

Biochemistry of Soil Organic Matter  
in Relation to Crop Production

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W. Flaig

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Lecture 3.

The Role of Nitrogen in Formation of Humic Substances

W. Flaig

1. Participation of nitrogenous compounds in the formation of humic substances.
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1. Participation of nitrogenous compounds in the formation of humic substances.

In cultures of microorganisms the formation of dark coloured nitrogenous humic like substances has been observed several years ago (BREMNER, FLAIG and KÜSTER 1955, KÜSTER 1952, 1956, LAATSCH, HOOPS and BIENEK 1952, von PLOTHO 1950, 1951, SCHEFFER, von PLOTHO and WELTE and others).

The content of hydrolysable  $\alpha$ -amino-nitrogen of the lignin fractions decreases during the humification of plant material, but the percentage to total nitrogen increases (see lecture 1). This can be explained in two ways. Either nitrogen as amino acids or peptides continues to condense with the lignin during humification, or the peptide chains are increasingly decomposed by the microorganisms. There is the possibility that both of these latter nitrogen reactions occur simultaneously.

Lignin degradation products can always be isolated during the course of humification. Furthermore it could be shown, that the formation of phenols or quinones is possible during oxidative degradation or by enzymatic dehydrogenation of lignin. In addition, it is known, that dark coloured polymers are formed during the oxidation of 1,2-di- or 1,2,3-tri-phenols together with amino acids in the presence of phenoloxidases, (STEINMETZ 1956, SWABY 1956, 1958, FLAIG 1956).

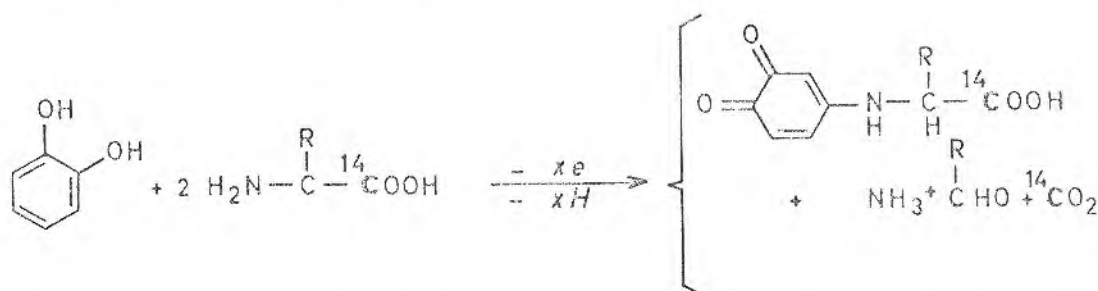


Fig. 1: Addition of amino acids on 1,2-diphenols or oxidative deamination during oxidation.

Investigations of the reactions occurring between amino acids and lignin degradation products in the presence of phenoloxidases showed, that besides the usual oxygen consumption during the addition of amino acids on quinones, by additional oxygen uptake the amino acids are deaminated and decarboxylated. These oxidative reactions are influenced by chemical structure, concentration and by pH. The investigations were made with labelled amino acids to elucidate the mechanism of the reaction. The labelled carbondioxide can be determined easily.

1.1 Conditions of the formation of N-free polymers.

Phenolic lignin degradation products are reactive compounds. Partially methylated o-diphenols and the o-diphenols themselves show some differences in the case of oxidative polymerisation.

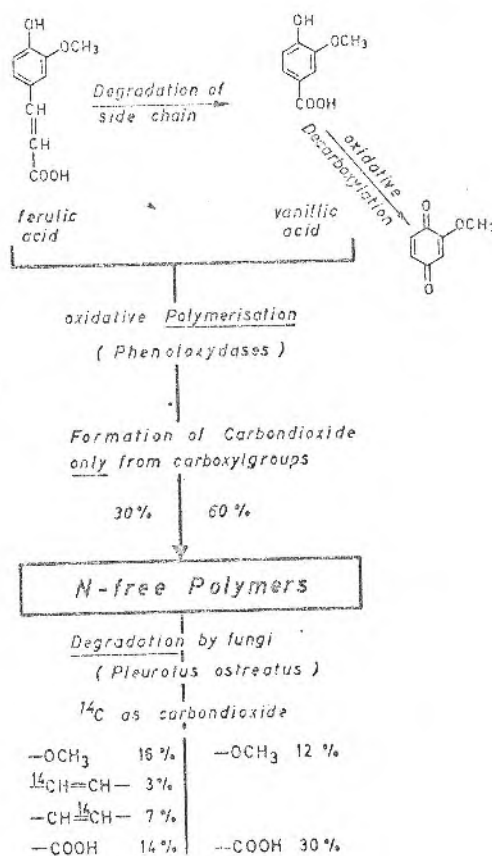


Fig. 2: Oxidative polymerisation of methoxyl substituted phenol carboxylic acids.

