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ANTIOXIDATIVE EFFECT OF MAILLARD REACTION PRODUCTS

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ANTIOXIDATIVE EFFECT OF MAILLARD REACTION PRODUCTS

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ABSTRACT

Lipid oxidation is a common deteriorative reaction in foods, limiting the storage time for many products. The main methods available for protecting the foods against lipid oxidation are: exclusion of air and light, addition of synthetic antioxidants, and storage at low temperatures. In addition, certain common food components possess antioxidative properties which might be utilized to a greater extent. In the present work the antioxidative effect of Maillard reaction products (MRP) was studied. Such products are frequently formed from sugars and amino compounds during heat treatment of food.

The antioxidative effect of the MRP was measured in model systems by three different methods, using emulsified linoleic acid as the substrate. Oxygen consumption was measured by a polarographic technique, when the lipid oxidation was accelerated by hemin. The formation of conjugated diene compounds during oxidation at 37°C was determined spectrophotometrically at 234 nm. Volatile compounds formed during oxidation, catalyzed by denatured horseradish peroxidase, were analyzed by a gas chromatographic headspace technique.

MRP obtained by reactions between sugars and amino acids, peptides or enzymic protein hydrolysates were investigated. The influence of reaction parameters such as reaction time, pH and concentration of the reactants, was studied. The choice of reactants, in particular the amino compound, was found to have a strong influence on the antioxidative effect of the MRP formed. Of the amino acids studied, the basic ones (histidine, lysine and arginine) formed the most antioxidative MRP. The antioxidative effect of MRP from dipeptides and sugar was found to be affected by the amino acid sequence of the dipeptide. When reacting dipeptides from histidine and glycine with xylose, stronger antioxidative effect was obtained when histidine was the N-terminal amino acid than when glycine was the N-terminal amino acid. Protein hydrolysates were also shown to form antioxidative MRP.

Finally, the effect of MRP on the storage stability of foods was studied in storage experiments. The addition of histidine and glucose to cookie dough was found to retard the development of rancid flavor in the cookies. The antioxidative effect was believed to arise from MRP formed during the baking. During frozen storage of sausage, preformed MRP added to the sausage batter were shown to retard lipid oxidation. MRP from histidine and glucose as well as MRP from enzymic hemoglobin hydrolysate and glucose were found to be effective.

KEYWORDS: Antioxidative effect, Maillard reaction products, sugars, amino acids, dipeptides, enzymic protein hydrolysates, cookies, sausage.
INTRODUCTION

Protection against lipid oxidation

Lipid oxidation is a major problem in food production and storage. The development of rancid off-flavor limits the storage time for many foods, even when their fat content is low. In particular, when foods are made stable against microbiological deterioration, often lipid oxidation instead becomes the main deteriorative reaction during storage. Many dry foods and frozen foods exemplify this.

Chemical, as well as technological aspects of lipid oxidation in foods were extensively reviewed at a symposium on "Lipids and their oxidation" held in 1961, the proceedings from which were published in a book edited by Schultz (1962). Since then, the chemistry of lipid oxidation has been reviewed by Cowan (1968) and by Lundberg and Järvi (1971). Chemical and kinetic aspects have been reviewed by Labuza (1971), Uti (1973), and Kochar and Meera (1975). Scott (1965) and Emanuel and Lyaskovskaya (1967) reviewed the subject of mechanisms of antioxidant function.

The oxidation of lipids in foods comprises a sequence of reactions. Briefly, the fatty acids (preferably polyunsaturated fatty acids) first react with oxygen, by a radical chain mechanism, forming fatty acid hydroperoxides. The hydroperoxides are subject to a number of further reactions, such as fission reactions, further oxidation, polymerization etc. Among the "secondary lipid oxidation products" so formed, are several volatile compounds, including aldehydes, ketones, alcohols and others. Some of these volatiles are responsible for the rancid flavor that constitutes the main problem of lipid oxidation in foods. The simultaneous loss of essential fatty acids is generally less significant. However, the possible toxic effects of oxidized lipids have also been discussed, mainly in connection with high temperature treatment, such as deep fat frying (Perkins 1976; Lang 1978).

Lipid oxidation is promoted by high temperature, light and several catalysts such as metals, hematin compounds and the enzyme lipoxygenase, which is present in many vegetabilia. The main methods available for protecting foods against lipid oxidation are consequently:
Storage at low temperature. It should, however, be mentioned that some frozen, salted meat products have shown "reversed stability", being more stable at -18°C than at -30°C (Poulsen and Lindeløv 1975). This is believed to be due to increased concentration of prooxidants in unfrozen liquid pools in the product.

- Exclusion of light by using appropriate packing material.

- Exclusion of oxygen by vacuum or inert gas packaging. An additional possibility is to include an "oxygen scavenger"-system in the package. Examples are (Karel 1974): (A) glucose oxidase together with the substrate glucose, and (B) hydrogen together with palladium or platinum for the catalyzed oxidation of the hydrogen gas to water.

- Utilization of antioxidants.

Antioxidants can work according to various mechanisms. Scott (1965) classified them into two main types, the first one operating by a radical chain-breaking mechanism; the second one operating by preventing the introduction of chain initiating radicals into the system. The most frequently used of the first type are phenols, such as BHA (Butylated Hydroxy Anisole), BHT (Butylated Hydroxy Toluene), esters of gallic acid (propyl-, octyl-, and dodecylgallate), and tocopherols. Four possible mechanisms for reaction of this type of antioxidant with the lipid radicals have been suggested (Stuckey 1962): (1) hydrogen donation, (2) electron donation, (3) addition to the lipid, and (4) formation of a complex with the lipid. The first one is considered to be the predominant mechanism of the common phenolic antioxidants.

The function of antioxidants of the second type is mainly to inactivate pro-oxidants, complex binding of metals, for example. Citric acid and ascorbic acid are examples of this type. The term "synergist" is sometimes used for this type of antioxidant, since it improves the effect of an antioxidant of the radical chain-breaking type, resulting in a stronger antioxidative effect, than would be expected from the combination of the two antioxidants.
Many foods normally contain antioxidative components, either naturally occurring in the raw materials or formed during the processing. Interest in these kinds of antioxidants has increased noticeably in recent years, probably as a result of the growing reluctance to use synthetic food additives. The present work started, therefore, with a review of the literature on the occurrence of natural or process-induced antioxidants (Lingnert 1972). Recently, the same subject was reviewed by Dewdney and Meara (1977).

In addition to tocopherols, well known antioxidants, the following classes of food components have been reported to include compounds with antioxidative properties:

- Amino acids
- Proteins
- Phospholipids
- Acids (ascorbic acid, citric acid)
- Plant phenols (e.g., pigments of flavonoid type)
- Maillard reaction products (MRP)

Several chemically unidentified food components, such as extracts from spices and other vegetable foods, have also been reported to possess antioxidative properties, the effect probably being derived from some of the components listed above. Certain microorganisms and fermented food products have also been found to be antioxidative.

In the present study, MRP were chosen for further investigation.

