OPTIMIZING THE REACTION CONDITIONS FOR THE SYNTHESIS OF ANTIOXIDATIVE SUBSTANCES FROM ARGININE AND XYLOSE

By

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I suppose every generation has its own group of heroes or persons which has touched or moved it by kindling a flame of awareness and concern within the souls of its constituents. It was in this way that the Beatles were heroes of my generation. They were heroes not only because they amplified a respect for love and peace, but also because they did things differently than others in their field. They were innovators. I do not believe they said anything that had not been said before, but they were innovative in the manner in which they said it. I was always amazed at how four individuals could produce such beautifully multi-faceted pieces of music all by themselves, and I realize now, of course, that not even the Beatles could have done what they did without " a little help from their friends." In that way only, can I say that I have something in common with the Beatles, because I too could not have completed this project without the help of several beautiful people. Drs. Mitchell and Nelson, for instance, provided me with valuable suggestions on how to present my results, and Dr. Dermer bestowed me with his priceless editorial skills so that the verbiage would not camouflage the content. Of course, if it had not been for Mrs. Pulliam's exquisite typing, I would still be pecking away, and if Dr. Lingnert had not so freely volunteered his expertise and advice on this research problem, there probably would not be a paper to type in the first place. As a matter of fact, when it comes to advice, I could not have asked for a better advisor than Dr. Waller. He never told me what to do but instead

offered numerous little gems of advice just when they were needed, not only in my research, but also in my writing. Just as important though, he was always open to my ideas no matter how crazy they may have seemed. He was almost like another parent to me. It was my parents, in fact, who gave me something that can't be measured. It is uncanny how a simple phrase like, " we know you can do it," can boost one's self esteem. Finally, taking second only to the Holy Trinity, Beth Polo blessed me with her sunshine and her rainbows and gave me hope when I was sure that only darkness would prevail.

To all of these wonderful individuals, I simply, but sincerely wish to say, thank you!

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INTRODUCTION

Man has always been preoccupied with the search for better life. Whether it be a plane that flies faster, or a pleasure that lasts longer, the search never seems to diminish. The field of food preservation has taken no exception to this obsession, and has found itself in the midst of a hunt for the ultimate food antioxidant. This is not surprising since a large portion of the food consumed by the developed countries of the world requires some form of preservation in order to retain its nutritive and aesthetic values. As long as the people of these countries continue to utilize foods requiring extended storage periods, the need for some form of preservation will be mandatory.

One of the primary components of food spoilage is the autoxidation of unsaturated fats. Unfortunately the acquisition of a compound possessing high antioxidative properties against this spoilage, and exhibiting minimum detrimental biological side effects, has eluded mankind. However, recently, the search has been focused on a set of compounds isolated from a reaction that naturally occurs everytime heated food undergoes browning. Known as the Maillard reaction, this synthesis could very well manifest itself as a primary source of safe and potent antioxidants.

The optimum conditions for producing antioxidants in several model Maillard systems are beginning to be elucidated. One potentially

promising system, however, has not been adequately explored. This is the antioxidant producing reaction between L-arginine and D(+)xylose. Therefore, the purpose of this paper is to report the results of a study designed to ascertain the optimum conditions necessary for producing a high yield of antioxidant from the Maillard reaction between L-arginine and D(+)xylose.

CHAPTER II

LITERATURE REVIEW

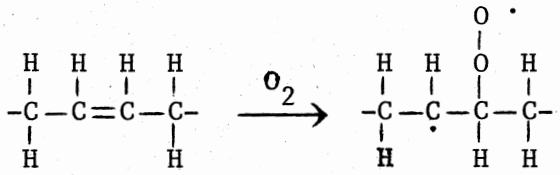
The autoxidation of foods has been a concern to people since the beginning of recorded history. The products of autoxidation of unsaturated fats are numerous, and account for the many obnoxious and sometimes toxic properties of rancid foods. The germination of bacterial spores, for example, can be inhibited by toxic compounds present in small amounts of autoxidized linoleic acid (1). Also it is generally believed that the malabsorption disease known as "tropical sprue" is caused by the consumption of rancid vegetable fat (2). Therefore, the understanding of the mechanism of autoxidation, and an understanding of a process for generating safe and effective inhibitors of it are important, not only for the aesthetic pleasure of humans, but for their general well being and good health as well.

Autoxidation

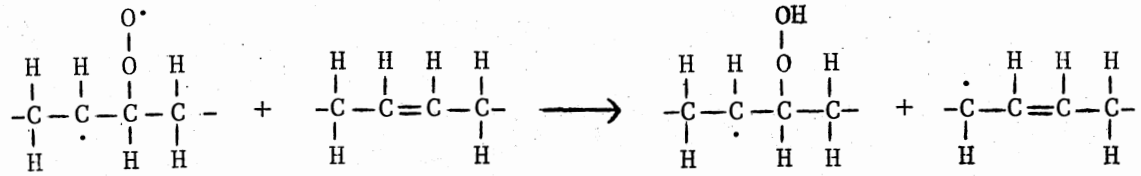
The current accepted mechanism for the autoxidation phenomenum involves the evolution of peroxides from free radicals (3) (4), and the subsequent initiation of chain branching (5) (6). Several extensive reviews have been published regarding this subject (7) (8) (9), and only a brief summary will be presented here.

The overall autoxidation process is a chain reaction involving initiation via the production of a free radical followed by a propogation

stage. It is in the initiation stage that peroxide radicals are formed, which in turn promote the propogation of other radicals, either by interacting with available substrate species or by breaking down in a process known as chain branching. By this means, the reaction is said to be autocatalytic, and will advance through a series of permutations until the propogation sequence is quenched either by inhibitors or by a depletion of reactive species. Although the initiation of the chain reaction is commonly facilitated by the presence of oxygen, it may also be enhanced by the presence of radiation or polyvalent metals. Unsaturated systems are quite susceptible to attack by oxygen. The double bonds of monounsaturated systems can, in fact, experience direct attack by oxygen, and undergo peroxide radical formation.



The peroxide radical thus formed can then go on to generate a second radical by abstracting an allylic hydrogen from another unsaturated molecule. The net result then, is the production of two radicals, one of which is a hydroperoxide.



With conjugated, polyunsaturated molecules, initiation can occur as it does in the monounsaturated molecule. More likely, however, the initiation will proceed by a process involving abstraction of an allylic

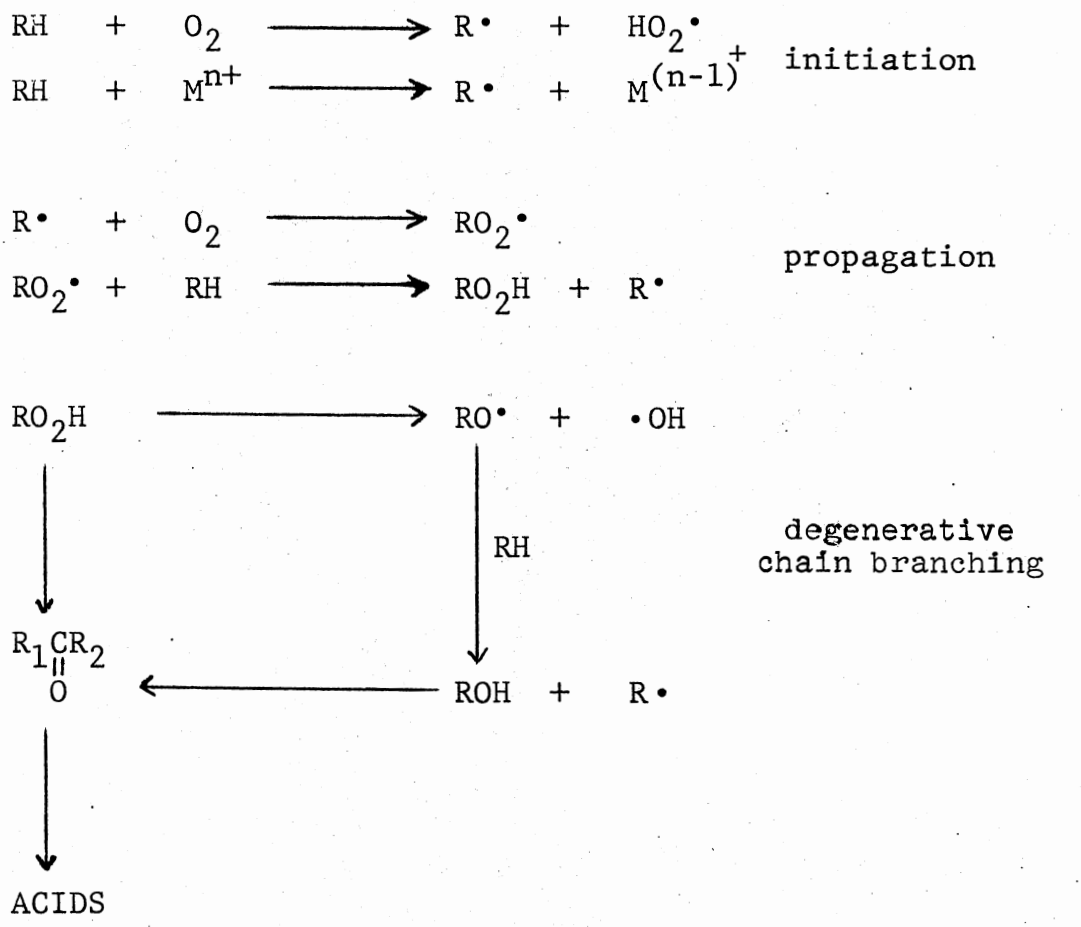
hydrogen, rather than a direct coupling of the oxygen with the unsaturated carbon. In either case, the initiation of a free radical soon leads to the generation of a multitude of secondary radicals, many of which are converted to hydroperoxides. These are subsequently broken down in a process known as degenerative chain branching to form a different family of radicals and thus perpetuate the chain reaction. However, they may also be converted to compounds such as epoxides, aldehydes, alcohols, ketones, and acids from which come the obnoxious odors and tastes of rancid foods. The chain reaction will continue until the free radical nature of the chain propagating molecule is destroyed. This is carried out most effectively by a compound which can donate a hydrogen atom to the radical without becoming a reactive radical itself. The presence of a specific amount of the inhibiting compound will result in a marked decrease in the rate of autoxidation. The rate will maintain its reduced value until the supply of the inhibiting compound is exhausted. The rate will then increase again.

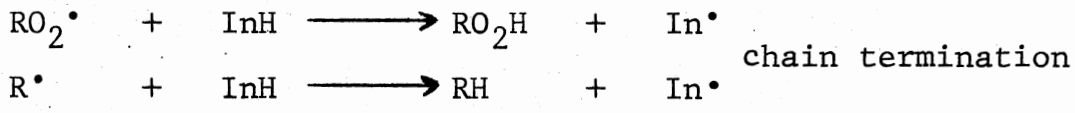
The overall scheme for the autoxidation mechanism involving initiation, propagation, degenerative branching, and termination is presented in Figure 1.

Antioxidants

Antioxidants are compounds which inhibit autoxidation either by preventing the propagation of the chain reaction via the termination mechanism described above or by preventing the initiation of radical formation in the first place. Compounds such as EDTA, citric acid, and ascorbic acid are typical examples of antioxidants which inhibit

Figure 1. General scheme for the Autoxidation Mechanism (8).


RH + O2 → R• + HO2•
RH + M^n+ → R• + M^(n-1)+
initiation
R• + O2 → RO2•
RO2• + RH → RO2H + R•
propagation
RO2H → RO• + •OH
RH
degenerative
chain branching
R1CR2
O
ROH + R•
ACIDS


RO2• + InH → RO2H + In•
R• + InH → RH + In•
chain termination

oxidation by preventing initiation. Because they easily form complexes with multivalent metals, they are quite effective in decreasing the amount of trace metals which are available to oxidize or facilitate the oxidation of an unsaturated molecule. On the other hand, phenolic compounds such as BHA (butylated-hydroxy-anisole), BHT (butylated-hydroxytoluene), and TBHQ (tert-butyl-hydroquinone) are classical examples of antioxidants which inhibit oxidation by blocking the propagation of the chain reaction. Because they can exist as nonreactive free radicals due to resonance stabilization, these antioxidants can effectively block the propagation of the chain reaction by donating a hydrogen atom to a free radical and thus prevent the formation of an entire subset of secondary free radicals. In some cases, a synergistic effect may be observed when an antioxidant which blocks propogation is combined with one which prevents initiation.

Workers from Japan and Sweden (10)(11)(12) have found that a multitude of antioxidants can be obtained from a rather unlikely source. Based on findings of Griffith and Johnson (13), that glucose added to cookie dough, increased the shelf life of the cookies, it was suggested and since confirmed that the glucose, a reducing sugar, was reacting with the various amino acids within the dough, and producing one or more compounds possessing antioxidative activity. The reaction between reducing sugars and amino containing compounds has been scrutinized by food chemists for years because it is one of the primary sources of browning and of evolution of distinct flavors, colors, and aromas during cooking. The fact that it may be the source of potent and yet safe antioxidants has until now been largely overlooked. However, before

discussing the work that has been conducted to improve the understanding of the antioxidative generating potential of this browning or Maillard reaction, it would be appropriate at this point to briefly review the reaction, its mechanism, and some of the conditions associated with it.

The Maillard Reaction

Esentially, there are three major types of browning reactions. One involves the intense heating of polyhydroxy carbonyls or sugars in a process referred to as caramelization, and can be catalyzed by either acids or bases. The second type occurs when compounds such as dehydrocarbonyls and polyphenols are oxidized to form di or polycarbonyl compounds. This oxidation can also proceed enzymatically, and is quite common in the food processing industry. The browning of freshly exposed fruits is a typical example, and is believed to involve the interaction of oxidases with tyrosine, catechols, and other similar molecules, to form quinones, which then go on to initiate the formation of pigmented compounds (14). The third major type of browning phenomenon, and the one to which this paper is addressed, involves the interaction of aldehydes, ketones, or reducing sugars with amino containing compounds, and is commonly referred to as the Maillard reaction. In nearly all instances, the browning observed in this reaction is ultimately the result of the formation of unsaturated polymers (15)(16).

