Muscle Membranal Lipid Peroxidation by an "Iron Redox Cycle" System: Initiation by Oxy Radicals and Site-Specific Mechanism

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KTBA (2-keto-4-(mercaptomethyl)butyric acid) degradation by iron–ascorbate or –cysteine generated ethylene. The generation of ethylene was enhanced by iron–ascorbate, especially in the presence of EDTA. As expected for a reaction involving hydroxyl radicals (HO·), ethylene production was inhibited by several HO· scavengers. The generation of ethylene from KTBA was also inhibited by superoxide dismutase (SOD) and catalase. Muscle membranal lipid peroxidation was stimulated by iron–ascorbate or iron–cysteine. Keeping iron constant, lipid peroxidation was found to be dependent on the concentration of ascorbate or cysteine. Lipid peroxidation was inhibited only slightly by HO· scavengers, and SOD or catalase failed to inhibit the reaction. However, lipid peroxidation was efficiently inhibited by antioxidants such as ascorbyl palmitate or BHT. EDTA, which enhanced the generation of the hydroxyl radical, inhibited almost totally the stimulation of membranal lipid peroxidation.

INTRODUCTION

Transition metals that are biologically ubiquitous with their labile d-electron system are well suited to catalyze redox reactions. These metals have a range of accessible oxidation states enabling them to transfer electrons. The redox potential for such a transfer can be varied by alteration of ligand-type geometry (Waters, 1971; Koppenol, 1978, 1983). Stable paramagnetic states, due to the presence of unpaired electrons, are common for transition metals, facilitating reaction with radical substances. They are thus able to react directly with oxygen and to remove the spin restriction between polyunsaturated fatty acid (PUFA) and oxygen, promoting lipid peroxidation.

Biological oxidation is due almost exclusively to metal ion promoted reactions, of which iron is the most abundant (Aisen and Liskowsky, 1980; Harrison and Hoare, 1980). A small pool of nonprotein-bound iron moving among transferrin, cell cytoplasm, mitochondria, and ferritin could provide "free iron" at concentrations of micromoles per liter (Gutteridge et al., 1981, 1982). Most recently we found in chicken and turkey muscle tissues a high amount of free iron (Hazan and Kanner, 1985).

Ferrous salts in aerobic aqueous solution produce superoxide anion, hydrogen peroxide, and hydroxyl radicals (Cohen and Sinet, 1980) by the following reactions:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-}\cdot \quad (1)$$

$$2O_2^{-}\cdot + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow HO\cdot + HO^- + Fe^{3+} \quad (3)$$

Addition of EDTA greatly accelerates the autoxidation of ferrous ion and hydroxyl radical generation (Cohen and Sinet, 1980; McCord and Day, 1978; Halliwell, 1978). The powerful oxidizing capability and extreme kinetic reactivity of the hydroxyl radical toward PUFA and other organic substances are well documented.

Ascorbic acids and cysteine are known reducing compounds present in biological tissues. They reduce very rapidly ferric to ferrous ions, producing a "redox cycle" (Winterbourn, 1979). In fact, reaction 3 is Fenton's reagent (Fenton, 1894), and the first redox cycling of Fenton's reagent was developed by Udenfriend et al. (1954) using ascorbic acid, metal ions, oxygen, hydrogen peroxide and EDTA for hydroxylation of aromatic substances.

It has long been known that iron can catalyze peroxidation and that this can be stimulated by the presence, in addition, of thiols and ascorbic acid (Ottolenghi, 1959; Wills, 1965; Kanner et al., 1977; Kanner, 1979). However, only recently has the involvement of hydroxyl radicals in these reactions been posulated, along with its contribution to the initiation of lipid peroxidation (Borg et al., 1978; Tien et al., 1982; Searle and Willson, 1983).

Ferrous ions can stimulate PUFA peroxidation by generating the hydroxyl radical from hydrogen peroxide but also by decomposing preformed lipid peroxides (LOOH) to form the alkoxyl (LO·) radical (eq 4–7).

$$LH + HO\cdot \rightarrow L\cdot + H_2O \quad (4)$$

$$L\cdot + O_2 \xrightarrow{LH} LOOH + L\cdot \quad (5)$$

$$LOOH + Fe^{2+} \rightarrow LO\cdot + HO^- + Fe^{3+} \quad (6)$$

$$LH + LO\cdot \rightarrow L\cdot + LOH \quad (7)$$

With regard to lipid peroxidation in muscle tissues, one of the most important questions concerns the source of the primary catalysts that initiate peroxidation in the muscle food in situ. Several authors suggest that such lipid peroxidation is stimulated by enzymic (Hultin, 1980) and nonenzymic reactions. Data have been presented suggesting that nonheme iron compounds are the major prooxidants in raw (Rhee et al., 1979) and cooked meat (Sato and Hegarty, 1971; Love and Pearson, 1974; Igene et al., 1979). Some researchers have reported that heme proteins seem to be the main prooxidants in muscle tissues (Younathan and Watts, 1959; Lee et al., 1975; Koizumi et al., 1976; Fisher and Deng, 1977), while others concluded that both heme and nonheme iron compounds are important (Wills, 1966; Kwoh, 1970; Lin, 1970; Lin and Watts, 1970).

