039	1.15	0.053	1.08	1.13
Phenylalanine	0.065	1.48	0.050	1.47	0.070	1.43	1.46
Lysine	0.143	3.25	0.103	3.03	0.150	3.06	3.11
Histidine	0.039	0.89	0.030	0.88	0.043	0.88	0.88
Ammonia	0.109	2.48	0.087	2.56	0.115	2.35	2.46
Arginine	0.059	1.34	0.045	1.32	0.065	1.33	1.33

Amino Acid Analysis

Tanned BSA: Base catalyzed oxidation of pyrocatechol, pH 9.5

Pyrocatechol:BSA-amino acid residue mole ratio of 2:1

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg.Value AA/Gly Ratio
Aspartic acid	0.105	2.92	0.103	3.22	0.107	3.15	3.10
Threonine	0.062	1.72	0.061	1.90	0.063	1.85	1.82
Serine	0.049	1.36	0.050	1.56	0.050	1.47	1.46
Glutamic acid	0.162	4.50	0.159	4.97	0.164	4.82	4.76
Proline	0.057	1.58	0.055	1.72	0.054	1.59	1.63
Glycine	0.036	1.00	0.032	1.00	0.034	1.00	1.00
Alanine	0.093	2.58	0.088	2.75	0.093	2.74	2.69
Half-cystine		0.42		0.41		0.41	0.41
Valine	0.079	2.19	0.074	2.31	0.077	2.26	2.25
Isoleucine	0.031	0.86	0.030	0.94	0.030	0.88	0.89
Leucine	0.126	3.50	0.123	3.42	0.125	3.68	3.67
Tyrosine	0.036	1.00	0.036	1.12	0.036	1.06	1.06
Phenylalanine	0.054	1.50	0.054	1.69	0.055	1.62	1.60
Lysine	0.080	2.22	0.083	2.59	0.082	2.41	2.41
Histidine	0.032	0.89	0.032	1.00	0.032	0.94	0.94
Ammonia	0.085	2.39	0.081	2.53	0.082	2.41	2.44
Arginine	0.037	1.03	0.036	1.22	0.038	1.12	1.12

Amino Acid Analysis

Tanned BSA: p-benzoquinone incubation, pH 8.0

p-benzoquinone:BSA-amino acid residue mole ratio of 1:10

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.086	2.96	0.079	3.04	0.083	2.86	2.95
Threonine	0.051	1.76	0.048	1.85	0.050	1.72	1.78
Serine	0.043	1.48	0.040	1.54	0.042	1.45	1.49
Glutamic acid	0.127	4.38	0.119	4.58	0.127	4.38	4.45
Proline	0.049	1.69	0.040	1.54	0.046	1.59	1.61
Glycine	0.029	1.00	0.026	1.00	0.029	1.00	1.00
Alanine	0.071	2.45	0.068	2.62	0.070	2.41	2.49
Half-cystine		0.31		0.36		0.33	0.33
Valine	--*	--*	0.060	2.31	0.054	1.86	2.09
Isoleucine	0.020	0.69	0.022	0.85	0.025	0.86	0.80
Leucine	0.098	3.38	0.093	3.58	0.098	3.38	3.45
Tyrosine	0.029	1.00	0.028	1.08	0.028	0.96	1.01
Phenylalanine	0.044	1.52	0.040	1.54	0.043	1.48	1.51
Lysine	0.083	2.86	0.080	3.08	0.095	3.28	3.07
Histidine	0.024	0.83	0.023	0.88	0.024	0.83	0.85
Ammonia	0.244	8.41	0.176	6.77	0.167	5.76	6.98
Arginine	0.036	1.24	0.034	1.31	0.037	1.28	1.28

