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REACTIONS BETWEEN AMINO ACID COMPOUNDS AND PHENOLS DURING OXIDATION

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INTRODUCTION

About 20 to 40 per cent of the nitrogen in the soil organic matter can be hydrolized to free amino acids; about 30 per cent is resistant against acid or alkaline hydrolysis (Bremner [1]).

In contrast to the high content of amino acids which are probably bound in peptide linkage (Scharpenseel and Krause [23]) is the low availability of nitrogen in soil organic matter to micro-organisms (Flaig and Schmidt [5]). Jansson [13] therefore speaks about the passive phase of soil nitrogen in organic linkage.

To explain this passive behaviour against attack of micro-organisms a fixation or binding of the α-amino nitrogen from amino acids or peptides has been considered. Some authors suppose a reaction with amino acids or peptides during oxidation of lignin, phenolic compounds from lignin degradation, or phenols from microbial metabolism. The reaction conditions for these reactions and how resulting products could fit the data about soil organic nitrogen, was the subject of the following work.

Phenols, which are known or supposed to be intermediates during microbial lignin decomposition (Flaig [4],) were oxidized in the presence of different amino acids, peptides or a protein. The oxidation was performed with phenol-oxidases from different fungi in a pH range from 5 to 8.

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Mason [20] compiled the known reaction between phenols and amino acid compounds during oxidation. Some newer contributions can be found by Gordon and Paleg [8].

MATERIALS AND METHODS

Phenols

The phenols were chemically pure compounds available commercially. The hydroxyhydroquinone was prepared from its triacetate and freshly sublimed before use.

Amino acids

The amino acids were also chemically pure commercial products. They were all α amino acids in the l- or dl-form.

Peptides

The peptides were from commercial sources or were synthesized according to usual methods (Greenstein and Winnitz [9]). They were found to be electrophoretically pure.

Protein

Serum albumin from beef was used in the crystalline form (Serva Entwicklungslabor, Heidelberg, Germany).

Enzymes

a) Phenoloxidases: Laccase from *Polystictus versicolor* was prepared and purified according to the method given by Malmström, Fahraeus and Mosbach [18] with slight modifications. $Q_{O2} = 10.000$ μl O_2/mg/h with catechol at pH 4.0. Mushroom polyphenoloxidase was prepared from mushroom spawn according to the method of Freudenberg and Richtzenhain [6] and of Frieden and Ottesen [7] to step 5 and lyophylized. $Q_{O2} = 12.000$ μl O_2/mg/h at pH 7.0 against catechol and it had a low cresolase activity.

b) Protease: For the degradation of the protein a preparation of Nagarse [10] (Nagase and Co., Ltd., Osaka, Japan) from *Bacillus subtilis* was used.

Warburg technique

Oxygen uptake was measured in vessels with one or two side arms. The total volume of the reaction mixture in the main compartment was always 3.0 ml, in the center well. 0.2 ml of 10 per cent NaOH was used to absorb CO_2. For pH control Sörensen phosphate buffer was used. The phenolase was added from the side arm at zero time. Laccase was used for experiments under acid conditions and mushroom phenolase for those under neutral and weak alkaline conditions. Sometimes for experiments run at pH 8 the second side arm of the Warburg vessels was filled with 2 *N* HCl to absorb the ammonia eventually released into the atmosphere, but since no de-

tectable amount of ammonia was found in the acid only one-armed vessels were used. When the oxygen uptake was completed the total solution mixture was acidified with HCl to pH 1. After standing overnight, the suspension was centrifuged to remove the precipitates. These were washed with 1 *N* HCl, dried in vacuum at 100°C and weighed. Kjeldahl nitrogen determinations were made on the precipitates and on an aliquot of the solution. Ammonia in the solution was determined by steam distillation of an aliquot with MgO. Amino nitrogen of the amino acids was determined with ninhydrin according to Bremner [2]. With some amino acids the ammonia obtained by this method did not correspond to the theoretical value; then we had to calculate with a factor. The α-amino-nitrogen of peptides was determined with a colorimetric ninhydrin method (Methods in Enzymology, Volume IV, p. 252, New York, 1959).