The Maillard reaction

Louis-Camille Maillard (1878-1936) was the first to systematically investigate the reactions between sugars and amino acids. He studied the effects of various sugars as well as of various amino acids and peptides on the development of brown color and on the formation of C\textsubscript{O\textsubscript{2}} during reaction, as performed at various temperatures (Maillard 1912 a,b; 1916; 1917). He also studied the composition of the brown substances formed, called "melanoidins". The summarizing term "Maillard reaction" has since been used for the complex series of reactions occurring between amino and carbonyl compounds.
The Maillard reaction is very common and most important in food technology, taking place, in particular, during heat treatment of food, but also during storage at room temperature. The reaction plays a dominating role in the color formation during, for example, frying and baking. The Maillard reaction is also called "non-enzymic browning", a term that also comprises the caramelization reactions (briefly, reactions involving merely sugars). Furthermore, the Maillard reaction is responsible for flavor formation, both desirable and undesirable, during food processing and storage (Hodge 1967; Reynolds 1970), and for nutritional losses (Carpenter 1973; Adrian 1974; Cheftel 1977), mainly by making lysine in the proteins biologically unavailable. Since lysine has an \( \epsilon \)-amino group, it can participate in the Maillard reaction even when bound in a protein. In addition, certain MRP have been reported to have antinutritional or even toxic effects (Adrian 1974).

The chemistry of the Maillard reaction was extensively reviewed by Hodge (1953), who also worked out an over-all scheme of possible reactions involved in the sequence, starting with the sugar and the amino acid and ending with the melanoidins. This scheme has since then become classical. Chemical aspects of the Maillard reaction have since been reviewed by Ellis (1959), Heyns and Paulsen (1960), Reynolds (1963; 1965; 1969), and Feeney et al. (1975). In spite of the large amount of work done on the Maillard reaction, only the first steps of the reaction sequence are fully elucidated. A large number of low-molecular weight, intermediate products have been isolated and identified. The mechanism of the formation of brown pigments is, however, not yet fully understood.

However, the following steps are generally recognized in the reaction sequence (Feeney et al. 1975):

1. The reversible formation of glycosylamine.
2. The rearrangement of the glycosylamine to the ketosamine, 1-amino-1-deoxyketose ("Arnadori rearrangement"), or to the aldosamine, 2-amino-2-deoxyaldose ("Heyns rearrangement").
3. The formation of a diketosamine or a diamino sugar.
4. Degradation of the amino sugar, usually initiated by the loss of one or more molecules of water to form amino or carbonyl intermediates.
5. Reaction of amino groups with the intermediates formed in Step 4 and subsequent polymerization of these products to brown pigments and other substances.
No satisfactory mechanism has yet been agreed upon for Step 5. Among the intermediates formed in Step 4 are what are known as reductones, briefly compounds containing an enediol structure. In addition to the reaction steps listed above, the Strecker degradation is of importance in the Maillard reaction, in particular with regard to flavor formation. The Strecker degradation involves the reaction of \( \alpha \)-amino acids to form aldehydes containing one carbon less than the amino acid, with the release of carbon dioxide.

**Antioxidative properties of Maillard reaction products**

The first reported observation on antioxidative properties of MRP was made by Franzke and Iwainsky (1954). They studied the antioxidative effect of products obtained through reactions of glycine or monosodium glutamate with glucose. The antioxidative effect was found to increase with increasing reaction time.

Griffith and Johnson (1957) found that the addition of 2.5% glucose to cookie dough (calculated on dough weight including water) at the expense of an equal amount of sucrose resulted in an increased browning during the baking and a greater stability against oxidative rancidity during the subsequent storage. They also showed that MRP from glycine and glucose exhibited antioxidative properties in model experiments with lard. The antioxidative properties were considered to be associated with the presence of reductones. Since then, pure reductones prepared from hexoses and secondary amines have been demonstrated to possess antioxidative properties (Evans et al. 1958; Cooney et al. 1958).

The work done on the antioxidative effect of MRP has since been dominated by Japanese research groups. Yamaguchi et al. (1964) found the storage stability of cookies to increase by addition of certain amino acids to the dough. Extracts from the cookies were effective in stabilizing fats against rancidity (Yamaguchi and Koyama 1967 a,b). The antioxidative effect of the MRP was reported to be comparable to that of BHA, higher than that of tocopherols, but lower than that of BHT, when comparing on the basis of weight (Yamaguchi and Koyama 1967 c; Yamaguchi and Fujimaki 1974 a, b, c). They also studied the decomposition of fat hydroperoxides by MRP from cetylamine and xylose (Yama-
guchi and Okada 1968) and investigated the antioxidative effect of several reductones (Yamaguchi 1969). No antioxidative effect was obtained in aqueous systems, but some reductones were found to be antioxidative in dehydrated systems. When fractionating MRP (obtained by heating 2 M glycine and 2 M xylose at 100°C for 2 h) by gel filtration and thin layer chromatography, they found a strong antioxidative effect in fractions containing melanoidins with a molecular weight of about 4500 (Yamaguchi and Fujimaki 1970 a, b; 1973 b). They also studied the changes of antioxidative effect during storage of MRP from xylose and amino acids or amines (Yamaguchi and Fujimaki 1973 a).

Kirigaya and co-workers studied the antioxidative effect of MRP from several amino acids and sugars (Kirigaya et al. 1968; 1969). Alanine, serine, threonine, histidine and arginine were found to form potent antioxidants. Of the sugars, the pentoses were most effective, especially xylose and sorbose. Fractionation of MRP from ammonia and glucose by gel filtration, ion-exchange chromatography and thin layer chromatography showed antioxidative activity to be distributed all over the colored fractions (Kirigaya et al. 1971). Comparisons at the same color intensity (A490 nm) showed that colored fractions in the lower molecular weight range had a stronger antioxidative effect than fractions in the higher molecular weight range. When melanoidins were oxidized with KMnO4, the brown color disappeared almost completely, while the products still were antioxidative (Kato et al. 1975). Reduction of melanoidins with NaBH4 caused fading of the brown color, as well as decreased antioxidative effect.

Tomita (1971 a, b; 1972 a, b) studied MRP from tryptophan and glucose, the combination that turned out to be most effective, when investigating several amino acids together with glucose. Addition of tryptophan and glucose to "fried rice cake chips" was found to improve the storage stability of this product (Tomita 1972 c).

Itoh et al. (1975) reported that dihydroxyacetone was superior to xylose, which in turn was superior to glucose in forming antioxidative MRP with several amino acids. The sugar - amino acid combinations were heated in corn oil at 175°C for 5 minutes.
Absolute ethanol extracts of MRP from glycine-glucose have been reported to possess strong antioxidative effect, although the color intensity was low (Hwang and Kim 1973; Lee et al. 1975 a, b.). Ethanol extracts from glucose-ammonia and glucose-glycine reaction mixtures were found to be equally antioxidative and prolongation of the reaction time from 2 h to 40 h had little effect on the antioxidative effect (Lee and Kim 1978).

Maleki (1973) reported that glycine-glucose reaction products inhibit the development of rancidity in corn oil.

Eichner (1975) studied the Maillard reaction in dehydrated foods and in dehydrated model systems. Intermediate reductone-like compounds, such as 1,2-enaminoles derived from Amadori products were claimed to possess antioxidative properties. By treating a glucose-lysine-Avicel model system containing sodium linoleate at a high temperature for a short time (80°C; 5 min.), lipid oxidation was inhibited during the subsequent storage at room temperature, without any development of visible browning.