*Undetermined due to amino acid analyzer malfunction

Amino Acid Analysis

Tanned BSA: p-benzoquinone incubation, pH 8.0

p-benzoquinone:BSA-amino acid residue mole ratio of 1:1

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.090	3.21	0.088	3.26	0.083	3.19	3.22
Threonine	0.052	1.86	0.052	1.93	0.046	1.77	1.85
Serine	0.043	1.54	0.044	1.63	0.038	1.46	1.54
Glutamic acid	0.127	4.54	0.135	5.00	0.118	4.54	4.69
Proline	0.045	1.61	0.053	1.96	0.046	1.77	1.78
Glycine	0.028	1.00	0.027	1.00	0.026	1.00	1.00
Alanine	0.071	2.54	0.077	2.85	0.066	2.54	2.64
Half-cystine		0.39		0.32		0.29	0.33
Valine	0.055	1.96	0.063	2.33	0.053	2.04	2.11
Isoleucine	0.022	0.78	0.023	0.85	0.019	0.73	0.79
Leucine	0.094	3.34	0.101	3.74	0.088	3.38	3.49
Tyrosine	0.030	1.07	0.031	1.15	0.029	1.12	1.11
Phenylalanine	0.042	1.50	0.045	1.67	0.040	1.54	1.57
Lysine	0.055	1.96	0.055	2.04	0.050	1.92	1.97
Histidine	0.015	0.54	0.014	0.52	0.012	0.46	0.51
Ammonia	0.191	6.82	0.119	4.41	0.203	7.81	6.35
Arginine	0.040	1.43	0.039	1.44	0.035	1.35	1.41

Amino Acid Analysis

Tanned BSA: p-benzoquinone incubation, pH 8.0

p-benzoquinone:BSA-amino acid analysis mole ratio of 2:1

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.084	2.90	0.081	3.00	0.079	2.92	2.94
Threonine	0.049	1.69	0.048	1.78	0.047	1.74	1.74
Serine	0.054	1.86	0.039	1.44	0.039	1.44	1.58
Glutamic acid	0.127	4.38	0.124	4.59	0.124	4.59	4.52
Proline	0.047	1.62	0.046	1.70	0.046	1.70	1.67
Glycine	0.029	1.00	0.027	1.00	0.027	1.00	1.00
Alanine	0.071	2.45	0.069	2.56	0.068	2.52	2.51
Half-cystine		0.28		0.29		0.30	0.29
Valine	0.059	2.03	0.060	2.22	0.058	2.15	2.13
Isoleucine	0.022	0.76	0.021	0.78	0.024	0.89	0.81
Leucine	0.095	3.28	0.094	3.48	0.096	3.56	3.44
Tyrosine	0.028	0.96	0.027	1.00	0.028	1.04	1.00
Phenylalanine	0.041	1.41	0.041	1.52	0.042	1.56	1.50
Lysine	0.046	1.59	0.045	1.67	0.046	1.70	1.65
Histidine	0.012	0.41	0.011	0.41	0.012	0.44	0.42
Ammonia	0.180	6.21	0.143	5.30	0.158	5.85	5.79
Arginine	0.035	1.21	0.034	1.26	0.036	1.33	1.27

Amino Acid Analysis

Tanned BSA: p-benzoquinone incubation, pH 8.0

p-benzoquinone:BSA-amino acid residue mole ratio of 5:1

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.087	3.22	0.082	3.04	0.092	2.97	3.07
Threonine	0.052	1.92	0.048	1.78	0.055	1.77	1.82
Serine	0.042	1.56	0.041	1.52	0.045	1.45	1.51
Glutamic acid	0.132	4.89	0.124	4.59	0.136	4.39	4.62
Proline	0.056	2.07	0.052	1.92	0.058	1.87	1.95
Glycine	0.027	1.00	0.027	1.00	0.031	1.00	1.00
Alanine	0.072	2.67	0.070	2.59	0.077	2.48	2.58
Half-cystine		0.23		0.25	2.37	0.24	0.24
Valine	0.066	2.44	0.064	2.37	0.071	2.29	2.37
Isoleucine	0.021	0.78	0.022	0.81	0.023	0.74	0.78
Leucine	0.100	3.70	0.094	3.48	0.103	3.32	3.50
Tyrosine	0.030	1.11	0.028	1.04	0.030	0.97	1.04
Phenylalanine	0.045	1.67	0.042	1.56	0.044	1.42	1.55
Lysine	0.041	1.52	0.041	1.52	0.042	1.35	1.46
Histidine	0.006	0.22	0.007	0.26	0.007	0.23	0.24
Ammonia	0.158	5.85	0.130	4.81	0.130	4.19	4.95
Arginine	0.037	1.37	0.038	1.41	0.038	1.22	1.33

Amino Acid Analysis

Standard amino acid mixture subjected to hydrolysis conditions in the presence of pyrocatechol and p-benzoquinone

Samples prepared to yield a theoretical concentration of 0.10 μ mole of each amino acid per ml. Amino acid analysis data given as μ moles amino acid/ml.