Over the years, since Maillard first reported the browning reaction between several reducing sugars and amino compounds (15), a multitude of plausible mechanisms have been postulated (18)(19)(20)(21)(22). Although the understanding of the final polymerization step still

remains unsolved, a fairly complete explanation of the complex events leading up to it can now be presented.

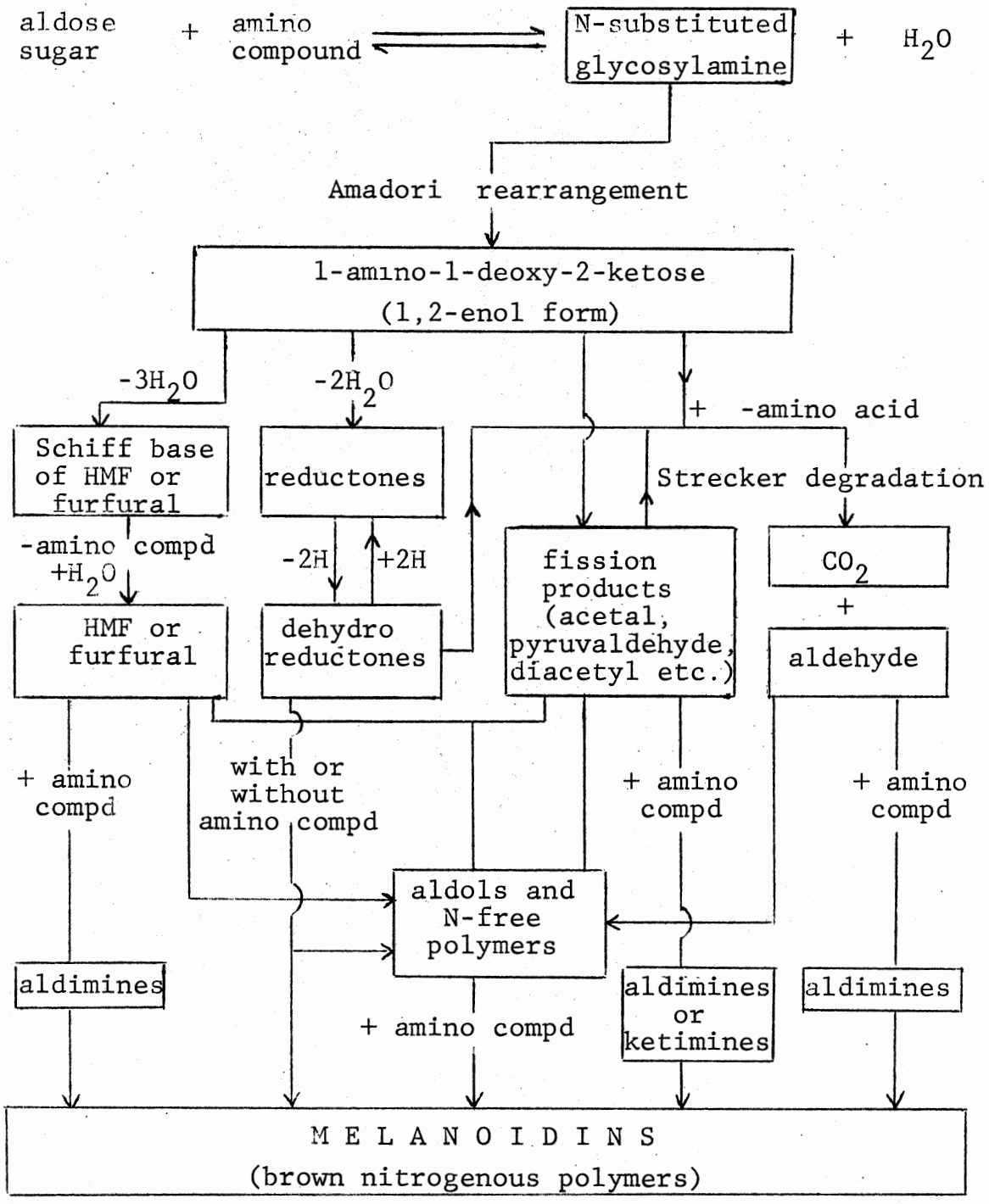
Mechanism

The Maillard reaction between a reducing sugar and an amino acid consists of a composite of many different reactions, the first of which is the condensation of the amine with the reducing sugar. The product of this then undergoes the classical Amadori rearrangement followed by a dehydration and subsequent fragmentation of the sugar. The amino acid portion is not without its contribution and proceeds, via a Strecker degradation, to part with its carboxyl function. The complexity of the overall reaction is insured as the products of these primary transformations react with one another resulting in the final stage of the Maillard reaction, which consists of a combination of aldol condensations followed by numerous aldehyde amine polymerizations, and subsequent formation of heterocyclic nitrogen compounds (Figure 2). The conditions of the reactions are quite important, and will be discussed in a separate section.

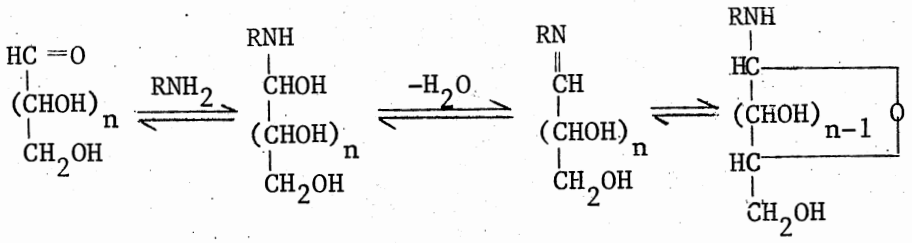
Condensation

The first step of the Maillard reaction involves the initial condensation of a reducing sugar with an amino compound. Haugaard, Tumerman, and Silvestri (23) determined that the condensation was reversible and that the reactants combined in a one to one molar ratio. However, as the reaction progressed, the condensation product irreversibly decomposed. Hannan and Lea (24)(25) also reported an initial one to one

Figure 2. Overall scheme depicting the Maillard Reaction (18).


aldose sugar
+
amino compound
N-substituted glycosylamine
+ H_2O
Amadori rearrangement
1-amino-1-deoxy-2-ketose (1,2-enol form)
$-3H_2O$
$-2H_2O$
+ -amino acid
Schiff base of HMF or furfural
reductones
Strecker degradation
-amino compd $+H_2O$
-2H
+2H
fission products (acetal, pyruvaldehyde, diacetyl etc.)
CO_2
+
HMF or furfural
dehydro reductones
aldehyde
+ amino compd
with or without amino compd
+ amino compd
+ amino compd
aldols and N-free polymers
aldimines
+ amino compd
aldimines or ketimines
aldimines
MELANOIDINS
(brown nitrogenous polymers)

reaction between glucose and α-N-acetyllysine, polylysine, and casein. Mohammad, Fraenkel-Conrat, and Olcott concurred by demonstrating that prior to browning, glucose and the free amino groups of bovine serum albumin combined in a one to one fashion (26). Katchalsky and Sharon (27) proposed a mechanism in which the condensation occurred by way of acid and base catalysis. They also found that the rate was in some manner dependent on the ease of opening of the pyranose ring, and therefore, was dependent on the amount of aldehyde present. Their postulation was that condensation proceeds by way of the opening of the ring form of the sugar, addition of the amine to the carbonyl group, and then subsequent elimination of a molecule of water to form the N-glycosylamine (27). This is presently the accepted mechanism for the sugar amine condensation.

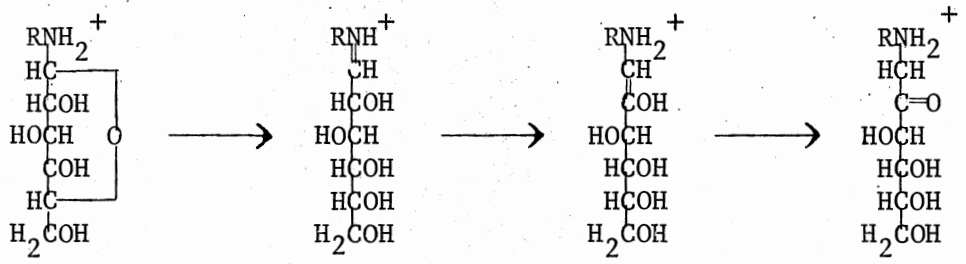


Amadori Rearrangement

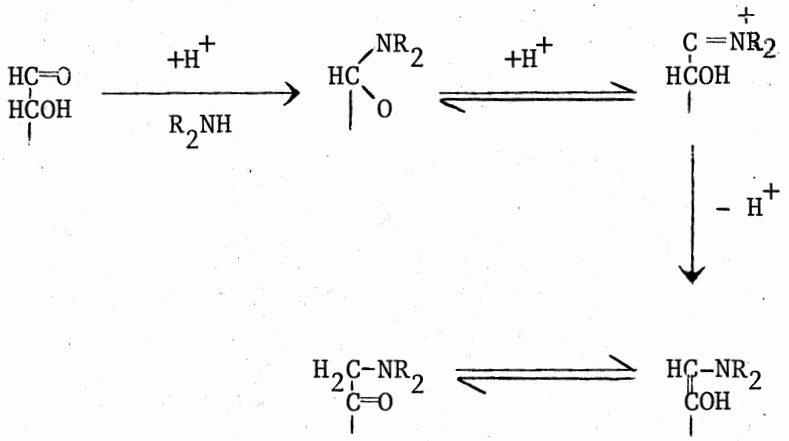
The second major step toward the completion of the browning reaction utilizes a rather simple, though not completely understood transposition of the carbonyl function of the sugar from the 1 carbon to the 2 carbon. The Amardori rearrangement, as it is known, was first implicated in the browning reaction in 1945 (28), but wasn't verified until 1952 by Gottschalk (29) and by Hodge and Rist (30). Strictly speaking, the Amadori rearrangement involves the isomerization of an

N-substituted glycosylamine to a 1-amino-1-deoxy-2-ketose. The most widely accepted mechanism is the one put forth by Weygand (31), and elaborated by Isbell and Frush (32).

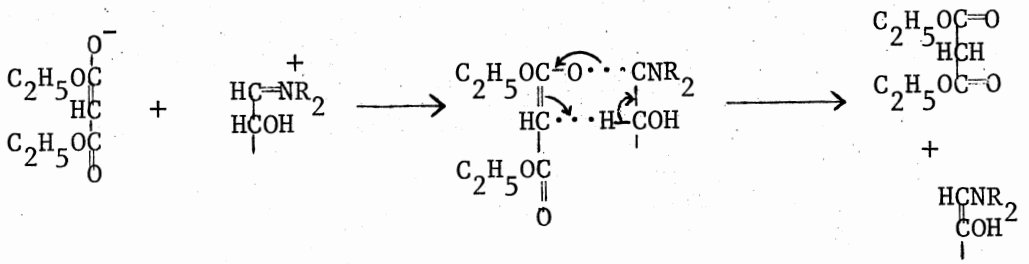
Weygand's model involved the addition of a proton from an acid catalyst, to the nitrogen atom of the glycosylamine, with subsequent formation of an open chair immonium salt (31). This was converted into the 1,2-enolic amine as a result of the abstraction of the hydrogen from the adjacent carbon atom number two. Deenolization then resulted in the formation of the two aldosylamine epimers and the 1-amino-1-deoxy-2-ketose (31).



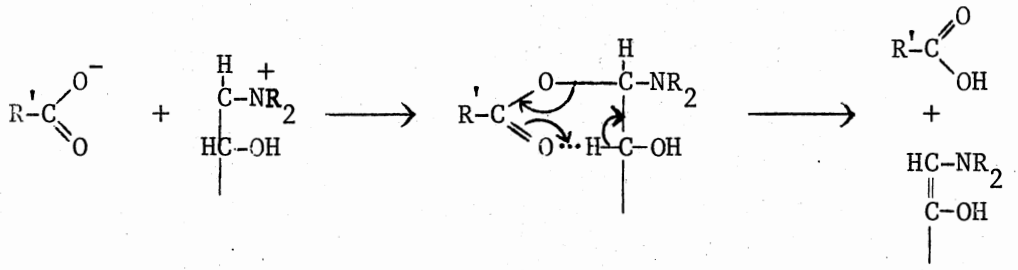
Although, they recognized the presence of the immonium ion in the rearrangement mechanism, Isbell and Frush (32) postulated that its existence was predicated by the action of acid catalyst on the ring oxygen atom, instead of on the nitrogen atom. The formation of the enol, and subsequent formation of the 1-amino-1-deoxyketose, involved the same mechanistic route expressed by Weygand.



Isbell and Frush went on to explain the reported catalytic effects of active methylene groups (30)(33) and carboxylic acids (34) on the production of Amadori compounds. They suggested that the rearrangements occurred, in the case of the methylene groups, through the addition of the enolic form of the methylenic compound to the immonium ion. The intermediate thus formed would then break apart to produce the keto form of the methylene compound and the enol form of the glycosylamine (32).



Carboxylic acids exerted their catalytic effect by condensing with the immonium ion, and then breaking away to leave behind the enolic glycosylamine.

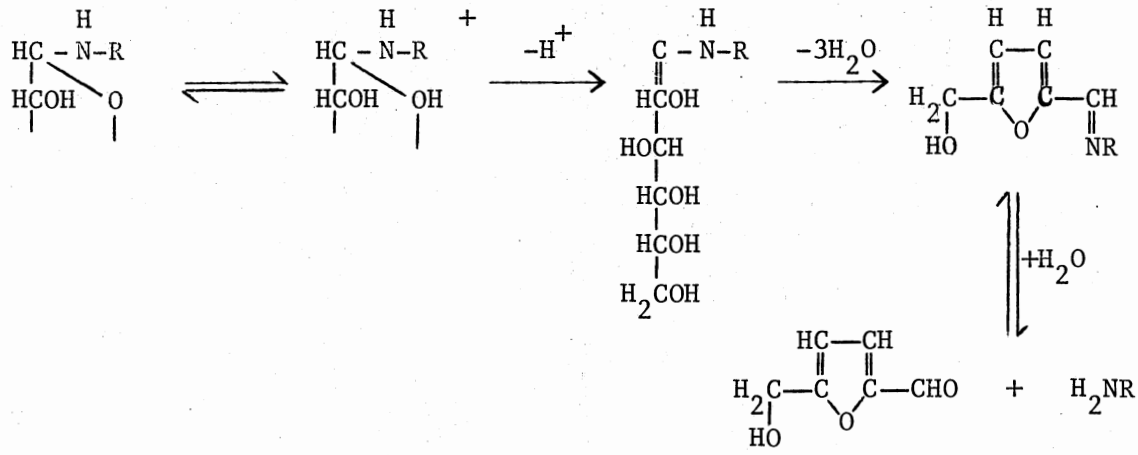


The formation of the Amadori rearrangement product represents a very critical linkage within the overall browning reaction. It is at this point that one or more of three major reactions occur. The active 1,2-enol of the Amadori rearrangement product may be subjected to dehydration to form furfurals and reductones. It may also undergo fission or fragmentation. Finally, it may participate in a Strecker degradation in which the amino acid moeity is converted, via a transamination type reaction, to an aldehyde of one carbon less than the amino acid. A close review of these three reactions is essential in appreciating the final phase of the Maillard reaction.