OBJECTIVE AND EXTENT OF THE PRESENT INVESTIGATION

The objective of the present investigation was to elucidate how some parameters involved in the Maillard reaction influence the formation of antioxidative products. The purpose was, furthermore, to demonstrate the possibilities of using antioxidative MRP in foods.

When studying the influence of different reaction parameters on the formation of antioxidative products, the measurement of antioxidative effect was performed in model systems. Since the lipid oxidation is composed of a sequence of reactions, a number of methods are possible, involving measurements at different stages of the oxidation sequence. This work, therefore, began by developing appropriate oxidizing systems and methods of measuring the lipid oxidation.

To ensure that antioxidative compounds, as evaluated in model systems, are capable of retarding lipid oxidation in complex foods, as well, application studies will have to be made. Sensory methods should then preferably be used to follow the development of rancidity. In this work, cookies and sausage were chosen as the foods for application studies of antioxidative MRP.
This thesis can naturally be divided into the following three parts:

1. Measurement of antioxidative effect (Publication I)
2. Model studies of the antioxidative effect of MRP (Publication II and III).
3. Applications of antioxidative MRP in foods (Publication IV and V).

MEASUREMENT OF ANTIOXIDATIVE EFFECT (Publication I)

When developing new antioxidants or when evaluating food components with regard to antioxidative properties, rapid and reliable methods for measurement of the antioxidative effect are needed. The measurements have to be done in model systems, using conditions that to the greatest possible extent allow prediction of the effect of the antioxidant in a food system during normal storage. Due to the fact that lipid oxidation constitutes a very dynamic system, and is affected by so many factors, the development of the appropriate model system and method of measurement is a difficult task, necessitating a great deal of compromise. In the end, the only fully reliable way to measure the practical antioxidative effect is to test in the actual food substance.

In brief, the following factors can be varied, when constructing a measuring system:

- The oxidizing system
- The method of accelerating the lipid oxidation
- The method of measuring the lipid oxidation.

The oxidizing system most often used is either a pure, polyunsaturated fatty acid (either free or esterified) or a well defined fat or triglyceride with a high degree of unsaturation. The lipid can be used as a single lipid phase, or emulsified in aqueous media. Linoleic acid (>99%) emulsified with the aid of Tween 20 in 0.1 M potassium phosphate buffer (pH 6.5 or in some cases pH 6.8) was used throughout this work. Linoleic acid was chosen because of its susceptibility to oxidation, allowing relatively short reaction times. Linoleic acid is, furthermore, nowadays used as the standard substrate for assaying the enzyme lipoxygenase. A high degree of purity was necessary to get reproducible results. An emulsified system was preferred to make it possible to use water soluble catalysts, as well as water soluble antioxidants. Moreover, most composed foods contain certain amounts of water.
The main methods available of accelerating the lipid oxidation are:

- Increased temperature
- Increased oxygen pressure
- Light
- Addition of catalysts (metals, hematin compounds, lipoxygenase).

Caution must be taken with either of the accelerating methods, since all of them are more or less prone to not only increase the reaction rate, but also to cause changes in the mechanism of the lipid oxidation. For instance light can, in the presence of suitable sensitisers, photochemically cause the formation of singlet oxygen, which may then play the dominating role in the initiating step of the lipid oxidation (Grosch 1975). Metals and hematin compounds are known to affect not only the free radical chain reaction, but also the further degradation of the hydroperoxides formed (Ingold 1962; Tappel 1962).

Increased temperature, and to some extent increased oxygen pressure, are the dominating acceleration means in the classical methods for rapid measurement of the antioxidative effect. Ragnarsson and Labuza (1977) found, however, that the overall activation energy, $E_a$, for lipid oxidation may be considerably higher in the presence than in the absence of antioxidants. Tests performed at 60-65°C thus lead to a sizable, but predictable, underestimation of the antioxidative effect. They considered data collected at as high temperatures as 100°C to be much less predictable.

The methods used in the present study were based on acceleration by a moderate increase of the temperature (37°C) or by the use of hematin compounds as catalysts.

The methods of measuring the lipid oxidation can, to simplify, be divided into three groups, with respect to the stage at which the lipid oxidation is measured:

- Measurement of the decrease of the reactants (oxygen or the lipid)
- Measurement of the primary products (hydroperoxides)
- Measurement of the secondary products (e.g., aldehydes)
In the first group, measurement of the oxygen consumption is very common, either by manometric methods or by the use of a polarographic oxygen electrode. The lipid consumption is less frequently measured. Rogstad and Reinton (1977), however, described a method for testing antioxidants, based on gas chromatographic measurement of residual, unreacted linoleic acid in an oxidizing linoleic acid emulsion. The main method of measurement to be assigned to the second group is the determination of the peroxide value. In the third group the determinations of the TBA-value (TBA = thiobarbituric acid), the carbonyl-value or the anisidine-value, as well as the analyses of specific aldehydes can be mentioned.

In this investigation, one method of measurement from each of the three groups mentioned above was chosen for evaluating antioxidative effect. Oxygen consumption was recorded by a polarographic method, the formation of conjugated diene compounds (linoleic acid hydroperoxides) was measured spectrophotometrically, and the formation of volatile compounds was measured by gas chromatography.

**Classical accelerated methods for measurement of antioxidative effect**

Among the most common accelerated methods for evaluation of antioxidative effect are the following:

The Active Oxygen Method (AOM) also called the Swift Stability Test. The method was originally proposed by King et al. (1933) and later slightly modified by Riemenschneider et al. (1943). The samples of lipid are kept in glass tubes and dry air is bubbled through. The temperature is maintained at approximately 98°C. Periodically, aliquots of the samples are withdrawn and the peroxide value is determined.

The Oxygen Bomb Method has long been used in the petroleum industry. Gearhart et al. (1957) were the first to use it for food lipids. The lipid oxidation is accelerated by using an oxygen pressure of about 0.7 MPa (100 psi) and a temperature of about 100°C. The oxygen pressure is recorded continuously.

Among Oxygen Absorption Methods is also the procedure described by Sylvester et al. (1942) and later modified by Martin (1961), in which the sample is kept at atmospheric pressure in oxygen at 100°C in flasks connected to mercury manometers. A similar design was proposed by
Eckey (1946), using, however, air as the surrounding atmosphere and maintaining the temperature at 80°C. In addition, various methods are based on manometric measurement of oxygen uptake, using the Warburg apparatus.

None of these classical methods was used in the present investigation, mainly since the high temperatures usually involved make the conditions too different from normal storage.

