PRELIMINARY ISOLATION AND CHARACTERIZATION OF AN ANTIOXIDATIVE COMPONENT OF THE MAILLARD REACTION BETWEEN ARGININE AND XYLOSE

By

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"Is this elevator going up?"

-Gail Renee Kleisly, 1979

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"Mark, promise me you'll carry on my fight against leisure suits."

-Z. Harris, 1979

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LIST OF ABBREVIATIONS

R	-	alkyl group
NH_4HCO_3	-	ammonium bicarbonate
BHA	-	butylated hydroxy anisole
BHT	-	butylated hydroxy toluene
^{13}C	-	carbon 13
^{14}C	-	carbon 14
CO_2	-	carbon dioxide
cm	-	centimeter
D_2O	-	deuterium oxide
HPLC	-	high performance liquid chromatography
IR	-	infrared
MRP	-	Maillard reaction products
MeOH	-	methanol
µCi	-	microcurie
µl	-	microliter
µm	-	micrometer
mg	-	milligram
ml	-	milliliter
M	-	molar
M.W.	-	molecular weight
nm	-	nanometer
N	-	normal

NMR - nuclear magnetic resonance

ODS - octadecylsilane

NaAc - sodium acetate

NaN_3 - sodium azide

U-^{14}C-arginine - uniformly labeled-^{14}C-arginine

U-^{14}C-xylose - uniformly labeled-^{14}C-xylose

CHAPTER I

INTRODUCTION

The development of rancid flavors in foods is largely due to oxidation of unsaturated lipids by air which is known as autoxidation. This reduces the storage time of the particular type of food according to the amount and type of unsaturated lipids. To prevent autoxidation from occurring, antioxidants are added to food products. There are naturally occurring antioxidants in some types of foods, but these are usually sufficient to protect the product for only a short period of time. Synthetic antioxidants have been developed which are more powerful and increase the storage time of foods considerably.

A new class of compounds has recently been found to have powerful antioxidative activity. These are products derived from the Maillard reaction; this reaction occurs between sugars and amino acids or peptides. These Maillard reaction products (MRP) are commonly found in most foods and have a characteristic brown color.

This research involves the preliminary isolation and characterization of a component of the MRP formed during the reaction of arginine and xylose which has considerable antioxidative activity. This component is an end product of the Maillard reaction and, up to this time, no end product, antioxidative or not, has been purified to any extent nor is any structure known. It is hoped that this study will ultimately

lead to the production of a new type of food preservative that is safer for human consumption than some of the synthetic antioxidants presently in use.

CHAPTER II

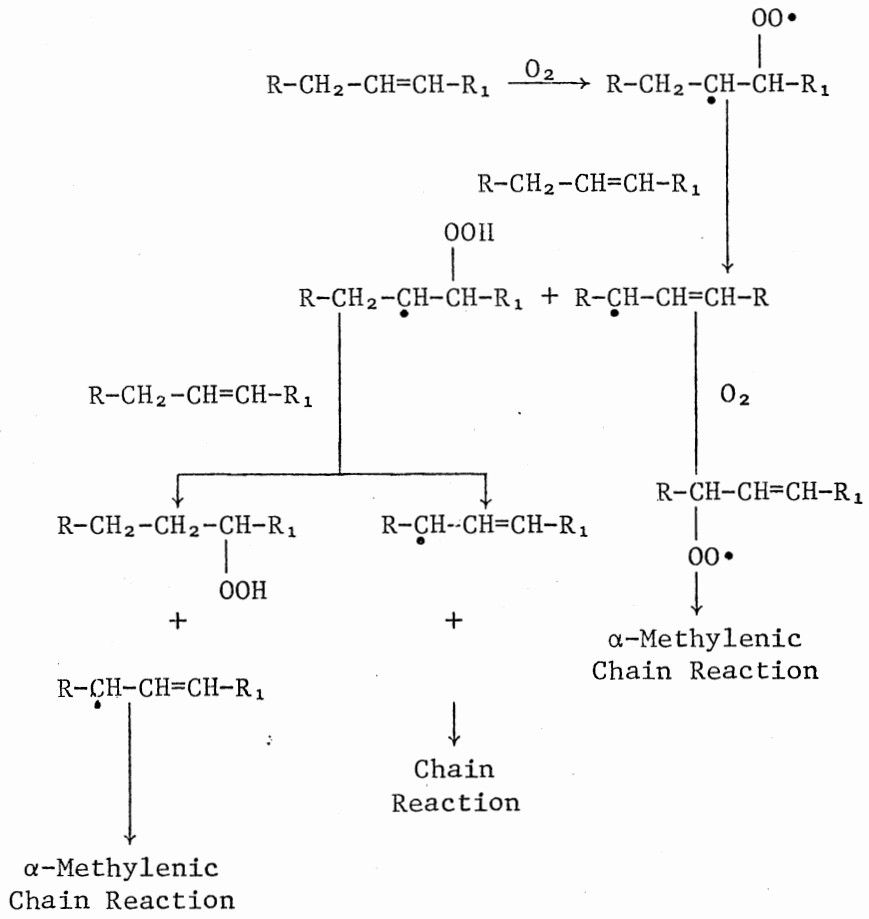
LITERATURE REVIEW

Antioxidants, which prevent the oxidative degradation of organic and biochemical compounds, are in widespread use today in many industries, particularly the food industry. In foods, one of the most prevalent types of oxidation is autoxidation. Unsaturated lipids are the most susceptible compounds in food products to autoxidation and when oxidized can produce rancid off-flavors which may make the product unacceptible. Since it is difficult to prevent food products from being exposed to, at least, a small amount of air during storage, autoxidation of unsaturated lipids is a major concern to the food industry.

Lipid Oxidation

Of the lipids, unsaturated fatty acids (and the larger molecules in which they are contained such as triglycerides, phospholipids and so on) are the most susceptible to autoxidation. The mechanism of autoxidation occurs by a free radical chain reaction (1). Initiation of the reaction in monounsaturated fatty acids (such as oleic acid) is thought to occur by direct attack by oxygen at the double bond in small amounts as shown in the first step in the mechanism in Figure 1. In nonconjugated polyunsaturated fatty acids (such as linoleic acid), initiation occurs with the abstraction of an allylic hydrogen as shown

Figure 1. Mechanism of Free Radical Autoxidation of Oleic Acid



R = $CH_3(CH_2)_6$

R_1 = $(CH_2)_7COOH$

in the first step of the mechanism shown in Figure 2. The propagation steps for both mechanisms are now thought to include the abstraction methylenic hydrogen alpha to the double bond.

Antioxidants

Antioxidants must inhibit some part of the free radical chain reaction mechanism of autoxidation. Scott (2) devised a means to classify antioxidants according to how they inhibit autoxidation and divides antioxidants into two classes, Type I and Type II. Type I antioxidants are known as free radical chain stoppers because they inhibit the propagation of free radicals in the mechanism. This is accomplished primarily by breaking the free radical chain reaction through the removal of either the alkylperoxy or alkyl radical by donation of a hydrogen atom from the antioxidant (3) as shown below:

$$ROO\bullet + AH \longrightarrow ROOH + A\bullet$$

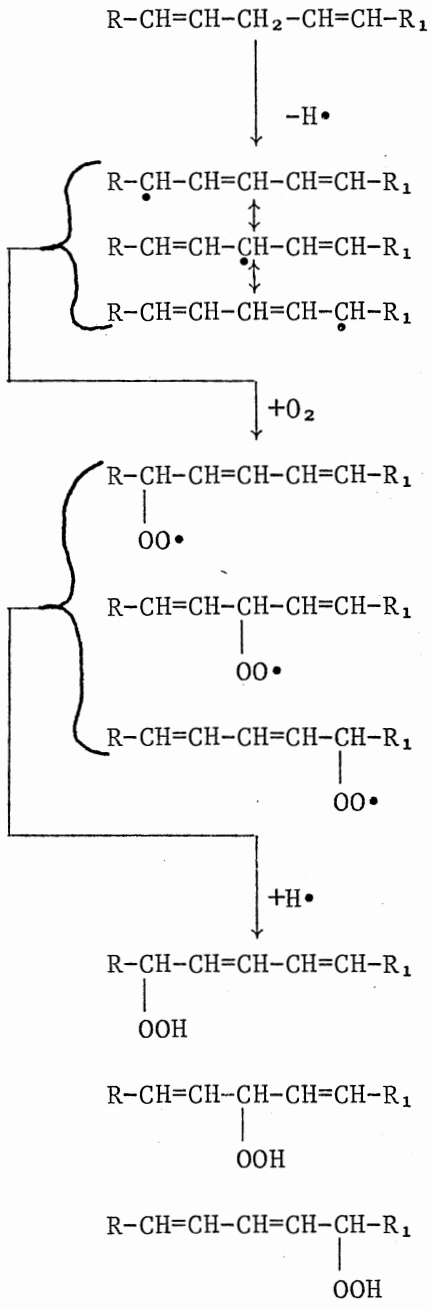
or

$$R\bullet + AH \longrightarrow RH + A\bullet$$

where ROO• is the alkylperoxy radical, R• is the alkyl radical and AH is the antioxidant. Some common examples of Type I antioxidants are shown in Figure 3.

Another means of preventing autoxidation is by controlling the source of production of free radicals in the initiation step. Compounds that do this are Type II antioxidants and most often accomplish this by complexing with metal catalysts (3). Some common examples of

Figure 2. Mechanism of Free Radical Autoxidation of Linoleic Acid



Valence Bond Forms for the Expected Intermediate

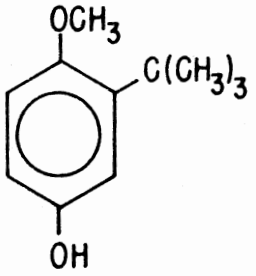
Three Possible Peroxy Radicals

Three Possible Hydroperoxy Products

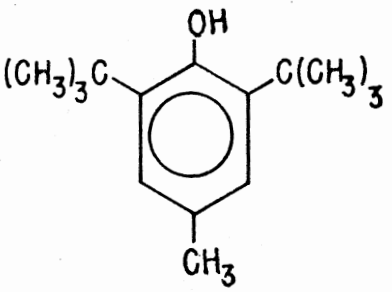
R = $CH_3CH_2CH{=}CH{-}CH_2-$

R_1= $(CH_2)_7COOH$

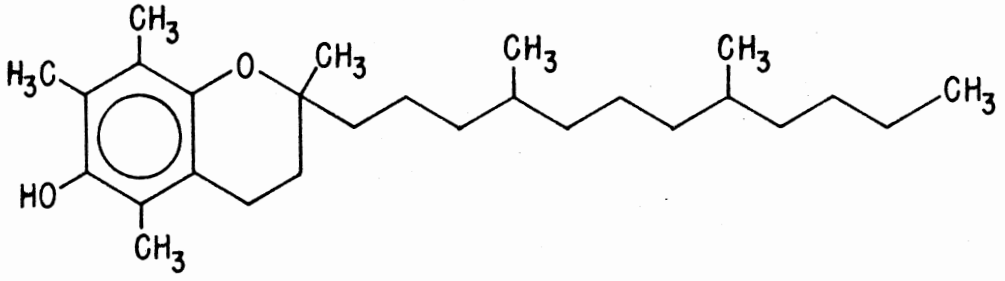
Figure 3. Structures of Common Examples of Type I Antioxidants



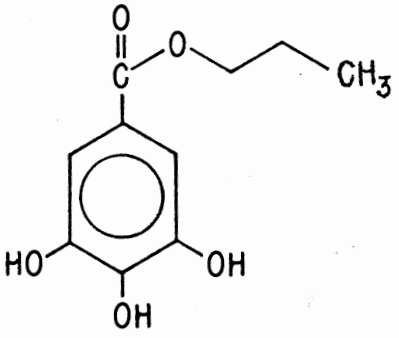
BHA



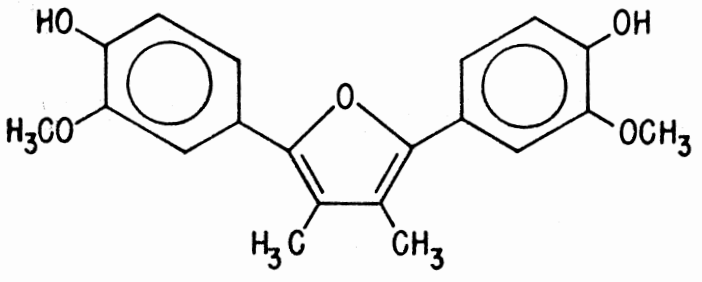
BHT



α – Tocopherol



Propyl Gallate



Gum Guaiac

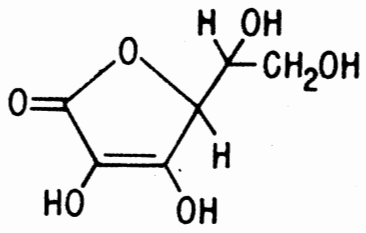
Type II antioxidants are shown in Figure 4.

Research investigating the possible uses of antioxidants has been going on since the early 1930's (4). These studies were concerned mainly with natural antioxidants such as propyl gallate and gum guaiac and thus were used quite extensively. Then in the early 1950's, two synthetic antioxidants, developed for use in the rubber industry originally, were tested and subsequently cleared for use in foods (5). These two antioxidants, BHA and BHT, were members of a class of compounds called the "hindered phenols". Hindered refers to the large R (usually t-butyl) group, ortho or meta to the hydroxyl function (see Figure 3). This group of antioxidants is more powerful than natural antioxidants (6) and today is a major type of antioxidant used in the food industry. Lingnert (7) has reviewed present known antioxidants used in foods and compounds that are known to have antioxidative activity but are not presently used in foods. Included in the latter list is an extensive review of antioxidative MRP.