Isotopic technique

Specifically labeled amino acids, commercially available (Radiochemical Center, Amersham, Great Britain), were used. The reaction products were converted to CO_2 by wet combustion [24] and precipitated as $BaCO_3$. An aliquot of 40 mg $BaCO_3$ was placed in a glass container in suspension with 600 mg silica gel in 20 ml toluenescintillator and the radiation measured in a scintillation counter (Tri-Carb Liquid Scintillation Spectrometer, Series 314 A, Packard Instruments Co., La Grange, Illinois, U.S.A.). The efficiency by this method was about 60% of the theoretical radioactivity.

Electrophoresis

To separate amino acids and peptides on paper a high voltage electrophoresis was used according to Wieland and Pfleiderer [27] (Hormuth and Vetter, Heidelberg, Germany).

EXPERIMENTAL RESULTS

I. STUDY OF THE REACTIONS BETWEEN PHENOLS AND AMINO ACIDS DURING OXIDATION

1. *Influence of amino acids on the oxygen uptake of phenols during oxidation*

The oxygen uptake of phenols during oxidation in presence of amino acids was seen to be influenced by *a*) the chemical structure of the phenolic compound, *b*) the structure and concentration of the amino acid which was added, and *c*) the pH-value in which the experiments were run. In agreement with James *et al.*[12] we found an additional oxygen uptake when catechol was oxidized in presence of different amino acids at pH 7 or 8. Also in agreement

with James *et al.* [12] we found with different amino acids differences in the additional oxygen uptake.

At pH values below 6.5 with catechol and all other phenols tested, no significant additional oxygen uptake was found by adding glycine or other aliphatic amino acids. Only with proline and tryptophan together with catechol was an increase in oxygen uptake found at pH values below 6.5; but no increase, sometimes even a small decrease, was found with histidine.

An influence on the oxygen uptake in presence of amino acids by phenols different from catechol was shown mainly by those diphenols related to catechol like protocatechuic acid and caffeic acid. With hydroquinone a large additional oxygen uptake was found at pH 7 and 8, but hydroxyhydroquinone and *p*-hydroxycinnamic acid resulted in only a small additional uptake at these pH values; *p*-hydroxybenzoic acid was only oxidized with laccase under acid conditions, mushroom phenolase at pH 6, 7, and 8 did not catalyze oxidation at all. Phenols containing methylated hydroxyl groups like guaiacol, vanillic acid, ferulic acid, and syringic acid showed almost no additional oxygen uptake with amino acids. pH-values varying from 5 to 8 appeared to have little or no influence on these results.

With the vicinal trihydroxybenzene compounds pyrogallol and gallic acid only a small effect on the oxygen uptake by adding amino acids was observed. All these experiments were made at an amino acid : phenol ratio 10 : 1.

2. *Binding and deamination of amino acids by oxidized phenols*

The additional oxygen uptake by oxidizing phenols in presence of amino acids was positively related to the amount of released ammonia. That different amino acids were diversely deaminated when they were oxidized together with catechol was shown by Kisch and Schuwirth [15]. Our studies with other *o*-diphenols with free hydroxylgroups, as protocatechuic, and caffeic acid, showed similar results. No deamination in the presence of different amino acids could be detected during the oxidation of methoxylated phenols, which also showed no additional oxygen uptake with glycine or other amino acids. In spite of the great additional oxygen uptake with amino acids, *p*-hydroquinone yielded almost no release of ammonia. With hydroxyhydroquinone a small amount

of ammonia was released only from gylcine or leucine, other trihydroxybenzene-compounds like pyrogallol or gallic acid showed also no noticeable deamination or binding of nitrogen.

James *et al.*[12] made the observation that for each extra atom of oxygen taken up by oxidizing catechol in presence of different amino acids with phenolase of belladonna, one molecule of ammonia was released from the amino group. In their experiments the molecular ratio of catechol to amino acids was 1 : 10. In Table 1 the additional oxygen uptake and ammonia release by oxidizing different phenols in presence of different amino acids with a molecular ratio of 1 : 5 at pH 7.0 is shown.