The methoxyl substituted lignin degradation products such as ferulic and vanillic acid polymerise in the presence of phenoloxylase from microorganisms. The dehydrogenative polymerisation occurs only when the monomers are substituted with a phenolic hydroxyl group in p-position of the side chain. In case of carboxyl-labelled compounds different quantities of active carbondioxide are split off from the two acids. No labelled carbondioxide is formed when the carbon atoms of the methoxyl groups of the two acids or the carbon atoms 2 and 3 of ferulic acid are labelled.

By oxidative polymerisation nitrogen free polymers are formed. No nucleophilic addition of amino acids occurs in the case of hydroxy-methoxy-compounds.

The polymers are degraded by white rot fungi, such as *Pleurotus ostreatus*. The carbon atoms of the carboxylic and methoxyl groups are more split off by the activity of the microorganisms than the carbon atoms 2 and 3 in the side chain of ferulic acid (HAIDER, LIM and FLAIG 1962, 1964).

Vanillic acid is transformed to methoxy-benzoquinone-1,4 by oxidative decarboxylation (SUNDMAN and HARO 1966) or to its dimer (FLAIG and HAIDER 1961a). The mentioned reactions are also interesting in connection with the uptake of lignin degradation products by the roots of plants, their translocation and transformation in plants and their physiological effect (summarized in: FLAIG, 1968) (lecture 7).

1.2 Conditions of the formation of nitrogenous polymers.

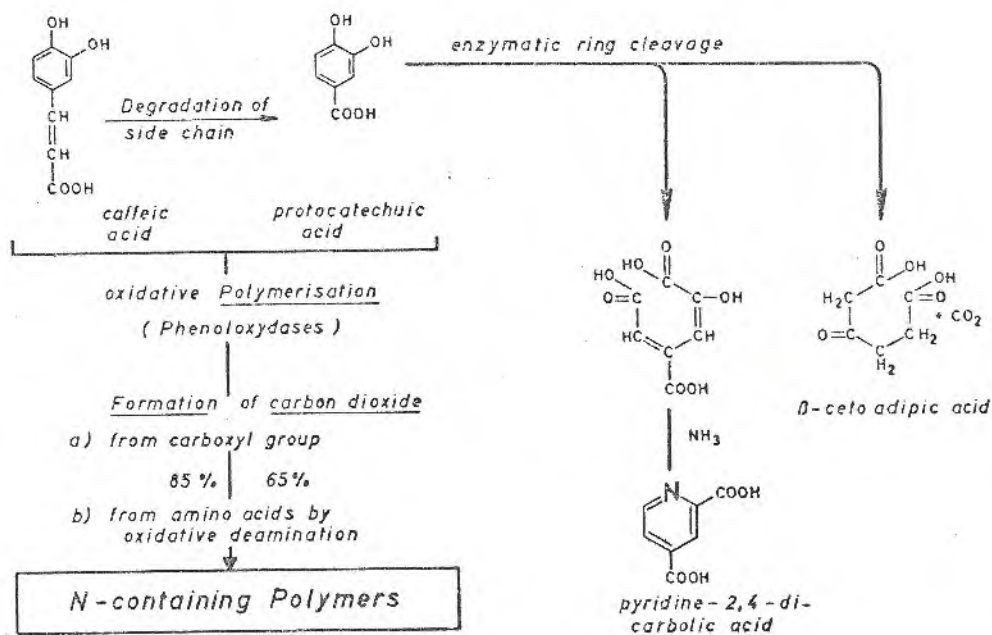


Fig. 3: Oxidative polymerisation of o-diphenolcarboxylic acids and their reaction with nitrogenous compounds.

Diphenylcarboxylic acids such as protocatechuic or caffeic acid polymerise also in the presence of phenoloxidas. But the released quantity of labelled carbondioxide is higher than in case of hydroxy-methoxy-derivatives. The formed polymers contain nitrogen, whilst the polymers in case of methoxyl substituted compounds were nitrogen free. A nucleophilic addition of amino acids occurs. In the presence of nitrogenous compounds from proteins the total released carbondioxide is partly derived from the carboxyl groups (case a) or from the amino acids and peptides (case b). The nitrogenous polymers are degraded much more slowly by microorganisms, this may be one of the reasons that such type of substances are accumulated in the soils.