Polarographic methods for measurement of antioxidative effect

The oxygen consumption in an oxidizing lipid system can be measured by polarographic technique, using an oxygen electrode. A number of methods for measurement of antioxidative effect based on this technique have been described. Various substrates and catalysts have been used. Hamilton and Tappel (1963) used a system consisting of 10 or 30% lipid (linoleic acid, methyl linoleate or lard) emulsified with the aid of an emulsifier (Myrij 53) in phosphate buffer (pH 7.2). Hemoglobin was used as the catalyst. The test was performed at 26-30°C. The method was slightly modified and standardized by Cort (1974), who used a 10% emulsion of safflower oil in water using 1% of the emulsifier. The safflower oil was first stripped of tocopherols by heating in a molecular still at 270°C in a rotary glass evaporator. Yagi (1970) used 25% safflower oil emulsified in water with the aid of Tween 40. The oxidation was performed at room temperature and catalyzed by FeSO₄. Berner et al. (1974) proposed a test system consisting of a lipid emulsion (animal fats or vegetable oils; 180 mg/50 ml) in phosphate buffer (pH 7.2) using Tween 20 as the emulsifier. The temperature was maintained at 45°C and a water solution of hemin was used as the catalyst.

A procedure originally described for measurement of lipoxygenase activity in closed systems (Eriksson and Svensson 1970) was, slightly modified, used in the present work. The substrate was 10 mM linoleic acid emulsified with the aid of Tween 20 in 0.1 M potassium phosphate buffer (pH 6.5). The oxidation took place in a reaction vessel (volume: approximately 6.5 ml) thermostated at 25°C. Hemin, dissolved in phosphate buffer: ethanol (1:1), was used as the catalyst. The main improvement involved in this system is the usage of the specially designed, closed reaction vessel, ensuring no dissolution of atmospheric oxygen into the linoleic acid during the experiment, allowing an accurate temperature
control, and avoiding variations of the volume. Furthermore, by using 50% alcohol in the hemin solution, this was made long-lastingly stable, as distinguished from the water solution of hemin used by Berner and co-workers, that was reported to change over time (Berner et al., 1974).

The main advantage of the polarographic methods is that they are very rapid as compared to classical methods, a test is most often completed within 10 minutes. The method is thus very convenient, particularly for the purpose of screening several substances with respect to antioxidative properties. When comparing the antioxidative effect of the antioxidants, certain care must, however, be used, since the results are dependent on the initial oxidation state of the linoleic acid emulsion. All comparisons must, therefore, be made at the same occasion using one and the same linoleic acid emulsion. Even then, the oxidation state of the linoleic acid emulsion should be checked, since changes due to autoxidation may occur during the experiment, even when keeping the emulsion in the refrigerator. Especially when the linoleic acid emulsion is freshly made, having a low content of conjugated dienes, small changes in the initial state of oxidation result in considerable variation in the measurement of the antioxidative effect. Smaller variations in the results are observed, when the emulsion is initially somewhat more oxidized. This is, however, obtained at the expense of the sensitivity. A compromise must therefore be made between reproducibility and sensitivity.

The results from the measurements can be expressed in different ways. Berner et al. (1974) compared the induction periods from experiments with added antioxidants with that of a control. Yagi (1970) measured the time needed for the consumption of 20% or 50% of the dissolved oxygen. Hamilton and Tappel (1963) used the time needed to consume 90% and Cort (1974) the time needed to consume 50% of the oxygen. In these two articles the antioxidative effect was expressed as a protective index (PI), defined as the time required for 90% or 50%, respectively, reduction of the oxygen in the antioxidant system divided by the corresponding time in the control. We used a similar method to express the antioxidative effect (A.E.), based on the time required for 50% reduction of the oxygen:
\[ A.E. = \frac{T_a - T_c}{T_c} \]

\( T_a \) = time elapsed for 50% reduction of the available gaseous oxygen in the sample containing the antioxidative addition,

\( T_c \) = corresponding time interval in the control.

While PI = 1 means "no antioxidative effect", in our equation A.E. = 0, when no antioxidative effect is obtained. Theoretically, the value of A.E. can vary from 0 to infinity, but in practice it seldom exceeds 10, since the method does not allow measurement of excessively low oxidation rates. Normally the antioxidant concentration was adjusted to give an A.E. of up to a maximal value of about 3.

**Spectrophotometric method for measurement of antioxidative effect**

The fact, that the oxidation of linoleic acid involves a double bond shift, resulting in the formation of conjugated double bonds, absorbing light in the UV region with absorption maximum at 234 nm, has previously been used in methods for measurement of lipoxygenase activity (Surrar 1964). In this work the oxidation of a linoleic acid emulsion, identical to that used in the polarographic method, was followed by measuring the absorption at 234 nm. When performing the oxidation at 37°C without any added catalyst, a reaction time of 15-20 h was found to be appropriate.

The method requires relatively small amounts of the antioxidants, and a large number of samples can be run simultaneously. It is, thus, well suited for measuring the antioxidative effect of, for instance, fractions from column chromatography, when separating food components with antioxidative properties.

The method can easily be modified to suit specific purposes. A catalyst can be included in the system and parameters, such as temperature, incubation time, and the concentration of linoleic acid in the emulsion can be varied. Since the hydroperoxides are intermediates in the lipid oxidation sequence, the concentration of them often passes through a maximum. It is, therefore, important to check the oxidation rate, when making modifications, to be able to choose the appropriate reaction time in each case.
Gas chromatographic method for measurement of antioxidative effect

Rancid flavor of foods is probably caused by a complex mixture of volatile, secondary products derived from the lipid oxidation. Since many such volatiles, differing in character and strength of odor, are formed at varying rates, the contribution of the individual compounds to rancid flavor is mostly unknown. In certain cases, however, the rancid flavor has been reported to correlate with the concentration of specific volatiles, for example, with the concentration of hexanal in explosion-puffed potatoes (Konstance et al. 1978) and in other low fat, dehydrated foods (Fritsch and Gale 1977), and with the concentration of pentane in vegetable oils and potato chips (Warner et al. 1974) as well as in certain nut-containing products (Bigallli 1977). The measurement of secondary lipid oxidation products, however, is mostly done with rather unspecific methods, like TBA-value or carbonyl-value determinations, which includes the measurement of several reaction products. The reaction of several aldehydes with TBA was systematically investigated by Marcuse and Johansson (1973). Since all aldehydes studied formed a yellow pigment with absorption maximum at 450 nm, while only 2,4-alkadienals and, to a lower extent, 2-alkenals formed a red pigment with absorption maximum at 530 nm, their TBA method was found capable of distinguishing to some extent different aldehyde classes.

Measurements directly on separated volatiles during lipid oxidation may be an alternative way of measuring the effect of antioxidants. This might allow a straightforward comparison of the results with the sensory phenomena caused by lipid oxidation, especially when better insight has been gained into the contribution of specific volatiles to the flavor.

For the present investigation a method of measurement based on a gas chromatographic headspace technique was developed. The substrate consisted of 3 mM linoleic acid emulsion in 0.1 M potassium phosphate buffer (pH 6.8). Tween 20 was used as the emulsifier. Heat activated horseradish peroxidase was used as the catalyst, due to its better long-lasting stability in the linoleic acid emulsion, as compared with hemin (Eriksson et al. 1971). The oxidation was carried out at 20°C with 400 ml of the linoleic acid emulsion in a one-liter flask. The flask was throughout the incubation purged with air at a flow rate of 10 ml/min. Occasionally during the incubation period, the air stream from the flask was allowed to pass through a trap (cooled by a mixture of solid carbon
dioxide and ethanol) in a pre-column concentration accessory connected to the gas chromatograph. The volatile compounds in the air stream, derived from the lipid oxidation, were then condensed. Usually 600 ml of headspace gas was sampled during 1 h. The condensed material was then injected onto the gas chromatographic column by directing the carrier gas through the trap and simultaneously replacing the cooling mixture surrounding the trap by an oil bath held at 140°C.