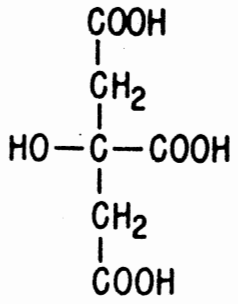
Maillard Reaction

Louis-Camille Maillard (8), the man for whom the Maillard reaction was named, was the first to study the reaction which occurred when combinations of sugars and amino acids were warmed. He noted that there were varying intensities of brown color developed and varying amounts of CO_2 given off depending on the type of amino acid or sugar used or the temperature at which the experiment was performed. The Maillard "reaction" is now known not to be a simple one step reaction but rather a complex series of reactions that take place between carbonyl containing compounds and amine containing compounds. The most commonly

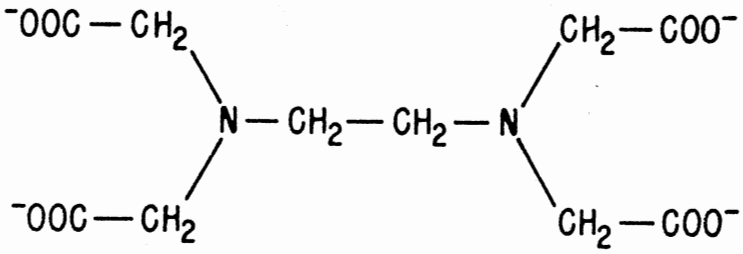
Figure 4. Structures of Common Examples of Type II Antioxidants



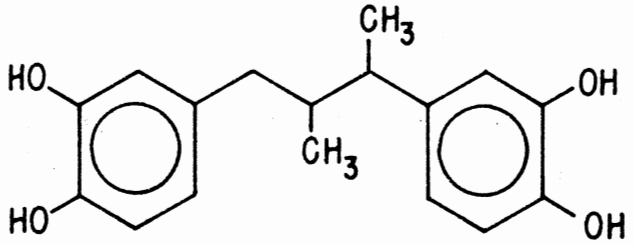
L – Ascorbic Acid



Citric Acid



EDTA



NDGA

studied examples though are still between reducing sugars and amino acids.

Hodge (9) reviewed the Maillard reaction in 1953 and gave a postulated mechanism that is still the best estimate of what actually occurs during the overall reaction. The first two steps are now well defined and the products have been isolated (10). The first step is the reversible condensation of the amino group with the carbonyl to form an N-substituted glycosyl amino acid. The next step involves a rearrangement of the glycosyl amine in either one of two ways depending on if the sugar was an aldose or a ketose. With an aldosamine, the product would be a 1-amino-1-deoxy-2-ketose which is the Amadori rearrangement (10). With the ketosamine, the product would be a 2-amino-2-deoxyaldose which is the Heynes rearrangement (11). Both the rearrangements are acid catalyzed with the carbonyl group of the amino acid providing the catalyst (10).

The next step is the formation of either a diketosamine or a diamino sugar. This is accomplished by the product of either the Amadori or Heynes rearrangement combining with another sugar or amino acid. The next step is the degradation of the diketosamine or diamino sugar through the loss of one or more molecules of water. This decomposition leads to numerous types of substituted furans, pyrroles, pyridines and other similar heterocyclic compounds which have been identified (11-13).

At this stage of the reaction no brown pigments have been produced. From this stage on, no mechanisms have been formulated to account for the formation of brown pigments or other types of compounds produced. Several groups (14, 15) have postulated that polymers of

the heterocyclic compounds are formed but as yet no brown pigments or end products have been isolated.

Antioxidative Properties of the Maillard Reaction

In 1954 Franzke and Swainsky (16) found that the products obtained from reacting glycine with glucose or monosodium glutamate with glucose had antioxidative activity. Since then several papers have been published on the antioxidative effects of MRP. However, there is a problem in comparing different studies reported in the literature in that the specific conditions of the reactions vary widely from group to group, and it has been found (17, 18) that this has a significant effect on the end products formed.

Griffith and Johnson (19) were the first to show MRP had antioxidative activity in food. They found that the substitution of glucose for 5% of the sucrose normally added to cookies not only caused a definite increase in the brown color of the cookies but also increased the stability of the cookies to oxidative rancidity. They further found that products from heating 0.2 M glycine and 0.2 M glucose together for 24 hours gave increased stability to both cookies and lard. The antioxidative activity was thought to be associated with the presence of reductones, which are resonance stabilized enediols. Later reductones purified by other groups (20, 21) from hexoses and secondary amines were demonstrated to have high antioxidative activity in both fats and oils.

In the early 1960s, the Japanese began research on the antioxidative effects of MRP and have since dominated the literature.

Yamaguchi's group (22) was the first to study MRP and found that addition of certain amino acids to the dough of biscuits and cookies caused a corresponding increase in stability. Later studies (23, 24) showed that extracts of the cookies were effective in protecting polyunsaturated fats from oxidation. Although never proven, they believed the extracts to be MRP of the reaction occurring between sugars and amino acids in the dough. When tested against known antioxidants, the extracts were more powerful than tocopherols, comparable to BHT but less powerful than BHA (25). Reductones formed from the reaction of xylose and cetylamine were found to be inactive in aqueous solutions but did have some antioxidative activity in dehydrated systems (26).

In 1968 Kirigaya's group (27) studied the reductones formed from the reaction of xylose with glycine and dihydroxyacetone with glycine. They did not find any antioxidative activity to be associated with the reductones, unlike previous groups. They did find a positive correlation between color intensity and antioxidative activity.

Separation of the active components of the MRP between xylose and glycine was attempted by Yamaguchi's group in 1973 (28). The MRP were separated first by gel filtration using Sephadex G-15, G-50 and then G-10 after which the antioxidative fraction was run on cellulose powder thin layer chromatography. The strongest antioxidative fraction was found to be in the fraction with a molecular weight of approximately 4500.

Itoh's group (29) compared the antioxidative activity of MRP formed when each of thirteen different amino acids were combined with glucose, xylose, and dihydroxyacetone. In all but two cases the dihydroxyacetone-amino acid combination was more potent than either

glucose or xylose amino acid combinations. In the two cases where this was not true, the antioxidative activity of even the most powerful combination was low. Also, in six cases the antioxidative activity was more powerful than was shown by a BHA standard. In a similar study done later by the same group (30), glyoxylate, glyoxal, methylglyoxal and dihydroxyacetone were each mixed with different amino acids and the antioxidative properties studied. Combinations using methylglyoxal and dihydroxyacetone both showed greater antioxidative activity than did a BHA standard.

Lee et al. (31, 32) studied the antioxidative characteristics of the ethanol extracts of the MRP between glycine and glucose. They found that the extracts had strong antioxidative activity even though color intensity was low. They also showed that by comparing the antioxidative activity between MRP formed after 2 hours of reacting and those formed after 40 hours, the shorter time was equally effective.

Several Japanese groups have looked at the effects of different factors on the antioxidative properties of the MRP. Gomu and Horikoshi (33) studied the interaction of metallic ions with the MRP from the reaction of glycine and glucose. These products were found to bind metal ions and coagulate optimally around pH 6 to 7 and EDTA would inhibit or eliminate this binding. Morita et al. (34) added oxidation catalysts, such as ascorbic acid, heme, and the monohydroperoxide fraction of methyl linoleate to a methyl linoleate solution containing the MRP of glucose and alanine. They determined that the glucose-alanine MRP inhibited oxygen uptake for three hours. A Japanese group (35) found that the antioxidative activity of MRP was increased when the original mixture was subjected to gamma

irradiation before reacting. While there was a similar increase in antioxidative activity when just the sugar solution was irradiated then reacted with the amino acid, no increase in activity was found when just the amino acid solution was irradiated.

Eichner (18) found that brown color in MRP did not necessarily mean they have high antioxidative activity. He found that at low water concentrations, the MRP formed had very little color but a high amount of reductones. When the MRP of a glucose-lysine mixture reacted at low water concentration was mixed with methyl linoleate and lyophilized, oxidation was inhibited for up to 200 hours.

Besides the Japanese, some of the most comprehensive studies of the Maillard reaction have been done by a group in Sweden at the Swedish Food Institute. To insure uniformity of their results, they first developed new methods to accurately and more sensitively measure antioxidative activity (36).

Various combinations of sugars and amino acids were studied under defined conditions (37). The amino acids used were L-arginine, L-cysteine, L-glutamic acid, L-histidine, L-lysine, and L-valine and the sugars used were D-fructose, D-glucose and D-xylose. Each of the amino acids and sugars were chosen because they had previously been shown to give rise to antioxidative MRP. The standard procedure used for synthesizing MRP was as follows: 5 millimoles of amino acid were combined with 10 millimoles of sugar in 5 mls of 0.1 M potassium phosphate buffer. The pH of the mixture was adjusted to 7 using potassium hydroxide or hydrochloric acid. The mixture was then refluxed for 5 hours. The resulting crude mixture would then be assayed for antioxidative activity without further purification.

Figure 5 shows the results of measuring the antioxidative activity of the various sugar-amino acid combinations by the oxygen consumption method of Svensson and Eriksson (38). The two best combinations were arginine-xylose and lysine-xylose. When antioxidative activity was measured by the gas chromatographic method (36), as shown in Figure 6, the best combinations were arginine-xylose and histidine with any of the three sugars. Another fact that can be seen from the two figures is that with arginine there is a substantial difference in the amount of antioxidative activity of the products depending upon which sugar was used in the reaction. However, with histidine, in both cases, any of the three sugars used gave approximately equal antioxidative activity.

From these results they decided to further study the histidine-glucose mixture and the factors affecting the synthesis. First the influence of the length of reaction time was tested and it was found that the antioxidative activity reached a maximum at 20 hours after which it decreased. The color intensity showed a similar pattern as can be seen in Figure 7. The pH of the solution rapidly decreased during the first five hours after which it stabilized at approximately pH 4.4 as shown in Figure 8. The effect of the initial pH of the reaction mixture before refluxing was looked at next. The results in Table I showed that both a very low and a very high initial pH gave MRP with low activity with the best results obtained between pH 7 and 9. They also studied the effect caused by the initial concentration of histidine and glucose and from the results shown in Table II there appears to be a threshold value for the concentration. Even though a 2:1 ratio of amino acid to sugar was used twice, the one with less

Figure 5. Comparison of Antioxidative Effect with Color Intensity of Maillard Reaction Products from Different Sugar-Amino Acid Combinations

The antioxidative effect was measured by the polarographic method and the larger the number the greater the antioxidative effect.

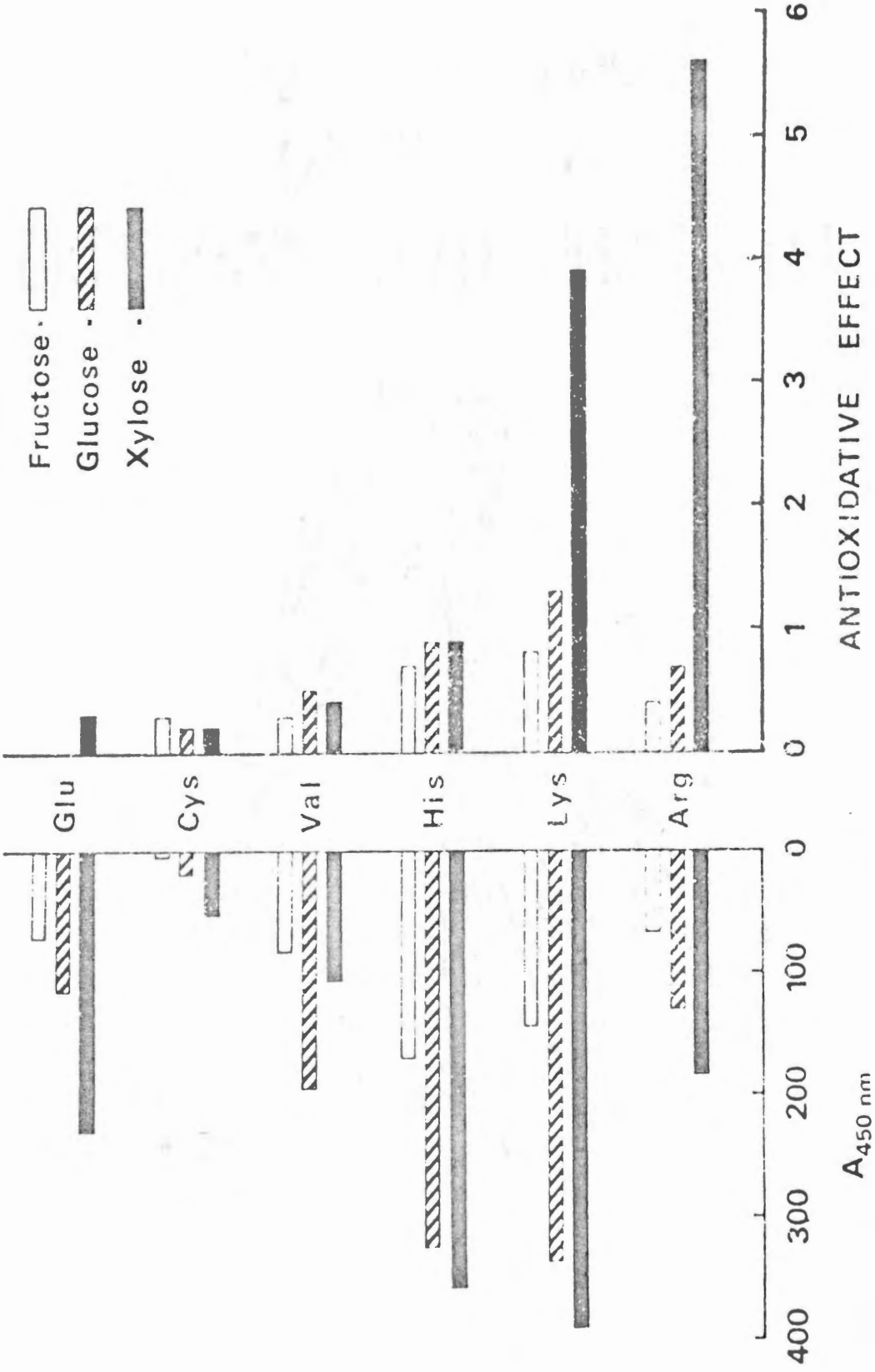

Fructose
Glucose
Xylose
Glu
Cys
Val
His
Lys
Arg
400
300
200
100
0
0
1
2
3
4
5
6
$A_{450\ nm}$
ANTIOXIDATIVE EFFECT

Figure 6. Antioxidative Effect of Maillard Reaction Products from Different Sugar-Amino Acid Combinations

Antioxidative effect was determined by the concentration of n-hexanal in a linoleic acid emulsion oxidized for 24 hours in the presence of the Maillard reaction products. The smaller the concentration, the larger the antioxidative effect.

