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Received for review November 24, 1975. Accepted April 20, 1976. This work was supported by Grant ES-00159. This is the third of a series of papers dealing with zinc metabolism; see Murthy et al. (1974).

Determination of Chromium in Several Proposed Standard Samples and of Zinc and Chromium in Wheat Milling and Beet Sugar Refining Samples

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The following chromium concentrations have been determined for the indicated proposed biological standards (values in nanograms per gram are given in parentheses): NBS bovine liver (50.1), NBS/NIOSH freeze-dried urine (2.42), Doisy's serum (2.55), Bowen's standard kale (344), and Tascosa wheat coarse ground (22.8) and fine ground (45.1). The refining processes for flour and sugar beets were examined to determine the steps at which zinc and chromium were removed. Values of zinc and chromium were determined to be 116 and 2.15 ng/g in refined sugar, and 5180 and 33 ng/g in refined flour.

A growing awareness of the importance of trace elements in biochemical processes and in nutrition has stimulated interest in the determination of their concentration in biological materials. The biologically important trace elements include zinc, which is known to be an essential nutrient and a cofactor in numerous enzyme systems (Parisi and Vallee, 1969), and chromium, which has been suggested to be essential for proper carbohydrate metabolism (Mertz, 1975). Zinc is found in sufficient quantities, in most biological materials, that its determination poses no special problems (Christian and Feldman, 1970). On the other hand, while numerous methods are available for chromium analysis on a macro scale (Bachman and Banks, 1969), its determination in biological samples has been difficult and unreliable (Parr, 1974). Much of this difficulty is due to the fact that this element occurs in extremely low levels in most biological materials. The controversy over the nutritional essentiality of chromium and the questions concerning its mechanism of action are not likely to be resolved until accurate measurement of its concentration in such materials can be made. Since chromium occurs in biological materials in the range of nanograms per gram, accurate determinations were not possible until recent years. Even with the application of modern analytical methods, such as atomic absorption, gas chromatography, and neutron activation, very little agreement among researchers has been produced. The variation among reported values for chromium in human blood and its constituents has been discussed by Underwood (1971), and similar discrepancies have been noted in values obtained from the analysis of other biological and environmental samples (McClendon, 1974). One is, therefore, led to question much of the published data concerning chromium in biological materials.

The removal of trace elements during food processing may affect adversely the nutrition of much of the population of the industrialized nations (Mertz, 1972). Refined sugar and flour are two products from which trace elements, including zinc and chromium, are largely removed during processing (Schroeder, 1971). The implications of this removal may be rather far reaching, as these food products are consumed in considerable amounts by a large segment of the population.

In the present study, procedures for sample preparation and analysis by the method of flameless atomic absorption are reported, along with chromium values for several proposed reference materials of biological origin. In addition, the refining processes for flour and sugar beets were examined to determine the steps at which zinc and chromium were removed.

EXPERIMENTAL SECTION

Materials. The following reagent grade chemicals were used: HNO_3 (Mallinckrodt), $HClO_4$ (Baker and Adamson), HCl (J. T. Baker), 2 M tetramethylammonium hydroxide (TMAH) in methanol (Southwestern Analytical Chemicals), $ZnSO_4$, and K_2CrO_4. All water used was doubly distilled and deionized. The following proposed biological reference standards were obtained from the sources indicated: bovine liver (National Bureau of Standards, SRM 1577), freeze-dried urine (NBS/NIOSH, supplied by J. O. Pierce, University of Missouri, Columbia, Mo., 1974), Doisy's serum (J. R. Doisy, State University of New York at Syracuse, 1974), Bowen's standard kale (H. J. M. Bowen, Reading University, Reading, Berks., U. K., 1974), and

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Tascosa wheat of two size fractions (E. Cary, U.S. Plant, Soil, and Nutrition Laboratory, Ithaca, N.Y., 1974). The wheat milling fractions were collected at Star Flour Mill, American Fork, Utah, and the sugar process samples were obtained at the Amalgamated Sugar Company Mini-Cassia plant near Rupert, Idaho.