TABLE 1

Additional oxygen uptake (exceeding the oxygen uptake of the control) in μatoms oxygen of 20 μmole phenolic compound in presence of 100 μmole of different amino acids in 3 ml buffer at pH 7.0, and the released ammonia in μmole. The oxidation was catalyzed with 100 μg mushroom phenolase. With hydroquinone no measurable amount of ammonia could be detected

Amino acids	Catechol		Protocatechuic		Caffeic acid		Hydro-quinone
	μatoms oxygen	μatoms NH_3-N	μatoms oxygen	μatoms NH_3-N	μatoms oxygen	μatoms NH_3-N	μatoms oxygen
Glycine	45	26	33	15	37	19	22
Alanine. . . .	15	8	4	3	18	13	9
Serine.	28	16	20	12	24	18	18
Valine	14	6	13	8	10	9	18
Methionine . .	18	10	14	9	8	4	22
Leucine. . . .	16	8	8	7	22	19	14
Threonine. . .	33	17	12	9	23	22	20
Proline	8	0	3	0	11	0	22
Tryptophan . .	28	8	27	18	38	30	22
Aspartic acid. .	15	7	3	3	5	5	5
Glutamic acid .	21	8	18	12	19	14	18
Lysine	18	7	18	12	19	15	22

Usually, but not always, the oxygen uptake and the deamintion were greater at pH 8.0.

Table 1 shows that the atomic ratio between additional oxygen uptake and released ammonia-N was greater than 1 when catechol was oxidized in the presence of amino acids with mushroom phenolase in a ratio 1 : 5; with protocatechuic acid and caffeic acid with some amino acids there is a nearer approach to one. To determine whether ammonia reacts after release with oxidation

products of the phenols, or if the additional uptake of oxygen is used in the binding of the amino acids, the following experiments were made:

One half mmole of catechol or hydroquinone were reacted with 2.5 mmole of various amino acids in 200 ml Erlenmeyer flasks in 60 ml buffered solution at pH 7 and 8 on a shaking machine for 24 hours at 20 to 22°C, with 3 mg mushroom phenolase to catalyze the reaction. After 24 hours the solution was acidified to pH 1 with HCl and, after standing overnight, centrifuged. The precipitates were washed with 1 *N* HCl, dried in vacuum at 100°C to constant weight and analyzed for nitrogen; the remaining solution combined with the washwater was analyzed as described before. The results of these experiments are given in Table 2 for pH 7. At pH 8.0 the results were similar except the ammonia content was sometimes a little higher.

TABLE 2

N found in solution and precipitate after acidification of reaction products of phenolic compounds oxidized in presence of amino acids. 0.5 mmole phenolic compound together with 2.5 mmole amino acid in 60 ml buffer pH 7.0 shaken for 24 hours together with 3 mg mushroom phenolase. Nitrogen is stated in μatom N

Amino acid	Catechol				Hydroquinone			
	μatom NH_3-N in sol.	Weights of ppt.*	N in ppt.	Difference N in sol.**	μatom NH_3-N in sol.	Weights of ppt.	N in ppt.	Difference N in sol.
Glycine . . .	452	75.1	304	170	40	43.9	140	160
Alanine . . .	120	59.3	230	60	28	52.7	149	110
Valine. . . .	92	82.5	220	30	34	65.3	207	—
Leucine . . .	214	88.3	213	100	50	112.0	389	60
Isoleucine . .	120	108.5	269	80	32	122.7	500	—
Threonine . .	319	66.4	175	290	62	45.0	65	200
Methionine. .	252	148.0	480	0	67	107.0	334	—
Aspartic acid.	170	95.2	236	77	61	60.5	106	20
Glutamic acid	180	99.3	255	95	54	81.8	116	140
Tryptophan .	140	334.8	1590	†	57	271.6	1341	†

* ppt. = precipitate.

** 'Difference nitrogen' means Kjeldahl-N in the solution minus ammonia-N minus α-amino N. This gives a measure of the amount of N which is bound in oxidation products of the phenols and cannot be precipitated by hydrochloric acid at pH 1.

† The amino-nitrogen of tryptophan could not be determined according to Bremner[2].

In order to see how ammonia would react with oxidation products of phenolic compounds similar experiments with ammonium sulfate instead of amino acids were made. Warburg experiments with different phenols detected no measurable effect on the oxygen

uptake in presence of different ammonia concentration at pH values varying from 6 to 8. In the experiments shown in Table 3 different equivalents of ammonia were added to give ratios 1 : 1, 1 : 2, and 1 : 5 of phenolic compound to ammonia.