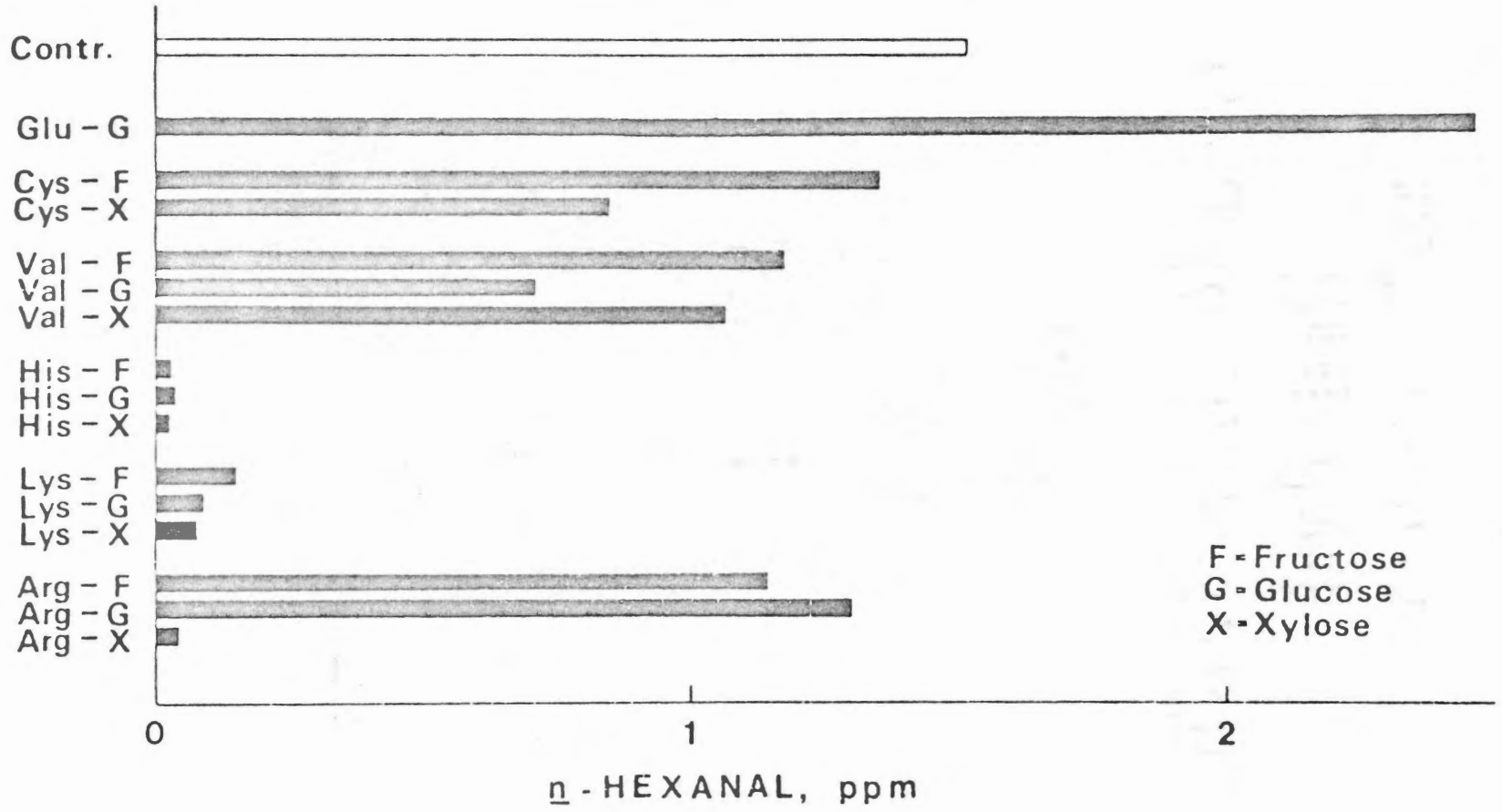

Contr.
Glu – G
Cys – F
Cys – X
Val – F
Val – G
Val – X
His – F
His – G
His – X
Lys – F
Lys – G
Lys – X
Arg – F
Arg – G
Arg – X
F = Fructose
G = Glucose
X = Xylose
0
1
2
n - HEXANAL, ppm

Figure 7. Development of Color and Antioxidative Effect During the Maillard Reaction of Histidine and Glucose

Color intensity was measured spectrophotometrically at 450 nm and antioxidative effect was measured by the polarographic method.

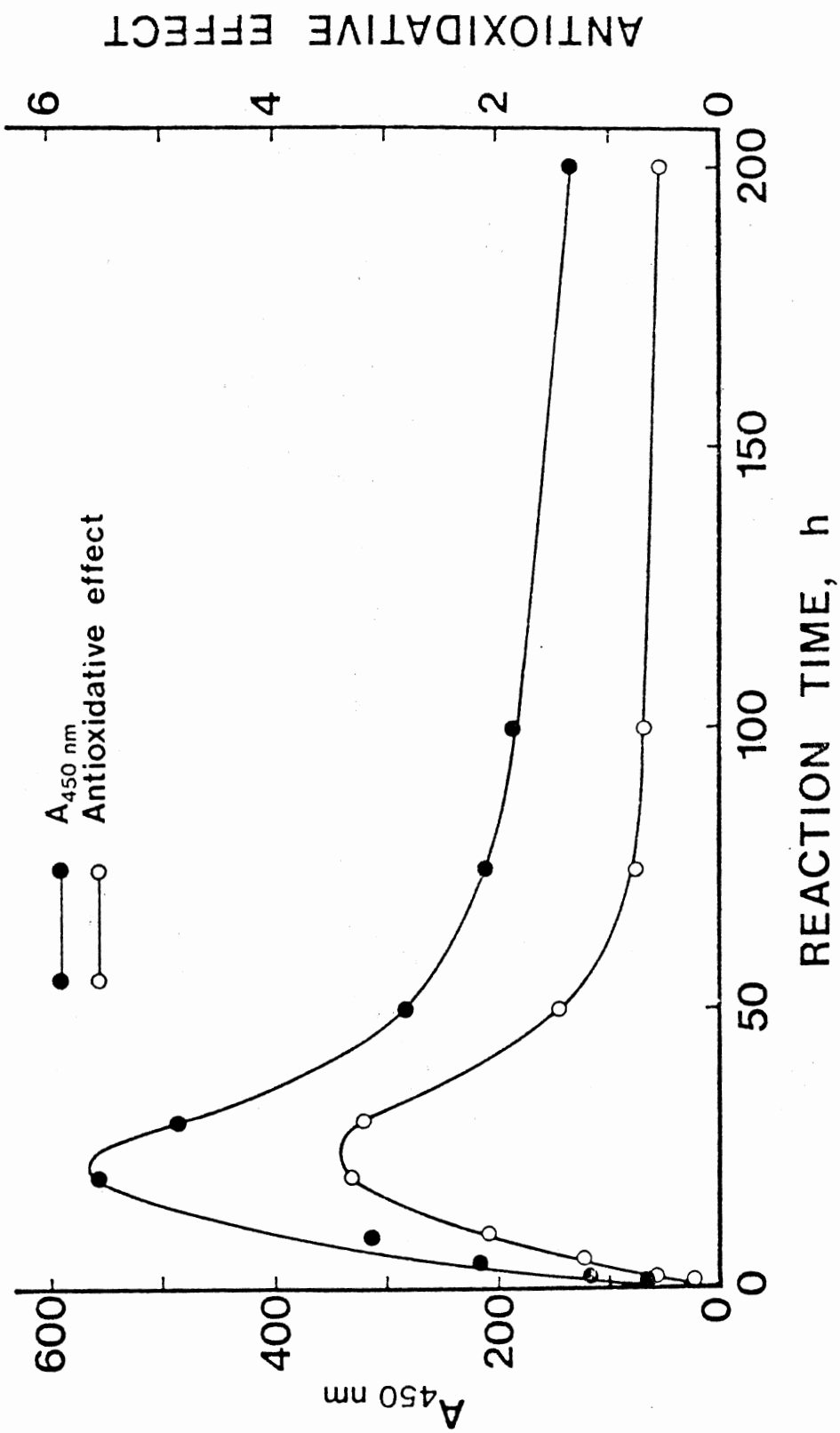

ANTIOXIDATIVE EFFECT
6
4
2
0
600
400
200
0
A450 nm
0
50
100
150
200
REACTION TIME, h
A450 nm
Antioxidative effect

Figure 8. Change in pH During the Maillard Reaction of Histidine and Glucose

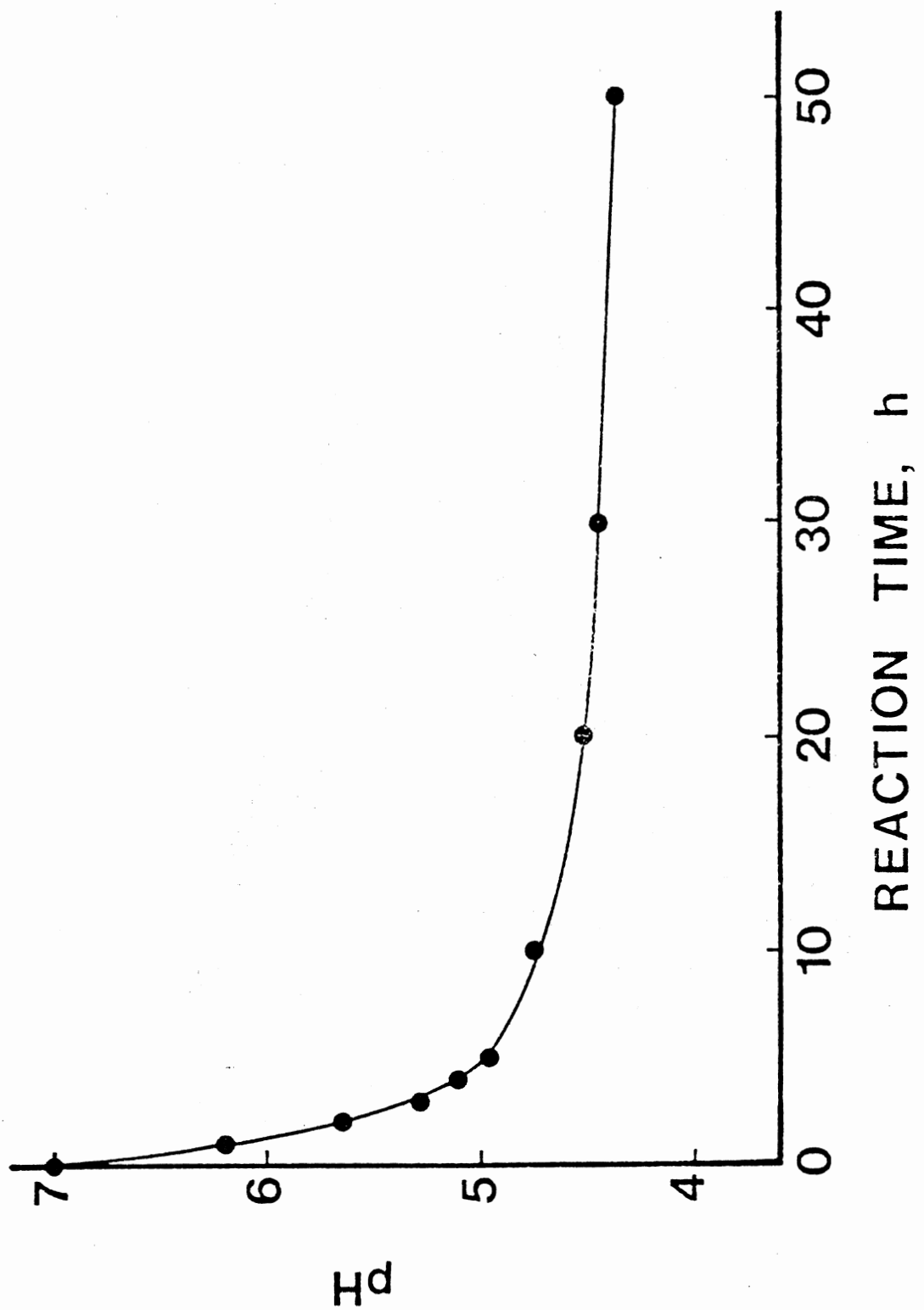

pH
7
6
5
4
0
10
20
30
40
50
REACTION TIME, h

TABLE I

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

Reaction Mixture[a]		Final pH	$A_{450\ nm}$	Antioxidative Effect[b]
Histidine (mmol)	Glucose (mmol)			
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.

material had less than half the antioxidative activity of the one with more material. The same effect was shown with a 2:1 ratio of sugar to amino acid but the highest antioxidative activity shown with the sugar in excess was not as great as with the amino acid in excess.