	Mole Ratios		
	Amino Acid: Pyrocatechol	Amino Acid: p-benzoquinone	
Amino acid	1:1	1:1	1:10
Aspartic acid	0.104	0.104	0.106
Threonine	0.101	0.102	0.106
Serine	0.095	0.095	0.102
Glutamic acid	0.109	0.108	0.112
Proline	0.112	0.109	0.092
Glycine	0.108	0.106	0.110
Alanine	0.103	0.106	0.106
Half-cystine	0.103	0.095	0.106
Valine	0.114	0.122	0.123
Methionine	0.099	0.095	0.087
Isoleucine	0.102	0.103	0.110
Leucine	0.108	0.104	0.111
Tyrosine	0.104	0.102	0.109
Phenylalanine	0.104	0.102	0.107
Lysine	0.106	0.108	0.108
Histidine	0.107	0.106	0.109
Arginine	0.108	0.108	0.111

Amino Acid Analysis

BSA Control (Enzymatic oxidation of pyrocatechol systems)

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.084	2.80	0.091	2.94	0.085	2.93	2.89
Threonine	0.049	1.63	0.054	1.74	0.050	1.72	1.70
Serine	0.042	1.40	0.044	1.42	0.041	1.41	1.41
Glutamic acid	0.116	3.87	0.129	4.16	0.118	4.07	4.03
Proline	0.041	1.37	0.048	1.55	0.053	1.83	1.58
Glycine	0.030	1.00	0.031	1.00	0.028	1.00	1.00
Alanine	0.065	2.17	0.075	2.42	0.070	2.41	2.33
Half-cystine		0.43		0.47		0.46	0.45
Valine	0.047	1.57	0.054	1.74	0.049	1.69	1.67
Isoleucine	0.023	0.85	0.025	0.81	0.022	0.76	0.81
Leucine	0.088	2.93	0.099	3.19	0.092	3.17	3.10
Tyrosine	0.030	1.00	0.032	1.03	0.031	1.07	1.03
Phenylalanine	0.040	1.33	0.044	1.42	0.043	1.48	1.41
Lysine	0.102	3.40	0.109	3.52	0.106	3.66	3.53
Histidine	0.027	0.90	0.030	0.97	0.028	0.97	0.95
Ammonia	0.236	7.87	0.161	5.19	0.151	5.21	6.09
Arginine	0.039	1.30	0.043	1.39	0.041	1.41	1.37

Amino Acid Analysis

Tanned BSA: Tyrosinase oxidation of pyrocatechol, pH 5.5

Pyrocatechol:BSA-amino acid residue mole ratio of 1:1

Enzyme level at 110 units (0.1 mg)

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.139	3.02	0.135	3.07	0.086	2.87	2.99
Threonine	0.084	1.83	0.079	1.80	0.052	1.73	1.79
Serine	0.065	1.41	0.065	1.48	0.045	1.50	1.46
Glutamic acid	0.201	4.37	0.193	4.39	0.125	4.17	4.31
Proline	0.077	1.67	0.076	1.73	0.054	1.80	1.73
Glycine	0.046	1.00	0.044	1.00	0.030	1.00	1.00
Alanine	0.112	2.43	0.108	2.45	0.071	2.37	2.42
Half-cystine		0.29		0.34		0.33	0.32
Valine	0.084	1.83	0.078	1.77	0.050	1.67	1.76
Isoleucine	0.035	0.76	0.035	0.80	0.022	0.73	0.76
Leucine	0.154	3.35	0.149	3.39	0.095	3.17	3.30
Tyrosine	0.046	1.00	0.046	1.04	0.030	1.00	1.01
Phenyl alanine	0.067	1.46	0.066	1.50	0.045	1.50	1.49
Lysine	0.133	2.89	0.132	3.00	0.085	2.83	2.91
Histidine	0.033	0.72	0.033	0.75	0.022	0.73	0.73
Ammonia	0.223	4.85	0.173	3.91	0.136	4.53	4.43
Arginine	0.064	1.39	0.063	1.43	0.040	1.33	1.38

Amino Acid Analysis

Tanned BSA: Tyrosinase oxidation of pyrocatechol, pH 7.0

Pyrocatechol:BSA-amino acid residue mole ratio of 1:1

Enzyme level at 110 units (0.1 mg)