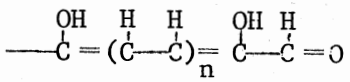
Dehydration

In 1952, Gottschalk clearly demonstrated that in mild acid media, the rearranged product of glucose and phenylalanine would go on to the Schiff's base of 5-hydroxymethylfurfural (35) (29). This occurred with the synchronous loss of three molecules of water, and was followed by

hydrolysis to form 5-hydroxymethylfurfural and the free amino acid. The conversion from the salt of the glycosylamine to the Schiff's base of the furfural was facilitated by the basic group of the amino acid.

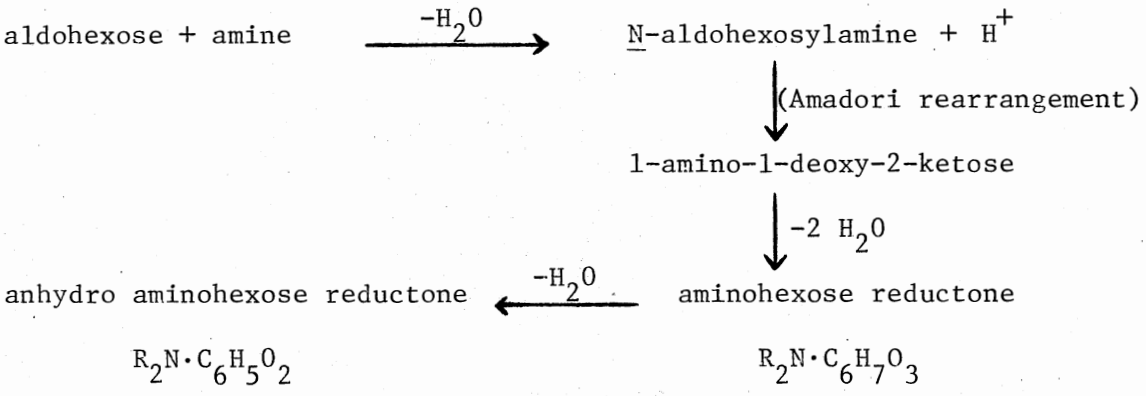


Besides undergoing dehydration to form furfurals, the sugar moeity of the 1-deoxyketosamine may also experience dehydration to form another class of compounds known as reductones. Reductones are resonance stabilized enediols that exhibit strong reducing activity in acid, base, or neutral media.



n=0,1

Hodge and Rist reported the production of an aminohexose reductone resulting from the dehydration of 1-amino-1-deoxy-2-ketose in the presence of an amino compound (33). Although they couldn't specify a structure, they did speculate that it could be a conjugated dienediol. Hodge later suggested that it might possess the structure of a furfural without ring closure, such as was known to occur with aromatic amines in the Stenhouse reaction (18). In 1956, Hodge and Fisher extended the reaction by isolating an anhydro aminohexose reductone (36). Unlike the colorless reductone precursor, this was bright yellow and formed a deep red product upon dehydrogenation. They proposed that the following sequence of reactions occurred in order to produce the anhydro reductone.

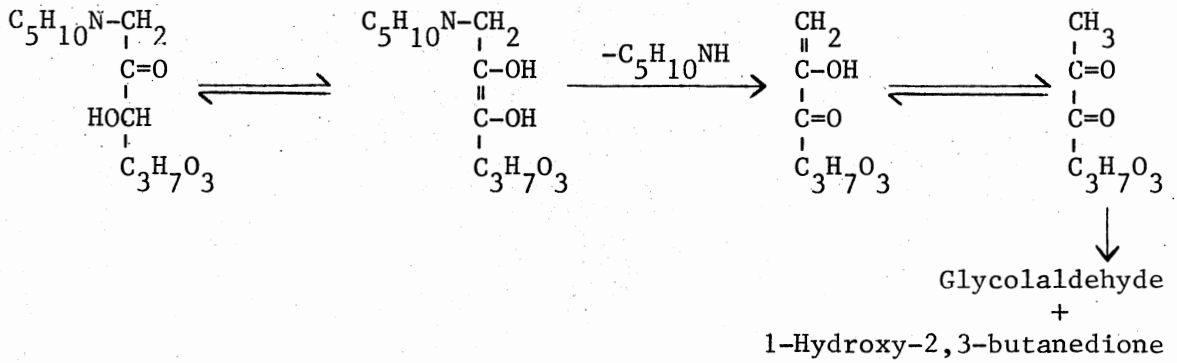


Both furfurals and reductones have been implicated as precursors in the production of brown products (37)(38)(39). However, before discussing the possible role that they may play in the browning reaction, two other major reactions of the 1-amino-1-deoxy-2-ketose still need to be reviewed.

Fragmentation

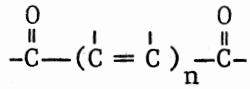
In neutral and acidic media, sugars have been reported to fragment

into compounds such as acetol, pyruvic acid, pyruvaldehyde, and diacetyl (40). Speck isolated these plus several other periodate-reducing compounds from a solution of sugars and amino acids (41). According to Hodge (18), the accepted mechanism for sugar fragmentation generally involves an amine catalyzed dealdolization centering around the weakened C-C bond α,β to the carbonyl group of the deoxyketose. In some cases, however, the amino group of the deoxyketose is apparently spontaneously discarded prior to fragmentation, and then acts as a catalyst for the sugar fission (42). The decomposition and resultant fragmentation of 1-deoxy-1-piperidinofructose could, therefore, conceivably follow this mechanism.



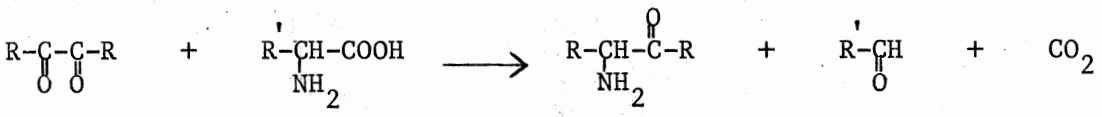
Strecker Degradation

Besides spontaneously breaking away from the deoxyketose, the amino moeity can also participate in a deamination type reaction known as the Strecker degradation (43). The reaction itself involves the decarboxylation and concurrent deamination and conversion to aldehyde of the amino acid by a dicarbonyl compound represented by the following general structure.



n = 0,1,2,...

Many of the compounds such as dehydrogenated reductones, produced from the 1-deoxyketose could easily participate in the Strecker degradation. It is not difficult to see that the evolution of CO_2, which is a common characteristic of the Maillard reaction, can easily be accounted for by this degradation.

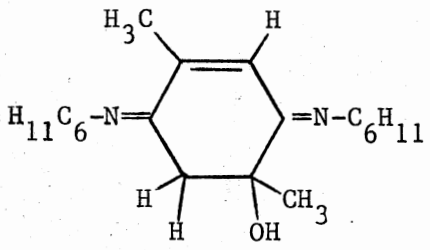


In fact, Wolfram et al. found that the carboxyl labeled amino acid was the source for nearly 100% of the CO_2 produced from the reaction between glucose and glycine (44). Although the Strecker degradation has unequivocally been implicated in the Maillard reaction, it must be pointed out that other evidence seems to support the suggestion that its presence is marginal and is not necessary for browning to occur (45)(46)(18). Whether or not this is true for the production of antioxidant is unknown at this time.

Final Stage

The final stage of the Maillard reaction, and the one in which the presence of brown coloration finally emerges, is still not understood. Through a series of aldol condensations, aldehyde-amine polymerizations, and formation of heterocyclic nitrogen compounds, the production of

brown nitrogenous polymers called melanoidins comes to completion. Very little can be said about the structure of these polymers or about the structure of the noncolored fluorescent precursors immediately preceding them. Carson provided evidence supporting the presence of aldol condensations, by isolating an intermediate in the melanoidin-producing reaction between diacetyl and cyclohexylamine (47)(48). Upon exposure to moist air at 25°C, the purified compound with the proposed structure represented below decomposed with coloring.

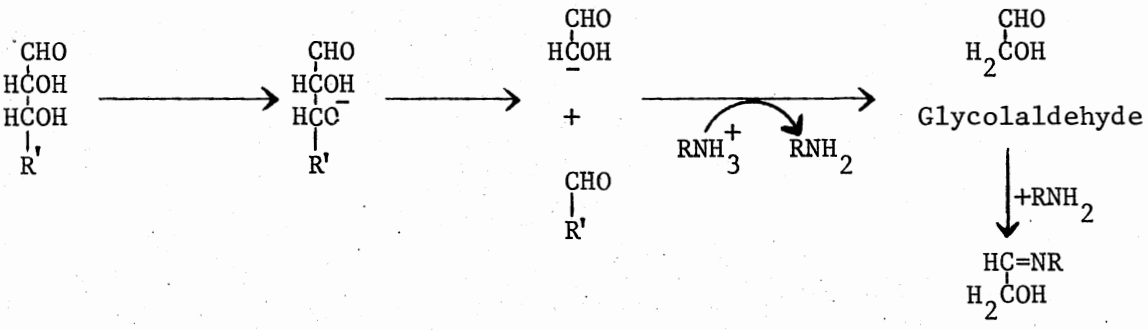


One compound isolated from this decomposition was N, N'-dicyclohexyl-2,5-dimethyl-p-benzoquinonimine, thus implying that quinones and quinonimines could also play a role in melanoidin formation. Aldehydes and amines are also known to react at low temperatures to form colored polymers (49), and aldehydes react with aldimines to form pyridines similar to those which have been distilled from melanoidins (50). Since aldehydes, aldimines, and amines are present prior to the formation of melanoidins, their probable contribution cannot be overlooked.

Alternate Pathways

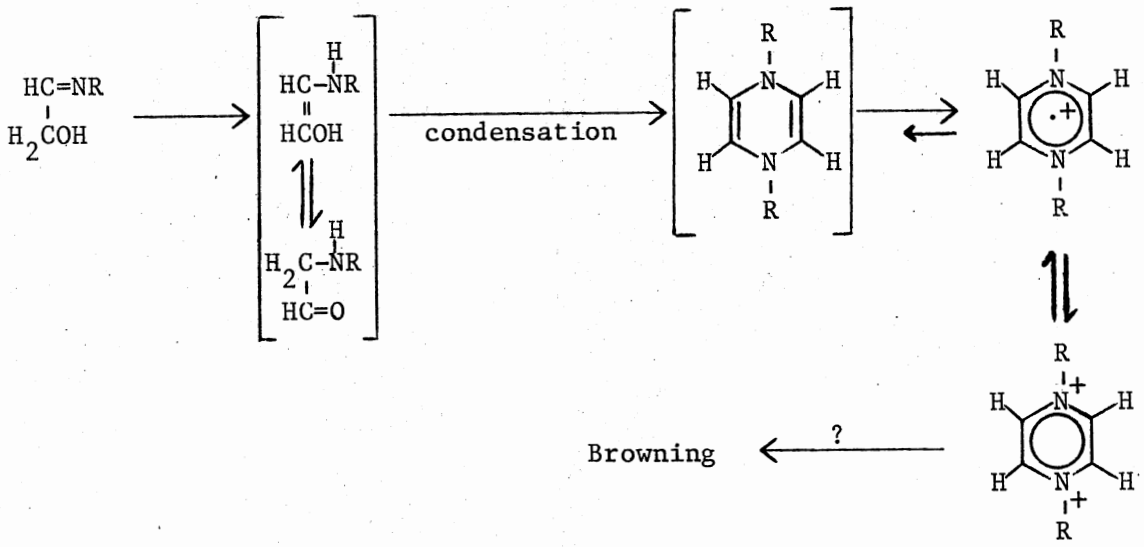
The preceding discussion has focused on what is believed to be the

major mechanism of the Maillard reaction. It should not be construed, however, that no other pathways exist. Recently, Namiki and Hayashi (51) have presented evidence which indeed suggests that an alternate pathway might also be involved in the very early stages of the Maillard reaction. Based on electron spin resonance studies and the identification of an N,N'-dialkylpyrazine radical isolated from the initial phase of the reaction, they have proposed that prior to condensation and Schiff's base formation, the sugar first undergoes fragmentation to produce glycolaldehyde. This then condenses with the amino compound to form the Schiff's base, glycolaldehyde-alkylimine.



The enaminol produced from the Amadori type rearrangement of the glycolaldehyde-alkylimine condenses to form an N,N'-dialkyldihydropyrazine.

The unstable N,N'-dialkyldihydropyrazine then is oxidized to form an N,N'-dialkylpyrazine salt, via the dialkylpyrazine radical (52).



Although the evidence is strong that the pyrazine salt contributes to the overall browning, the significance of its contribution and the mechanism by which it does so have not been determined.

Very little else can be said about the mechanisms of the Maillard reaction except that the amounts and kinds of products finally produced depend entirely on the various parameters and conditions associated with the reaction. An attempt to examine all of the parameters influencing the Maillard reaction would extend beyond the scope of this review. However, those conditions which are pertinent to the thrust of this investigation certainly merit further discussion.

Conditions

Type of Reactants

The particular type of sugar and amino acid present in the reaction medium will influence the rate and extent of browning. Although they do vary significantly depending on the other reaction conditions, a few generalities concerning the reactivity of the sugar and the amino acid have been established.

Pentoses are more reactive than hexoses when reacted with amino acids at 95^{o}C, and ribose, arabinose and xylose are especially effective in producing brown coloration (53)(54). Sugars without a reducing group, however, fail to produce any noticeable browning (55).