Procedure. Digestion vessels, volumetric apparatus, polypropylene bottles for sample storage, and all other apparatus used in handling samples were cleaned in hot concentrated HNO_3 and rinsed 4 to 6 times with water. By this procedure, we were able to minimize zinc and chromium contamination. For example, in those cases involving wet digestion of samples, digest blanks of 6–7 ng/g of zinc and 3–4 ng/g of chromium were obtained. In the cases of those samples (sugar juices, urine) which were not digested prior to determination, blank values of 2 ng/g of zinc and 0.5 ng/g of chromium were found. Analysis of the digestion acids, HNO_3 and $HClO_4$, gave chromium values of 13.7 and 0.3 ng/g, respectively, indicating that the primary source of chromium contamination was the HNO_3.

Standard stock solutions containing 10^6 mg/g of zinc and chromium were prepared by dissolving $ZnSO_4$ and K_2CrO_4, respectively, in water. Working solutions were prepared daily by appropriate dilution of the stock solutions.

The solid samples (bovine liver, standard kale, Tascosa wheat, and solids from the sugarbeet process) were handled as follows. Samples of ≤1 g, wet weight, were weighed into polystyrene weighing trays and transferred to a 100-ml Kimax beaker. One to two milliliters of water, 3 ml of HNO_3, and 1 ml of $HClO_4$ per gram of sample were added, and the mixture was heated under the hood at about 70 °C with occasional swirling until brown NO_2 gas was no longer evolved. An additional 0.5 ml of HNO_3 was added where necessary to complete digestion. Care was taken to prevent reduction of volume below 1 ml, as fires and charring may result from dehydration of $HClO_4$. A sample which begins to char will clear soon after addition of 0.25–0.5 ml of HNO_3. After cooling, the digestion solutions were diluted to an exact volume (5 or 10 ml) with water, and stored in polypropylene sample bottles.

In order to confirm the reliability of the above wet ashing procedure, bovine liver samples were also digested in TMAH. After addition of 2 ml of water and 2 ml of methanolic TMAH, the samples were heated at about 70 °C with addition of sufficient water to keep the mixture fluid. Approximately 2 h was required for digestion of all solid material. The resulting coffee-colored solutions were diluted and stored as described above.

Wheat milling fractions were dry-ashed in oxygen plasma (Nielson, 1975). Aluminum foil boats were formed upon a plexiglas template, cleaned with cold HNO_3, and dried in the oven at 150 °C. Approximately 0.1–0.2-g samples were weighed into the boats, and ashed at 1000 mTorr oxygen pressure for 5 to 6 h. The residue was dissolved in 1.0 ml of 1.0 M HCl, diluted to 5.0 ml, and stored in polypropylene bottles. Serum samples were run as received. Freeze-dried urine was reconstituted in water and kept under refrigeration until used. The sugar process juices were diluted 1:10 (weight to volume) with water containing 1 ml of 1.0 M HCl per 10 ml of H_2O, and stored in polypropylene bottles. Sugar solutions were prepared by dissolving 1 g of the crystalline product in water, adding 1 ml of 1.0 M HCl, and diluting to 10 ml with water.

All samples were analyzed within 24 h after preparation using a Perkin-Elmer 305A atomic absorption spectrophotometer equipped with an HGA-2000 graphite furnace, and a Sargent-Welch Model SRF strip-chart recorder.

Table I. Chromium Concentrations in Proposed Standard Reference Materials

Material	Cr, ng/g[e]
Bovine liver[a]	50.1 ± 4.8
Freeze-dried urine[b]	2.42 ± 0.45
Doisy's serum[c]	2.55 ± 0.40
Bowen's kale[d]	344 ± 71
Coarse[d] ground	22.8 ± 6.5
Tascosa wheat, fine[d] ground	45.1 ± 3.7

[a] Average of 9 samples run 3 times each. [b] Average of 12 values. [c] Average of 3 samples run 3 times each. [d] Average of 6 samples run 3 times each. [e] ± values given as standard deviation.

Twenty-microliter aliquots of solution were introduced into the graphite furnace using an Eppendorf micropipet. The analysis sequence followed for zinc was: drying, 25 s at ca. 100 °C; charring, 30 s at ca. 500 °C; atomization, 10 s at ca. 2000 °C. Analysis for chromium followed the "double atomization" technique of Davidson and Secrest (1972) which was modified to give the following program: sample drying, 25 s at ca. 100 °C; charring, 20 s at ca. 1350 °C; first atomization, or second charring, 30 s at ca. 1600 °C; final atomization, 12 s at ca. 2700 °C. No interference was observed in the analysis of the samples for either Zn or Cr. In the flameless AA technique negligible absorption by Zn (2139 nm) or Cr (3579 nm) is observed at the wavelength of the other element. In addition, Zn is atomized at a lower temperature (2000 °C) compared to Cr (2700 °C). The procedure was checked frequently using calibration standards prepared from reagent grade $ZnSO_4$ and K_2CrO_4. Agreement within 6% was obtained when the method of standard additions was used to check the calibration standards.