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.084	3.11	0.091	2.94	0.083	2.96	3.00
Threonine	0.050	1.85	0.055	1.77	0.051	1.82	1.81
Serine	0.041	1.52	0.045	1.45	0.043	1.54	1.50
Glutamic acid	0.118	4.37	0.132	4.26	0.122	4.36	4.33
Proline	0.051	1.89	0.051	1.64	0.050	1.78	1.77
Glycine	0.027	1.00	0.031	1.00	0.028	1.00	1.00
Alanine	0.066	2.44	0.074	2.39	0.069	2.46	2.43
Half-cystine		0.31		0.31		0.30	0.31
Valine	0.049	1.81	0.061	1.97	0.047	1.68	1.82
Isoleucine	0.022	0.82	0.023	0.74	0.022	0.79	0.78
Leucine	0.090	3.33	0.096	3.10	0.092	3.28	3.24
Tyrosine	0.028	1.04	0.032	1.03	0.031	1.11	1.06
Phenylalanine	0.042	1.56	0.044	1.42	0.043	1.54	1.51
Lysine	0.075	2.78	0.078	2.52	0.072	2.57	2.62
Histidine	0.022	0.81	0.025	0.81	0.022	0.78	0.80
Ammonia	0.131	4.85	0.129	4.16	0.126	4.50	4.50
Arginine	0.038	1.41	0.042	1.35	0.040	1.43	1.40

Amino Acid Analysis

Tanned BSA: Tyrosinase oxidation of pyrocatechol, pH 7.5

Pyrocatechol:BSA-amino acid residue mole ratio of 1:1

Enzyme level at 110 units (0.1 mg)

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.092	2.79	0.092	2.97	2.88
Threonine	0.055	1.67	0.054	1.74	1.71
Serine	0.048	1.45	0.046	1.48	1.46
Glutamic acid	0.135	4.09	0.133	4.29	4.19
Proline	0.056	1.70	0.056	1.81	1.76
Glycine	0.033	1.00	0.031	1.00	1.00
Alanine	0.077	2.33	0.075	2.42	2.38
Half-cystine		0.32		0.32	0.32
Valine	0.060	1.82	0.058	1.87	1.84
Isoleucine	0.025	0.76	0.024	0.77	0.76
Leucine	0.102	3.09	0.094	3.03	3.06
Tyrosine	0.035	1.06	0.035	1.13	1.10
Phenylalanine	0.048	1.45	0.047	1.52	1.48
Lysine	0.074	2.24	0.072	2.32	2.28
Histidine	0.024	0.73	0.023	0.74	0.74
Ammonia	0.149	4.52	0.122	3.94	4.23
Arginine	0.040	1.21	0.039	1.26	1.24

Amino Acid Analysis

Tanned BSA : Tyrosinase oxidation of pyrocatechol, pH 8.0

Pyrocatechol:BSA amino acid residue mole ratio of 1:1

Enzyme level at 110 units (0.1 mg)

Amino acid	μ moles ml	AA/Gly Ratio	μ moles ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.088	2.93	0.092	2.97	2.95
Threonine	0.054	1.70	0.053	1.71	1.70
Serine	0.042	1.40	0.045	1.45	1.42
Glutamic acid	0.125	4.17	0.131	4.22	4.20
Proline	0.047	1.57	0.052	1.68	1.62
Glycine	0.030	1.00	0.031	1.00	1.00
Alanine	0.073	2.43	0.075	2.42	2.42
Half-cystine		0.30		0.31	0.30
Valine	0.054	1.80	0.057	1.84	1.82
Isoleucine	0.022	0.73	0.023	0.74	0.074
Leucine	0.094	3.13	0.095	3.06	3.10
Tyrosine	0.031	1.03	0.034	1.10	1.06
Phenylalanine	0.045	1.50	0.045	1.45	1.48
Lysine	0.071	2.37	0.072	2.32	2.34
Histidine	0.025	0.83	0.026	0.84	0.84
Ammonia	0.131	4.37	0.228	7.36	5.86
Arginine	0.039	1.30	0.039	1.26	1.28

Amino Acid Analysis

Tanned BSA: Tyrosinase oxidation of pyrocatechol, pH 5.5

Pyrocatechol:BSA-amino acid residue mole ratio of 1:1

Enzyme level at 220 units (0.2 mg) and 55 units (0.05 mg)