As further reaction the cleavage of the aromatic ring of protocatechuic acid occurs and  $\beta$ -ceto adipic acid is formed during enzymatic oxidation (STANIER and INGRAHAM 1954, Mc DONALDS, STANIER and INGRAHAM 1954,

GROSS, GRAFFORD and TATUM 1956, OTTEY and TATUM 1956, FLAIG and HAIDER 1961 a ). This aliphatic acid is easily available for the microorganisms. The formation of protocatechuic acid and the cleavage of its ring is an important reaction for the transformation of phenolic compounds from plants and microorganism during humification because some reactions lead by demethylation and hydroxylation to this o-diphenolic acid. By this way the aromatic compounds disappear from the mixture of compounds, which participate in formation of humic substances.

TRIPET, DAGLEY and STOPHER (1960) found an enzymatic cleavage of protocatechuic acid with cell-free extracts from *Pseudomonas* sp., whereby the semialdehyd of muconic acid is formed. This reacts with ammonia on a not enzymatic way to pyridine-2,4-dicarboxylic acid. This reaction may be considered as one example for the fixation of nitrogen in heterocyclic compounds.

### 1.3 Different reactivity of phenols participating in nitrogen fixation.

Among the identified phenols which are formed either by oxidative or reductive cleavage of humic acids, or isolated from soils or synthesized by microorganisms, there exist two types of phenolic compounds, which are of interest in studies of the structure and therefore also of the chemical and physical properties of humic acids.

After demethylation of lignin degradation products with side chains of 1 or 3 carbon atoms different phenols are formed which possess two or several hydroxyl groups in o-position. Dimerisation and polymerisation occur during oxidation. Many microbial synthesized phenols



have two or three hydroxyl groups in m-position. By hydroxylation also phenols are formed with OH-groups in o-position.

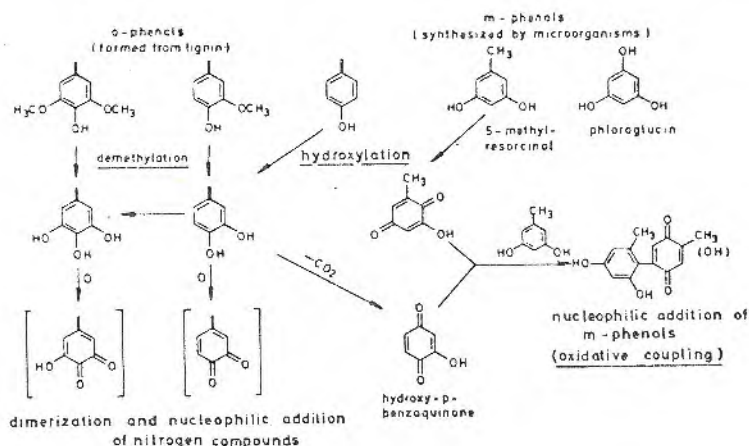


Fig. 4: Transformations of polyphenols under oxidising conditions.

The compounds with OH-groups in o- or m-position differ widely in their reactivity. Catechol, pyrogallol, hydroxyhydroquinone derivatives add nucleophilically derivatives of resorcinol and phloroglucinol (MUSSO et al. 1965) as well as proteins or their products of hydrolysis during oxidation to quinonoid intermediates. Phenols with OH-groups in m-position do not. Phenolcarboxylic acids are transformed by decarboxylation to hydroquinone derivatives and by further oxidation to quinones.

The differences in reactivity are important with respect to the function of nitrogen in the molecule of humic acids. The oxidative coupling leads to ramification and increases aromaticity.

2. About the linkage of amino acids, peptides and proteins in humic acids.

2.1 Nucleophilic addition of nitrogen compounds by phenols during oxidation.

a) Nucleophilic addition of proteins, peptides and amino acids by oxidized phenols

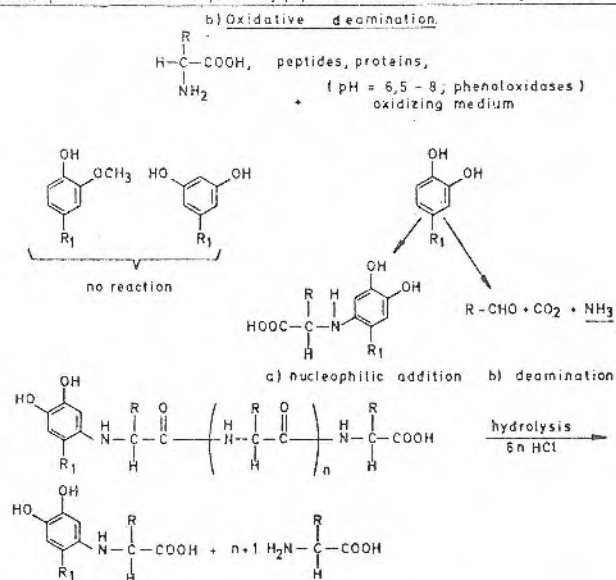


Fig. 5: a) Nucleophilic addition of proteins, peptides and amino acids by phenols during oxidation.  
b) Oxidative deamination.