RESULTS AND DISCUSSION

The lack of a suitable standard reference material for chromium in biological materials has been discussed earlier. Our results for several proposed standard reference materials are given in Table I. The value given for bovine liver is the average of results obtained from both acid and TMAH digest procedures. These materials are being analyzed concurrently in other laboratories, by a variety of analytical methods, with the hope of establishing a suitable reference standard for chromium in biological materials. The samples selected represent a wide variety of sample types. Both solid and liquid samples are included, and a wide range of chromium concentrations is indicated. It is intended that a variety of standards be available for matching with many different types of samples of unknown chromium content. Inquiries concerning the attempt to establish biological reference standards for chromium should be directed to Dr. J. O. Pierce, Director, Environmental Trace Substances Research Center, University of Missouri, Columbia, Mo. 65201.

Results have been reported for certain of these materials. Cary (E. E. Cary, personal communication, 1975), using atomic absorption (Cary and Olson, 1975), determined the chromium concentration of NBS bovine liver to be 80 ng/g while Pierce (J. O. Pierce, personal communication, 1975) reports a value of 44.9 ng/g determined by neutron activation analysis. The latter value falls just outside the standard deviation limits of our value, 50.1 ± 4.8 ng/g. Other workers at NBS (McClendon, 1974) report values determined by neutron activation analysis of 163 and 210 ng/g depending upon whether the sample was prepared by destructive or nondestructive methods, respectively. Widely divergent values ranging from 5 to 1600 ng/g of

Table II. Literature Values (ng/g) for Zinc and Chromium in Wheat and Flour

Reference	Wheat		Germ		Middlings		Tailings		Bran		Flour	
	Zn	Cr	Zn	Cr	Zn	Cr	Zn	Cr	Zn	Cr	Zn	Cr
Lorenz et al., 1974	26 100				27 000				64 000		4100	
Toepfer et al., 1973		280		230		380				380		230
Schroeder et al., 1970		50		70		60		70		70		30
Zook et al., 1970	24 000	380									6300	220
Schroeder, 1968	31 500	1750	133 000	1270	106 000	2220	3 600	150	100 000	2180	8900	230
Waggle et al., 1967	41 900		116 700		110 000		64 700		116 700		6400	
Czerniejewski et al., 1964	31 000		100 800		100 000		105 300		99 000		6400	
Present study, Table III	23 460	73	20 355	11	~7 000	36	20 810	289			5180	33

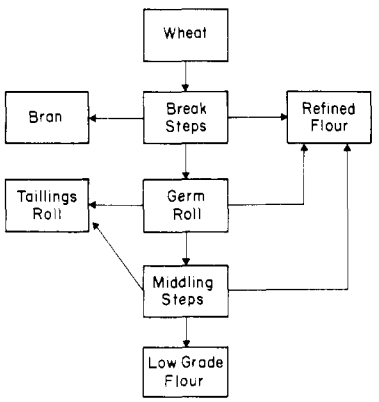

Figure 1. Flow diagram for wheat milling process.

Table III. Zinc and Chromium Concentrations in Wheat Milling Fractions

Sample[a]	Zn, ng/g[b,c]	Cr, ng/g[b,c]
Wheat kernel	23 460 ± 1580	73 ± 7
1st break	27 100 ± 5280	66 ± 12
2nd break	30 070 ± 4430	56 ± 16
3rd break	29 500 ± 2570	121 ± 33
4th break	81 885 ± 2450	310 ± 122
Germ roll	20 355 ± 4140	11 ± 2
1st middlings	8 570 ± 130	19 ± 3
2nd middlings	6 570 ± 270	14 ± 6
3rd middlings	7 330 ± 254	65 ± 2
4th middlings	8 380 ± 320	41 ± 9
Tailings roll	20 810 ± 1230	289 ± 58
Refined flour	5 180 ± 140	33 ± 9