The Swedish group also studied the antioxidative effects of the histidine-glucose MRP in cookies (39) and sausage (40). The cookie study was divided into four groups: one contained no additives which was the control, the second contained unreacted L-histidine monohydrochloride (0.1%) and D-glucose (1.0%) added to the dough, the third contained 0.1% histidine-glucose MRP and the last contained a combination of BHA and BHT (0.0016%) in amounts usually used for industrial food preservation. Sensory evaluation showed that the cookies containing the histidine-glucose MRP lasted as long as those containing the BHA and BHT. Surprisingly, the cookies containing the unreacted histidine and glucose lasted nearly twice as long. In the sausage study there were six groups: the control with no additiion, sausage containing 0.08% histidine-glucose MRP, sausage containing 0.16% histidine-glucose MRP, sausage containing 0.08% histidine and 0.32% glucose, sausage containing 0.16% histidine and 0.32% glucose and sausage containing MRP from hemoglobin hydrolysate reacted with glucose. In these studies sensory evaluation showed that the histidine-glucose MRP gave the best protection from formation of rancid flavor.

Some structural analysis was attempted on the histidine-glucose product (41). The molecular weight was determined to be 1500 ± 300 by gel filtration chromatography and HPLC. Elemental analysis showed 37.2% carbon, 5.8% hydrogen, 12.9% nitrogen, and 44% oxygen.

For an antioxidant to be used in food products, it must first be tested to see that it is safe for consumption. Another group in Sweden (42) performed short-medium term toxicity tests on the histidine-glucose and arginine-xylose MRP. Results showed that neither MRP effected the digestibility nor retention of dietary protein. Some growth inhibition was noticed but this was attributed to a decrease in food intake most probably caused by the poor palatability of the diet. Thus no antinutritional or toxic properties were noticed in the four week study even though the doses administered were 25 times larger than would normally be added to effect antioxidative ability.

CHAPTER III

MATERIALS AND METHODS

The L-arginine monohydrochloride and D-xylose used were obtained from Sigma Chemical Company (St. Louis, Mo.). The initial mixture consisted of 0.05 moles of L-arginine monohydrochloride and 0.025 moles of D-xylose in 25 mls of 0.1 M potassium phosphate buffer pH 7.2. The pH of the mixture was adjusted to 8.0 using 1 M potassium hydroxide. The mixture was then refluxed at 100°C for 20 hours.

Assays

The assay used was the spectrophotometric method of assaying antioxidative activity developed by Lingnert et al. (36). First a linoleic acid emulsion was made by putting 10 mls of 1 M potassium phosphate monobasic in a glass beaker and agitating with a magnetic stirrer then adding dropwise 0.5 mls of Twen 20 from Sigma Chemical Company (St. Louis, Mo.) followed by 0.5 mls of linoleic acid from Nu Chek Prep (Elysian, Minn.) also dropwise. Then 3 pellets of potassium hydroxide are added and allowed to dissolve completely before adding 15 mls of the 1 M potassium phosphate monobasic. The mixture is brought to a total volume of 250 mls with water and the pH adjusted to 6.5 using concentrated hydrochloric acid.

A 2.0 ml aliquot of the linoleic acid emulsion was placed into a number of test tubes along with 200 µl of the sample to be tested

leaving one tube containing only the linoleic acid emulsion as a control. Then from each of the tubes, a 200 µl aliquot was removed and the remaining portion incubated 15 to 20 hours in the dark at 37°C. The 200 µl aliquot was added to a test tube containing 2 mls of 100% methanol and 6 mls of 60% methanol:water. The absorbance at 234 nm of each of these tubes was read on a Varian model 635 spectrophotometer.

At the end of the incubation period another 200 µl aliquot of each tube was taken, added to the methanol and methanol:water solution as before and the absorbance at 234 nm read. The difference in absorbance (ΔA_{234}) was calculated by subtracting the reading taken before incubation from the reading taken after incubation. The antioxidative activity (A.O. act.) was calculated as follows:

$$\text{A.O. act. (\%)} = \frac{\Delta A_{234_C} - \Delta A_{234_S}}{\Delta A_{234_C}} \times 100$$

where ΔA_{234_C} is the ΔA_{234} of the control tube and ΔA_{234_S} is the ΔA_{234} of the sample tube. The linoleic acid emulsion was made fresh daily.

Dialysis

Dialysis was carried out in dialysis tubing from Spectrum Medical Industries (Los Angeles, Calif.). Three different types of tubing were used with molecular weight cutoffs of 1000, 3500, and 6000-8000. Each dialysis was done on the arginine-xylose complex just after refluxing for 24 hours against a total of 12 liters of water using 6 liters for approximately 12 hours then changing the outside fluid to fresh water.

Gel Filtration

All gel filtration media (Sephadex G-15, G-25, and G-50) was obtained from Sigma Chemical Company (St. Louis, Mo.). Each media was allowed to swell overnight in the eluting solvent. Columns were packed by gravity flow and allowed to equilibrate with three column volumes of eluting solvent. All columns were run at room temperature and when not in use were preserved with 0.02% NaN_3. Fractions were collected using an ISCO model 328 fraction collector and monitored at 254 nm and 450 nm using a Varian model 635 spectrophotometer.

High Performance Liquid Chromatography

Most of the HPLC was done on a Waters Associates reverse-phase μbondapak ODS (10 μm bead size) column (30 x 0.39 cm) connected to a Waters Associate model 6000A chromatographic pump via a Whatman reverse-phase μbondapak ODS (30-38 μm bead size) precolumn (6 x 0.39 cm). The effluent absorbance was monitored at 254 nm by a Waters Associates model 440 absorbance detector. All other HPLC experiments were done using the same columns but on a Dupont model 830 liquid chromatography system and the effluent absorbance was monitored by a Beckman model 25 spectrophotometer.

Labeled Experiments

The L-U-^{14}C-arginine monohydrochloride and D-U-^{14}C-xylose used in these experiments were obtained from Amersham Corporation (Arlington Heights, Ill.). Two different experiments were run, one using 2 μCi of labeled arginine and the other using 2 μCi of labeled xylose. The

arginine-xylose MRP was synthesized as usual with the labeled compound included, and then the active component was isolated following the usual isolation scheme (see Results, isolation). Aliquots were taken at each step of the isolation procedure and counted on a Packard model PL Prias liquid scintillation counter. Each aliquot was dissolved in 3 mls of Packard Instagel liquid scintillation cocktail before counting.

Ultraviolet Spectroscopy

UV spectroscopy was done on a Perkin-Elmer model 559 microcomputer controlled spectrophotometer.

Infrared Spectroscopy

The IR spectrum was obtained by the Fourier-transformed infrared photoacoustic spectroscopy technique described by Rockley (42). The spectrum was taken of the lyophilized material of the active peak from HPLC on a Digilab FTS-2DC interfaced to a Data general NOVA 3/12. The material used was the same as that used for the UV spectrum.

Nuclear Magnetic Resonance Spectroscopy

Proton and ^{13}C NMR spectra were run on a Varian model XL-100(15) instrument interfaced to a Nicolet Technology Corporation TT-100A Fourier transform accessory. Solutions of standard L-arginine monohydrochloride and standard D-xylose (Sigma Chemical Company, St. Louis, Mo.) were dissolved in 0.5 ml of D_2O [100.00 atom % from Aldrick Chemical Company (Milwaukee, Wis.)] to a final concentration of 400 mg/ml. The proton NMR analysis needed only one scan to obtain a good

spectrum while the ^{13}C NMR analysis needed 360 scans.

Due to the small quantities of the final active component from HPLC, the material was dissolved in 30 µl of the 100.00 atom % D_2O and placed in a 1.7 x 90 mm capillary tube to be run. The proton NMR spectrum was run overnight (approximately 16 hours) accumulating 1700 scans while the ^{13}C spectrum needed to be run for approximately 64 hours accumulating 36,000 scans to obtain a good spectrum. TSP-deuterated (sodium-3-trimethyl-silyl propionate-2,2,3,3-d_4) was used as a marker.

CHAPTER IV

RESULTS AND DISCUSSION

Synthesis of Arginine-Xylose MRP

When the arginine and xylose were mixed together in the phosphate buffer, the pH was found to be 6.06. Dissolving either the arginine or the xylose alone in the buffer at the same concentration as used in synthesis, the pH was 6.79 and 8.86, respectively, showing some reacting taking place immediately upon mixing. Within a few minutes after boiling was started, the solution turned from clear to a faint yellow color which darkened progressively until, at 15 minutes, the solution was a dark yellow color. After this point, the solution started to turn brown and continually darkened until, at 30 minutes, the solution was so dark no further change could be visibly noticed. After the boiling was complete, the solution was assayed for antioxidative activity. An aliquot of 50 µl would give 100% activity and an amount as small as 20 µl gave 98% activity. This proved that the synthesis did indeed give rise to a powerful antioxidative compound or compounds.

Isolation of the Active Component

Since the few attempts at isolating an active component of MRP had used gel filtration (29, 41), it was decided that the first step in isolating the arginine-xylose MRP active component would be a

Sephadex G-15 column. The result seen in Figure 10A was a very broad band of active material. Also, the active material still had a high salt concentration from the buffer used in the synthesis which had not separated on the column. Since the gel filtration had not desalted the mixture, dialysis against distilled water was tried. Using 1000 molecular weight cutoff tubing, the mixture was desalted very well (conductivity went from 54 m MHO before dialysis to 32 μ MHO after dialysis) while retaining the activity in the dialysis tubing. After dialysis, the mixture was lyophilized and it was found that a considerable amount of material had been lost by weight from the original reaction mixture through dialysis. Thus the dialysis step proved to not only remove salts from the active component but other materials as well.

A portion of this lyophilized material from dialysis was then subjected to gel filtration chromatography. After several different types of Sephadex columns were tried (see Gel Filtration), it was found that Sephadex G-50 gave the best results. The material from gel filtration was then subjected to HPLC. The final isolation scheme from synthesis is shown in Figure 9. Each portion of the isolation will now be discussed in more detail.

Dialysis

The first dialysis was done solely to desalt the newly synthesized material. During dialysis material would come through the tubing enough to turn the outside fluid a fairly dark brown color. After 12 hours the outside fluid would be replaced with fresh distilled water and at the end of dialysis this too would be brown in color. When the

Figure 9. Flow Scheme of Synthesis and Isolation of the Antioxidative Component from the Maillard Reaction of Arginine and Xylose

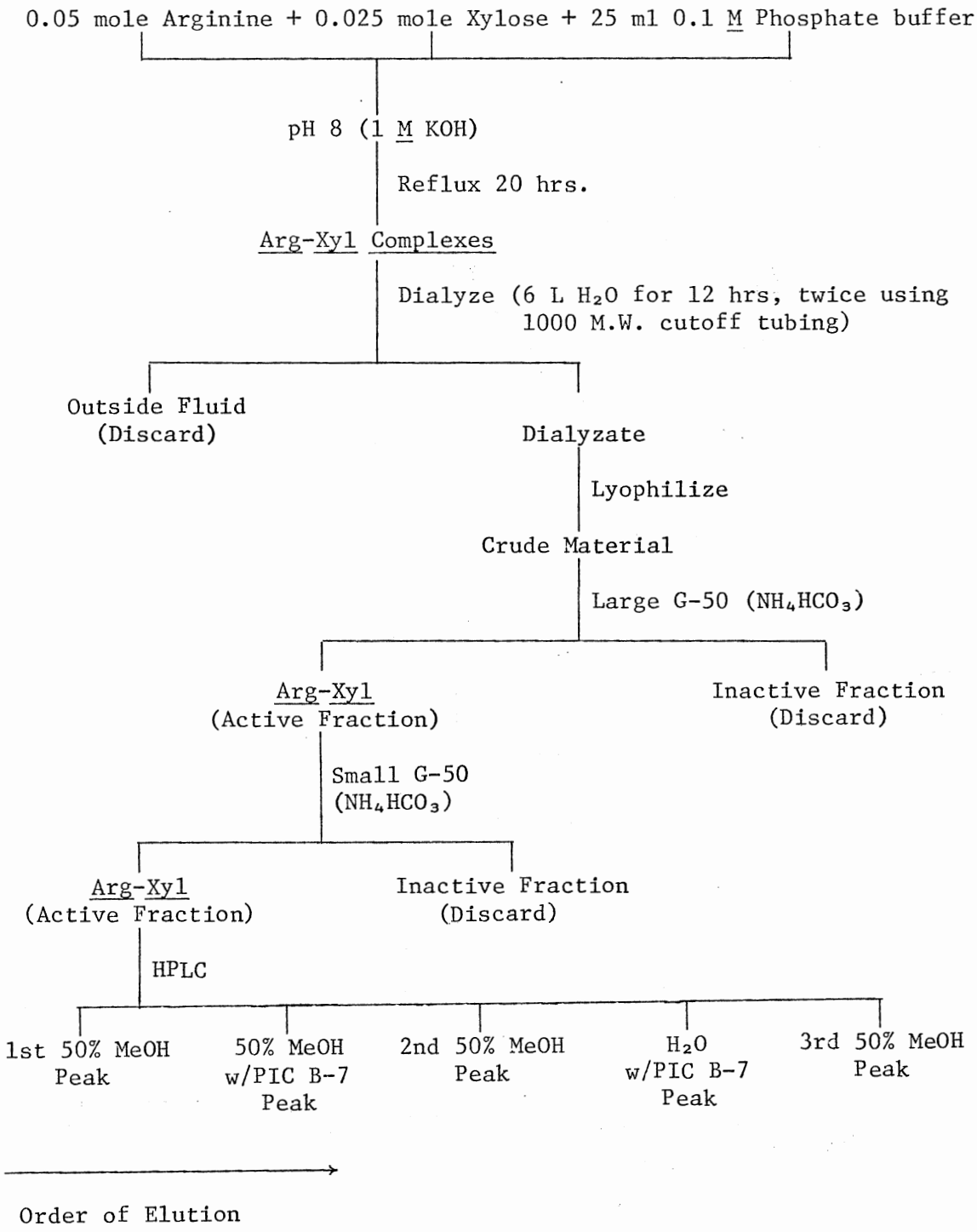

0.05 mole Arginine + 0.025 mole Xylose + 25 ml 0.1 M Phosphate buffer
pH 8 (1 M KOH)
Reflux 20 hrs.
Arg-Xyl Complexes
Dialyze (6 L H_2O for 12 hrs, twice using 1000 M.W. cutoff tubing)
Outside Fluid (Discard)
Dialyzate
Lyophilize
Crude Material
Large G-50 (NH_4HCO_3)
Arg-Xyl (Active Fraction)
Inactive Fraction (Discard)
Small G-50 (NH_4HCO_3)
Arg-Xyl (Active Fraction)
Inactive Fraction (Discard)
HPLC
1st 50% MeOH Peak
50% MeOH w/PIC B-7 Peak
2nd 50% MeOH Peak
H_2O w/PIC B-7 Peak
3rd 50% MeOH Peak
Order of Elution