Extensive studies have been made with labelled amino acids about the possible linkage of proteins and of their products of hydrolysis to phenols during oxidation (HAIDER, FREDERICK and FLAIG 1965. Some lignin degradation products isolated from decomposing lignin from soils or some phenols of microbial origin can add nitrogenous compounds derived from proteins at variable rate and in different amounts during oxidation in the presence of phenoloxidases. Summarizing, we could establish, that guajacol and resorcinol derivatives however do not add amino acids in a pH-range of 6.5 to 8.0.

The rate of nucleophilic addition (a) is the highest in the case of catechol and hydroxyquinone derivatives. Addition by pyrogallol derivatives occurs also to a smaller extent, dependent on the chemical constitution of the derivatives. By means of labelled compounds, it could be proved that the amino acids are added intact. An addition also occurs with peptides and proteins.

Oxidative deamination (b) is discussed later.

Furthermore, during oxidation in the presence of phenol oxidase and nitrogen compounds, catechol derivatives polymerise to nitrogenous polymers which have properties comparable to those of humic acids. In contrast, as mentioned before, the polymers of guajacol derivatives do not contain nitrogen (FLAIG and HAIDER 1961) (comp. 1.1 with 1.2).

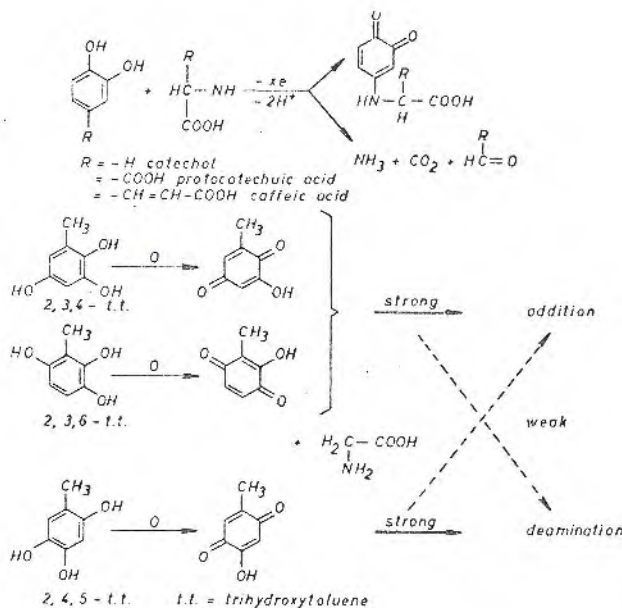
After nucleophilic addition the amino acids cannot be hydrolyzed from the corresponding addition products by 6 N hydrochloric acid. In case of addition products formed with proteins and peptides, only the amino acids could be hydrolyzed with the exception of the N-terminal amino acids in which the amino group has reacted with the oxidised phenol (HAIDER, FREDERICK and FLAIG 1965, HAIDER and MARTIN 1970). Therefore not all non-hydrolyzable nitrogen is bound in heterocyclic form as it is often supposed.

## 2.2 Oxidative deamination of amino acids

By oxidative deamination the amino acids are transformed in ammonia, carbondioxide and in a carbonyl compound.

If both reactions, addition and deamination, occur together more than 1 Mol oxygen is uptaken (HAIDER, FREDERICK and FLAIG 1965). One mole-

cule quinone gives one addition product, but deaminates several Mols of amino acids.



**Fig. 6:** Addition and oxidative deamination of amino acids by o-diphenols during oxidation in the presence of phenol oxidases.

The ratio of the two reactions such as the addition of an amino acid and its deamination depends upon the constitution of the added phenol.

2,3,5- and 2,3,6-Trihydroxytoluene or their oxidation products effect a strong addition but a weak deamination, whilst in the presence of 2,4,5-trihydroxytoluene the deamination occurs more strongly than the addition of an amino acid (HAIDER and MARTIN 1967). Two of these compounds 2,4,5- and 2,3,5-trihydroxytoluene are formed in the culture media of *Epicoccum nigrum*.

### 2.3 Properties of substances precipitable with acids (humic acids).

Precipitates can be isolated by addition of mineral acids to the reaction solution of phenols and amino acids. The precipitates are

the main part of the addition products.