[a] Sample descriptions are given in the text. [b] Average of two samples run three times each. [c] ± values given as standard deviation.

chromium have been reported upon analysis by a variety of methods (Parr, 1974). This lack of agreement in the case of bovine liver reemphasizes the need for careful work on these standard materials. The Cr concentration of freeze-dried urine determined by neutron activation was reported to be 26 ng/g (Gills et al., 1974), significantly higher than our value of 2.42. The suggested value of 2.5 ng/g for Doisy's serum (R. J. Doisy, personal communication, 1975) agrees well with our value of 2.55. Our value for Cr in Bowen's Kale, 344 ng/g, is in good agreement with those reported earlier, 330 ng/g (Bowen, 1969) and 370 ng/g (Cary and Olson, 1975).

Several studies have been conducted to compare trace element levels in wheat milling fractions and flour. Some of the values found in the literature for zinc (Lorenz et al., 1974; Zook et al., 1970; Schroeder, 1968; Waggle et al., 1967; Czerniejewski et al., 1964) and chromium (Zook et al., 1970; Schroeder, 1968; Toepfer et al., 1973; Schroeder et al., 1970) in hard wheats and their products together with related results from the present study are shown in Table II. Our results for zinc in wheat and flour agree well with those of other workers. Our values for several milling fractions fall well below most of those reported, but a good deal of variation may be noted among published values. Handling and milling procedures could contribute to differences in the trace element content of the samples taken; however, it is also probable that significant variations in trace element levels exist among wheats grown in different localities, due to differences in soil mineral composition. Variations in Cr content of 3 to 43 ng/g have been reported (Welch and Cary) for hard red wheat seed samples (both spring and winter types) grown on a variety of soil types throughout the midwestern United States.

The zinc and chromium contents of wheat milling fractions are given in Table III. A schematic representation of the milling procedure is given in Figure 1. The break steps represent successive grinds of the wheat, between which refined flour is sifted out and collected. After the fourth "break," the hulls and coarse material, or bran, are removed and used as animal feed. After each break, larger particles of the wheat kernel, cleaned of bran, go to the germ roll, where they are distributed to the middling steps according to particle size. Flour is sifted off after each grinding step. The dross from the germ roll and middlings, which represent successively finer grinds, is collected in the tailings roll and used for animal feed. The data in Table III show that zinc and chromium are found in greatest amounts in the hulls and coarse outer parts of the wheat. This finding agrees well with other data concerning trace element distribution in the wheat kernel (Morris et al., 1945).

It is nutritionally significant that white flour, which is a staple in the diet of the bulk of the population in industrial nations, has been largely depleted of chromium, zinc, and presumably other essential trace elements. The increased zinc and chromium concentrations in successive break steps (Table III) could result from elemental concentration in the hulls and coarse material or from the stainless steel grinding equipment or a combination of these. The amounts of trace elements added to the refined flour as a result of the grinding process are unknown. It has been reported that stainless steel grinding equipment can measurably increase the chromium concentration of ground materials (Cary and Allaway, 1971). Although little is known about the form in which trace elements are found in foods, their addition during the grinding process could result in their being present in a less nutritionally available form.

Published data on trace elements in sugar are less extensive than those cited for wheat. Carpenter and Bichsel (1969) have investigated sugar process juices for several trace elements, but did not include zinc or chromium. Reported values for zinc in refined sugar range from 200 ng/g (Schroeder, 1971) to 1120 ng/g (Mee and Hilton, 1969), our value, 116 ng/g, being closest to the lower of these. Chromium values for refined sugar are found to be even more divergent. Published values include: 80 ng/g (Schroeder, 1968), 20–30 ng/g (Schroeder, 1969), 150–300 ng/g for various sugar samples (Schroeder et al., 1970), 20

Table IV. Zinc and Chromium Concentrations in Sugar Process Samples

Sample[a]	Zn, ng/g[b,c]	Cr, ng/g[b,c]
Raw water	99 ± 9	8 ± 0.4
Raw sugarbeet	689 ± 70	11 ± 1.5
Cossettes	1194 ± 62	129 ± 12.2
Pressed pulp	1615 ± 117	312 ± 37.1
Diffusion juice	857 ± 140	90 ± 14.5
1st carbonation	413 ± 13	37 ± 3.0
2nd carbonation	307 ± 45	13 ± 1.4
Carbonation sludge	3878 ± 110	1955 ± 170.6
Thin juice	336 ± 35	9 ± 2.3
Thick juice	1426 ± 126	39 ± 4.1
Standard liquor	957 ± 70	68 ± 9.6
Refined sugar	116 ± 6	2 ± 0.3
Intermediate green syrup	4097 ± 336	301 ± 30.5
Ion exchange effluent	3400 ± 967	310 ± 16.2
Molasses	7657 ± 742	1641 ± 146.0
Molasses pulp	7893 ± 183	1280 ± 79.6