Careful analysis of the data on lipid peroxidation in muscle tissues suggests that many of the results deal with the stimulation of hydroperoxide breakdown (propagation and branching) and not with the initiation step.

Most of the studies on the mechanisms of lipid peroxidation have been conducted in oils or lipid emulsions (Logani and Davies, 1980). Mechanisms established in those systems may differ from those in organized cells, subcellular organelles, and tissues. Model systems containing microsomes from fresh liver (Hochstein and Ernster, 1963; Koster and Slee, 1980; Tien et al., 1982) or muscle tissues (Hultin, 1980; Kanner and Harel, 1985) were

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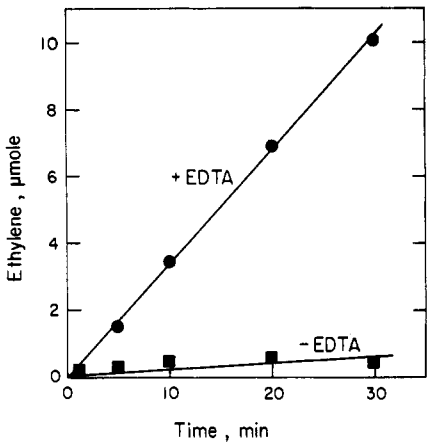

Figure 1. Ethylene generation from KTBA degradation by iron–ascorbate with and without EDTA: ■, Fe^{3+} (10 μM) + ascorbate (200 μM); ●, Fe^{3+} (10 μM) + ascorbate (200 μM) + EDTA (100 μM).

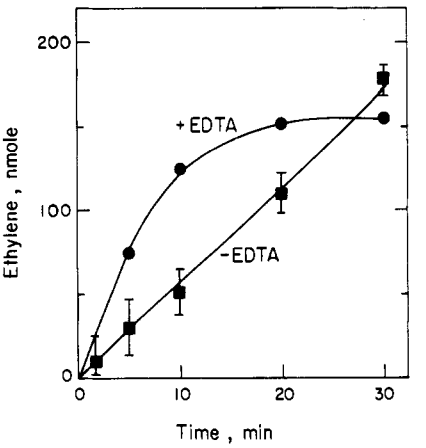

Figure 2. Ethylene generation from KTBA degradation by iron–cysteine with and without EDTA: ■, Fe^{3+} (10 μM) + cysteine (200 μM); ●, Fe^{3+} (10 μM) + cysteine (200 μM) + EDTA (100 μM).

used to study the process of initiation of lipid peroxidation. In the present study, we investigated the involvement of hydroxyl radicals produced by an "iron redox cycle" system in the initiation of muscle membranal lipid peroxidation. This research was conducted in order to extend our knowledge of the initiation and stimulation of lipid peroxidation in muscle tissues.

MATERIALS AND METHODS

Ascorbic acid and ascorbyl palmitate were purchased from Merck (Darmstadt, West Germany); cysteine, 2-keto-4-(mercaptomethyl)butyric acid (KTBA), *dl*-α-tocopherol, L-histidine, and thiobarbituric acid from Sigma Chemical Co. (St. Louis, MO); and dimethyl sulfoxide (Me_2SO), D-mannitol, and ferric chloride from BDH Chemicals Ltd. (Poole, England). Degradation of KTBA, 1 mM (in 50 mM acetate buffer, pH 7.0) to ethylene was conducted in a glass tube closed with a serum cap lined with Teflon and incubated in a shaking bath at 25 °C. The total volume of the gas phase and the liquid phase in each ampule was measured. The ethylene production in the reaction was identified with the use of a 6-ft Prapak Q column and a flame ionization detector in a Packard gas chromatograph. The amount of ethylene was calculated from a standard sample.

Isolation of the microsomal fraction from muscle tissues was done by a procedure described previously (Apgar and Hultin, 1982; Kanner and Harel, 1985).

Protein determinations were conducted by the modified Lowry procedure (Markwell et al., 1978), using BSA as standard.