Tab. 1: Distribution of the nitrogen in the reaction of 2500  $\mu$  mole glycine with 500  $\mu$  mole catechol (2500  $\mu$  atom N; 3 mg mushroom phenoloxydase)

	$\alpha$ -NH <sub>2</sub> -N	NH <sub>3</sub> -N	N-diff.*	N-prec.
nitrogen in $\mu$ atom in solution and in precipitate	1574	452	170	304

\* Kjehldahl-N minus (NH<sub>2</sub>-N + NH<sub>3</sub>-N)

Precipitate	weight in mg	weight % of total appl.subst.	% N	N in prec. in % of total N	% NH <sub>3</sub> -N of total N
	75,1	31	5,6	12	18

(According to HAIDER, FREDERICK and FLAIG 1965)

In one example the distribution of the nitrogen in the precipitate and in the solution is depicted. Under the mentioned conditions about one molecule of glycine is condensed with one molecule oxidised catechol, because the sum of the nitrogen in the precipitate and N-diff.-(that is the nitrogen in the solution, which is not  $\alpha$ -NH<sub>2</sub>-N and not NH<sub>3</sub>-N) is nearly 500  $\mu$  atom like the amount of the applied catechol. The nitrogen N-diff. belongs to soluble addition products, which cannot be precipitated by mineral acids. The number for NH<sub>3</sub>-N in the solution demonstrates, that a further molecule glycine is deaminated. The value of  $\alpha$ -NH<sub>2</sub>-N in the solution belongs to the not yet transformed glycine. The sum of all N-fractions is about 2500  $\mu$  Moles = the added amount of nitrogen.

The nitrogen content of the precipitate is 5,6 %; 12 % of the nitrogen applied in form of glycine are fixed in the precipitate and 18 % are released as ammonia. The numbers are representative for the most investigated acids.

Some properties of the precipitates are comparable with those of humic acids. The fixed amino acids of the reaction products with the phenols cannot be hydrolyzed with 6 n HCl. After electrophoresis or paper chromatography of hydrolysates no spots are observed, which belong to the applied amino acid. This result means that not all unhydrolysable nitrogen of the humic acids is heterocyclic bound. Some other linkages of nitrogen must have occurred perhaps in form of aromatic amines.

2.4 Distribution of the carbon skeleton and nitrogen as constituents of the amino acids in the reaction products and in the solution.

Some further experiments have been made, to elucidate, how the amino acids are transformed, and where the parts of the transformed amino acids remain during both reactions, during the addition and the deamination. For this purpose different <sup>14</sup>C-labelled amino acids have been used (HAIDER, FREDERICK and FLAIG 1965).

Tab. 2: Oxidative deamination of differently labelled amino acids during the oxidation of catechol in the presence of mushroom phenoloxidase (pH = 7). Distribution of the carbon <sup>14</sup> and the nitrogen in different fractions.

Amino acid	% added activity in		% added nitrogen in	
	CO <sub>2</sub>	precipitate	NH <sub>3</sub> in solution	precipitate
$\begin{array}{c} \text{NH}_2 \\   \\ \text{HC}-^{14}\text{COOH} \end{array}$	31	14	20	18
$\begin{array}{c} \text{NH}_2 \\   \\ \text{H}^{14}\text{C}-\text{COOH} \end{array}$	1	15	21	18
$\text{HOOC} - (\text{CH}_2)_2 - \begin{array}{c} \text{NH}_2 \\   \\ \text{CH}-^{14}\text{COOH} \end{array}$	21	6	16	10
$\text{HOOC} - (\text{CH}_2)_2 - \begin{array}{c} \text{NH}_2 \\   \\ \text{CH}-\text{COOH} \end{array}$	0,2	8	16	10