From several amino acids, glycine, aspartic acid, and arginine are excellent promoters of browning when reacted with furfural. Of these, arginine is the most effective (56). Likewise, arginine seems to produce the most intense color of several amino acids when reacted with glucose or with xylose (57).

In general, the sugar has a more pronounced effect on the rate of browning than does the amino acid. However, the amino acid seems to exert more influence on the extent of browning (58). In fact, although changes in concentration can greatly alter the relative outcomes, there appears to be a strong correlation between the basic strength of the amino compound and the extent of browning resulting from its reacting with sugar (59).

Concentration

The total sugar and amino acid concentration influence the rate of browning in a complex manner. High concentrations in the range of 70-80% (w/v of liquid solvent) favor the formation of ketosamines; the converse is true for the decomposition and subsequent polymerization of the ketosamines (44) (60). Optimization, therefore, involves an attempt to locate a concentration which compromises the need for an initial high concentration and a final low concentration. This balance, of course, will vary from one reaction system to the next. For instance, with a 5:1 mixture of glycine and xylose, respectively, Wolfrom found that the maximum amount of browning is obtained with a 70% w/v solution (61). On the other hand, Kiely et al. (62) found that with 1:1 mixtures of twenty amino acids and eight different sugars, an 85% w/v solution yielded the maximum rate of browning. This strongly suggests that the optimum concentration of the reactants needs to be fairly high in order to arrive at a rapid rate of browning. However, yields of up to 40% of brown pigments have been isolated from sugar-amino acid solutions with concentrations as low as 10% w/v (44).

Time

Generally, the intensity of browning is proportional to the square of the time (23). However, as usual, for every rule there is at least one exception. Lingnert et al. (11) found that a glucose and histidine system exhibited maximum browning ($A_{450\ nm}$) after twenty hours. By the time the reaction had progressed one hundred hours, though, the $A_{450\ nm}$ had diminished to one-sixth of the maximum.

Temperature

The rate of browning increases with increasing temperature. Although browning continues to be enhanced by temperatures above 100°C, some of this browning could be a result, not of the Maillard reaction, but of the thermal decomposition of carbohydrates, since this is known to occur at temperatures as low as 100°C (63). Also, the concentration of the solution will influence the effect of temperature on the rate. Reactions in dilute and highly concentrated solutions are characterized by a higher activation energy than are the concentrated solutions (64).

Pressure

Although it is known that pyrolysis of carbohydrates will proceed more rapidly under reduced pressure (65), it is not known what the effects of increased pressure will be on the rate and the extent of the Maillard reaction. It has been established, however, that brown polymers isolated from the reaction and subsequently subjected to hydrogen at 200 atm., will break down to form nonpigmented low molecular weight compounds, some of which are starting material (66). The implication of this is that under high pressure in a hydrogen environment, the Maillard reaction will be inhibited. The effect of moderate pressure in oxygen or nitrogen environments apparently has not been fully explored.

Oxygen

Reports on the influence of oxygen on the browning of sugar-amine systems are, at first glance, inconsistent and difficult to interpret.

Some say that oxygen enhances browning (67)(68), others say that it inhibits browning (69), and still others claim that the presence of oxygen has no effect either way on the rate of browning (70). Apparently, all three cases are true, and the particular influence demonstrated by oxygen depends on the pH, the concentrations of reactants, and the specific sugar and amine employed.

pH

A common characteristic of the Maillard reaction is the rapid drop in pH as the browning commences. The rate of browning subsequently decreases at the same time that the pH decline reaches a plateau. This implies that the initial pH and the pH of the reaction as it progresses, could also be influential factors in relation to the rate and extent of browning. In fact, this is true. The rate and extent of browning increases as the initial pH of the reaction is increased and subsequently maintained by a buffer. Although browning can occur even at a pH as low as 0.65, significant browning only seems to occur at a pH of 4.0 or above (71). Also, the optimum pH will vary from one sugar-amine system to another. For example, the glucose-glycine reaction yields maximum browning at a pH of 8.0. On the other hand, several arginine, lysine, and histidine sugar combinations yield maximum browning at a pH of 4.0 (62).

Buffers

In order to maintain a fairly constant pH, acetate or phosphate buffers are commonly employed in Maillard reactions, even though no

work appears to have been conducted to ascertain what would constitute an optimum buffer and buffer concentration. However, there is evidence which indicates that the presence of a buffer does definitely enhance the production of browning products (72). Whether or not a phosphate buffer is any better than an acetate or a tris buffer is questionable, although there is evidence to suggest that the presence of phosphate accelerates the browning reaction (72). Whether or not this is due to its buffering effect or to some other effect is not clear.

Miscellaneous

Hodge (33) found that in order to obtain maximum yields of the ketose derivative, 20%-50% by volume of active methylene compounds needed to be used. It is interesting to note that the rearranged product of N-D-glucosyl-p-toluidine, though, was not obtained in an ethanol-ethylmalonate solution until a small amount of pipiridine was added, thus implying that other base catalysts might equally be effective in promoting the reaction.

Although neutral salts do not appear to affect the composition of pyrolysis products of a number of mono, oligo, and polysaccharides (73), Smith et al. (74) reported that calcium chloride inhibited browning and was especially effective when combined with sulfite.

Certain metals also influence the browning reaction. Fe^{+2} has been reported to accelerate browning (67), Mn^{+2} inhibit it (69), and Cu^{+2} has been reported to do both (68) (75).

Antioxidative Products

Originally, the antioxidative activity generated by some Maillard reaction systems was thought to be associated with reductones produced in the reactions (13) (76). Later, however, Kirigaya and his colleagues (77) found that, although a xylose-glycine system produced antioxidative activity, none of the activity was associated with the reductones that were formed. As a compliment to this finding, Yamaguchi (78) reported that the antioxidative activity of the xylose-glycine reaction was confined primarily to a 4500 molecular weight fraction. Since then, the association of high antioxidative activity with the presence of high molecular weight compounds generated in the Maillard reaction has been confirmed (79) (80).

In the same way that a specific set of conditions is required for producing the maximum amount of browning in the Maillard reaction, an optimum combination of conditions is also required for obtaining the maximum level of antioxidative activity. The most extensive attempt at discerning what factors influence the rate and extent of antioxidant production was conducted by Lingnert and Eriksson (11). They found that only certain combinations of sugars and amino acids would generate appreciable amounts of antioxidant. Histidine, arginine, and lysine all produced high amounts of antioxidant when reacted with xylose. Histidine and lysine also produced high levels of antioxidant when they were reacted with glucose or fructose.

The histidine-glucose reaction was further investigated by Lingnert and Eriksson (11). They not only looked at the effect of total sugar-amino acid concentration, but also at the effect of various molar combinations

of the sugar and the amino acid on the extent of antioxidant production. The results are displayed in Table I, and show that essentially, the amount of antioxidant produced increases as the total concentration increases.

They also looked at the effect of different initial pH's on the outcome of the reaction. Table II summarizes the result of that study, and indicates that although a pH between 5.0 and 9.0 will yield appreciable results, the yield is not much greater than that obtained at a pH of 3.0. The high alkaline reaction, however, apparently is not conducive to obtaining maximum yields.

Prior to this present investigation, the only work conducted on the arginine-xylose system was that of Foster's (80). He found that unlike the histidine-glucose reaction which produced a maximum amount of antioxidant at twenty hours, the arginine-xylose system produced a maximum amount of antioxidant by three hours. The generation of antioxidant paralleled the decline in pH and the increase in ($A_{450\ nm}$). The reaction itself was carried out using a 2:1 ratio of arginine (2.0 M) to xylose (1.0 M), buffered with 0.1 M phosphate (pH 7.2) adjusted to pH 8.0 with potassium hydroxide. No other reaction parameters were investigated in Foster's study. In fact, the effects of pH, the presence or absence of buffer, pressure, 0_2, N_2, and the addition of various organic additives on the rate and extent of antioxidant produced in the arginine xylose reaction, apparently, have not been investigated until now.

TABLE I

THE INFLUENCE OF HISTIDINE AND GLUCOSE CONCENTRATION ON FINAL pH, COLOR ($A_{450\ nm}$) AND ANTIOXIDATIVE EFFECT OF MAILLARD REACTION PRODUCTS FROM HISTIDINE-GLUCOSE

Reaction Mixture[a]				
Histidine (mmol)	Glucose (mmol)	Final pH	$A_{450\ nm}$	Antioxidative Effect[b]
0.0	5.0	5.9	8	0.0
5.0	0.0	7.0	0	0.0
2.5	5.0	5.2	158	1.0
5.0	2.5	6.4	106	1.1
5.0	5.0	5.8	165	1.6
5.0	10.0	4.8	324	1.9
10.0	5.0	6.5	161	2.4

[a]The amounts of histidine and glucose indicated were refluxed in 5 ml 0.1 M potassium phosphate buffer, pH 7.0, for 5 h.

[b]The antioxidative effect was measured by the polarographic method.

TABLE II

INFLUENCE OF INITIAL pH ON COLOR (A_{450}) AND ANTIOXIDATIVE EFFECT OF MAILLARD REACTION PRODUCTS FROM HISTIDINE-GLUCOSE

Initial pH	Final pH	$A_{450\ nm}$	Antioxidative Effect
3.0	2.8	174	1.7
5.0	3.0	285	1.9
7.0	5.0	279	2.0
9.0	5.5	248	2.0
11.0	7.3	122	1.2

[a]The antioxidative effect was measured by the oxygen consumption method.

CHAPTER III

MATERIALS AND METHODS

Unless stated otherwise all reactions were carried out in 250 ml round bottom flasks containing 0.1 mole of L-arginine hydrochloride (Sigma grade, Sigma Chemical Co., St. Louis, Mo.) and 0.05 mole of D(+)xylose (grade II Sigma Chemical Co.) in 50 ml of water. All resultant samples were stored at -18^{o}C until the antioxidative activity was to be assayed for.

Tris Buffer

A 1.0 M solution of Tris-(hydroxymethyl)-amino methane (Sigma) was adjusted to a pH of either 7.0 or 8.0 with potassium hydroxide and conc. HCl.

Organic Additives

Pyridine (Reagent grade ACS) was obtained from Eastman Kodak (Rochester, N.Y.). Geraniol was supplied by Chemicals Procurement Laboratories (College Point, N.Y.) and nonanol was purchased from K & K Laboratories (Jamaica, N.Y.).

Pressure Reactions

The Parr Pressure Reaction Apparatus (Parr Instrument Co., Moline, Illinois) was used for all pressure and gas studies. A specially

designed teflon stopper was used in place of the usual rubber stopper so that the commercial antioxidants impregnated in the rubber would not contaminate the reaction mixture. Standard 99.5% oxygen, Lamp grade nitrogen, and Type-1 grade-E breathing-air were obtained from Sooner Supplies (Shawnee, Okla.).

Assays

Both the micro (spectrophotometric) and the macro (oxygen electrode) assays developed by Lingnert et al. (12) were utilized to monitor levels of antioxidative activity.

In the micro assay, a linoleic acid emulsion was made by slowly introducing 0.5 ml of linoleic acid (99% Nu Chek Prep. Inc. Elysian, Minn.) to a solution of 10 ml of 1.0 M K_2HPO_4 and 0.5 ml of Tween 20 (Sigma Chemical Company), and to this was added two or three pellets of potassium hydroxide followed by 15 ml of 1.0 M K_2HPO_4 and 225 ml of triple distilled water. The pH was then adjusted to 6.5 with conc. HCl. Two milliliters of the emulsion were placed in a test tube along with 10 µl of a sample to be assayed, and from this 200 µl were withdrawn and added to a test tube to which was added 2 ml of 100% methanol and 6 ml of 60% methanol. The remainder of the 2 ml of emulsion and sample mixture were then placed in a 37°C water bath and incubated 15 to 20 hours. Meanwhile, the absorbance of the methanol solution to which the 200 µl of sample had been added, was measured at 234 nm. After incubating for 15 to 20 hours, 200 µl of the linoleic acid and sample mixture were also measured for absorbance at 234 nm in the same manner that the nonincubated mixture had been. Antioxidative activity (A.O.A.)

was calculated using the equation,

$$\text{A.O.A.} = \frac{\Delta A_{234_c} - \Delta A_{234_s}}{\Delta A_{234_c}}$$

where ΔA_{234_c} is the difference in absorbance between a preincubated and an incubated control, and ΔA_{234_s} is the analogous change in absorbance of the sample.

With the macro method an emulsion of linoleic acid was prepared using the same procedure as described above. A hemin solution was also prepared by dissolving 5 μmole of bovine hemin (Sigma Chemical Company) in 500 ml of a one-to-one mixture of 0.02 M potassium phosphate buffer (pH 7.0) and 95% ethanol.

The assay was carried out by adding 4 ml of the linoleic acid emulsion to a test tube containing 100-200 μl of an appropriate dilution of the sample to be assayed. This was pipeted into a specially built oxygen consumption chamber, and 0.2 ml of the hemin catalyst was then added. Oxygen consumption, which is indicative of oxidation of the linoleic acid, was followed using a VSI Model 53 Oxygen Monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio) and was simultaneously recorded on chart paper. The length of time required to consume 50% of the oxygen originally contained in the solution was utilized in determining the activity by means of the following formula:

$$\text{A.O.A.} = \frac{t_s - t_\ell}{t_\ell}$$

where t_s is the time required for 50% of the oxygen to be consumed in the sample being assayed and t_ℓ is the time required for 50% of the

oxygen to be consumed in the linoleic acid emulsion to which no sample material had been added.