	220 Units		55 Units			Avg. Value
Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	AA/Gly Ratio
Aspartic acid	0.122	2.84	0.090	3.10	0.091	3.03	3.06
Threonine	0.073	1.70	0.053	1.83	0.053	1.77	1.80
Serine	0.059	1.37	0.044	1.52	0.043	1.43	1.48
Glutamic acid	0.173	4.02	0.132	4.55	0.127	4.23	4.39
Proline	0.072	1.67	0.058	2.00	0.053	1.77	1.81
Glycine	0.043	1.00	0.029	1.00	0.030	1.00	1.00
Alanine	0.098	2.28	0.073	2.52	0.075	2.50	2.51
Half-cystine		0.31		0.27		0.35	0.31
Valine	0.066	1.53	0.053	1.83	0.056	1.87	1.85
Isoleucine	0.032	0.74	0.022	0.76	0.022	0.73	0.74
Leucine	0.128	2.98	0.093	3.21	0.094	3.13	3.17
Tyrosine	0.039	0.91	0.031	1.07	0.032	1.07	1.07
Phenylalanine	0.059	1.37	0.044	1.52	0.046	1.53	1.52
Lysine	0.106	2.46	0.088	3.03	0.089	2.97	3.00
Histidine	0.027	0.63	0.023	0.79	0.024	0.80	0.80
Ammonia	0.172	4.00	0.120	4.14	0.122	4.07	4.10
Arginine	0.055	1.28	0.038	1.31	0.039	1.30	1.31

Amino Acid Analysis

Tanned BSA: Tyrosinase oxidation of pyrocatechol, pH 5.5

Pyrocatechol:BSA-amino acid residue mole ratio of 1:1

Enzyme level at 11 units (0.01 mg)

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.091	3.03	0.088	3.03	3.03
Threonine	0.053	1.77	0.051	1.76	1.76
Serine	0.043	1.43	0.043	1.48	1.46
Glutamic acid	0.130	4.33	0.128	4.41	4.37
Proline	0.052	1.73	0.051	1.76	1.74
Glycine	0.030	1.00	0.029	1.00	1.00
Alanine	0.074	2.47	0.073	2.52	2.50
Half-cystine		0.42		0.43	0.42
Valine	0.063	2.10	0.053	1.83	1.96
Isoleucine	0.023	0.77	0.022	0.76	0.76
Leucine	0.094	3.13	0.093	3.21	3.17
Tyrosine	0.033	1.10	0.032	1.10	1.10
Phenylalanine	0.045	1.50	0.045	1.55	1.52
Lysine	0.100	3.33	0.098	3.38	3.36
Histidine	0.027	0.90	0.026	0.90	0.90
Ammonia	0.114	3.80	0.105	3.62	3.71
Arginine	0.040	1.33	0.039	1.34	1.34

Amino Acid Analysis

Tanned BSA: Tyrosinase oxidation of pyrocatechol, pH 5.5

Pyrocatechol:BSA-amino acid residue mole ratio of 1:10

Enzyme level at 110 units (0.1 mg)

Amino acid	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	μ moles / ml	AA/Gly Ratio	Avg. Value AA/Gly Ratio
Aspartic acid	0.104	2.97	0.111	3.17	0.118	3.37	3.17
Threonine	0.062	1.77	0.065	1.86	0.066	1.88	1.84
Serine	0.054	1.54	0.056	1.60	0.058	1.66	1.60
Glutamic acid	0.147	4.20	0.146	4.17	0.153	4.37	4.25
Proline	0.061	1.74	0.051	1.46	0.054	1.54	1.58
Glycine	0.035	1.00	0.035	1.00	0.035	1.00	1.00
Alanine	0.086	2.46	0.086	2.46	0.087	2.46	2.46
Half-cystine		0.47		0.46		0.48	0.47
Valine	0.057	1.63	0.048	1.37	0.057	1.63	1.54
Isoleucine	0.022	0.63	0.025	0.71	0.023	0.66	0.67
Leucine	0.109	3.11	0.111	3.17	0.112	3.20	3.16
Tyrosine	0.034	0.97	0.037	1.06	0.036	1.03	1.02
Phenylalanine	0.050	1.43	0.053	1.51	0.054	1.54	1.49
Lysine	0.112	3.20	0.118	3.37	0.121	3.46	3.34
Histidine	0.028	0.80	0.028	0.80	0.032	0.91	0.84
Ammonia	0.194	5.54	0.125	3.57	0.134	3.83	4.31
Arginine	0.048	1.37	0.043	1.23	0.046	1.31	1.30

Amino Acid Analysis: Gas-liquid chromatographic analysis of N-trifluoroacetyl-L-tryptophan methyl ester (N-TFA-L-tryptophan (ME) subjected to enzymatically oxidized pyrocatechol.