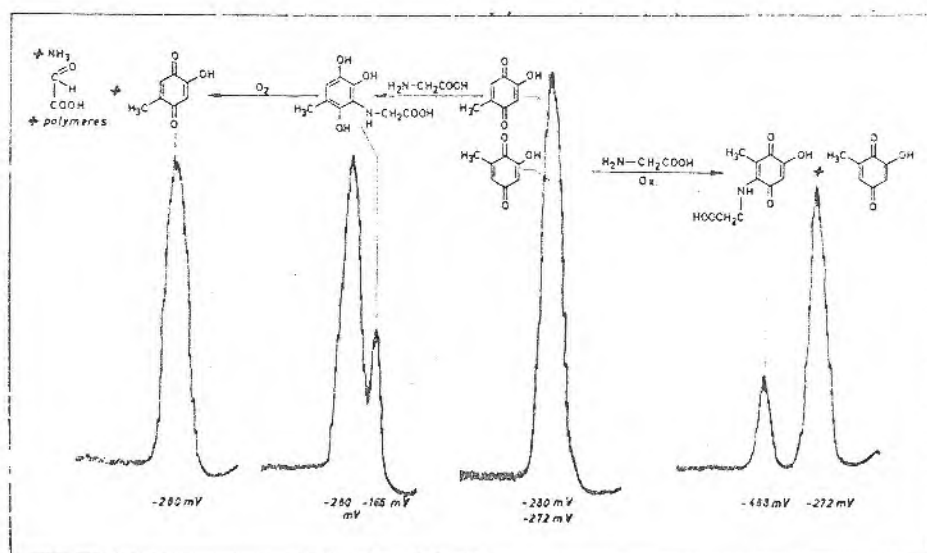
Catechol has been oxidised in the presence of amino acids labelled at different C-atoms and in the presence of phenoloxidase. (HAIDER, FREDERICK and FLAIG, 1965). When the oxidation was finished the reaction solution has been acidified till pH = 1. The released carbon-dioxide was absorbed in sodium hydroxide solution and the precipitation was isolated. The summarized results are the following:

1. The larger amount of released labelled carbondioxide in the case of carboxyl labelled amino acids in comparison to those, which are labelled in another carbon atom, is a measure for the deamination, because the activity in the precipitates formed in the presence of the two different labelled amino acids are in every case the same. Furthermore the activity in the precipitates formed with differently labelled amino acids demonstrates, that the amino acids are added as a whole in the oxidation product without a loss of carbondioxide.
2. The quantity of the released carbondioxide in the carboxyl labelled amino acids was larger than the amount of the ammonia nitrogen in the solution. Furthermore the nitrogen content in the precipitation was larger than their content of labelled carbon. These data indicate, that a part of the nitrogen in the precipitate is not bound in form of an amino acid. Further condensation must have taken place, by which nitrogen is bound in another way.

Experiments with labelled di- or tripeptides demonstrated, that not only nucleophilic addition of a peptide by a quinone but also deamination occurred. When the addition products have been hydrolyzed, all the amino acids were identified except the N-terminal, which is bound at the ring.

## 2.5 Mechanism of nucleophilic addition and oxidative deamination of amino acids by phenols.

We were interested in mechanism of the influence of molecular structure of the different phenols on addition or deamination of amino acids. The reaction between different substituted trihydroxytoluenes and glycine in oxidative medium was investigated by polarographic methods. (RIEMER 1970, FLAIG and RIEMER 1971).



**Fig. 7:** Reaction of the oxidation products of different substituted trihydroxytoluenes with glycine; polarographic measurements.

The oxidation products of 2,3,5-trihydroxytoluene (right) adds amino acids in p-position to the phenolic hydroxyl group.

The addition product is very stable. Its half-wave potential is 216 mV more than this of the initial compound. Deamination does not occur in this case.

The stability of this addition product is caused by the increase of the p-quinoid part in the resonance hybride by interaction of the



electron donating substituents across the ring system.

In contrary to this, a slow reaction occurs with glycine in the case of 2,4,5-trihydroxytoluene (left) after oxidation to the corresponding quinone. The addition product has a more positive half-wave potential than the initial product. In the addition product with the higher half-wave potential the o-quinoid part of the resonance hybrid is increased and decomposes by further addition of oxygen, whereby 2-hydroxy-5-methyl-benzoquinone-1,4 is formed again as well as ammonia, glyoxylic acid and polymers.

According to the polarographic investigations the reaction mechanism of addition of glycine to 2-hydroxy-5-methyl-benzoquinone-1,4 and the following oxidative deamination is depicted in the following scheme:

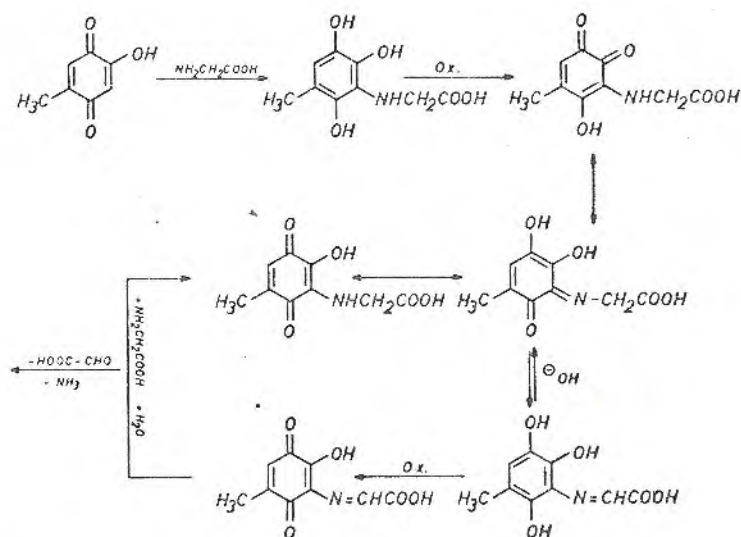


Fig. 8: Mechanism of oxidative deamination of glycine by 2-hydroxy-5-methyl-benzoquinone-1,4.