CHAPTER IV

RESULTS AND DISCUSSION

Influence of Time

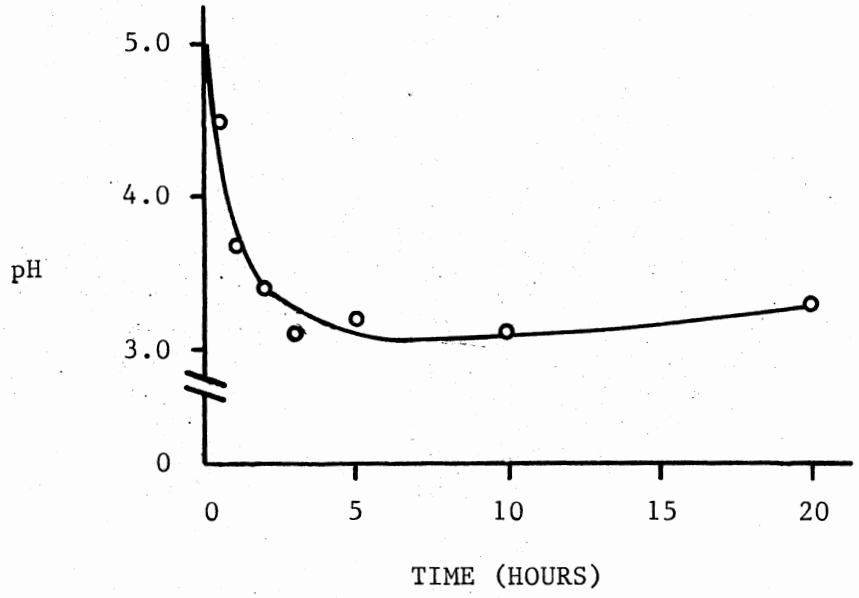
When the arginine and xylose were combined with 50 ml of water, the change in pH and antioxidative activity with time were monitored by either the micro assay or by the macro assay using appropriate dilutions of the crude sample. Figure 3 shows that the pH was at a minimum value between two and five hours. Indication of the time required to produce maximum antioxidative activity varied depending on which assay was used to monitor it. The micro assay revealed that maximum activity was encountered by ten hours and that the rate of production began to decline after only five hours. With the macro assay however, the maximum activity wasn't obtained until twenty hours, even though the rate of production also began to decline after five hours, as with the micro method. This is shown in Figure 4. These results indicated that in a nonbuffered system, the optimum time lies somewhere between ten and twenty hours.

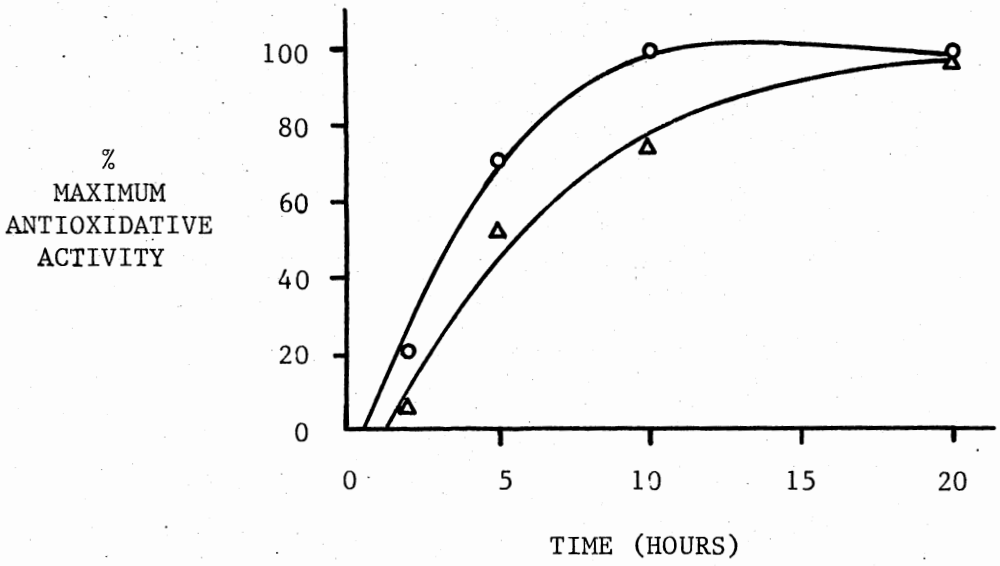
Effect of Tris Buffer

To determine if a buffer has any effect on the rate and extent of antioxidant production, three reactions were carried out. One reaction involved the combination of arginine and xylose in nonbuffered water, whereas the other two reactions involved the combination of arginine

Figure 3. Average change in pH with time of the nonbuffered arginine-xylose reaction.

Figure 4. Average change in % maximum antioxidative activity with time of the nonbuffered arginine-xylose reaction.
Legend: -o- micro assay; -Δ- macro assay.


5.0
4.0
3.0
0
pH
0
5
10
15
20
TIME (HOURS)


%
MAXIMUM
ANTIOXIDATIVE
ACTIVITY
100
80
60
40
20
0
0
5
10
15
20
TIME (HOURS)

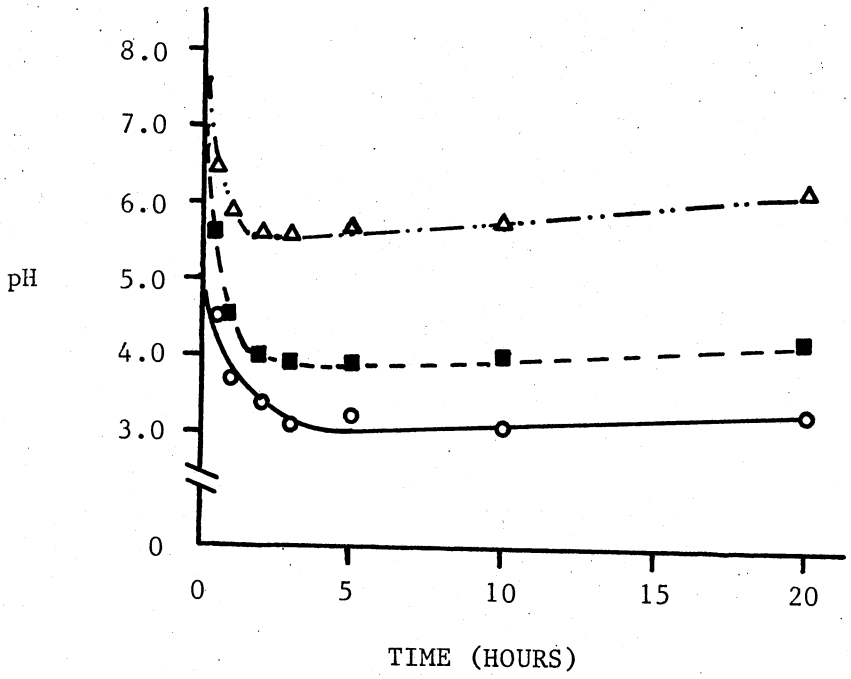
and xylose in either 1.0 M Tris pH 7.0 or 1.0 M Tris pH 8.0. Only the micro assay was utilized to monitor the progress of these reactions. Figure 5 indicates that the buffer had negligible impact on the maintainance of pH, and Figure 6 shows that although all three systems obtained maximum activity by ten hours, the Tris 8.0 reaction was characterized by a higher initial rate than the Tris 7.0 or the nonbuffered system. Optimum dilutions equivalent to 1 µl of crude sample were used for the assay of these reactions since it was determined that the assay was not sensitive enough to measure 0.1 µl equivalents and that 10 µl aliquots were too concentrated to reveal any differences in activity from one sample to the next.

Effect of Initial pH

In order to investigate what influence the initial pH has on the production of antioxidant, a preliminary study was initiated in which two reactions were carried out. Whereas the first reaction consisted of the usual nonbuffered arginine-xylose mixture, the second reaction consisted of the nonbuffered arginine-xylose mixture to which was added 3 ml of conc. HCl. Both systems were refluxed for twenty hours and 2 ml aliquots were withdrawn at various times so that the change in pH and antioxidative activity could be monitored. The change in pH with time can be seen in Figure 7 and as in the case of the previous nonbuffered and Tris 7.0 and Tris 8.0 buffered systems, the pH attained a minimum value by three hours. Figure 8 illustrates the change in antioxidative activity with time of the two reactions. It was decided to pursue further the effect of initial pH and so, the above procedure was repeated. However, this time, five separate systems were used instead of

Figure 5. Average change in pH with time of the nonbuffered, Tris 7.0, and Tris 8.0 arginine-xylose reactions.
Legend: —o— nonbuffered; —■— Tris 7.0; —△— Tris 8.0.

Figure 6. Change in % antioxidative activity with time of the non-buffered, Tris 7.0 and Tris 8.0 arginine-xylose reactions.
Legend: —o— nonbuffered; —■— Tris 7.0; —△— Tris 8.0.


pH
8.0
7.0
6.0
5.0
4.0
3.0
0
0
5
10
15
20
TIME (HOURS)

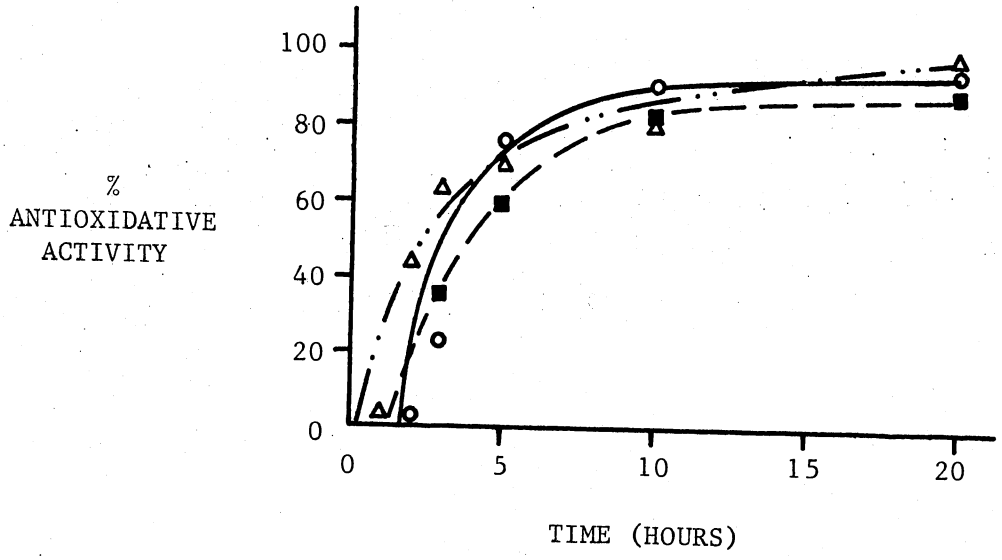
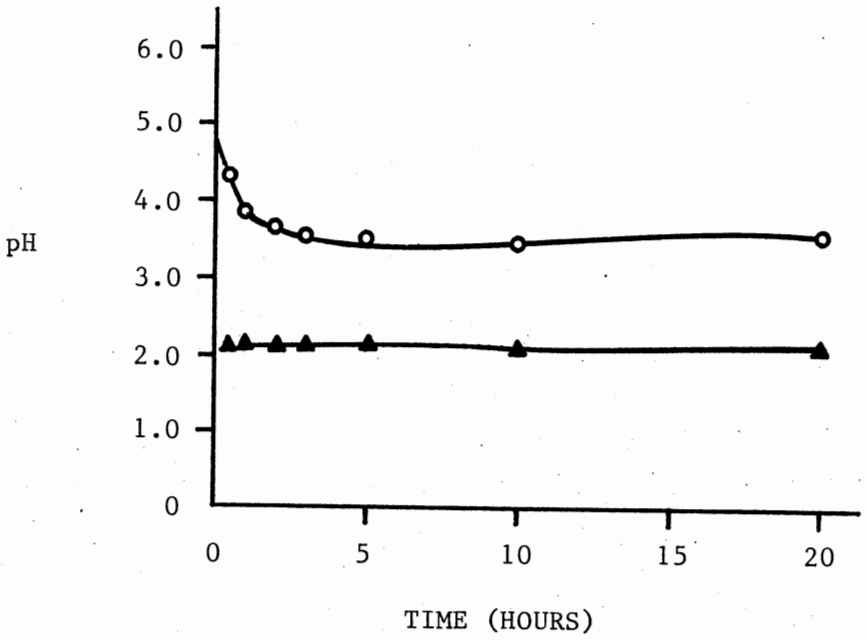
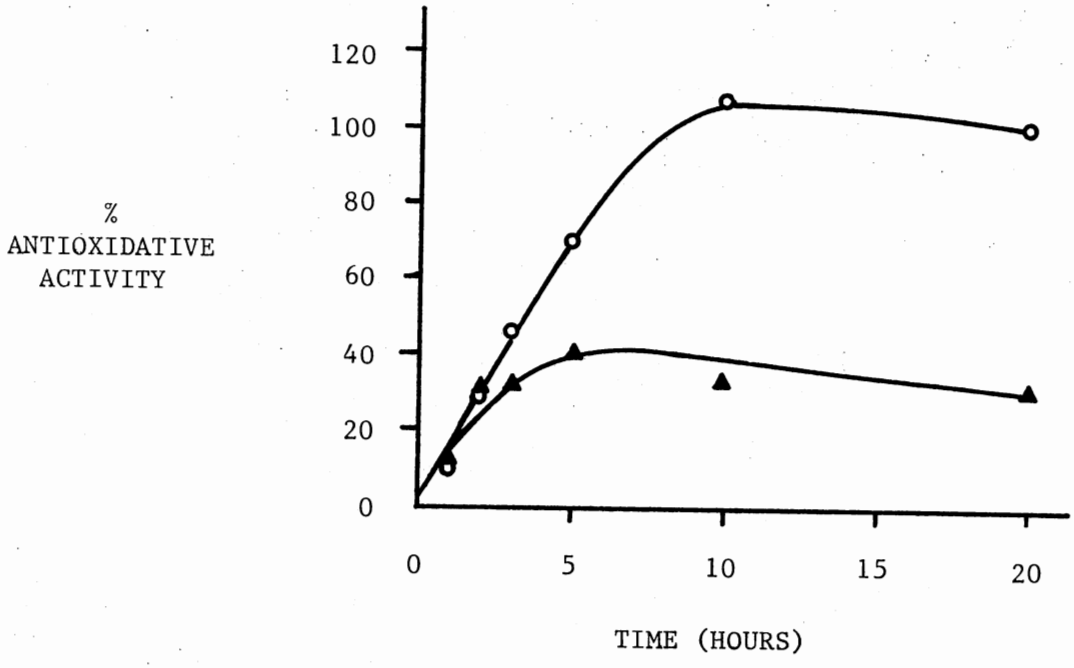

%
ANTIOXIDATIVE
ACTIVITY
100
80
60
40
20
0
0
5
10
15
20
TIME (HOURS)

Figure 7. Change in pH with time of two nonbuffered arginine-xylose systems possessing different initial pH values.