Sample	Corrected integrator units	Sample	Corrected integrator units
N-TFA-L-tryptophan ME Control		Pyrocatechol:N-TFA-L-tryptophan ME @ 1:1	
1	14.5	a-1	12.0
2	15.0	2	11.4
3	16.2 avg = 15.2	3	11.2
		4	9.4 avg. = 11.0
Pyrocatechol:N-TFA-L-tryptophan ME @ 1:10			
a- 1	11.6	b-1	12.3
2	14.4	2	11.9
3	13.5	3	10.0
4	13.8 avg = 13.3	4	11.1 avg. = 11.3
b- 1	12.1		
2	13.0		
3	15.6 avg = 13.6		
c- 1	13.7		
2	13.0		
3	12.4 avg = 13.0		

Amino Acid Analysis: Gas-liquid chromatographic analysis of N-trifluoroacetyl-DL-methionine methyl ester (N-TFA-DL-methionine ME) subjected to enzymatically oxidized pyrocatechol.

Sample	Corrected integrator units	Sample	Corrected integrator units
N-TFA-DL-methionine ME Control		Pyrocatechol:N-TFA -DL-methionine ME @ 1:1	
1	15.7	a-1	12.0
2	15.3	2	12.0 avg.=12.0
3	16.2 avg. = 15.7		
		b-1	12.9
Pyrocatechol:N-TFA-DL-methionine ME @ 1:10		2	13.1
		3	11.8
a-1	15.2	4	13.2 avg.=12.8
2	14.9		
3	14.9 avg. = 15.0		
b-1	13.9		
2	13.3		
3	15.2		
4	15.5 avg. = 14.5		
c-1	14.2		

APPENDIX III. Analysis Data: trypsin and pepsin digestibility of tanned BSA

I. Trypsin digestibility of control and tanned BSA samples

CONTROL		TANNED BSA+					
		base catalyzed oxidation of pyrocatechol		enzymatic oxidation of pyrocatechol			
Sample	μ moles NaOH* uptake/mg BSA/ 10 min.		Sample	μ moles NaOH* uptake/mg BSA/ 10 min.		Sample	μ moles NaOH* uptake/mg BSA/ 10 min.
1a	0.212	Pyrocatechol:BSA amino acid residue mole ratio of 1:10	1a	0.033	Pyrocatechol:BSA-amino acid residue mole ratio of 1:10	1a	0
b	0.232		b	0.033		b	0
2a	0.208		2a	0.021		2a	0
b	0.208		b	0.021		b	0
c	0.198						
			3a	0.016		3a	0.01
3a	0.196		b	0.036		b	0
b	0.196						
c	0.196	Pyrocatechol:BSA-amino acid residue mole ratio of 1:1	1a	0.040			
			b	0.057			
			2a	0.011			
			b	0.023			
			c	0.011			
			3a	0.048			
			b	0.006			
			c	0.030			

*Corrected for base uptake by: a) Sample alone; b) trypsin alone; c) pH adjustment when enzyme added.

+For base catalyzed oxidation of pyrocatechol; pH 8.0
For enzymatic oxidation of pyrocatechol; pH 5.5, 110 units mushroom tyrosinase (1100 units/mg).

II. Pepsin digestibility of control and tanned BSA samples.

CONTROL		TANNED BSA+					
		base catalyzed oxidation of pyrocatechol			enzymatic oxidation of pyrocatechol		
Sample	μ moles HCl uptake/mg BSA/ 5 min		Sample	μ moles HCl* uptake/mg BSA/5 min		Sample	μ moles HCl* uptake/mg BSA/ 5 min
1	0.785	Pyrocatechol:BSA-amino acid residue mole ratio of 1:10	1a	0.540	Pyrocatechol:BSA-amino acid residue mole ratio of 1:10	1a	0.410
2	0.816		b	0.720		b	0.500
3	0.789						
4	0.771		2a	0.610		2a	0.460
5	0.846		b	0.380		b	0.460
6	0.785						
7	0.869		3a	0.600		3a	0.440
8	0.921		b	0.610		b	0.360
						4a	0.460
		Pyrocatechol:BSA-amino acid residue mole ratio of 1:1	1a	0.560			
			b	0.360		5a	0.430
			2a	0.610		b	0.370
			3a	0.530		6a	0.480
			b	0.560			

*Corrected for acid uptake by: a) sample alone; b) pepsin alone; c) system resulting from enzyme addition

+For base catalyzed oxidation of pyrocatechol; pH 8.0
For enzymatic oxidation of pyrocatechol; pH 5.5, 110 units mushroom tyrosinase (1100 units/mg)