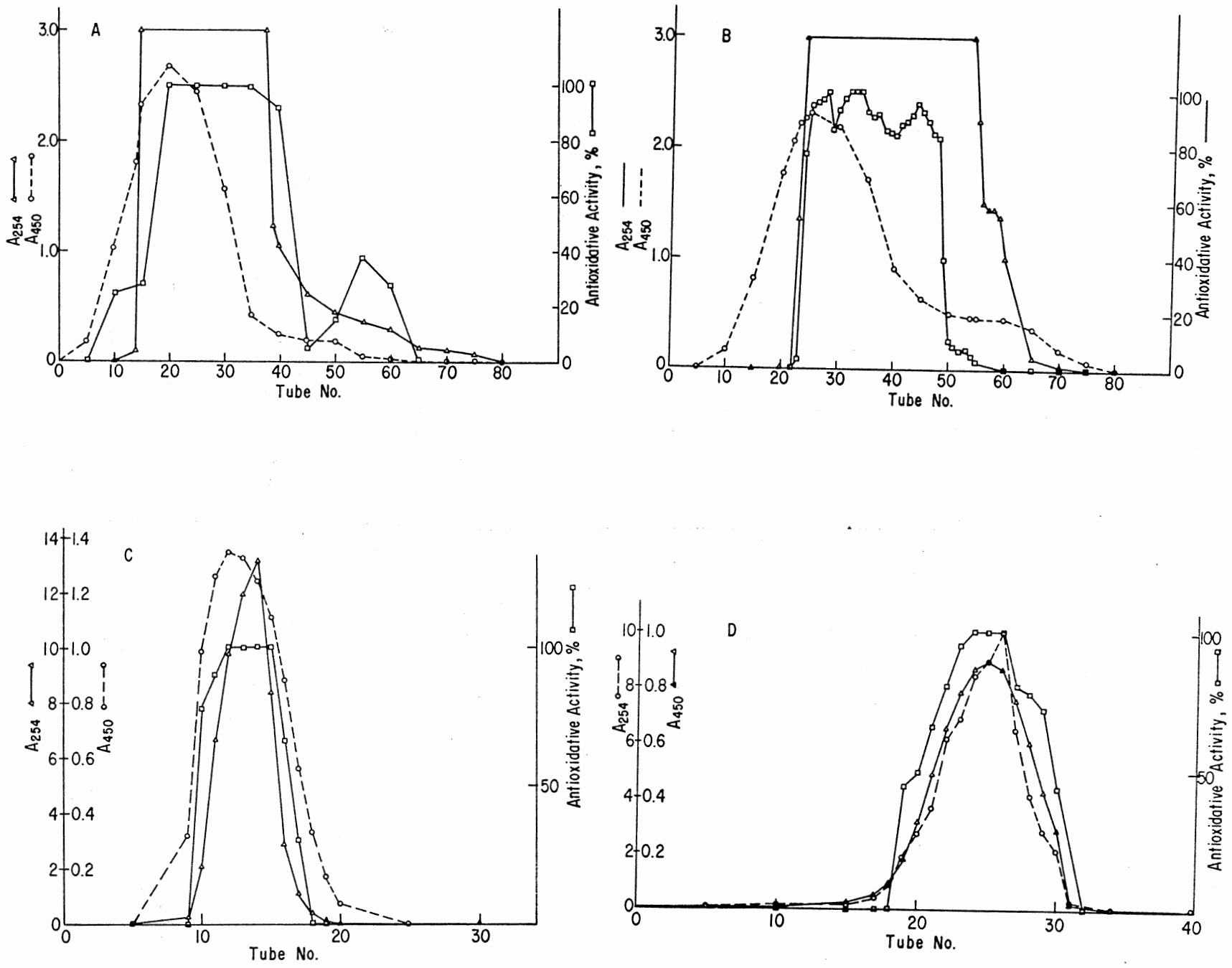
Figure 10. Elution Diagrams of Different Types of Gel Filtration Used in Isolating the Arginine-Xylose Antioxidative Component

A. Sephadex G-15 column (36.8 x 2.7 cm) eluted with 1 M phosphate buffer and 10 ml fractions collected.

B. Sephadex G-25 column (36.8 x 2.7 cm) eluted with distilled H_2O and 10 ml fractions collected.

C. Sephadex G-50 column (96 x 1.5 cm) eluted with 1% NH_4HCO_3 and 10 ml fractions collected.

D. Sephadex G-50 column (54 x 1.5 cm) eluted with 1% NH_4HCO_3 and 2 ml fractions collected.


A
B
C
D
A_{254}
A_{450}
Antioxidative Activity, %
Tube No.

dialyzate was lyophilized, only about 1 gram of material was recovered. This represents only 7% by weight of the original amount of arginine and xylose used in the synthesis. The dialyzate retained most of the activity showing that there was a considerable increase in purity of the active component.

Dialysis was done in increasingly larger M.W. cutoff tubing using the same procedure. A 200 µl aliquot would be assayed from the dialyzate of each size tubing. The 1000 M.W. cutoff tubing gave 100% activity, 3500 M.W. cutoff tubing gave 86% activity and the 6000-8000 M.W. cutoff tubing gave only 56% activity. It was therefore decided to use the 1000 M.W. cutoff tubing for dialysis since it retained the active component better than the other two types of tubing used.

Gel Filtration

Originally, a Sephadex G-15 column was used to separate the active and inactive fractions of the mixture. As can be seen in Figure 10A, the active peak was very broad (each fraction being 10 mls) giving very little separation. Sephadex G-25 was tried next using water instead of phosphate buffer in order to deep the salt concentration down. Figure 10B shows that the G-25 column seemed to give three peaks of activity. However, when material from each peak was run on HPLC, they all gave the same pattern showing that there was only one broad peak as with the G-15. Rechromatography of either the G-15 or G-25 active peaks did not sharpen the peaks appreciably.

Charged solvents, whose salts would volatilize during lyophilization, were tried with G-50 columns to see if they had any effect on separation. Both 1 N acetic acid and 1% NH_4HCO_3 were used. The 1 N

acetic acid gave a much narrower band of colored material than either the G-15 or the G-25. However, the acetic acid interferred with the assay so that it was impossible to tell where the active material was. The 1% NH_4HCO_3 gave similar results and did not interfere with the assay. Rechromatography on a smaller column, collecting 2 ml instead of 10 ml fractions resulted in a sharper peak. The results from the 1_1 NH_4HCO_3 columns can be seen in Figures 10C and 10D. Approximately 100 mg of crude material from dialysis were run on the large G-50 and after the small G-50 column run only 9 mg of active material were recovered.

On all Sephadex columns shown in Figure 10, except on the small G-50, a band of brown material would bind to the column. It could only be washed off with 1 N acetic acid or similar solution so it is not known whether or not it is active. However, it comprised only about 6% of the total material put on a large G-50 column so it was discarded. It was assumed that the reason no material was bound to the small G-50 column was that all the material that interacts with the Sephadex was bound to the large G-50 column.

High Performance Liquid Chromatography

Initial HPLC experiments were run using the crude material from dialysis and were eluted with a water-MeOH combination of 85% H_2O:15% MeOH giving the pattern shown in Figure 11A. Since the column separated on the basis of polarity, 0.01 N sodium acetate was added to the eluting solvent to alter the separation. The pattern did indedd change drastically as seen in Figure 11B.

All further HPLC runs were on the active material from small G-50

Figure 11. Elution Patterns of Different Methods of Running HPLC

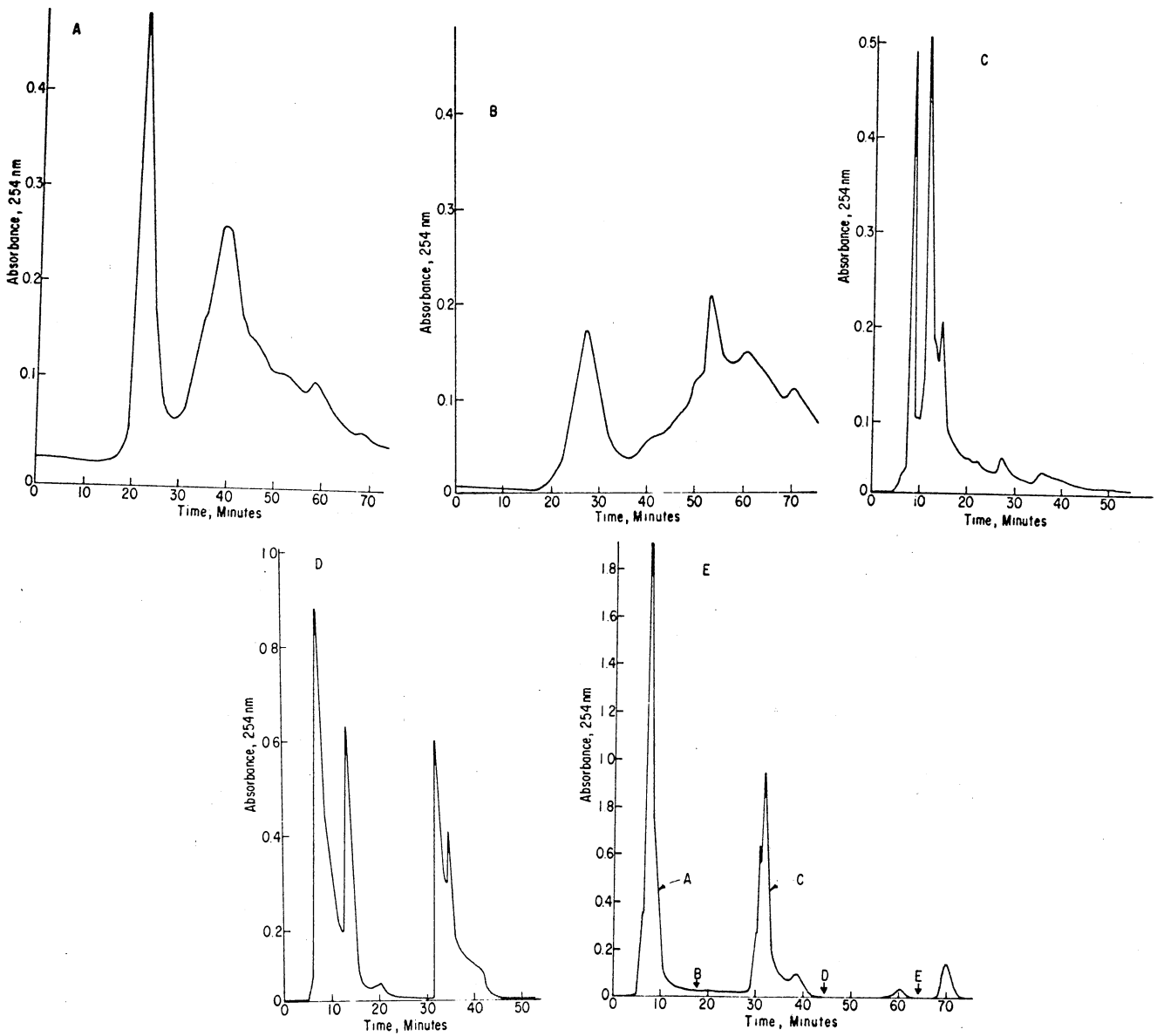
A. 1 mg of crude material from dialysis eluted with 85% H_2O:15% MeOH at 0.5 ml/min.

B. 1 mg of crude material from dialysis eluted with 85% H_2O:15% MeOH containing 0.01 N NaAc at 0.5 ml/min.

C. 0.5 mg of active material from small G-50 column eluted with H_2O containing PIC B-7 at 0.5 ml/min.

D. 0.5 mg of active material from small G-50 column eluted with H_2O containing PIC B-7 first then switching to 50% MeOH containing PIC B-7 at 24 min. with a flow rate of 0.5 ml/min.

E. 1 mg of active material from small G-50. Starting conditions were 0.5 ml/min flow rate eluting with 50% MeOH, at point A flow rate was increased to 2 ml/min, at point B switched to 50% MeOH containing PIC B-7 at 0.5 ml/min, at point C switched back to 50% MeOH at 2 ml/min, at point D switched to H_2O containing PIC B-7 at 0.5 ml/min. and at point E switched back to 50% MeOH at 2 ml/min.