[a] Sample descriptions are given in the text. [b] Average of two samples run three times each. [c] ± values given as standard deviation.

ng/g (Toepfer et al., 1973), and 7–20 ng/g, depending upon the method of preparation (Wolf et al., 1974). Our value for chromium in refined beet sugar, 2 ng/g, is markedly lower than most of those cited. It is of interest that, with the exception of the most recent study (Wolf et al., 1974), standard Cr samples were not available to these workers for calibration of their equipment, and the atomic absorption equipment used in several of the Cr analyses was of the flame rather than the flameless type.

In their recent work Wolf and coworkers (1974) analyzed refined and other sugar samples by flameless atomic absorption both after ashing in oxygen plasma and by direct introduction into the graphite furnace. They report chromium values of 20 and 7 ng/g for refined sugar, 64 and 31 ng/g for brown sugar, and 266 and 29 ng/g for molasses after analysis by these two methods, respectively. These researchers suggest that a considerable amount of the naturally occurring chromium in sugar samples is lost before analysis when such samples are injected directly into the graphite furnace. They cite the markedly lower values for all samples as well as the similarity between values obtained by direct analysis of brown sugar and molasses as evidence. They suggest, therefore, preashing in oxygen plasma to circumvent this difficulty. We report chromium concentrations of 2 and 1641 ng/g, respectively, in sugar and molasses, and a sample of brown sugar analyzed in our laboratory had 355 ng/g of chromium. While our value for refined sugar is low by comparison, we found a high chromium concentration in molasses, comparable to the value of 1210 ng/g reported by Schroeder (Schroeder, 1971), and an intermediate level in brown sugar, as would be expected. These data plus the predictable increase in chromium through the series of sugar process juices, discussed below, indicate that we did not encounter this loss. Our value for brown sugar is higher than that reported by Wolf and associates, but these authors have noted an expected difference in brown sugar chromium levels, dependent upon production and handling procedures (Wolf et al., 1974). Our value for beet molasses, a crude and unpalatable preparation, cannot be compared to their results for household molasses.

The zinc and chromium concentrations of sugar process samples are given in Table IV. The washed sugarbeets are sliced into cossettes, and the sugar is removed by countercurrent diffusion resulting in diffusion juice. The remaining solids are pressed and dried giving pressed pulp. Addition to the diffusion juice of an aqueous suspension of $Ca(OH)_2$ and CO_2 to precipitate $CaCO_3$ constitutes the carbonation steps, where much of the zinc and chromium is removed. Carbonation sludge is removed at this point. Following treatment with SO_2 to decolorize the product, the thin juice is concentrated fivefold to give thick juice. Low grade sugar is next added to give standard liquor, from which white refined sugar is crystallized after low-pressure boiling. The syrup which is spun off after crystallization is again boiled and seeded to promote crystallization. The spin-off, or intermediate green syrup, is treated with cation exchange resin, which removes a substantial amount of the zinc present and then boiled and seeded for a third crystallization. The second and third crystallization steps provide low grade sugar for enrichment of the thick juice. The effluent syrup from the third crystallization is molasses from which no more sugar can be crystallized economically. Molasses is added to the dried pressed pulp giving molasses pulp, a useful livestock feed. While a reduction in the concentrations of chromium and zinc is seen at carbonation, and of zinc at ion exchange, high levels of both metals are seen in the molasses. This indicates that the process of crystal formation is primarily responsible for the low levels of trace elements found in the highly purified white sugar. As in the case of wheat grinding, contact with stainless steel in operations such as beet slicing may contribute measurable chromium and possibly zinc. The trace element content of sugarbeets undoubtedly varies with the soil conditions from field to field. The raw water supply, sometimes taken from various sources of available ground water, may contribute varying amounts of trace elements. These factors, plus the heterogeneous nature of individual juice batches, being a mixture of primary flow, added low grade sugars, and recycled batches, complicate greatly the calculation of the actual amounts of zinc and chromium being processed at a given operation, or through the plant as a whole. However, the end product contains very little zinc or chromium.