Microsomes for lipid peroxidation assay were incubated in air in a shaking water bath at 37 °C. The reaction mixture contained 1 mg of microsomal proteins/mL and 4 mL of 50 mM acetate buffer, pH 7.0.

Thiobarbituric acid reactive substances were determined by a procedure of Bidlack et al. (1973). The results are reported as nanomoles of malondialdehyde (MDA) per milligram of protein, using a molar extinction coefficient of $\epsilon_{532} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

The accumulation of conjugated dienes produced during lipid peroxidation was monitored by the increase in A_{233} (Buege and Aust, 1978).

Results are the means of triplicates, and in the figures each error bar (I) denotes the standard deviation.

RESULTS

The incubation of KTBA in the presence of ascorbate or cysteine with ferric ion generated ethylene. Generation of ethylene was enhanced by the addition of EDTA, especially in the presence of ascorbate (Figures 1 and 2). As expected for a reaction involving HO· radicals, ethylene production was inhibited by several hydroxyl radical scavengers. Ethanol, Me_2SO, formate, and histidine were found to be very efficient in scavenging HO· and preventing KTBA decomposition to ethylene. The generation of ethylene from KTBA was also inhibited by superoxide dismutase and catalase and partially by bovine serum albumin or microsomes (Tables I and II).

Table I. Effect of Several Enzymes, Proteins, and Hydroxyl Radical Scavengers on the Generation of Ethylene by Ferric Chloride, Ascorbate, and Cysteine

treatment	ethylene, μmol/30 min	% inhibn
control (Fe^{3+} 10 μM; ascorbate 200 μM)	0.43	
control + microsomes (1 mg protein/mL)	0.27	37.2
control + superoxide dismutase (200 units)	0.11	74.4
control + catalase (200 units)	0.01	97.7
control + mannitol (100 mM)	0.13	69.8
control + ethanol (400 mM)	0.08	81.4
control (Fe^{3+} 10 μM; cysteine 200 μM)	0.19	
control + catalase (200 units)	0.04	90.7
control + mannitol (100 mM)	0.03	93.0

Table II. Effect of Several Enzymes, Proteins, and Hydroxyl Radical Scavengers on the Generation of Ethylene by Ferric Chloride, Ascorbate, and EDTA

treatment	ethylene, μmol/30 min	% inhibn
control (Fe^{3+} 10 μM; ascorbate 200 μM; EDTA 100 μM)	10.00	
control + microsome (1 mg protein/mL)	7.89	21.1
control + bovine serum albumin (0.5 mg/mL)	8.57	14.3
control + superoxide dismutase[a] (200 units)	5.68	43.1
control + catalase[a] (200 units)	0.19	98.1
control + L-histidine (25 mM)	1.35	86.5
control + Na formate (50 mM)	1.43	85.7
control + Me_2SO (200 mM)	0.14	98.5
control + ethanol (200 mM)	0.35	96.4

[a] Autoclaved SOD and catalase gave results similar to control.

Lipid peroxidation was determined by the accumulation of thiobarbituric acid reactive compounds (TBA-RS as malondialdehyde) and conjugated dienes. A high correlation was achieved between the accumulation of TBA-RS and conjugated dienes. The amount of TBA-RS and conjugated dienes that accumulated in the model system

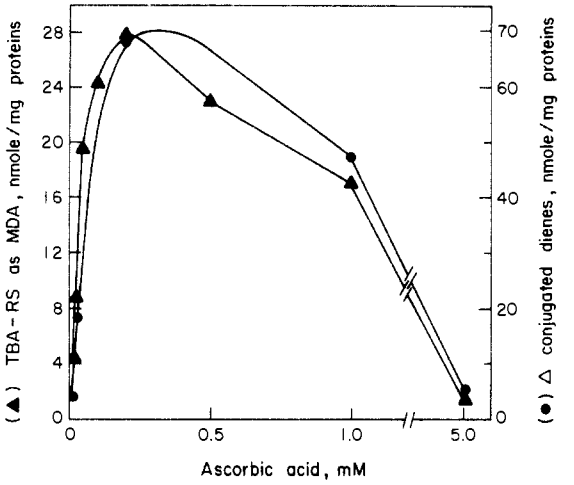

Figure 3. Membranal lipid peroxidation as affected by ascorbate concentration (Fe^{3+} constant—10 μM).