TABLE 3

Nitrogen bound from ammonium sulfate during oxidation of phenolic compounds. 500 μmole phenolic compound oxidized together with 500, 1000, and 2500 μ equivalents of $(NH_4)_2SO_4$ in 60 ml buffer pH 7.0 and 8.0. All nitrogen values are given in μatom N

NH_3 μ-equiv.	Catechol				Hydroquinone			
	pH 7		pH 8		pH 7		pH 8	
	N in ppt. μatom	diff.* N in sol. μatom	N in ppt. μatom	diff.* N in sol. μatom	N in ppt. μatom	diff.* N in sol. μatom	N in ppt. μatom	diff.* N in sol. μatom
500	47	18	55	31	17	41	26	41
1000	52	11	75	34	18	57	42	48
2500	64	20	95	36	50	56	64	63

* 'Diff. N in sol.' means the difference of total Kjeldahl nitrogen and ammonia-N in solution.

Experiments run with hydroxyhydroquinone under these conditions showed essentially the same results.

3. *Studies about the reaction products of amino acids with oxidized phenols*

Only small amounts of free amino groups or of free ammonia were detected in the precipitates obtained from the experiments shown in Tables 2 and 3 by the Van Slyke method or with MgO. Other aliquots were hydrolyzed with 6 *N* HCl in sealed tubes at 120°C for 14 hours. The dried hydrolisation products were redissolved in water and pherographed on paper with a high-voltage electrophoresis. On the pherograms many spots were detected by fluorescence under ultraviolet light, which had moved toward the negative pole in an acid buffer of pH 1.9. Beside a very faint spot of the used amino acid, no positive spots could be detected after spraying with ninhydrin.

The next objective was to get information about what happened to the carbon skeleton of amino acids both when they were bound in the reaction products and when deamination took place. There-

fore, some experiments with specific C^{14}-labelled amino acids were made in a Warburg apparatus in the usual way.

The vessels were filled with 2.5 ml of a solution which contained 50 μmoles of catechol or hydroquinone and 150 μmoles of the amino acids in buffer at pH 7 or 8, the reaction was started by adding a solution of 150 μg of phenolase in 0.5 ml water or buffer. Per vessel, 0.1 μc of the specific labeled amino acid was used. In the center well of the vessels 0.2 ml of 10% sodium hydroxide was placed to absorb the CO_2. When the oxygen uptake was completed, the solution in the main compartment was acidified to pH 5 to release the retained CO_2 during shaking for another two hours. After the sodium hydroxide was changed, the solution in the main compartment was brought to pH 1 with hydrochloric acid. After shaking two hours and standing overnight, all solutions were taken out. The solutions of the main compartments were centrifuged; the precipitates were washed, centrifuged again, and dried. The amount of isotopic carbon in the precipitates, centrifuged solutions and the different sodium hydroxide solutions was determined, and the nitrogen content was determined in the different fractions. Results of these experiments are shown for pH 7 in Table 4.

TABLE 4

Distribution of C^{14} and N from labeled amino acids present during oxidation of catechol and hydroquinone at pH 7. 3 ml solution containing 50 μmole catechol or hydroquinone together with 150 μmole of labeled amino acid (0.1 μc) oxidized by action of 250 μg phenolase in buffer pH 7.0

Phenol used	Fraction analyzed **	Amino acid present				
		Glycine 1, 2-C^{14} %	Glycine 2-C^{14} %	Valine 1-C^{14} %	Glutamic acid 1-C^{14} %	Glutamic acid 3, 4-C^{14} %
Catechol	Activity in 1st NaOH	31 *	1.1	14.1	21.2	0.2
	Activity in 2nd NaOH	1.8 *	0.2	0.7	2.5	0.1
	NH_3-N in solution	20.2	20.8	10.0	15.5	15.5
	Activity in ppt.	13.8	14.8	8.3	5.8	7.6
	N in ppt.	17.5	18.2	10.1	9.6	10.0
Hydroquinone	Activity in 1st NaOH	1.4 *	0.3	1.3	1.8	0.1
	Activity in 2nd NaOH	1.2 *	0.1	0.7	1.4	0.1
	NH_3-N in solution	1.1	1.0	0.5	1.5	1.3
	Activity in ppt.	14.2	14.8	8.5	4.8	5.0
	N in ppt.	15.5	15.9	8.6	5.5	5.8

* These values have been doubled to represent the % of carboxyl groups which were decarboxylated, since the activity of the carboxyl group is only half of the total activity in the molecule.