The first step is the addition of the amino acid on the quinone and oxidation to the corresponding o-quinone with the more positive half-wave potential. A tautomerisation to a pH-dependent equilibrium occurs.

Further oxidation leads to oxidative deamination by nucleophilic displacement with a further molecule of an amino acid. The former glyoxylic acid has been identified by polarographic measurements.

The catalytic effect of quinones with different molecular structure on nucleophilic addition and on oxidative deamination seems to be not only interesting for the formation of humic substances, but also in the connection with the physiological effect of phenols or quinones on plant metabolism, their uptake, translocation and transformation in the plant or their fixation as insoluble products in the roots.

3. Autoxidation of phenol derivatives in the presence of ammonia at pH-values about 8.

3.1 1,2- or 1,4-Diphenols.

Till now only the addition reactions in oxidising medium have been reported, which were catalysed by phenoloxidases and which occur in a range of pH-value between 6 and 8. At higher pH-values the phenols with at least two hydroxyl groups in o- or p-position react with ammonia to dark coloured nitrogenous polymers.

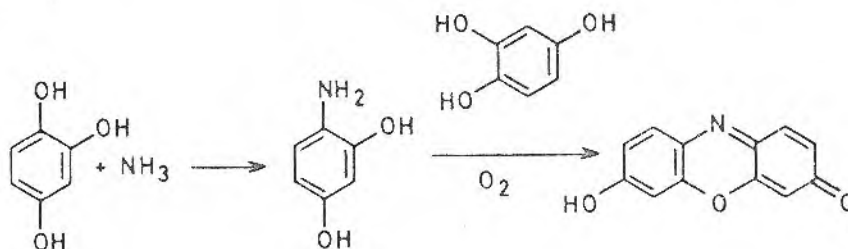


Fig. 9: Formation of phenoxazine derivatives.

In the absence of air and in the presence of ammonia a hydroxyl group can be replaced in hydroxyhydroquinone by an amino group (LANTZ and

MICHEL 1961). The aminoresorcinol thus formed reacts in the presence of oxygen with a further hydroxyhydroquinone to produce 7-hydroxyphenoxazine.

### 3.2 1,3-Diphenols

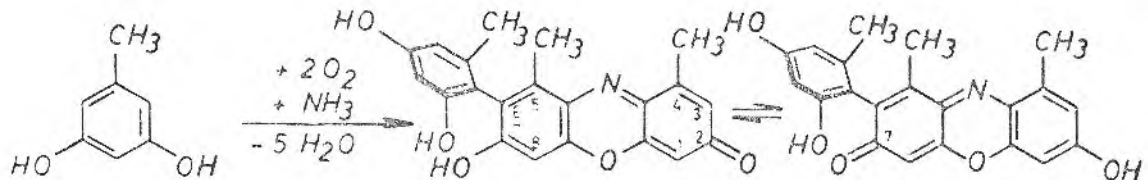


Fig. 10: Reaction of resorcinol derivatives (e.g. orcinol) during oxidation with ammonia. (Formation of phenoxazines; orcein dyestuffs) (MUSSO 1961).