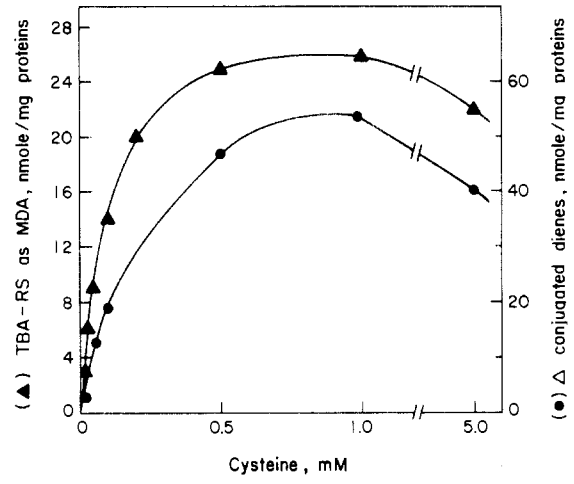

Figure 4. Membranal lipid peroxidation as affected by cysteine concentration (Fe^{3+} constant—10 μM).

containing ferric chloride and microsomes was found to be dependent on the concentration of ascorbic acid or cysteine. Maximal TBA-RS was accumulated at a concentration of 200 μM ascorbate or 1.0 mM cysteine. A high concentration of either compound inhibits the production of TBA-RS and conjugated dienes (Figures 3 and 4).

Membranal lipid peroxidation dependent on the reaction of the iron–ascorbate couple was found to be only very slightly partially inhibited by hydroxyl radical scavengers such as Me_2SO and histidine. Catalase and SOD failed to inhibit iron–ascorbate-dependent membranal lipid peroxidation. However, lipid peroxidation was inhibited efficiently by antioxidants such as ascorbyl palmitate and butylated hydroxytoluene. EDTA, which enhanced the generation of the hydroxyl radicals, inhibited almost totally the stimulation of membranal lipid peroxidation (Table III).

Table III. Membranal Lipid Peroxidation Stimulated by Iron–Ascorbic Acid as Affected by Several Hydroxyl Radical Scavengers and Antioxidants

treatment	TBA-RS as MDA, nmol/mg protein (20 min)	% inhibn
Fe^{3+} (10 μM)	1.6	
control (Fe^{3+} 10 μM; ascorbate 200 μM)	31.5 ± 0.6	
control + D-mannitol (100 mM)	32.5	0
control + L-histidine (25 mM)	28.0	11.0
control + ethanol (400 mM)	30.0	4.8
control + Me_2SO (200 mM)	29.7	5.7
control + α-tocopherol (25 μM)	27.1	14.1
control + BHT (25 μM)	1.1	96.4
control + ascorbyl palmitate (25 μM)	0.6	98.0
control + EDTA (100 μM)	1.3	95.9

DISCUSSION

Ascorbic acid and thiols are, respectively, strong two- or one-electron reducing agents, which are readily oxidized in one-electron steps by metal ions and metal complexes in their higher valence states. Reduced iron in the presence of oxygen generates superoxide anions (Cohen and Sinet, 1980) that are further reduced by dismutation to hydrogen peroxide. The involvement of superoxide and hydrogen peroxide in the reaction between ascorbic acid and ferric chloride was demonstrated by the inhibition of KTBA oxidation and ethylene generation with superoxide dismutase or catalase. It seems that superoxide dismutase inhibited the generation of HO· and KTBA oxidation by reducing the interaction of ascorbic acid semiquinone with O_2^- and the production of hydrogen peroxide by almost 50%. The rate of this reaction was found to be 2.6×10^8 M^{-1} s^{-1} (Bielski, 1983) (reaction 8). The importance of

$$A^-\cdot + O_2^-\cdot \xrightarrow{2H^+} A + H_2O_2 \quad (8)$$

$$O_2^-\cdot + O_2^-\cdot \xrightarrow[2H]{SOD} H_2O_2 + O_2 \quad (9)$$

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad (10)$$

A^- = semiquinone radical; A = dehydroascorbic acid

H_2O_2 as the precursor of HO· is evidenced by the fact that catalase inhibited iron–ascorbate or EDTA–iron–ascorbate stimulation of KTBA oxidation (reaction 10). Hydroxyl radical scavengers are substances with a high reactivity with the hydroxyl radical, the rate constants generally being above 10^9 M^{-1} s^{-1}. The oxidation of "target" molecules (PUFA, DNA, nucleosides, deoxy sugars) is often prevented by scavengers such as mannitol, formate, benzoate, thiourea, Me_2SO, and ethanol (Anbar and Neta, 1976; Czapski, 1984).