A large amount of volatile compounds was recorded, and the formation of individual compounds during the oxidation could be followed. Mostly, the antioxidative effect was evaluated by studying the effect on the formation of n-hexanal, even though other volatiles were studied as well, for example n-pentanal, n-hept-trans-2-enal and n-oct-trans-2-enal. Essentially the same results were obtained with either of these compounds. However, it was observed that different compounds were formed according to different kinetics. Further studies of this phenomenon may be valuable for the understanding of qualitative flavor changes during lipid oxidation.

This method of measuring the antioxidative effect is rather time-consuming and laborious. However, it has shown good reproducibility, and is therefore suitable for comparisons between antioxidants, allowing the different antioxidants to be tested on different occasions. The method is mainly intended to be used as a complement to one or both of the previously discussed methods. Since the different methods are based on measurement at different stages of the lipid oxidation sequence, the use of two or three of them makes the results far more reliable.

MODEL STUDIES OF THE ANTIOXIDATIVE EFFECT OF MAILLARD REACTION PRODUCTS

The formation of antioxidative MRP from the reaction between various sugars and amino acids, dipeptides or protein hydrolysates under well-defined conditions was studied in model systems. The standard procedure for synthesis of the MRP was as follows: 5 mmol amino acid and 10 mmol sugar were refluxed in 5 ml 0.1 M potassium phosphate buffer, pH 7.0, for 5 h. Potassium hydroxide or hydrochloric acid was used to adjust the initial pH. When reacting protein hydrolysates with sugars, 1.0 g of protein hydrolysate was used instead and when reacting peptides and sugars, the concentrations were reduced to one fifth of those stated above for amino acids and sugars.
The crude reaction mixtures were used without any further fractionation, when evaluating their antioxidative effect. The reaction mixtures were generally characterized by measuring the final pH and the absorption at 450 nm (used as a measure of the brown color) and, when applicable, by determining the amounts of residual, unreacted glucose, fructose, arginine and lysine. The sugars as well as the amino acids were determined by enzymatic methods.

**Antioxidative effect of Maillard reaction products from different amino acid - sugar combinations (Publication II)**

The influence of individual amino acids and sugars on the antioxidative effect of the MRP formed was studied by reacting six different amino acids (L-arginine, L-cysteine, L-glutamic acid, L-histidine, L-lysine, and L-valine) with three different sugars (D-fructose, D-glucose, and D-xylose).

The antioxidative effect, as measured by the polarographic method, and the color ($A_{450\text{ nm}}$) of the reaction mixtures are shown in Figure 1. The

![Figure 1](image_url)  

**FIG. 1** Antioxidative effect (as measured by the polarographic method) and color ($A_{450\text{ nm}}$) of MRP from different amino acid - sugar combinations. (Based on Table 1 in Publication II.)
ability of the different amino acid - sugar combinations to form antioxidative products was found to be highly variable. By far the most antioxidative, as measured by this method, were the combinations arginine - xylose and lysine - xylose. Glutamic acid reacted with either glucose or fructose, on the other hand, had no antioxidative effect under these conditions. Of the combinations with glucose and fructose, those with lysine and histidine were the most antioxidative.

Considerable differences in the color formation were also observed. All the Maillard reaction mixtures containing fructose were less colored than the corresponding reaction mixtures with either glucose or xylose. In most cases the combinations with fructose were also somewhat less antioxidative than the combinations with glucose or xylose. Otherwise, no general correlation between color formation and antioxidative effect could be found.

The antioxidative effect of amino acid - sugar combinations, as measured by the gas chromatographic method, is shown in Figure 2, where n-hexanal was chosen as the indicator of lipid oxidation. Certain differences from

![Graph showing concentration of n-hexanal in linoleic acid emulsion oxidized for 25 h in the presence of MRP from different amino acid - sugar combinations. (Based on Table 2 in Publication II.)]
the results obtained with the polarographic method can be observed. Especially the combinations arginine-xylose and lysine-xylose had less effect when measured with this method. As for other combinations, the results obtained with the two methods of measurement are, on the whole, consistent. For instance, the glutamic acid - glucose combination was not found to be antioxidative with the gas chromatographic method either.

In summary, the basic amino acids are the ones most capable of forming antioxidative products under the conditions used in the present investigation. In the case of arginine, this is at least true for the combination with xylose. With regard to the other amino acids, the choice of sugar seems to be of minor importance.

The lesser extent of color formation in combinations with fructose, in comparison with that in combinations with the other two sugars, was in several cases also accompanied by a lower antioxidative effect, possibly due to the inferior reactivity of fructose. Both arginine and lysine remained unreacted in larger amounts after the reaction with fructose than after reaction with either glucose or xylose. More fructose than glucose remained unreacted also, irrespective of which amino acid was used. Isomerization between the sugars occurred during the reaction period, giving certain amounts of fructose, when starting with glucose and vice versa. This is a well known phenomenon in carbohydrate chemistry. The isomerization to mannose is also possible. However, this sugar was not determined in this study. Finally, the reactions with fructose were also characterized by a smaller pH-drop.

Generally, the differences in antioxidant formation between the different amino acid - sugar combinations can hardly be explained by differences in overall reactivity, as judged from the determinations of remaining sugars and amino acids in the reaction mixtures.

Obviously, the results from the two methods of measurement of antioxidative effect diverged in some cases, as becomes evident when comparing Figure 1 and Figure 2. While arginine-xylose is superior to, for example, histidine-glucose in retarding the oxygen consumption, the hexanal formation is to a greater extent inhibited by histidine-glucose. A speculative explanation of this is that there are differences in the antioxidative mechanism between these MRP. They may direct the secondary reactions
of the lipid oxidation in different ways. This observation points, in any case, to the importance of using more than one method of measurement, when evaluating the antioxidative effect. However, it still remains to be found out, which of the two results is most accordant with the effect in foods during normal storage.

Kirigaya et al. (1969) have previously studied the effect of various amino acids and sugars on the antioxidative effect of the MRP. Aqueous solutions of 2 M amino acid and 2 M sugar were reacted at 100°C for 5 h. Their reaction mixtures were dialyzed and the inner solutions were lyophilized and tested for antioxidative effect in a 40% ethanol solution of a mixture of linoleic acid and linolenic acid. The increase of the peroxide value during storage was followed. Several amino acids were reacted with glucose and several reducing sugars were reacted with glycine. Of the amino acids studied, the following formed MRP with the strongest antioxidative effect: DL-alanine, β-alanine, DL-serine, L-threonine, L-histidine and L-arginine. When comparing with the present work, good agreement is observed in the cases when the same amino acids were used. Kirigaya and co-workers observed, however, a certain antioxidative effect of glutamic acid - glucose, in contrast with the present results. Of all the sugars studied, L-xyllose and L-sorbose were found to be the most effective ones in forming antioxidative MRP. Glycine-fructose gave stronger antioxidative effect than glycine-glucose, possibly since the comparison was made on the basis of weight, using dialyzed MRP.