ACKNOWLEDGMENT

The authors wish to thank Steven B. Larsen for his technical assistance.

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Received for review September 11, 1975. Accepted February 17, 1976.

Formation of Free-Radical Products by the Reaction of Dehydroascorbic Acid with Amino Acids

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Short time heating of a mixture of dehydroascorbic acid with amino acid in water or ethanol gives fairly stable radical products. The ESR spectrum obtained in ethanol was composed of two kinds of spectra, a triplet (spectrum A) and a quintet–doublet (spectrum B), while that obtained in the aqueous system was composed of spectrum A and another multiplet (spectrum C). The radical products were separately detected on TLC, where that of spectrum A (R-A) was in a blue spot and that of spectrum C (R-C) was in the vicinity of the red pigment. Based on the chemical and spectral properties of these radical products, the structures and mechanism of formation of them are discussed in relation to those of the red pigment.

The browning reaction of dehydroascorbic acid (DHA) with amino acid is well known as it develops a wine red color and causes a deterioration in the quality of some foods. There have been a number of studies on this color development; recently the structure and the mechanism of formation of the red pigment have been proposed (Kurata et al., 1973; Ranganna and Setty, 1974). Additionally, there have been some studies on the development of free-radical products in the reaction of amino compounds with carbonyl compounds relating to ascorbic acid, for example, the stable radicals in melanoidin as the browning reaction products from glucose and glycine (Mitsuda et al., 1965), the unstable free radicals in the reaction of ninhydrin with amino compounds (Orr, 1965; Yeferov et al., 1970), and a radical intermediate in a redox reaction between ascorbic acid and dehydroascorbic acid (Laroff et al., 1972).

Recently, the authors have found the development of free radicals in various amino–carbonyl reactions. Sugar–amino acid or amine systems gave unstable free-radical products in an early stage of the reaction (Namiki and Hayashi, 1975). On the other hand, the reactions of DHA with various amino acids or amines have been found to provide fairly stable free radicals of a different type (Namiki et al., 1974; Yano et al., 1974); this paper is concerned with the details of the formation and isolation of the radical products from DHA and amino acids, and moreover with some speculation on their structure and formation mechanism, in relation to those of the red pigment.

MATERIALS AND METHODS

DHA was prepared from L-ascorbic acid (AsA) using *p*-benzoquinone as an oxidizing reagent (Euler and Hasselquist, 1954; Müller-Mulat, 1970). Barium 2,3-diketogulonate was prepared from DHA (Kenyon and Munro, 1948), and the free acid was obtained by desalting with Dowex 50-W. AsA, amino acids, and other reagents used were guaranteed grade.

The reactions of DHA with amino acids were done in Pyrex test tubes using distilled water or purified 95% ethanol as a medium, and the ESR spectrum was measured in a quartz tube with a JES-ME-1X ESR spectrometer. The splitting constant and g value of the ESR spectrum were determined by means of potassium peroxylamine disulfonate as a standard. Since the ESR signal is recorded as the first derivative, the concentrations of free-radical products were measured for convenience as the intensity relative to the standard, polycrystalline Mn^{2+}. Thin-layer chromatography (TLC) was performed on microcrystalline cellulose (Avicel) with ethyl acetate–pyridine–water (7–10:4:3) as solvent. Ninhydrin and 2,4-dinitrophenylhydrazine were used for visualization of colorless substances. The densitometry was performed with a Shimazu dual-wavelength TLC scanner CS-900. Uv and visible spectra of solutions were measured with a Hitachi EPS-3T spectrometer.

RESULTS AND DISCUSSION

Development of ESR Spectra of the Reaction Mixtures of DHA and Amino Acids. A mixture of DHA and α-Ala (each 1 M) in 95% ethanol was heated in a boiling water bath. The reaction mixture turned pink immediately, then wine red, and gradually brown red with further heating. Simultaneously, the characteristic ESR signal developed and increased rapidly at an early stage of the reaction (Figure 1a); prolonged heating caused little change in the shape of the spectrum.

The same reaction in an aqueous system gave similar changes in the development of color and ESR signal, although both proceeded far more quickly than those in

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