Figure 8. Change in % antioxidative activity with time of two nonbuffered arginine-xylose systems possessing different initial pH values. Legend: ▲ pH 2.0; ○ pH 5.0.


pH
6.0
5.0
4.0
3.0
2.0
1.0
0
0
5
10
15
20
TIME (HOURS)


%
ANTIOXIDATIVE
ACTIVITY
120
100
80
60
40
20
0
0
5
10
15
20
TIME (HOURS)

only two. This way the influence of a broader range of initial pH values could be studied. Figures 9 and 10 respectively depict the changes in pH with time and the level of antioxidant activity produced after 20 hours. Notice, that in all five reactions, the pH approached a minimum value sometime prior to five hours. The first study on the effect of initial pH revealed that by twenty hours the reaction possessing an initial pH of 5.0 produced over twice as much activity as the reaction possessing an initial pH of 2.0. In the extended investigation, the two reactions characterized by initial pH values of 5.0 and 7.0 generated the maximum amount of antioxidant activity. In both of these systems the pH reached a minimum value between 3.5 and 4.0 and remained fairly constant throughout the rest of the reaction.

Influence of Temperature

The nonbuffered arginine-xylose system was allowed to react at 4 different temperatures (4°, 25°, 37°, 100°C) over a time span of 50 hours. Aliquots were withdrawn at selected intervals so that the change in antioxidative activity could be followed. A fifth solution was stored at -18°C and was used as a control. From Figure 11 it can be seen that of the four reactions investigated, only the one subjected to 100°C generated measurable antioxidative activity.

Influence of Organic Additives

To determine if certain organic compounds could be introduced to the arginine-xylose reaction and enhance the production of antioxidative activity, mixtures were set up containing 0.1 mole of arginine,

Figure 9. Change in pH with time of five nonbuffered arginine-xylose systems possessing different initial pH values.

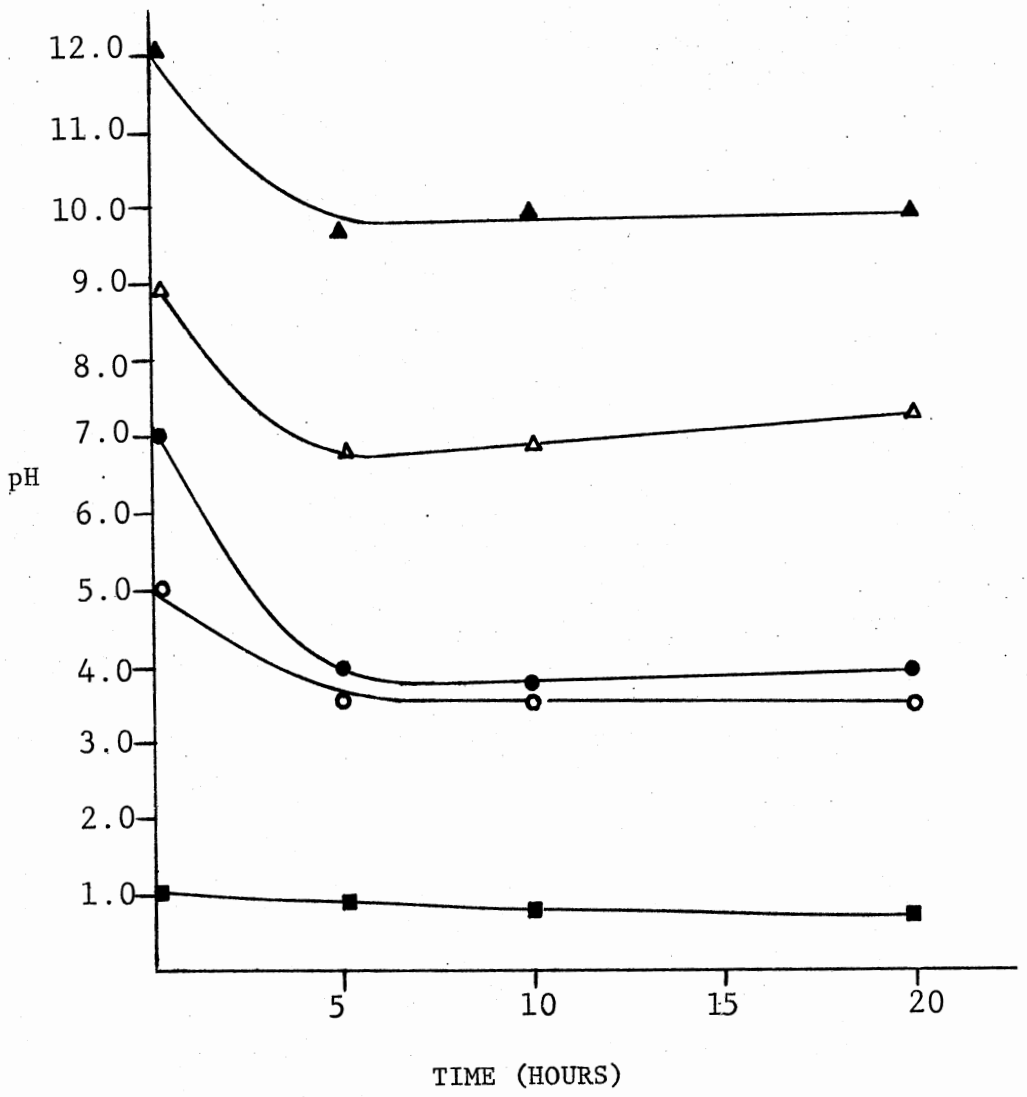

12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
pH
5
10
15
20
TIME (HOURS)

Figure 10. Antioxidative activity produced after twenty hours in five nonbuffered arginine-xylose systems possessing different initial pH values.

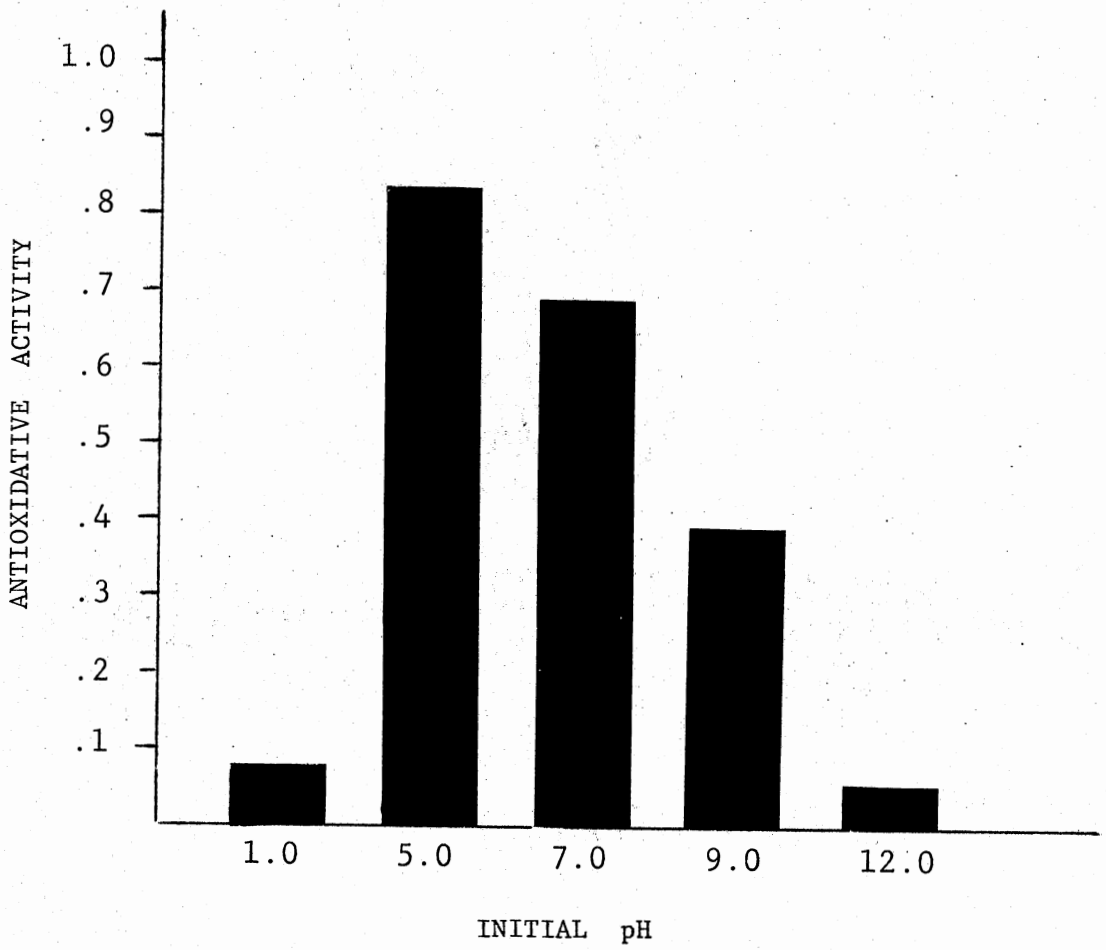
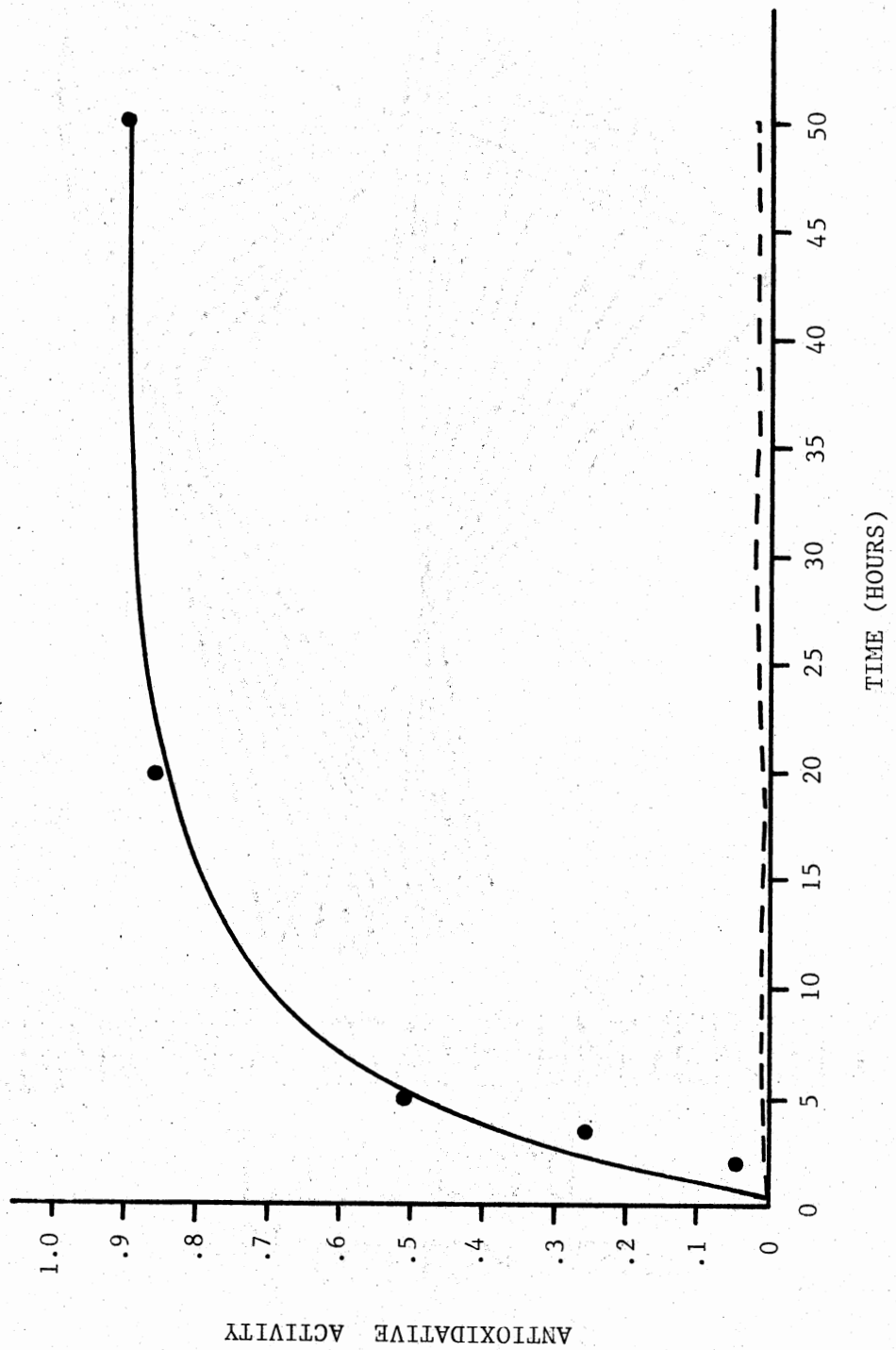

ANTIOXIDATIVE ACTIVITY
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
1.0
5.0
7.0
9.0
12.0
INITIAL pH

Figure 11. Temperature related changes in antioxidative activity with time of the arginine-xylose reaction.
Legend: ——— 100°C; ---- 37°C, 25°C, 4°C, -18°C.


ANTIOXIDATIVE ACTIVITY
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0
5
10
15
20
25
30
35
40
45
50
TIME (HOURS)

0.05 mole of xylose and one of the following: 50 ml ethanol, 50 ml acetone, 45 ml n-pentanol, 9 ml benzyl alcohol, 1 ml nonanol, 1 ml geraniol, 50 ml tetrahydrofuran (THF), 50 ml carbon tetrachloride (CCl_4), 50 ml pyridine. The volumes were then adjusted to 100 ml with water and the mixtures were then refluxed for five hours and assayed for antioxidative activity. An unbuffered arginine-xylose system was also refluxed and was used as a control each time the experiment was run. The THF, CCl_4 and pyridine reactions were conducted at a later time than the others and the antioxidative activity was determined using the macro assay. The micro assay was used to determine the activity of the remaining systems and as shown in Figure 12 only the benzyl alcohol mixture produced activity equivalent to the control. Figure 13 shows that both the THF and the CCl_4 mixtures generated less activity, but the pyridine mixture displayed a marked increase in activity compared to the control. It was first thought that since the crude reaction mixture assay contained free pyridine, perhaps the pyridine alone was contributing to the enhancement of activity. Therefore, 50 ml of pyridine were assayed for antioxidative activity but proved to be highly prooxidative. It seemed plausible that pyridine, perhaps by combining with other antioxidants generated in the reaction, might produce a superior antioxidative effect. An appropriate amount of pyridine therefore, was added to a control sample just prior to being assayed. Again, a prooxidative effect was exhibited. Finally, equal weights of lyophilized control and lyophilized arginine-xylose-pyridine reaction mixture were assayed separately and the pyridine system still displayed an antioxidative activity twice that of the control (it should be noted

Figure 12. Comparison of the effects of different organic compounds on the production of antioxidative activity in the arginine-xylose reaction.