** Values related to C^{14} and N-content of the used amount of amino acid.

Table 4 shows that more CO_2 from the carboxyl group was evolved than NH_3 from the amino group by the action of oxidized catechol. In order to see how the different reaction products would vary with different amounts of amino acids and constant amounts of phenols, some experiments were made; Table 5 shows the results of catechol with different amounts of glycine. Evolved CO_2 was determined with carboxyl labeled glycine.

TABLE 5

Effect of varying the ratio between catechol and glycine on oxygen uptake, CO_2 and NH_3 released and precipitate. 50 μmole catechol in presence of increasing amounts of glycine in 3 ml buffer pH 7.0 oxidized by action of 250 μg phenolase. Nitrogen is indicated in μatoms N

Ratio catechol to glycine (μmole)	Additional oxygen uptake (μatom)	NH_3-N released (μatoms)	CO_2 released (μmole)	weight of ppt. (mg)	Nin ppt.	Diff. * Nin sol.
50 : 10	9	1.4	5.0	6.9	7.1	0
50 : 20	22	3.6	9.3	7.7	14.8	2
50 : 50	31	7.8	23.0	8.2	22.0	8
50 : 100	58	23.5	38.0	8.3	26.8	12
50 : 200	63	38.7	43.5	3.6	18.5	18
50 : 500	92	77.2	72.2	3.0	12.8	25
50 : 1000	106	103.5	101.0	1.6	9.5	40
50 : 2000	100	102.4	101.5	0.9	5.8	45

* See footnote in Table 2.

Discussion of the results from Part I

The reaction between the α-amino group of amino acids and oxidized phenols began to start at pH values higher than 6.5. Only those phenols which have no methylated hydroxyl group reacted when oxidized in presence of amino acids. The increase in oxygen uptake by oxidizing phenols in presence of amino acids was positively related to deamination and the release of ammonia; but with hydroquinone almost no deamination could be detected (Table 1). Differences in the content of nitrogen of the oxidation products could be detected with different amino acids and also with different phenols. The highest amount of nitrogen was found in oxidation products of dihydroxyphenols like catechol, protocatechuic acid, caffeic acid and hydroquinone when oxidized in presence of amino acids. The highest nitrogen content in the reaction products seemed to be 1 atom of nitrogen per phenolic unit.

With C^{14}-labeled amino acids (Table 4) it could be shown that amino acids were bound as a whole molecule in the oxidation products. Also, the carboxyl group of reacted amino acids is not split off after acidifying to pH 1, as it was supposed by Jackson and Kendal [11]. The deamination of amino acids through action of oxidized *o*-diphenols was accompanied by decarboxylation. Therefore, the reaction products of amino acids after deamination cannot be α-ketoacids as mentioned by James *et al.*[12] and Trautner and Roberts [26].

For ratios of amino acid to catechol below 5 : 1, the release of CO_2 from the carboxyl group of amino acids was greater than the release of ammonia from the amino group, while with higher concentrations of amino acids NH_3 to CO_2 is around one (Table 5). Table 4 shows also that with C^{14}-labeled amino acids the content of nitrogen in the precipitates was greater than the content of radioactive carbon. Reaction of the released ammonia with oxidized phenols was not the reason for these results. As a theory one might suppose that at low amino acid concentrations, a reaction of the intermediate formed aminohydroquinone with other quinones takes place to form phenoxazones [3 17 21], while higher amino acid concentrations result mainly in a nucleophilic addition on the quinone.

The observation of James *et al.*[12] that for each extra atom of oxygen taken up by catechol when oxidized together with amino acids, one molecule of ammonia is released from the amino group is only valid for high amino acid concentrations (Table 5). The relatively low oxygen uptake in the range of high amino acid concentrations seems to be explained by the presumption of Mason [13] that the nucleophilic addition product which results from *o*-quinone and amino acid is not oxidized by phenolase but only by a surplus of *o*-quinone. This might be indicated also by the decrease in precipitability with hydrochloric acid of oxidation products in the range of high amino acid concentration (Table 5).