A similar investigation was reported by Tomita (1971a), who studied MRP from glucose and various amino acids. Solutions of 10 mM glucose and 10 mM amino acid were heated at 120°C for 1 h. The antioxidative effect of the reaction mixtures were measured by a method similar to the one used by Kirigaya et al. (1969). MRP from L-tryptophan-glucose were found to be the most antioxidative. The order in which the other amino acids followed was dependent on the concentration used in the test. Even this investigation supported, however, the view that potent antioxidants are formed from L-histidine. Obvious disagreement is observed between the results obtained by Tomita and those obtained by Kirigaya et al. This is possibly due to the fact that different conditions were used for the synthesis of the MRP.
From the present work it is, however, evident, that it is not sufficient to investigate MRP from many amino acids and one sugar and from many sugars and one amino acid. It can not be taken for granted, that the most efficient amino acid when reacted with glucose is the most efficient when reacted with other sugars as well. The combinations with arginine exemplify this, MRP from arginine-xylose being highly antioxidative, while arginine-glucose and arginine-fructose form MRP with a relatively weak antioxidative effect.

**Antioxidative Maillard reaction products from histidine and glucose**  
(Publication II)

Since MRP from histidine and either of the sugars showed strong antioxidative effect, irrespective of the method of measurement used, the combination histidine-glucose was chosen for further studies of the influence of some reaction parameters on the antioxidative effect.

**Influence of reaction time**

Starting with 100 ml of reaction mixture, the reaction between histidine and glucose was allowed to proceed for 200 h. At intervals, samples were withdrawn for the measurement of antioxidative effect (by the polarographic method), color ($A_{450 \text{ nm}}$), and pH. The antioxidative compounds and the pigments seemed to be formed simultaneously, both going through a maximum after about 20 h of reaction. The decrease in color on further reaction is due mainly to precipitation of pigmented material of a high molecular weight, since all measurements were made on the soluble part of the reaction mixture. It is, however, uncertain to what extent the same explanation is true for the decrease in the antioxidative effect as well, since other experiments did not confirm any connection between the formation of brown pigments and antioxidative effect. (See e.g. the influence of the molar ratio of histidine to glucose below.)

A rapid decrease in pH was observed during the first five hours, after which time pH stabilized at about 4.4.

**Influence of pH**

The reaction between histidine and glucose was performed in water solutions adjusted with NaOH or HCl to initial pH 3.0, 5.0, 7.0, 9.0, and 11.0, respectively. The antioxidative effect and the absorption at 450 nm
FIG. 3 The influence of initial pH on the color ($A_{450 \text{ nm}}$) and the antioxidative effect (as measured by the polarographic method) of MRP from histidine and glucose. (Based on Table 3 in Publication II.)

of the reaction mixtures after a reaction time of 5 h is shown in Figure 3. Antioxidative products were formed over the whole of the pH-range. The strongest antioxidative effect, as well as the highest color intensity, was, however, obtained at intermediate pH. It should, however, be kept in mind that considerable changes occurred in pH during the reaction. When starting at pH 9.0 the final pH was 5.5, compared to pH 5.0, which was obtained, when the initial pH was 7.0. The differences in pH during the reaction were thus smaller than is shown by the initial pH-values.

Kirisaya et al. (1969) reported that high initial pH in the reaction gave stronger antioxidative effect, when comparing at constant weight after dialysis and lyophilization of the reaction mixtures. Since no data obtained with unfractionated reaction products were reported, these results are difficult to compare with those in the present study. However, Tomita (1971 b), using tryptophan-glucose and lysine-glucose also reported that a high initial pH (pH 7 - 11) favored the formation of antioxidative products, when measuring the effect of the crude reaction mixtures. The discrepancy as compared with the present results may be explained by differences in the reaction conditions. (Tomita used $10^{-2}$ M solutions, that were heated at 120°C for 1 h.)
Influence of molar ratio of histidine to glucose

The antioxidative effect, as measured by the polarographic method, and the color ($A_{450\text{ nm}}$) of Maillard reaction mixtures obtained by reaction between histidine and glucose at different molar ratios is shown in Figure 4. When the glucose concentration was increased, keeping the histidine concentration constant, both the antioxidative effect and the color was increased. When, on the other hand, the glucose concentration was kept constant, variations in the histidine concentration had no effect on the color formation. The antioxidative effect was, however, also increased by increased histidine concentration. In summary, the color formation seemed almost entirely dependent on the glucose concentration, while the antioxidative effect seemed to be more dependent on the histidine than on the glucose concentration. The reaction paths important for the formation of antioxidative compounds, obviously differ from those important for the formation of colored compounds.

![Histidine: Glucose](image)

**FIG. 4** The influence of histidine and glucose concentration on the color ($A_{450\text{ nm}}$) and the antioxidative effect (as measured by the polarographic method) of the MRP formed. (Based on Table 4 in Publication II.)
Tomita (1971b) in the above mentioned work found that both the antioxidative effect and the color intensity increased upon increasing the glucose concentration while keeping the tryptophan concentration constant. The influence of the tryptophan concentration was not studied. Kirigaya et al. (1969) found that a high molar ratio of glycine to xylose was favorable for the formation of antioxidative products, when comparing dialyzed reaction products on an equal weight basis.

**Antioxidative effect of Maillard reaction products from dipeptides and sugars** (Publication III)

The ability of peptides to form antioxidative MRP was investigated. Since glycine was found to be a poor antioxidant-forming amino acid, and L-histidine was known to form MRP with strong antioxidative effect, the two possible dipeptides of these amino acids were chosen for this study. Xylose was chosen as the sugar. Figure 5 shows the antioxidative effect of MRP obtained from xylose and either these peptides, the individual amino acids, or a mixture of the free amino acids. The antioxidative effect was measured by the gas chromatographic method, using n-hexanal as an indicator of lipid oxidation. Considerable antioxidative effect was

![Amino compound reacted with xylose (2 mmol):](image)

**FIG. 5** Concentration of n-hexanal in linoleic acid emulsion oxidized for 25 h in the presence of MRP from xylose and dipeptides or amino acids. (Based on Table 1 in Publication III.)
obtained, when reacting a mixture of the two amino acids with xylose, in spite of the fact that MRP from xylose and glycine alone were even found slightly pro-oxidative. Glycylhistidine was equally effective as the mixture of the amino acids in forming antioxidative products with xylose, while L-histidylglycine was even more effective. The antioxidative effect of MRP from L-histidylglycine and xylose was, in fact, found to be comparable to that of MRP from histidine and xylose, even though the latter combination contained the double amount of histidine (equals the molar sum of histidine + glycine in the dipeptide). It can thus be concluded that potent antioxidants are able to form also when reacting peptides with sugars, the antioxidative effect being dependent not only on which amino acids constitute the peptide, but also on their sequence. It is at least probable that the N-terminal amino acid is important, since its α-amino group can be used in the Maillard reaction.