A
B
C
D
E
Absorbance, 254 nm
Time, Minutes
A
B
C
D
E

columns using the paired ion reagent PIC B-7 (a trade name for heptane sulfonic acid) obtained from Waters Associates (Milford, Mass.) at a concentration of 0.005 M. Initial runs using the PIC B-7 were made using just water with PIC B-7 in giving the pattern shown in Figure 11C. However, the material from the small G-50 column was brown and the material collected from the HPLC was clear. When the column was washed with 50% MeOH:H_2O to remove any PIC B-7 from the column, a large peak containing the brown material came off (peak not shown in Figure 11C). To elute the brown material from the column after eluting with water containing PIC B-7, 50% MeOH:H_2O with PIC B-7 was run through the column giving the pattern shown in Figure 11 D. Antioxidative activity was found only in the first peak eluted after switching to the 50% MeOH:H_2O with PIC B-7 solution.

The conditions that gave the best results are shown and described in Figure 11E. Of all the peaks shown, only the peak at 31 minutes had any considerable activity. The peak was collected without including any material from the two shoulders on the front of the peak.

Labeled Experiments

Radioactively labeled arginine-xylose MRP was synthesized using either U-^{14}C-L-arginine monohydrochloride or U-^{14}C-D-xylose, and the active component was isolated by the isolation scheme shown in Figure 9. Table III shows the amount of radioactivity remaining at each step of the isolation. The loss of radioactivity during refluxing could be due to loss of the carboxyl group of arginine as CO_2 which has been postulated to occur (9). Both the arginine and the xylose show considerable loss during dialysis (67% and 60%, respectively) which is

TABLE III

TRACER STUDY OF SYNTHESIS AND ISOLATION OF THE ANTIOXIDATIVE COMPONENT USING U-^{14}C-ARGININE AND U-^{14}C-XYLOSE

Step	DPM		DPM Loss (Nonactive Material)		Mg of Active Material		DPM/mg of Active Material	
	U-^{14}C-Arg	U-^{14}C-Xyl	U-^{14}C-Arg	U-^{14}C-Xyl	U-^{14}C-Arg	U-^{14}C-Xyl	U-^{14}C-Arg	U-^{14}C-Xyl
Original Arginine-Xylose Mixture (Before Refluxing)	4.13×10^6	3.57×10^6	-	-	-	-	-	-
Arginine-Xylose Antioxidative Mixture (After Refluxing)	3.00×10^6	3.41×10^6	1.13×10^6	1.6×10^3	-	-	-	-
Crude Material (after dialysis)	1.12×10^6	1.36×10^6	1.87×10^6	2.05×10^6	736	922	1.52×10^3	1.48×10^3
Large Sephadex G-50 Column	1.14×10^5	1.32×10^5	3.24×10^4	2.00×10^3	80	81	1.19×10^3	1.52×10^3
Small Sephadex G-50 Column	2.99×10^4	7.99×10^4	1.80×10^4	4.70×10^3	9.48	9.14	3.15×10^3	8.75×10^3
HPLC: *								
0-20 min	-	-	1.31×10^4	2.19×10^4	-	-	-	-
20-28 min	-	-	5.62×10^2	1.27×10^3	-	-	-	-
28-30 min	-	-	6.11×10^2	2.80×10^3	-	-	-	-
30-32 min	1.27×10^3	3.18×10^3	-	-	0.47	0.50	2.71×10^3	6.37×10^3
32-42 min	-	-	4.30×10^3	1.22×10^4	-	-	-	-
42-57 min	-	-	1.68×10^2	9.08×10^2	-	-	-	-
57-62 min	-	-	1.65×10^2	2.17×10^3	-	-	-	-
62-72	-	-	8.53×10^2	5.97×10^3	-	-	-	-

*Times listed below are times during which effluent was collected and correlate to times on Fig. 11E.

indicative of the fact that the active component is a minor portion of the total MRP produced.

Not all the material from dialysis was run on the large G-50 columns which is why there is such a large difference in the amount of radioactivity between the two. Only 97 mg of the labeled arginine and 102 mg of the labeled xylose were run in their respective experiments. A much larger amount of radioactivity was lost from the arginine experiment at this stage than was lost from the xylose experiment. After separation on the large G-50 column, 40 mg of each was used for rechromatography on the small G-50 column. The arginine experiment lost considerably more radioactivity than the xylose experiment (see Table III) but in each there was an increase in radioactivity/mg.

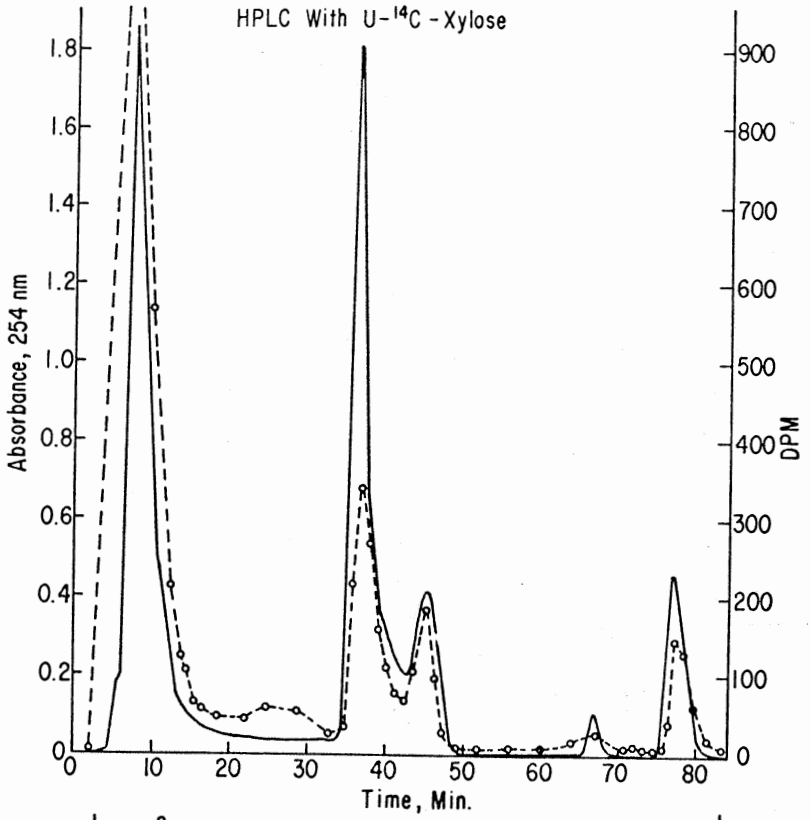
The active material from the small G-50 was then run on HPLC in two different ways. In one method, 2 ml aliquots were collected throughout an entire run. In the other method the rest of the active material from the small G-50 was run and the same sections were collected in each run. Table III shows the results from the second method while the results from the first method are shown in Figure 12. All results are consistent showing that the final active component isolated from HPLC is only approximately 7% of the material from the small G-50. Surprisingly, the first major peak (including all shoulders) contained 50% of the material from the small G-50 but had no antioxidative activity.

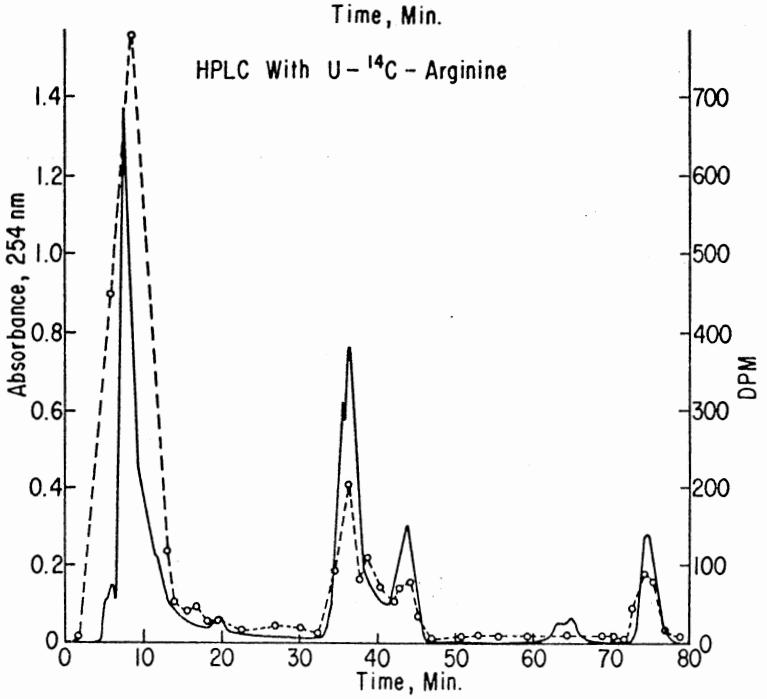
Timed Synthesis

A synthesis was done to monitor the changes in pH, color, and

Figure 12. Elution Patterns of HPLC of Radioactively Labeled Active Material from Small G-50 Column

The regular lines show absorbance at 254 nm and the dashed lines with circles show DPMs.


HPLC With U-14C - Xylose
Absorbance, 254 nm
DPM
Time, Min.
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
100
200
300
400
500
600
700
800
900
10
20
30
40
50
60
70
80


HPLC With U - 14C - Arginine
Absorbance, 254 nm
DPM
Time, Min.
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
100
200
300
400
500
600
700
10
20
30
40
50
60
70
80

antioxidative activity throughout the 20 hours of refluxing. The results shown in Figure 13 show that pH drops rapidly finally leveling out at 4.3. The activity of the undiluted material showed that the maximum amount of activity is reached after only 3 hours. The undiluted solution could have been too concentrated to show any further increase after 3 hours so a 1:10 dilution was made and the assay was run again. As can be seen, the results are the same as the undiluted solution showing the activity does not peak and go down during the 20 hours of synthesis. Not shown in Figure 13 is the increase in color intensity measured spectrophotometrically at 450 nm. There was noted a slow increase in color until a maximum was reached after 11 hours. Thus, colored compounds were still being made after the antioxidative activity had reached its maximum.

Infrared Spectroscopy

The IR spectrum shown in Figure 14 has approximately 7 major peaks. All assignments for peaks are based on comparisons to published IR correlation charts and published IR spectrum.

The first real peak in the near IR range is at approximately 3441 cm^{-1}. This is most likely due to N-H stretch and is more likely to be secondary rather than primary because a primary N-H would show a strong band around 1600-1650 cm^{-1} which is not present. The next peak is the set of three peaks from 2800-2900 cm^{-1}. These, or one or two of these, peaks are from C-H stretch. The set of two small peaks at about 2330 and 2360 cm^{-1} are due to water in the sample. The peak at 1657 cm^{-1} could be due to C=O stretch and probably is an aliphatic ketone rather than aromatic or an aldehyde. The peak is not as strong as most C=O

Figure 13. Change of pH and Antioxidative Activity During Synthesis of Arginine-Xylose Mixture

Line with triangles is change in pH, dashed line with squares is change in activity of undiluted material and lines with circles is change in activity of material diluted 1:10 with distilled H_2O. Six ml aliquots were taken every 15 minutes for the first hour, then every hour afterwards.