II. CONTRIBUTIONS ABOUT THE REACTION OF PHENOLS DURING OXIDATION WITH PEPTIDES AND A PROTEIN

Previously Kisch [14] and Mason [19] made the observation that some di- and tripeptides were deaminated by the action of catechol during oxidation. To study how phenols react with peptides during oxidation, some experiments were made with peptides similar to those described before with amino acids. The results from glycylglycine and diglycylglycine in the presence of catechol, caffeic acid, hydroxyhydroquinone and hydroquinone at pH 6, 7, and 8 were similar to those from glycine with these phenols. With higher peptide concentrations usually no precipitates could be obtained with hydrochloric acid. In Table 6 the results for catechol and these two peptides are shown. The results with other phenols were similar, but hydroxyhydroquinone had a stronger deaminative action on these peptides than on glycine (Kisch [14]). As with amino acids, hydroquinone showed no deamination.

TABLE 6

Reaction products of peptides and phenols during oxidation.
20 μmole of phenolic compound together with increasing amounts of glycylglycine or diglycylglycine in 3 ml buffer pH 7.0 oxidized by action of 150 μg mushroom phenolase. All nitrogen values are given in μmole NH_3; difference nitrogen means Kjeldahl nitrogen in the solution minus ammonia-N minus (α-amino nitrogen multiplied with 2 or 3) depending on the number of nitrogen atoms in the peptide. α-Amino nitrogen was determined colorimetrically with ninhydrin

Peptide	(μmole)	Catechol				
		Additional oxygen uptake (μatom)	NH_3-N released in sol.	Weight of ppt. (mg)	N in ppt.	Diff. N in sol.
Glycylglycine	20	18	3	2.7	12	10
	40	24	6	3.1	12	14
	80	44	8	3.0	11	27
	160	56	17	2.2	8	31
Diglycylglycine	20	18	9	2.6	22	7
	40	24	14	2.6	20	32
	80	30	19	2.3	17	35
	160	49	21	1.8	14	40

According to the nitrogen content in the precipitable and non-precipitable reaction products between peptides and oxidized phenolic compounds the highest molecular ratio of peptide to

phenolic unit was 1 : 1, this indicates that only the α-amino group was in reaction with oxidized phenols and the nitrogen of the peptide group does not participate.

To study the effect of a peptide chain with different amino acids on the activity of the amino group in peptides we made experiments with the peptides: Glycyl-alanine; glycyl-valyl-leucine; glycyl-valyl-isoleucine; glycyl-methionyl-isoleucine; leucyl-glycine; tryptophanyl-alanine together with catechol, caffeic acid, hydroxyhydroquinone and hydroquinone. The results of these experiments were that the rest of the peptides did not influence significantly the activity of the amino group. In the peptides with glycine as N-terminal group, no great difference was found in deamination as well as in formation of nitrogen containing reaction products between the oxidized phenols and the peptides.

Nitrogen in soil organic matter can be hydrolyzed with mineral acids partly to amino acids. Could amino acids also be hydrolyzed from the reaction products between oxidized phenols and peptides. Figure 1 shows an example of these experiments. The HCl-precipitable reaction product which resulted from caffeic acid and glycyl-

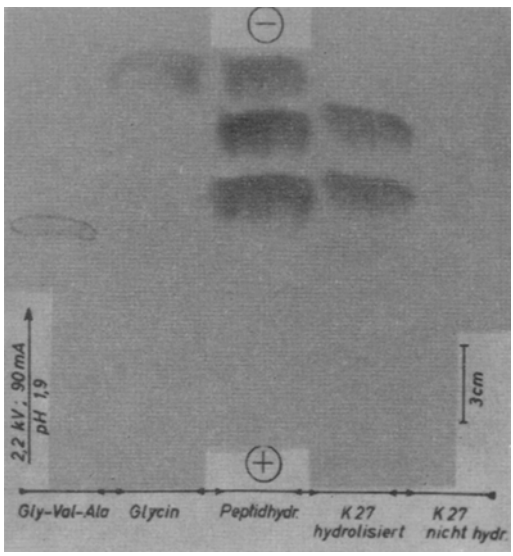

Fig. 1. Electropherogram of a hydrolysate of the reaction product K 27 between oxidized caffeic acid and glycyl-valyl-alanine; comparison substances are plotted in the same concentrations.

valyl-leucine were hydrolyzed with 6 *N* HCl. After evaporating, the residue was dissolved in water and aliquots from it separated on paper with a high voltage electrophoresis (K 27 hydr.). An electrophoretic pattern was developed from glycine and a hydrolysate of the pure peptides in the same way.