Antioxidative effect of Maillard reaction products from enzymic protein hydrolysates and sugars (Publication III)

Since the dipeptides showed the ability to form MRP with strong antioxidative effect, it was of interest to investigate the possibilities of using protein hydrolysates as the amino source. If the antioxidative effect of MRP is to be utilized in foods, the use of hydrolysates obtained from proteins commonly used in foods would be a possible (and perhaps less expensive) alternative to the use of pure amino acids. MRP from glucose and some protein hydrolysates obtained by enzymic hydrolysis of proteinous food by-products were, therefore, tested in this study. The different hydrolysates were chosen arbitrarily, and they were not characterized regarding amino acid composition, peptide chain lengths, terminal amino acids etc. The purpose was merely to get an idea of their potential ability to form antioxidants by the Maillard reaction.

Certain protein hydrolysates have previously been reported to be antioxidative per se (Yamaguchi et al. 1975a, b; Bishov and Henick 1975). Some antioxidative effect was also demonstrated by protein hydrolysates used in this investigation. However, the effect was considerably improved when reacting the hydrolysates with glucose. All the hydrolysates tested (hydrolyzed brewer's grains, autolyzed malt sprouts and hydrolyzed hemoglobin) were found capable of forming antioxidative MRP. As compared
with MRP from e.g. histidine-glucose, higher concentrations of those from the protein hydrolysates were, however, required for equal antioxidative effect. At low concentrations they even indicated pro-oxidative properties. By the proper choice of, for example, proteolytic enzyme, hydrolysis conditions and possibly by separation methods, it should, however, be possible to optimize the properties of the hydrolysate with regard to the content of peptides of suitable chain lengths and with the appropriate terminal amino acids, which favor the formation of antioxidative products in reaction with sugars.

APPLICATION OF ANTIOXIDATIVE MAillard REACTION PRODUCTS IN FOODS

If the antioxidative properties of MRP are to be utilized in foods, two essentially different methods of application are possible:

- Potent antioxidants, synthesized from the appropriate sugars and amino compounds and possibly purified by fractionation, can be used as a normal food additive.

- Since the Maillard reaction is very common in food processing, the formation of antioxidative MRP may be optimized in many foods by controlling the process parameters and the recipe. The appropriate protein hydrolysates, amino acids or sugars can, for example, be included in the recipe of foods, which are normally subject to sufficient heat treatment.

Few studies on the antioxidative effect of MRP in foods have been reported. Griffith and Johnson (1957) found, that the replacement of part of the sucrose by glucose in the dough of cookies resulted in darker cookies with improved stability against rancid flavor development. This effect was ascribed to low molecular reductones formed by the Maillard reaction during baking. Yamaguchi et al. (1964), also studying cookie stability, found that the addition of amino acids to the dough improved the keeping quality. Tomita (1972c) reported the stability of "fried rice cake chips" to be improved by the addition of tryptophan and glucose. Since the processing included heating at 120°C for 30 min and deep fat frying at 180°C for 3 min, MRP formed were assumed to be responsible for the antioxidative effect. All these examples are, thus, to be assigned to the second method of application stated above.
Sato et al. (1973), however, found that the rapid development of oxidized flavor in refrigerated cooked beef, so-called "warmed-over flavor" (WOF), was inhibited by retorted solutions of sugars and amino acids, which illustrates the first method of utilizing antioxidative MRP. Recently, Einerson and Reineccius (1977) reported similar results from experiments with turkey meat.

In this work cookies and sausage were chosen as the food products for application studies, trying both the addition of preformed antioxidative MRP and the addition of a sugar together with an amino acid.

**Application in cookies (Publication IV)**

Two variations of a basic type of cookie, differing only in type of fat used, were baked. One contained lard as the fat component and the other one a shortening of vegetable origin. Of each variant of basic cookie the following samples were baked:

A. Cookies with no added antioxidants (control).

B. Cookies baked from doughs containing 0.1% L-histidine monohydrochloride monohydrate and 1.0% D-glucose. The addition of glucose was made at the expense of the same amount in weight of sucrose.

C. Cookies baked from doughs containing 0.1% MRP, obtained by refluxing L-histidine monohydrochloride monohydrate and D-glucose for 5 h. (The concentration stated, 0.1%, is based on the original amounts of the reactants in the Maillard reaction mixture).

D. Cookies baked from doughs containing approximately 2 ppm BHA /BHT (1:1), corresponding to 0.1 g BHA + BHT per 1000 g fat, which is the highest concentration allowed in Sweden.

The cookies were stored at 30°C in heat-sealed, laminated aluminum pouches, and were regularly analyzed by sensory evaluation and by gas chromatographic analysis with respect to volatile compounds formed.

Of the two variants of basic cookie, the one containing vegetable fat was found to be considerably more stable, possibly due to higher content of tocopherols or, perhaps, better initial quality with respect to the oxidation state.
No significant antioxidative effect of the preformed MRP was observed by sensory evaluation or by gas chromatographic analysis in the experiment with cookies containing lard. A weak retardation of the development of rancid flavor, as well as of the formation of n-hexanal, was caused by the addition of BHA/BHT. By far the most protective was, however, the addition of histidine and glucose. Although the increase in the concentration of n-hexanal in all cases occurred later than the increase of the intensity of rancid flavor, indicating that n-hexanal was not the most important one of the volatiles causing the rancid flavor, good agreement was noticed between the results of the two measurements. Occasionally performed determinations of the peroxide value also supported these results.

Although storage for a longer period of time would have been desirable in the experiment with cookies containing vegetable fat, the results obtained indicated good agreement with those obtained in the experiment with cookies containing lard.

A proximate question is, thus, whether the added histidine per se or reaction products formed during the baking were responsible for the antioxidative effect in the cookies. In the latter case the antioxidative reaction products could theoretically be formed by reaction between histidine and glucose or by reaction of either histidine or glucose with other reactants in the cookie dough. Histidine could be effective as a carbonyl scavanger by reacting with, for instance, volatile, odorous aldehydes and ketones, which might partly explain the ability of added histidine to retard the development of rancid flavor. Antioxidative effect of histidine alone has been observed previously (Marcuse and Fredriksson 1969; Tjio and Karel 1969). However, we have not noticed any antioxidative effect of histidine at such low concentrations as those used when testing MRP with the polarographic method. With the gas chromatographic method, histidine was observed to be slightly antioxidative, when assaying at ten times the MRP concentration commonly used (Lingnert, unpublished results).

Since only 30% of the added histidine could be recovered as free histidine in the cookies after the baking a reasonable assumption is that MRP from histidine contributed to the antioxidative effect. To what extent the added histidine reacted with either the added glucose or with other
carbonyl compounds present in the dough is still a question. Thus, no final proof can be presented by now that a histidine-glucose reaction product is responsible for the antioxidative effect in stored cookies. The results of Griffith and Johnson (1957) who added only glucose to the dough indicate that products obtained by reaction of glucose with other reactants than the added histidine also can contribute to the antioxidative effect. However, their results support the assumption that MRP formed during the baking were the active antioxidants.

If mainly histidine-glucose reaction products were responsible for the antioxidative effect in the cookies, it should be asked why preformed MRP did not exert any antioxidative activity. It may be that the concentration of antioxidative MRP was in fact lower when adding preformed MRP than when adding free histidine. Since about three times as much histidine, totally, was added to the cookie dough in the latter case, and only 30% could be recovered as free histidine in the cookies after the baking, these cookies contained about twice as much "reacted histidine" as those with added MRP. It is also possible that the reaction conditions during the baking, for example the low water activity, are favorable for the formation of antioxidative MRP.