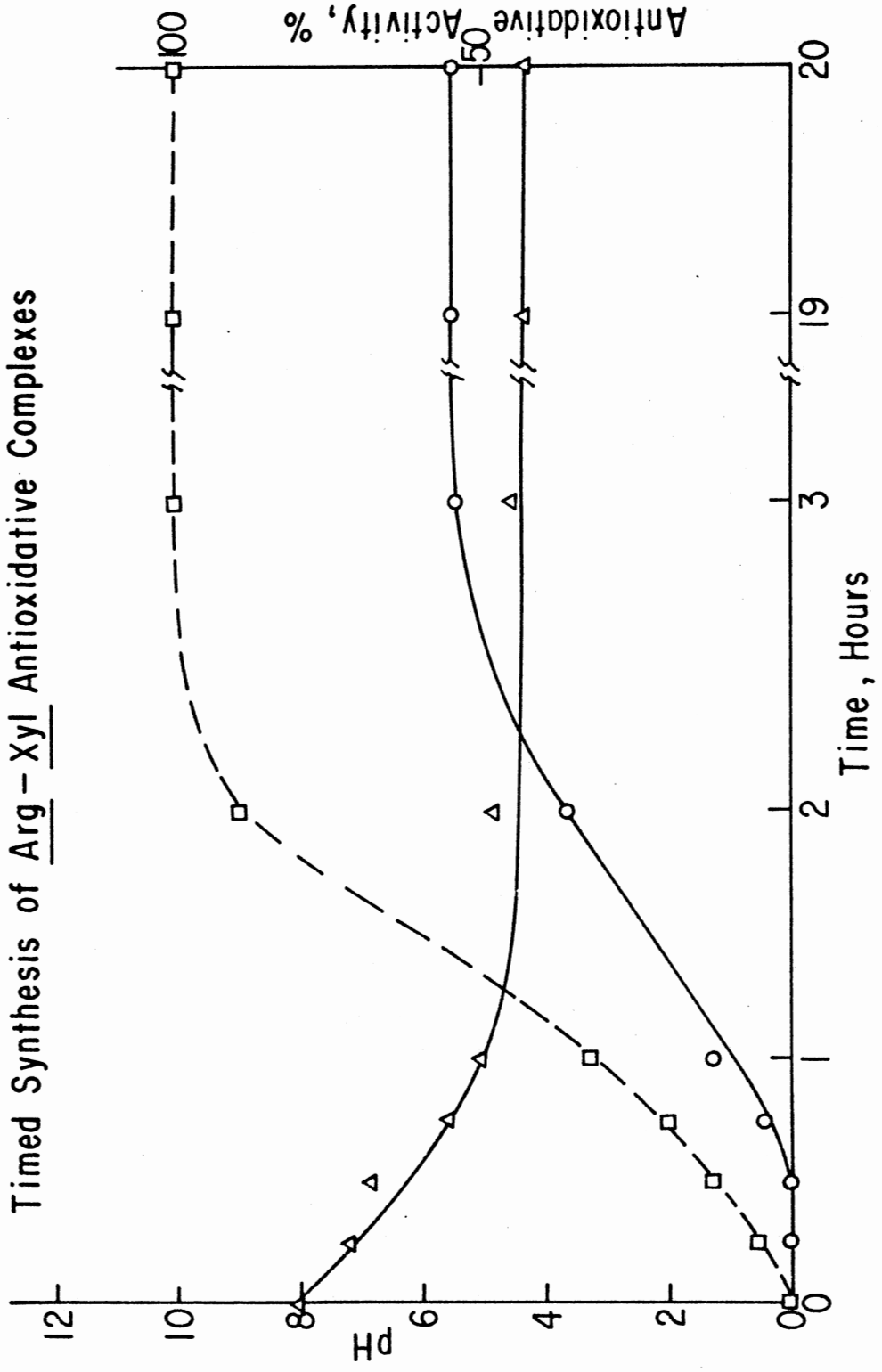
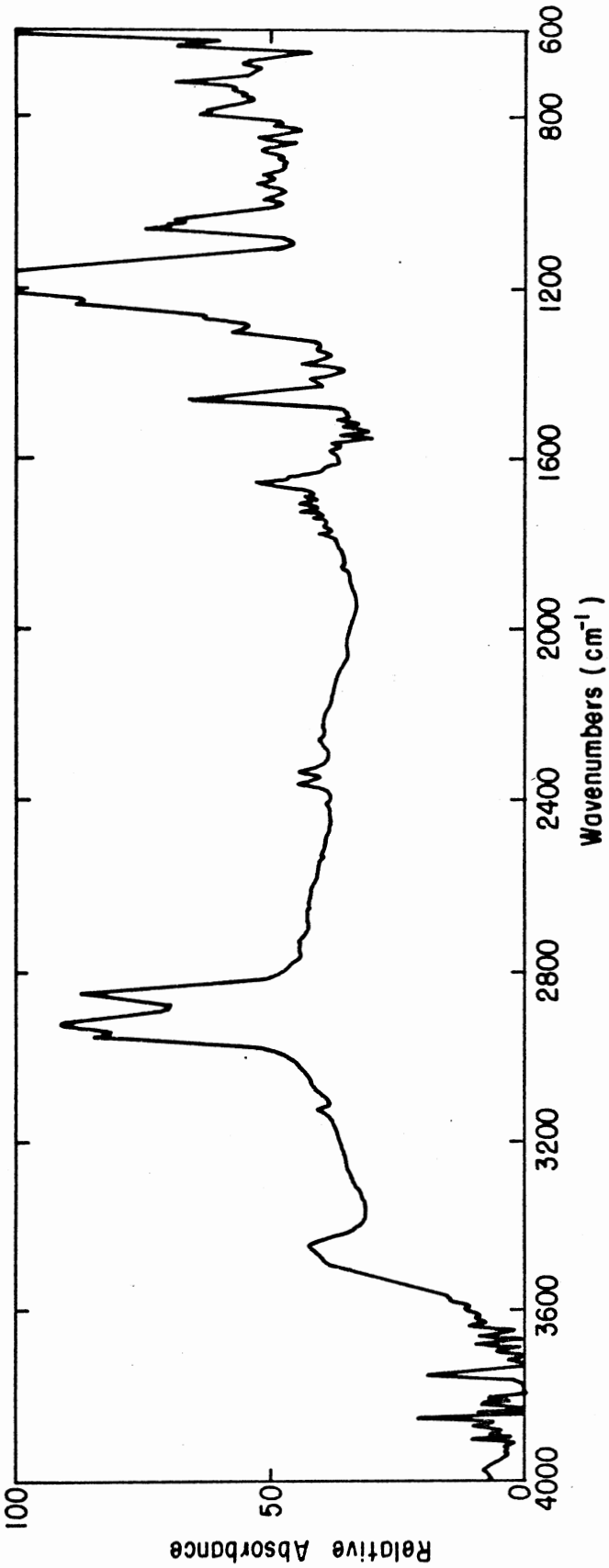

Timed Synthesis of Arg-Xyl Antioxidative Complexes
pH
Antioxidative Activity, %
Time, Hours
0
2
4
6
8
10
12
50
100
1
2
3
19
20

Figure 14. Infrared Spectrum of Active Component from HPLC

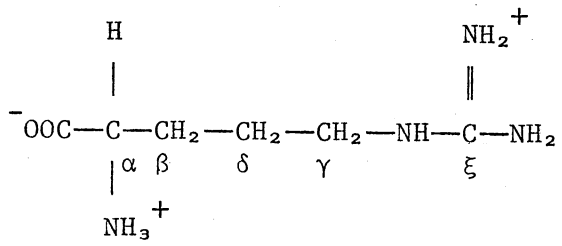

Relative Absorbance
100
50
0
4000
3600
3200
2800
2400
2000
1600
1200
800
600
Wavenumbers (cm⁻¹)

peaks in that area but aliphatic ketones show another medium peak around 1000-1100 cm^{-1} and the spectrum does have a peak at about 1064 cm^{-1}. The peak at approximately 1456 cm^{-1} could be due to C-H bend.

The strongest peak in the spectrum at about 1200 cm^{-1} could be due to either C-O or C-N stretch and since there is no other indication of either an ether linkage or a hydroxyl group in the spectrum, the peak is probably due to C-N stretch. The most prominent fact that can be derived from the spectrum is that there is no indication of any hydroxyl groups which usually when present show very strong peak at 3500 cm^{-1}. The fact was very surprising since one of the starting materials, namely xylose, contained four hydroxyls.

Nuclear Magnetic Resonance Spectroscopy

Figure 15 shows the ^{13}C NMR spectra of standard arginine, standard xylose, and the active component. As can be seen, the peaks in the active component spectrum are shifted upfield from either of the standard spectra and are very complex. There is no sign of the carbonyl carbon or ξ carbons of arginine in the active component which in the



standard arginine spectrum appeared at 176.9 and 156.2 ppm downfield from the deuterated-TSP marker, respectively. However, these peaks could be present but buried in the noise of the active component

Figure 15. ^{13}C NMR Spectra of Standard Arginine (Top), Standard Xylose (Middle) and Active Component from HPLC (Bottom)

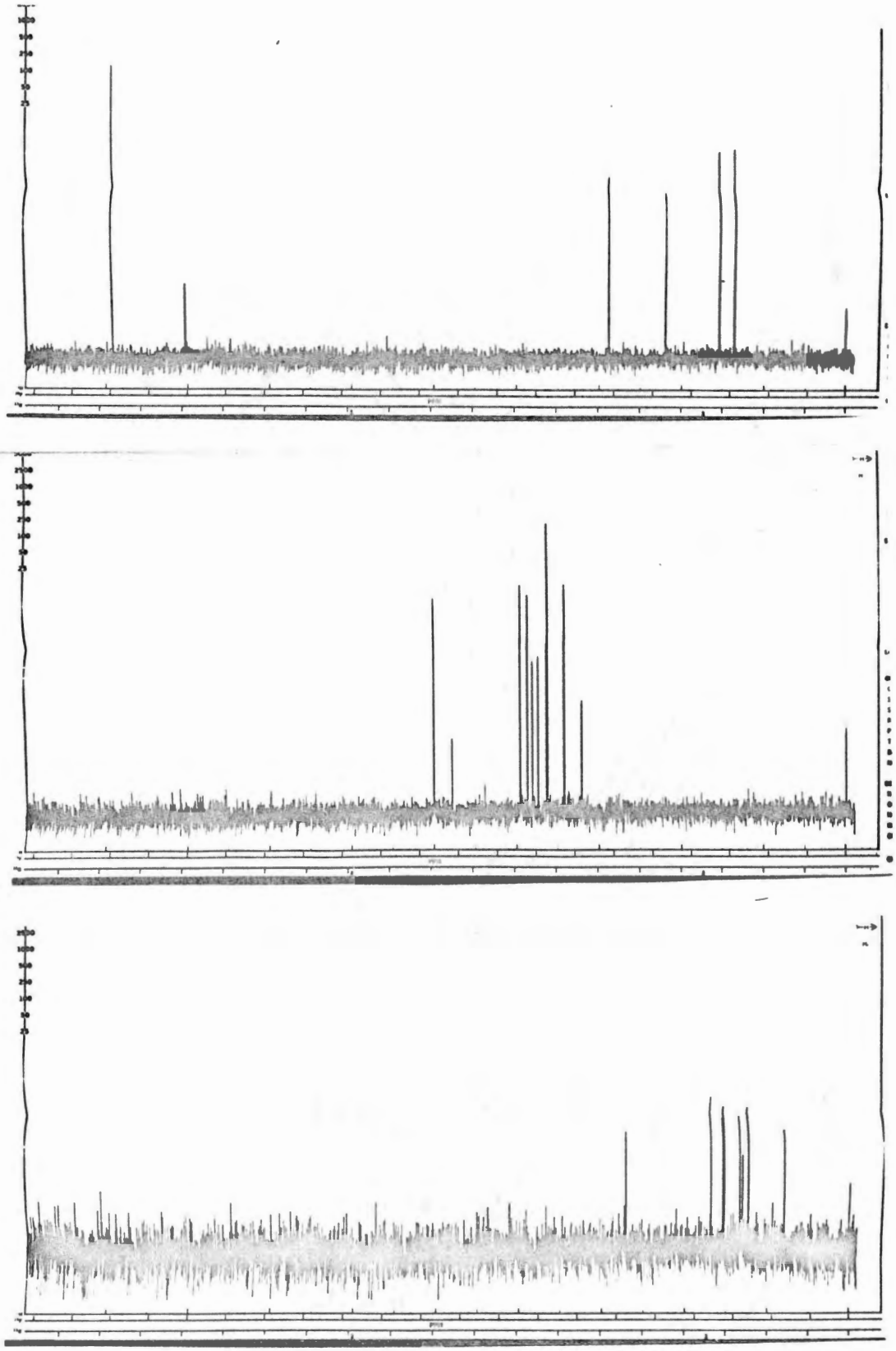
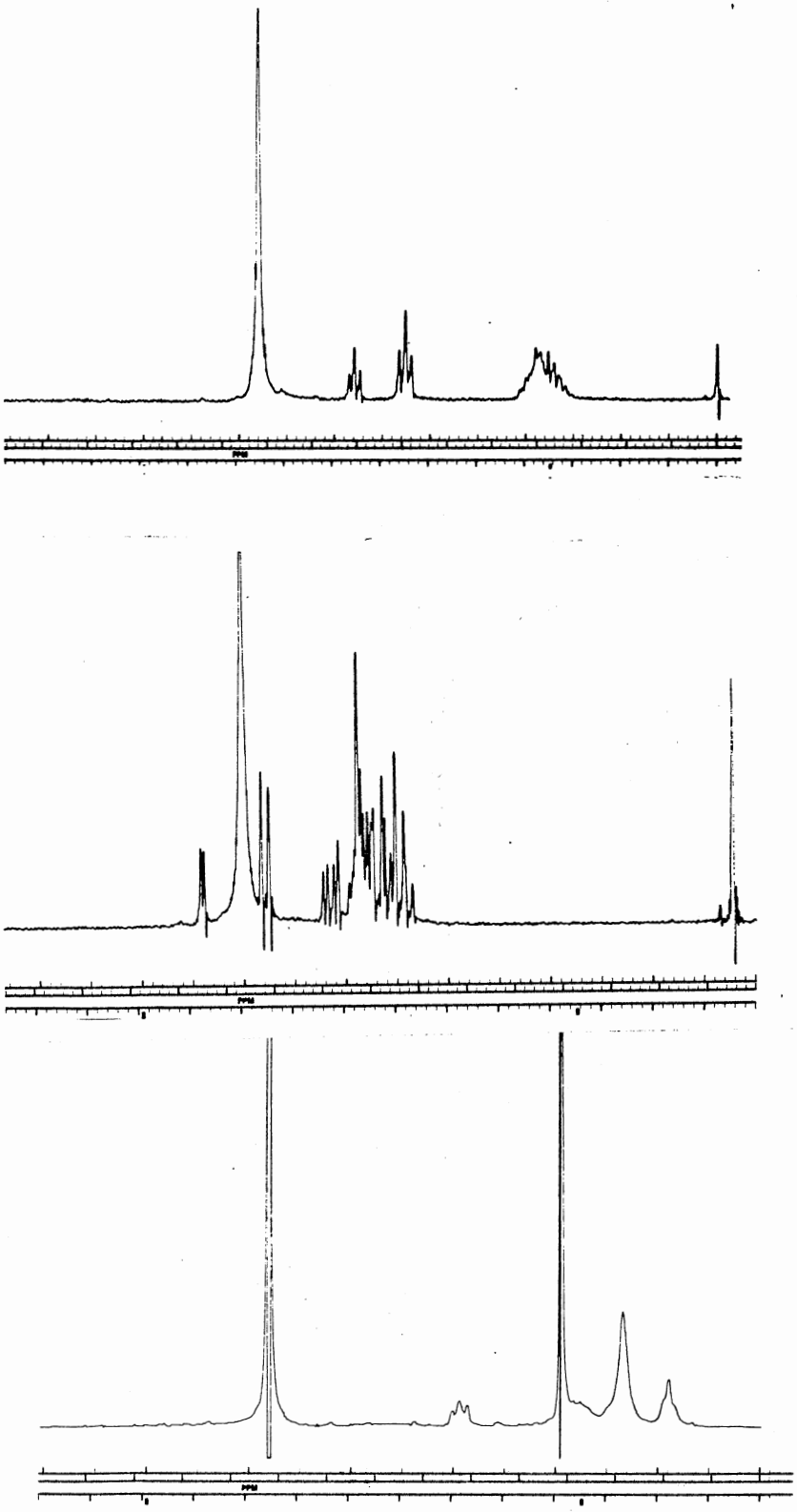


Figure 16. ^{1}H NMR Spectra of Standard Arginine (Top), Standard Xylose (Middle) and Active Component from HPLC (Bottom)



spectrum. Also, as with the IR analysis, there is no indication of hydroxyls on any of the carbons in the active component spectrum when comparing it to the standard xylose spectrum where thses carbons appeared between 78 and 63 ppm downfield from the deuterated-TSP marker. The proton NMR spectra shown in Figure 16 show similar results although no specific conclusions can be made form them since the active component spectra is not compared to the deuterated-TSP marker. One point that can be made from the ^{13}C spectra is that the active component shows a tremendous and significant change from the starting materials.