In the hydrolysate from the reaction product the N-terminal amino acid glycine was absent; this can be understood if the peptide reacts with its amino group. The amino acids which have their position following the N-terminal amino acid seemed to be hydrolyzed normally.

Mason [19] studied the oxygen uptake from catechol during oxidation in presence of serum albumin. He observed an increase in oxygen uptake if the molecular ratio of catechol to serum albumin was greater than 3 : 1.

Our results agreed with those of Mason and similar observations were made with other phenolic compounds.

Some experiments were made with serum albumin and different phenols to study the activity of the α-amino group of the N-terminal amino acid and the ε-amino group of the lysine residues in the protein.

According to Thompson [25] this protein has a molecular weight of 65.000; its N-terminal amino acid is aspartic acid. According to Wold [28] it has 14 lysine residues in the molecule and the amount of cysteine seemed to be small (around one mole per mole of serum albumin).

In Warburg vessels 10 μmole of catechol or hydroquinone were oxidized together with 1, 5, or 10 μmoles of crystalline serum albumin at pH 7 or 8. After the reaction the duplicates were combined and aliquots were reacted with dinitrofluorobenzene according to the method of Sanger and Thompson [22], and the dinitrophenylprotein was separated. After hydrolysis the resulting DNP*-amino acids were chromatographed on paper and the DNP-aspartic acid and DNP-lysine eluted and quantitatively determined in a colorimeter. Results of these experiments are given for pH 7 in Table 7.

The results shown in Table 7 indicate that both types of amino groups in serum albumin can react with oxidation products of

* DNP = Dinitrophenyl.

TABLE 7

Recovery of original aspartic acid and lysine from serum albumin after reaction during phenol oxidation.

10 μmole catechol or hydroquinone were oxidized in presence of 1, 5, or 10 μmole serum albumin from beef in 3 ml buffer pH 7.0 by action of 100 μg mushroom phenolase. After reaction the protein was converted to DNP-protein, this was hydrolyzed and the resulted DNP-amino acids chromatographed and determined with the colorimeter. The optical density was compared with the DNP-amino acids resulting from the same amount of unreacted serum albumin

Ratio phenolic compound to serum albumin	Catechol		Hydroquinone	
	% of original DNP-aspartic acid	% of original DNP-ε-lysine	% of original DNP-aspartic acid	% of original DNP-ε-lysine
10 : 1	10	40	5	45
10 : 5	19	90	12	90
10 : 10	23	98	15	100

phenols, but it seems that the first is more active than the latter.

To determine whether the protein part in the reaction product between serum albumin and these oxidized phenols was stable against the action of a proteinase, the protein was degraded in other aliquots from the previously described Warburg experiments by action of Nagarse [10], a proteinase from *Bacillus subtilis*. The proteolysis was measured according to Kunitz [16] where the protein which was undigested by action of a proteinase was precipitated with trichloroacetic acid and the optical density of the supernatent solution was measured at 280 mμ, the absorption maximum of tyrosine and tryptophane. Proteolysis of the protein took place in the reaction products. The velocity of proteolysis of the reacted serum albumin seemed to be no slower than an unreacted control.

SUMMARY

About 30 per cent of organic soil nitrogen can be hydrolized with HCl to amino acids; about 30 per cent is nonhydrolizable. In contrast to this high content of amino acid nitrogen is the small availability of the nitrogen to micro-organisms. In light of the theory proposing a reaction between the α-amino group of amino acids or peptides and quinones formed during oxidation of lignin degradation products or other phenolic compound, different types of phenols were oxidized by phenolases in presence of amino acid compounds.

It could be shown that the reaction of binding of nitrogen started at pH values higher than 6.5, and that only such phenols reacted which had no

methoxylated hydroxyl groups. The reaction of some phenols during oxidation in presence of amino acids was accompanied by deamination and decarboxylation of the latter.

The reaction products of phenols with amino acids were stable against hydrolysis. Using peptides it was found that all amino acids, except the N-terminal which is bound to oxidized phenols, could be hydrolyzed normally.

With serum albumin it could be shown that there is a reaction with the amino group of the N-terminal amino acid and also with the ε-amino group of lysine residues with phenols during oxidation. The reacted protein seemed to be degraded normally with a protease of *Bacillus subtilis*.

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