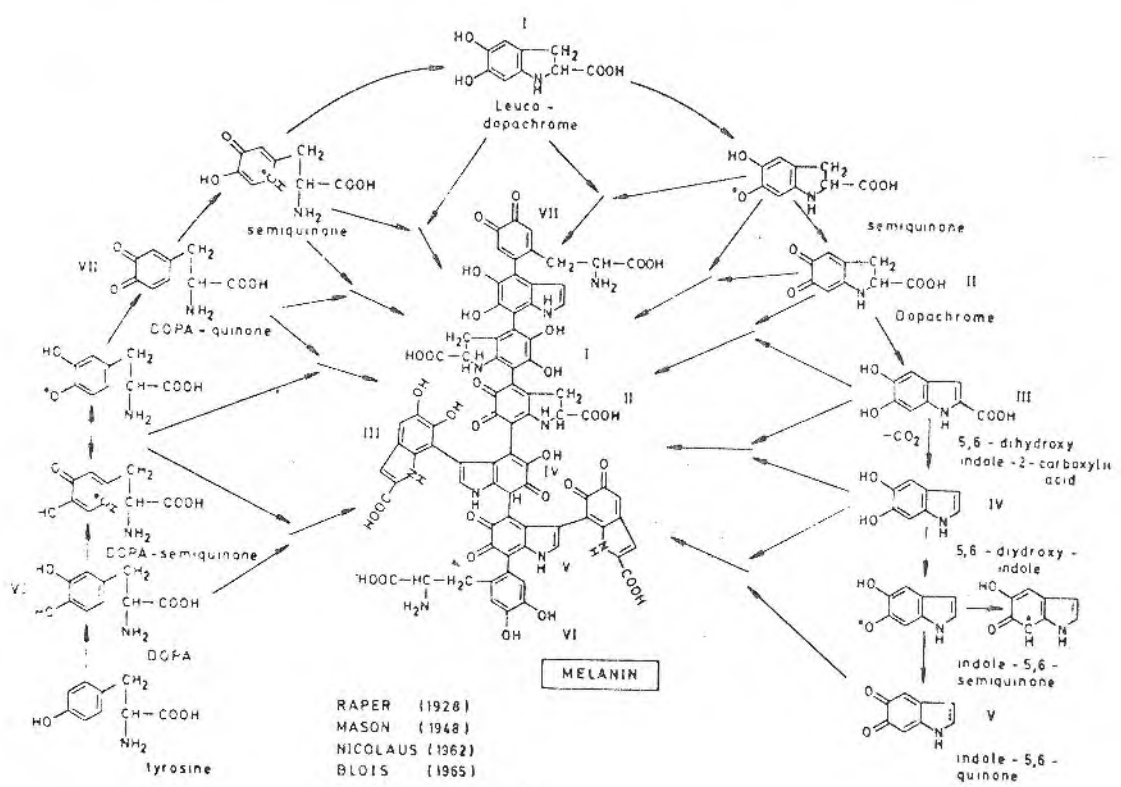
Before it has been mentioned, that 3,5-dihydroxytoluene (orcinol) was found in the culture media of *Epicoccum nigrum* in the humic acids after reductive cleavage. Also such types of substances react with ammonia in an oxidising medium but only at higher pH-values than 8. MUSSO et al. (1961, 1963, 1965) investigated these reactions to elucidate the constitution of the orcein dyestuffs. For instance  $\alpha$ -hydroxy-orcein is a tautomeric mixture of the derivatives from phenoxazine-2 and phenoxazine-7, which are also formed through quinonoid intermediate by the addition of ammonia at orcinol.

#### 4. Random polymerisation of tyrosine to melanin as a model for the participation of heterocyclic nitrogen in formation of the spheric shaped humic acids.

A further possibility of the formation of dark coloured humic substances is the participation of aromatic amino acids from the protein of the

microorganisms. For example the oxidation of tyrosine could lead through the dopaquinone VII (RAPER 1927) to dark coloured polymers (CROMARTIE and HARLEY-MASON 1953).

The proposed random polymerisation of tyrosine to melanin (BLOIS 1965, MASON 1948, NICOLAUS 1962, RAPER 1928) can be used for the explanation of the formation of humic acids. Both are dark coloured, higher molecular substances with a spherical shape. The polymerisation occurs in each case through quinonoid intermediates, such as semiquinone free radicals.



**Fig. 11:** Hypothetical scheme of melanin synthesis assuming the participation of semiquinone free radicals.

The random polymerisation to melanin starts from tyrosine and all intermediates participate in the formation of the polymer. Melanin is not only formed by the polymerisation of the end product of oxidation of tyrosine, indole-5,6-quinone.

Similar but much more complicated processes occur during the formation of humic substances, since not one but several phenolic components and various nitrogenous compounds take part in the formation of the polymers.

The formation of dark coloured substances in structurally intact dead plant tissues at the beginning of humification could be explained in a similar way (KONONOVA 1961).

This contribution to the participation of nitrogenous compounds in the formation of humic substances concerned much fundamental research work to this problem.

One of the functions of humus is its ability to contribute to soil productivity as a slow acting nitrogen source. Many field experiments and experiments with  $^{15}\text{-N}$  have been made to find out the dynamics of nitrogen transformations and nitrogen economy in soil. But all the observations, which have been made, cannot be explained without knowledge about their causal connections. We are here only at the beginning of research work. But the fact, that phenolic constituents of humus catalyse the liberation of ammonia from organic compounds such as amino acids, a product of dead soil organisms, seems to be a step for further elucidation of nitrogen economy in soil. Some of the mentioned reactions gave valuable suggestions for synthetic, slow releasing nitrogen fertilizers.

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