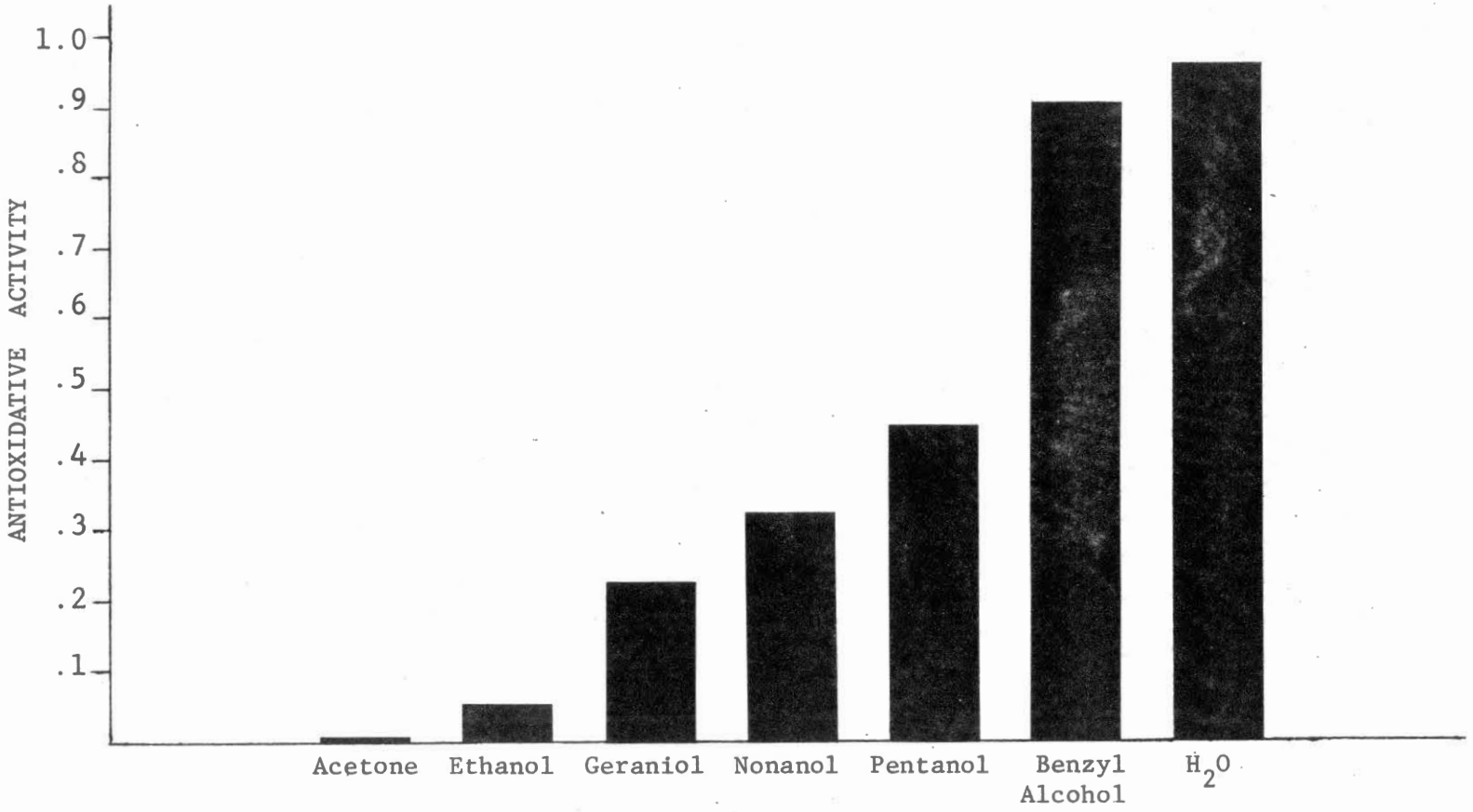
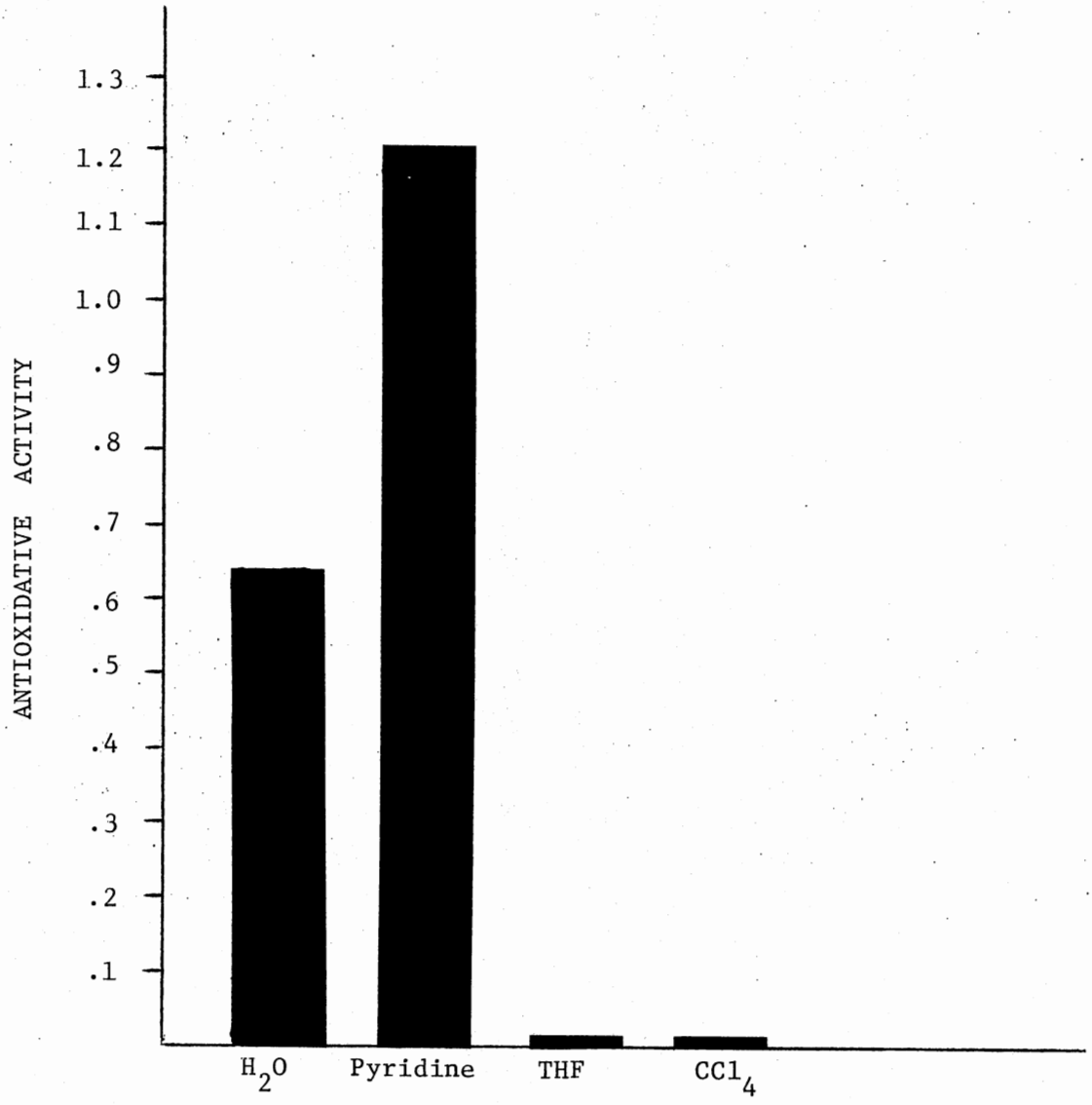

ANTIOXIDATIVE ACTIVITY
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Acetone
Ethanol
Geraniol
Nonanol
Pentanol
Benzyl Alcohol
H_2O

Figure 13. Comparison of the effects of pyridine, tetrahydrofuran and carbon tetrachloride on the production of antioxidative activity in the arginine-xylose reaction.


ANTIOXIDATIVE ACTIVITY
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
H_2O
Pyridine
THF
CCl_4

that actually, the pyridine mixture was washed with 1.0 N acetic acid, and lyophillized three times in order to remove the pyridine, prior to being weighed and assayed).

In order to obtain a more clear perspective on the difference between the pyridine reaction and the control reaction, both mixtures were refluxed for 70 hours, and aliquots were withdrawn at various times so that the change in activity could be monitored. The control consisted of 0.1 mole of arginine, and 0.05 mole of xylose, both of which were dissolved in 50 ml of water and the pyridine system contained the same amount of arginine and xylose as the control but contained 25 ml of pyridine and 25 ml of water. Figure 14 clearly shows that throughout the entire time, the pyridine system generated over double the activity of the control. The control reached a maximum at around 20 hours, but the pyridine system did not reach maximum activity until around 50 hours. Finally, Figure 15 shows that nearly twice as much control needed to be used in order to produce an antioxidative effect equivalent to that generated by the pyridine system. Thus, either pyridine may have served as a catalyst, or it may have been incorporated into new antioxidative compounds.

To gain an insight into the composition of the reaction products, a C, H, O, N analysis was performed by Galbraith Laboratories, Knoxville, Tennessee and the results indicated that the arginine-xylose reaction products (AX) contained 22.7% O, 20.7% N, 37.1% C, and 7.2% H, and the arginine-xylose-pyridine reaction products (AXP) contained 24.4% O, 17.7% N, 40.9% C, and 7.1% H. The differences were clearly evident in that the AXP contained more carbon and oxygen and less nitrogen and hydrogen than did the AX. Since it was likely that 95-97% of

Figure 14. Comparison of the change in antioxidative activity with time between the water and the pyridine-water systems. Legend: —o— water; —▲— pyridine-water.

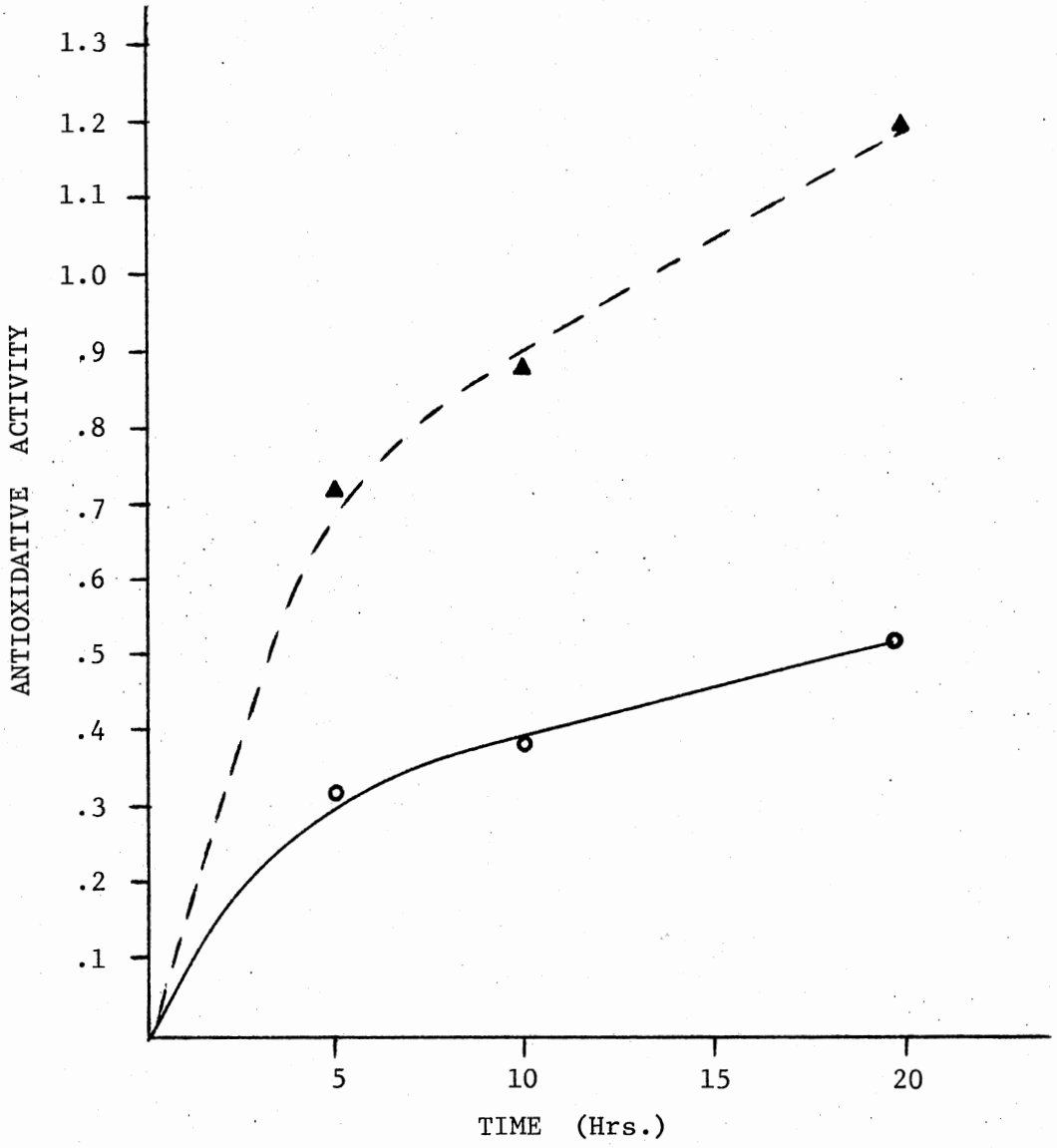
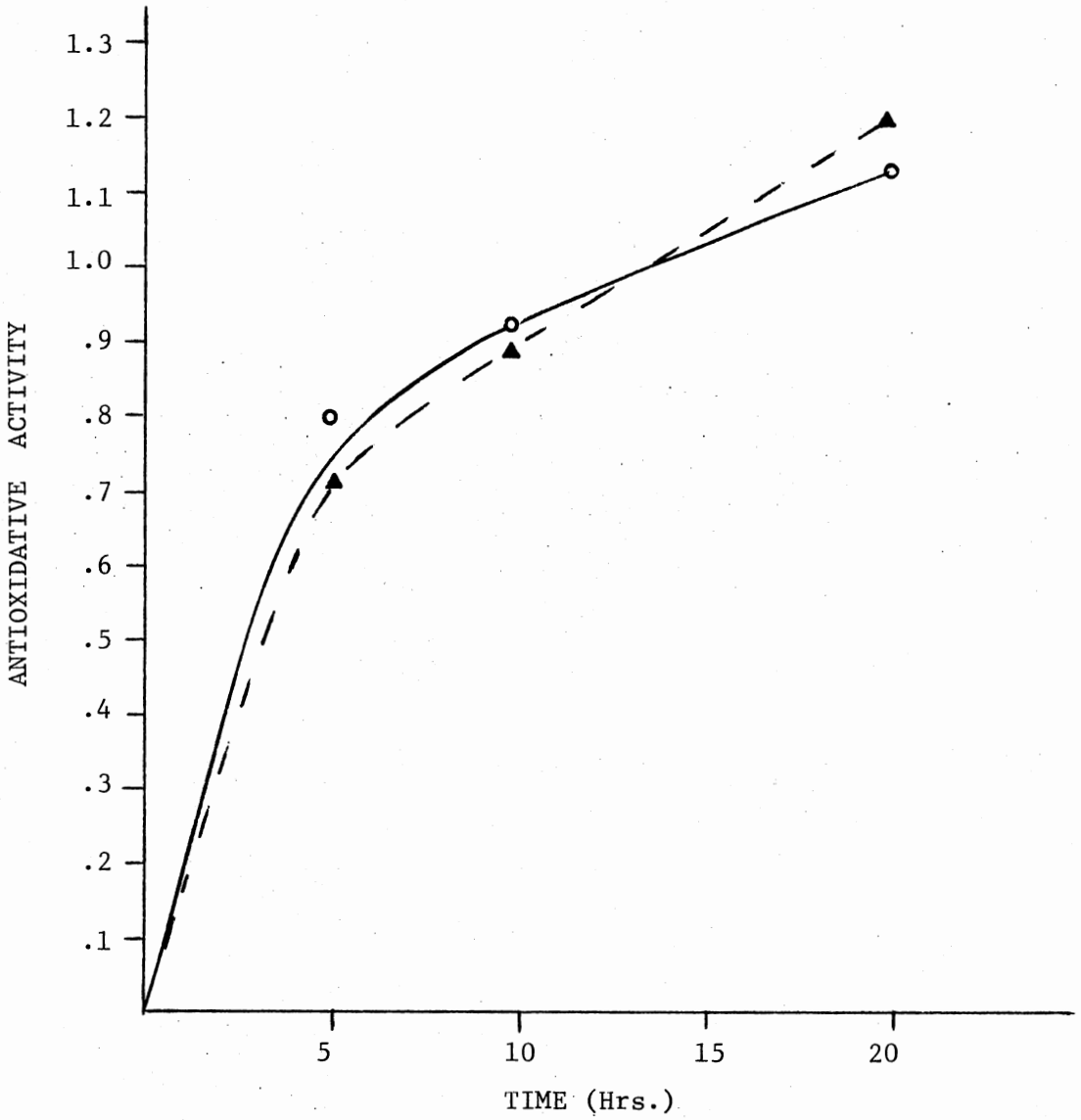