Ultraviolet Spectroscopy

The UV spectrum is shown in Figure 17. From the second derivative spectrum it can be seen that there are 6 peaks which are at 197, 212, 231, 247, 251, and 258 nm. The quanidinium group in arginine has a λ_{max} at 205 nm (arginine itself has a λ_{max} at 207 nm) and since there is no peak near 205 nm this suggests that the quanidinium group is absent in the active component. At this time it is not known what types of functional groups the peaks in the spectrum represents or if the spectrum indicates the presence of more than one compound in the active component.

Relative Potency of the Active Component

The antioxidative activity of the arginine-xylose active component was compared to that of the crude material to determine if isolation had increased or decreased the activity. The comparison shown in the

Figure 17. Ultraviolet Spectrum of Active Component from HPLC

Line A is regular UV spectrum and line B is second derivative curve.

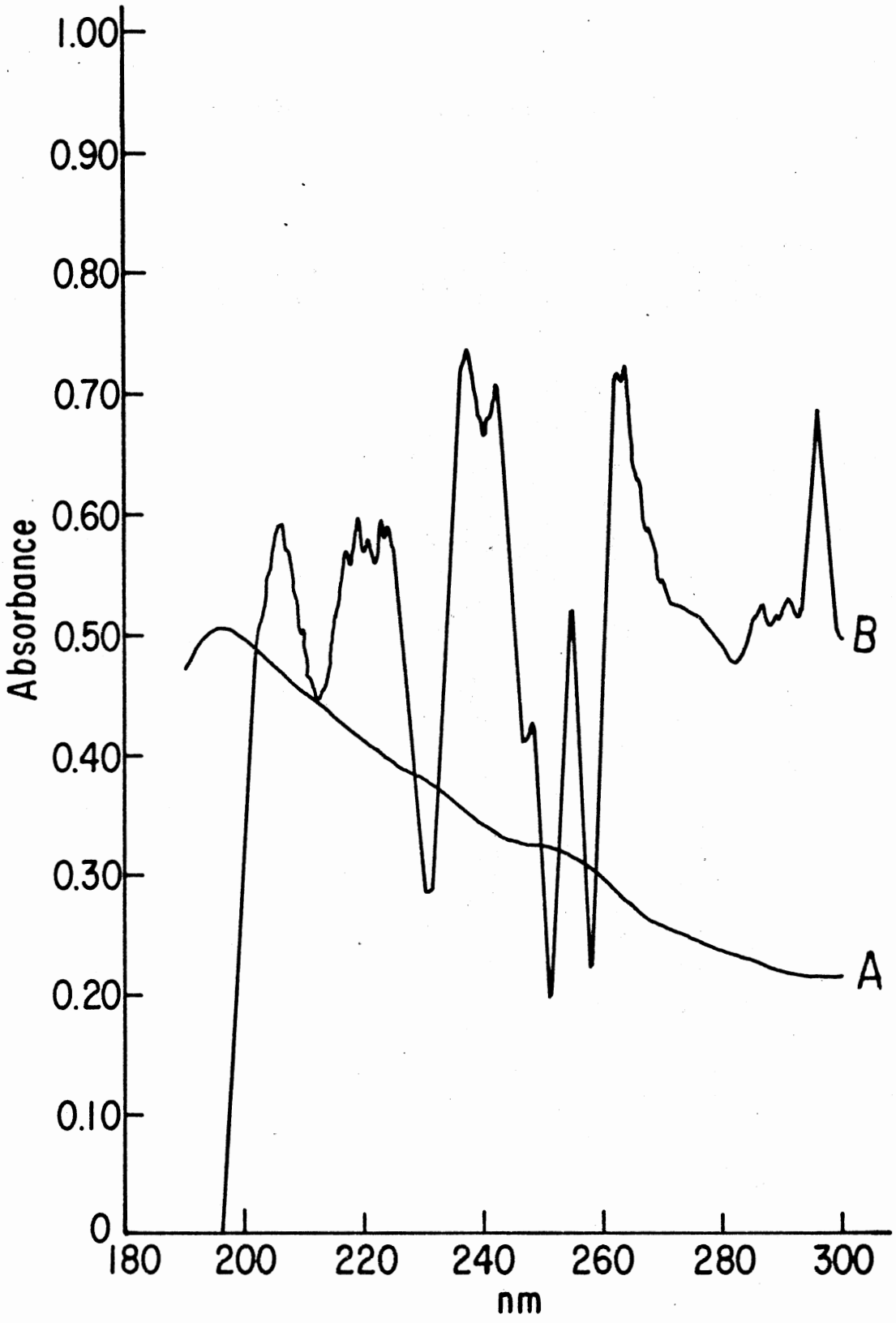
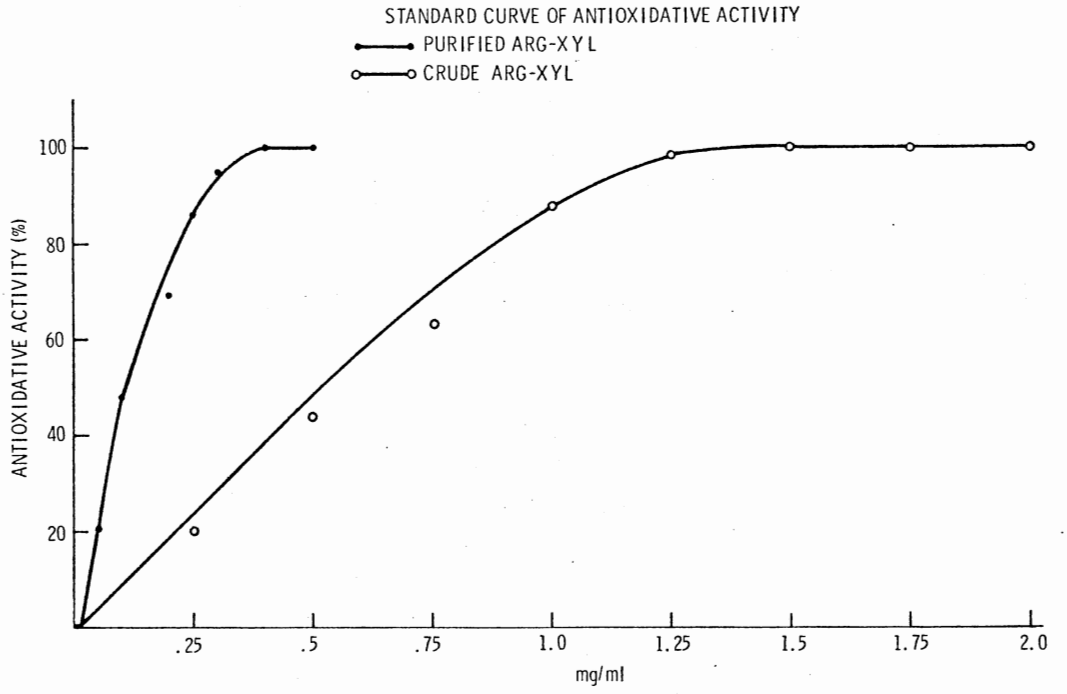
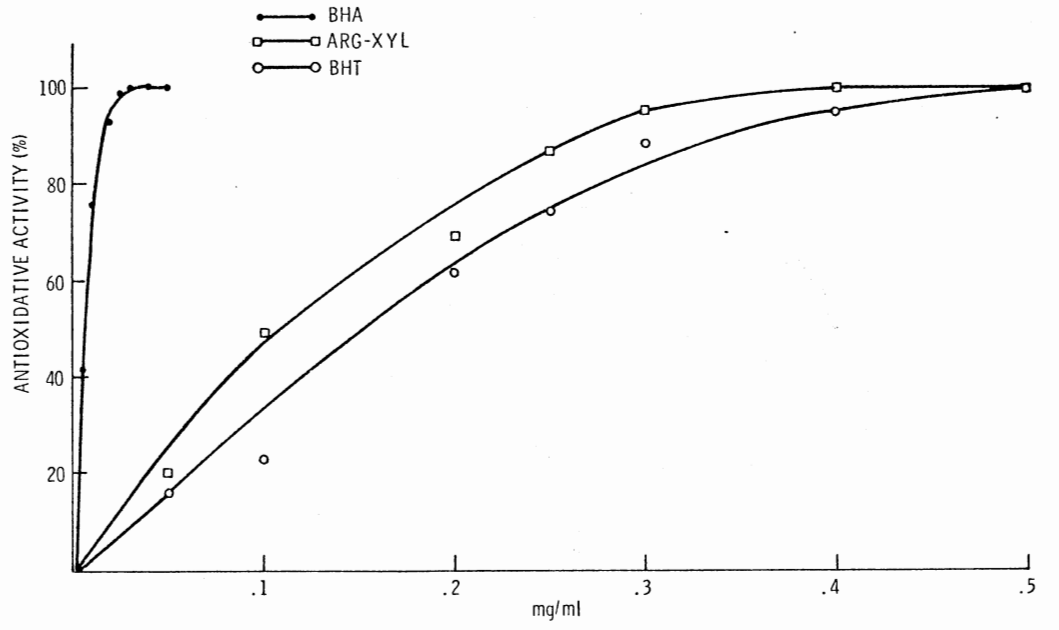

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
Absorbance
180
200
220
240
260
280
300
nm
A
B

Figure 18. Comparison of Antioxidative Activity of Active Component to Crude Material and to BHA and BHT


STANDARD CURVE OF ANTIOXIDATIVE ACTIVITY
PURIFIED ARG-XYL
CRUDE ARG-XYL
ANTIOXIDATIVE ACTIVITY (%)
100
80
60
40
20
.25
.5
.75
1.0
1.25
1.5
1.75
2.0
mg/ml


BHA
ARG-XYL
BHT
ANTIOXIDATIVE ACTIVITY (%)
100
80
60
40
20
.1
.2
.3
.4
.5
mg/ml

top graph of Figure 18, shows the isolated active component is over three times more potent than the crude material reaching 100% activity at only 0.4 mg/ml while 100% activity was not reached with the crude material until 1.25 mg/ml. In the comparison of the active component to BHA and BHT (bottom graph of Figure 18), it was forun to be about 10 fold less potent than BHA but 1.25 times as potent as BHT.

CHAPTER V

SUMMARY AND CONCLUSIONS

There has only been one other published report (37) that includes any mention of antioxidative activity in the arginine-xylose MRP. This is the first report to concentrate solely on the antioxidative aspects of the arginine-xylose MRP. An antioxidative component of considerable activity has been isolated from the total MRP produced and, although it is not yet completely purified and its structure is not known, it is known that it is significantly different than the starting materials arginine and xylose.

There has been much controversy about whether or not antioxidative activity in MRP is associated with the brown pigments. The arginine-xylose active component showed no indication of being associated with the brown pigments. The original reaction mixture is a deep dark brown color and much of this color is lost during dialysis. The crude material from dialysis is still dark brown but the material becomes lighter in color with each isolation step and the effluent from the active peak off the HPLC is only faintly yellow. The lyophilized material obtained from this effluent is an off-white color and would probably be white if the material was completely purified. Another major controversy is whether or not the antioxidative properties of MRP is associated with reductone compounds known to be produced in the mixture. However, the arginine-xylose active component was not tested to determine whether

it contained any reductones.

The molecular weight of the active component as determined by dialysis is in the same range as other published molecular weights for MRP (28, 41). However, the molecular weight was determined very crudely and could vary substantially upon more careful analysis. One indication of this is that the only postulated structures to account for some high molecular weights are polymers which include furan rings derived from the sugars. No evidence was found in the spectral studies to indicate the presence of a furan ring or any other type of aromatic compound.

In comparing the timed synthesis studies done using arginine and xylose to those done by Lingnert and Eriksson (37) using histidine and glucose, there is considerable variation. Whereas the pH of the arginine-xylose mixture dropped rapidly and leveled off after only 3 hours, the pH of the histidine-glucose mixture did not level off until about 20 hours. Also, there was no evidence that antioxidative activity dropped after reaching a maximum with the arginine-xylose as it did with the histidine-glucose, and the maximum activity was reached much sooner with the arginine-xylose.

Little was learned about the structure of the active component due to lack of quantity of material which prevented doing more than spectral studies which required only small amounts of material. The fact that the active component was such a small portion of the overall MRP produced was a surprising fact. This could indicate that it is a product reached after many reactions and that most of the reactants are tied up in initial products or that it is a product of a minor reaction in the overall Maillard reaction process. This, however, will only be

answered when the overall Maillard reaction is understood much better.

The only chemical study done was to see if the active component gives a ninhydrin test. Both the crude material from dialysis and the active component were tested and neither showed a positive ninhydrin test result. This indicates that there are probably no free amino groups in the active component and that the alpha amino groups from arginine must be blocked in either a peptide linkage with the carboxyl group of another arginine molecule or with a xylose in a C-N linkage with the aldehyde carbon.

Although final purification and structural identification of the actual compound or compounds possessing the antioxidative activity has not yet been accomplished, considerable knowledge of the active component has been obtained and final isolation and identification should be forthcoming.

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