**Application in sausage (Publication V)**

Sausages of frankfurter type were produced from batter containing the following additions:

A. No additions (control)

B. 0.08% MRP obtained by refluxing L-histidine monohydrochloride monohydrate and D-glucose for 5 h. (The concentration stated, 0.08%, is based on the original amounts of histidine and glucose in the Maillard reaction mixture.)

C. 0.16% MRP as above.

D. 0.08% L-histidine (added as L-histidine monohydrochloride monohydrate) and 0.32% D-glucose.

E. 0.16% L-histidine and 0.32% D-glucose.

F. 0.16% MRP obtained by refluxing an enzymic hemoglobin hydrolysate and D-glucose for 5 h. (The concentration calculated as above.)
The sausages (5 cm in diameter and approximately 50 cm long) were wrapped separately in aluminum foil, frozen and stored at -20°C. During the storage period samples were withdrawn for sensory evaluation, gas chromatographic analysis of volatile compounds, and determination of the peroxide value.

In this experiment the sensory evaluation showed the preformed MRP capable of retarding the lipid oxidation. All the three samples with added MRP were found to develop rancid flavor at a lower rate and were even judged to be less rancid at the start of the experiment as compared with the other three samples. The explanation may be that lipid oxidation to some extent had already taken place during the production of those sausages, which did not contain any added antioxidative MRP. This early onset of lipid oxidation may be similar to the formation of WOF, since the process included heating in 75°C water for 45 minutes, followed by keeping at 0°C for one day. According to a recent review these conditions are favorable for the development of WOF (Pearson et al. 1977).

Neither the gas chromatographic analyses, nor the peroxide value determinations could support the results from the sensory evaluations in this experiment. None of these chemical analyses was found to be a sufficiently sensitive measure of rancidity in this product. Due to difficulties in uniforming large amounts of inhomogeneous raw materials, such as beef, tallow, and backfat, great variations within one and the same sample of sausage were, moreover, obtained. In the sensory evaluation these variations were suppressed due to the large number of analyses (9 - 11 judges judging three replicates of each sample from at least two different sausages). The variations were, however, highly troublesome in the chemical analyses, of which only two replicates were made. Considerably more replicates would be necessary to establish the possible, small differences between the samples.

The experiment confirms that MRP obtained from protein hydrolysates, as well, may exert antioxidative properties in foods. The heat treatment included in the process was, however, obviously insufficient for the production of antioxidative MRP from the added histidine and glucose.
ADDITIONAL REMARKS

This work has pointed at the possibilities of utilizing the antioxidative properties of MRP in foods. Both of the essentially different methods of application (see p. 30) have been illustrated. In the cookies, the antioxidants were formed from added amino acids and sugars during the baking, while in the sausage, preformed MRP were needed, since the heat treatment included in the process was insufficient.

It should be mentioned, however, that the first method must be used with discretion, in particular, if adding reducing sugars to the product, since this will involve an obvious risk of impairing the nutritional value of the product as a result of reactions between the sugar and the essential amino acids in the proteins. These nutritional consequences should, therefore, always be kept under control, when using this method.

Although MRP have been consumed ever since man learned to cook food, the possibility of MRP having antinutritional or even toxic effects cannot be precluded. Reports on detrimental effects of this kind were reviewed by Adrian (1974). High amounts of "premelanoids" (a term including all soluble MRP) have been found to principally reduce the digestibility and metabolic value of food nitrogen not subjected to heat treatment. Experiments have shown that the PER (Protein Efficiency Ratio) can fall 20 - 40%. Deoxyfructosyl-tryptophane has been reported to inhibit small-intestinal disacharidase activities (Lee et al. 1977) and to affect liver poly-ribosomes (Chichester 1974).

Particularly in the use of preformed, antioxidative MRP, the possible antinutritional effects should be considered. Evaluations of toxic effects should preferably be done. MRP from histidine-glucose and arginine-xylose, produced according to the standard procedure in this work, have been investigated in limited feeding experiments with growing rats, in order to get an idea of possible acute toxicity (Asp et al. 1979). A standard diet containing 10% casein as the protein source was fed to the rats for four weeks. About twenty-five times more MRP than is considered necessary with respect to the antioxidative effect were added to the diet. Both kinds of MRP caused a slight decrease of food intake and growth, probably due to palatability factors. There was no inhibition of
the utilization of dietary protein or other signs of antinutritional or toxic effects detectable during these feeding experiments. Considerably more extensive studies are, of course, needed to prove the safety of these MRP.

The structure of the antioxidative MRP remains to be elucidated, as does the mechanism of their antioxidative effect. Griffith and Johnson (1957) considered the antioxidative compounds to be reductones of relatively low molecular weight, as did Eichner (1975), who suggested that 1,2-enaminoles derived from the Amadori-compounds were responsible for the antioxidative effect. Pure reductones prepared from secondary amines have been demonstrated to be antioxidative (Evans et al. 1958). Yamaguchi (1969) found some reductones to be antioxidative in dehydrated systems, but no effect was obtained in aqueous systems. Antioxidative material, believed to be MRP, extracted from retorted turkey meat was investigated by Eneron and Reineccius (1978). Fractionation by gel filtration indicated a molecular weight of the antioxidant of between 200 and 500. Kirigaya et al. (1968), on the other hand, found that the nondialyzable fractions played the most important role. Yamaguchi and Fujimaki (1973 b) reported a fraction of melanoidins with a molecular weight of about 4500 to be strongly antioxidative. This is in accordance with ultrafiltration experiments on the histidine-glucose reaction mixture used in this study. Using membranes with well defined molecular weight cut-offs, these experiments showed the molecular weight of the antioxidative compounds to be between 1 000 and 10 000 (Lingnert, unpublished results).

Regarding the antioxidative mechanism, MRP have been reported to form complexes with metals (Kajimoto et al. 1975; Kajimoto and Yoshida 1975; Gomyo and Horikoshi 1976). The ability to complex metals is, however, not likely to be the main antioxidative mechanism of the MRP. Probably, they also affect the free radical chain reaction in some way. The Maillard reaction has been found to involve stable free radicals, as well (Namiki and Hayashi 1975; Hayashi et al. 1977). It is possible that these interact with the lipid free radicals, causing an inhibition of the lipid oxidation.

Further research is, however, needed in order to characterize the structure of the antioxidative compounds. An understanding of the structure would facilitate the elucidation of the antioxidative mechanism.
CONCLUSIONS

From the present work it is evident that:

- it is advisable to use more than one method of measuring antioxidative effect.

- the antioxidative effect of MRP is strongly affected by the choice of sugar and amino compound.

- of the amino acids tested, the basic ones form the most antioxidative MRP.

- the antioxidative effect of MRP from sugars and dipeptides is affected by the amino acid sequence in the peptide.

- enzymic protein hydrolysates can form antioxidative MRP by reaction with sugar.

- antioxidative MRP are effective in retarding the development of rancidity in foods, either when added preformed to the food, or when formed from added reactants in the foods during processes involving sufficient heat treatment.
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