ANTIOXIDATIVE ACTIVITY
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
TIME (Hrs.)

Figure 15. Comparison of the change in antioxidative activity with time between 2 µl of the water and 1 µl of the pyridine-water systems.
Legend: —o— water; —▲— pyridine-water.


ANTIOXIDATIVE ACTIVITY
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
TIME (Hrs.)

the products were non-antioxidative (79), the 3-5% yield of the anti-oxidative activity normally present, might have been increased in the pyridine reaction which would be the situation if pyridine served as a catalyst. If the antioxidative compound present in the AX mixture contained a greater percentage of carbon and oxygen than nitrogen and hydrogen, then an increase in its yield would have been reflected by an increase in the carbon and oxygen content relative to nitrogen, which was the case when pyridine was used in the reaction. If, however, the pyridine had become a partner in the reaction and in doing so generated a new antioxidative compound, then it would have been less likely (though possible) that the new product contained a ratio of elements consistent with the analysis. The situation clearly indicated a need for research leading to the synthesis, purification, and identification of the antioxidant(s) produced in the reactions.

Effects of Oxygen, Nitrogen and Pressure

Thirty ml of nonbuffered arginine-xylose were allowed to react in a 50 ml Parr vessel for ten hours at a temperature of 100^{o}C either at one or five atmospheres of pressure. Prior to the commencement of the reaction, air, nitrogen, or oxygen was bubbled through the solution for several seconds. The control vessel was then fitted with a reflux column through which the appropriate gas was continuously introduced at 1 atm., whereas the pressurized vessel was brought to 5 atm. with the appropriate gas and shaken in the Parr apparatus for the length of the reaction. Aliquots were then assayed for antioxidative activity, and Table III and Figure 16 summarize those results. Because of the large variation in the measurement of activity, no definite conclusions could

be drawn from this study, except that it appeared that moderate pressure had little if any effect on the results of the arginine-xylose antioxidative reaction under an air environment.

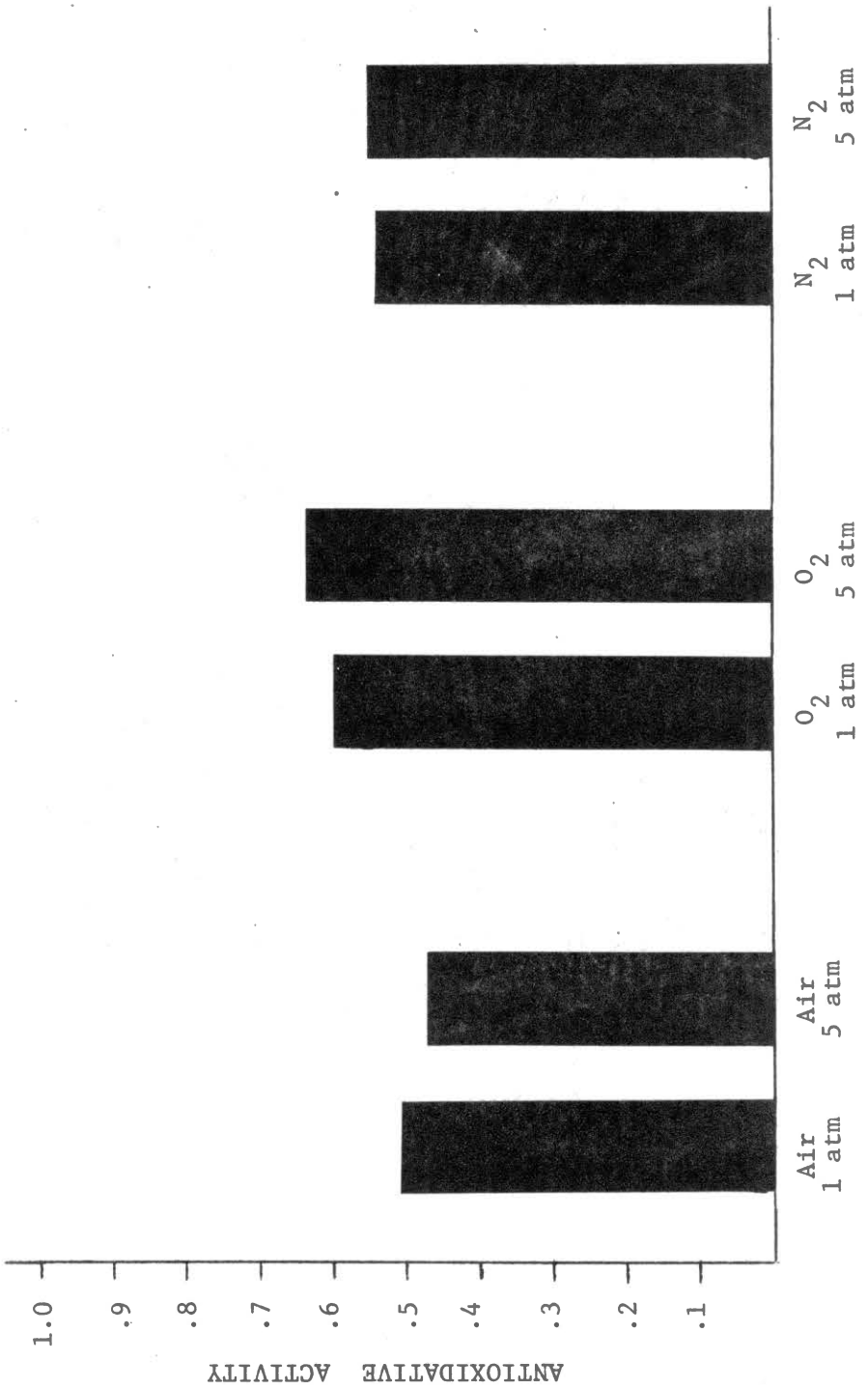
TABLE III

ANTIOXIDATIVE ACTIVITIES OF THE ARGININE-XYLOSE REACTION CONDUCTED IN O_2, N_2, AND AIR AT 1 ATM. AND 5 ATM.

	Antioxidative Activity*		
	Sample 1	Sample 2	$\overline{X}$
Air - 1.0 atm.	2.20	2.81	2.51
Air - 5.0 atm.	2.58	2.07	2.33
O_2 - 1.0 atm.	2.87	3.08	2.98
O_2 - 5.0 atm.	2.07	4.26	3.17
N_2 - 1.0 atm.	2.16	3.19	2.68
N_2 - 5.0 atm.	1.64	3.82	2.73

*Determined by the macro assay.

Figure 16. Effects of Oxygen, Nitogen, and air at 1 atm. and 5 atm. on the antioxidative activity of the arginine-xylose reaction.


ANTIOXIDATIVE ACTIVITY
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Air 1 atm
Air 5 atm
O_2 1 atm
O_2 5 atm
N_2 1 atm
N_2 5 atm

CHAPTER V

SUMMARY AND CONCLUSIONS

The few attempts to define the optimum conditions for generating antioxidative activity in the Maillard reaction have resulted in varying conclusions. Lingnert and Eriksson (11) found that maximum activity was obtained with the histidine-glucose reaction after a reflux period of twenty hours. Foster (80), however, reported that the arginine-xylose reaction yielded maximum activity after only three hours. Although Foster and Lingnert used a phosphate buffer in their reactions, they both reported that it exerted negligible influence on the maintainance of pH. Lingnert also reported that except for extreme acidic or basic conditions, the amount of activity produced from the reaction changed very little over a pH range between 3.0 and 9.0. Neither Lingnert nor Foster investigated the influence of pressure or the presence of organic compounds on the production of antioxidative activity.

The present investigation indicated that the arginine-xylose reaction yielded maximum antioxidative activity sometime between ten and twenty hours. The maximum time varied depending on whether the micro or the macro assay was employed. The micro assay generally revealed that maximum activity was obtained by ten hours, whereas the macro assay revealed that maximum activity was obtained by twenty hours. The difference in results from one assay system to the other may have been due to the fact that the two assays were measuring different stages of

autoxidation and consequently were reflecting either the presence of different mechanisms of antioxidative activity, or the presence of more than one antioxidant in the sample aliquot. More than likely, however, the difference was due to a greater sensitivity of the macro assay in measuring small changes in activity between a ten and a twenty hour reaction sample.

Although the presence of buffers seemed to accelerate the initial rate of the reaction, it exerted no influence on the amount of final activity produced. Unfortunately, only one concentration of Tris was used and it is quite conceivable that any enhancement of activity resulting from the buffering, could have been countered by a possible inhibitory action of the Tris.

In agreement with Lingnert and Eriksson (11), maximum antioxidative activity was obtained throughout a initial pH range between 5.0 and 9.0. Of the initial pH's studied, solutions with initial pH values of 5.0 and 7.0 resulted in the generation of maximum activity. By five hours both of these solutions reached a constant pH level between 3.5 and 4.0. Although the antioxidative activity continued to climb to a maximum for another five to fifteen hours, the rate at which it was produced began to decrease at the same time that the pH reached a minimum which was at five hours. Although, it is tempting, therefore, to conclude that the optimum pH lies somewhere between 5.0 and 3.0, caution must be exercised in doing so, since the effect of an initial pH of 3.0 was not included in this study.

Although the catalytic effects of certain nitrogenous compounds on the rate of browning have been reported (32) no one has looked at the effects of an organic base catalyst on the production of antioxidative

activity. The present study revealed that pyridine more than doubled the activity. No attempts however, were made to isolate and purify the antioxidative component of the pyridine enhanced reaction, and therefore, no conclusion could be made as to whether the pyridine was acting merely as a catalyst or if it was actually involved in producing a different antioxidant possessing higher activity than the regular arginine-xylose produced antioxidant.

The temperatures varied between 90^{o} and 100^{o} in the pressure reactions, which may account for some of the variation in antioxidative activity. Because of the large variation in activity among samples, definite conclusions could not be drawn from this experiment. However, the indication was that little if any influence by pressure was exerted.

Although this study has succeeded in clarifying a few of the optimum conditions of the arginine-xylose reaction, obviously more research needs to be done. The effects of other buffers, the molar ratio of arginine to xylose, and the total concentration of both arginine and xylose need to be investigated. In addition, the influence of other base catalysts on the outcome of the reaction merits further study. The mechanism by which pyridine enhances the outcome of the reaction to produce accelerated antioxidative activity is not understood. Employing the use of isotopically labeled pyridine may provide some insight as to whether or not the mechanism is catalytic in nature or if the pyridine is partially or totally incorporated into a new antioxidative compound. Finally, a more conclusive study on the effect of pressure and various gases could prove to be fruitful in extending the optimum level of antioxidative activity produced in this reaction.

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