The oxidation of KTBA to ethylene was inhibited by hydroxyl radical scavengers, which indicates the production of hydroxyl radicals during the reduction of iron ions by ascorbate or cysteine in the presence of oxygen. EDTA was found to change only slightly the rate of ascorbic acid oxidation (results not shown). However, it enhanced the generation of HO· radicals by almost 2/-fold. It is known that the redox potential of the Fe^{3+}/Fe^{2+} pair can vary by complexing ligands (Schwarzenbach and Heller, 1951; Bottari and Anderegg, 1976; Richter and Waddell, 1983). EDTA reduces the redox potential of Fe^{2+} (Koppenol 1978), and this increases the rate constant transfer of the electron from Fe^{2+} to H_2O_2 and decomposition of the latter to HO·.

Iron in the presence of ascorbic acid stimulated membranal lipid peroxidation. EDTA, which in the presence of iron–ascorbate stimulated hydroxyl radical dependent KTBA oxidation, prevented membranal lipid peroxidation. When the concentration of EDTA exceeds that of iron ions, as in our model system, lipid peroxidation is substantially inhibited (Wills, 1965; Tien et al., 1982; Gutteridge, 1984). It seems that EDTA in excess deactivates the iron ion by surrounding it with tightly bound ligands that cannot be replaced by reagents such as hydroperoxides (Waters, 1971). However, EDTA in excess did not prevent the interaction of iron with ascorbate or the decomposition of hydrogen peroxide to hydroxyl radical: on the contrary,

it enhanced this reaction. Hydroxyl radicals that were generated by ferrous–EDTA in the presence of H_2O_2 could initiate lipid peroxidation (Gutteridge, 1984). In our model system, EDTA in excess seemed to prevent iron penetration through the membrane.

EDTA could remove iron from low molecular target biological complexes (Gutteridge et al., 1981). Hydroxyl radicals are generated in the presence of EDTA–iron–ascorbate in the exogenous environment of the membrane. The constant rate reaction of the hydroxyl radicals with most substances is about 10^8–10^9 M^{-1} s^{-1}. For this reason, the radical will not move far away from its site of formation. Microsomes inhibited almost 30% of the KTBA oxidation by the EDTA–iron–ascorbate system. It seems that this inhibition is derived from the reaction of hydroxyl radicals with exogenous membranal proteins and not with unsaturated fatty acids. Our results are in agreement with other observations (Hodgson and Fridovich, 1975; Oberley and Buettner, 1979; Samuni et al., 1981) that a site-specific or site-directed mechanism is important for hydroxyl radicals attack of target molecules.

Gutteridge (1984) attempted to distinguish between the damage done to deoxy sugars by HO· formed in "free" solution and that by a site-specific mechanism. The results suggest that, in the absence of EDTA, iron ions bind to the carbohydrate target molecules and bring about a site-specific reaction on the carbohydrate target molecules. This reaction is poorly inhibited by most HO· scavengers. In the presence of EDTA, however, iron is removed from these binding sites to produce HO· in free solution. Even at a high concentration, these can be readily intercepted by the addition of HO· scavengers. In our model system, HO· scavengers prevented KTBA oxidation by iron–ascorbate also in the absence of EDTA, but they failed to prevent lipid peroxidation. The physical state of membrane lipids seems to affect the penetration of HO· scavengers into the membrane and their effectiveness in preventing lipid peroxidation (Girotti and Thomas, 1984). For the same reason, exogenously added SOD and catalase are not expected to have any effect on the prevention of lipid peroxidation induced by the iron–ascorbate system.

Summing up our data, it seems that iron–ascorbate-dependent membranal peroxidation was derived from the attack of a very small amount of HO· radicals or ferryl species formed at the site of the unsaturated fatty acids double bounds, uninhabitable by HO· scavengers.

Ferrous ions formed during the oxidation of ascorbic acid stimulate peroxidation by catalyzing the decomposition of preformed hydroperoxides to alkoxyl radicals (LO·) that can initiate lipid peroxidation (Kanner et al., 1977; Gutteridge, 1984). The greatest stimulation of dependent iron–ascorbate membranal peroxidation was derived from the decomposition of hydroperoxides, which are very efficiently inhibited by antioxidants such as ascorbyl palmitate or butylated hydroxytoluene. Hydroxyl radicals formed in the exogenous microsomal environment do not have the capability to initiate membranal lipid peroxidation. The special arrangement of the membrane constituents provides it with a structural antioxidant capability that prevents spontaneous lipid peroxidation by exogenous species.

According to this study, relatively stable, nontoxic compounds like ascorbic acid, thiols, and "free iron", in contrast to reactive radicals such as HO·, would be able to diffuse into the membrane and thus be channeled to sites where they could be converted to highly active compounds.

Registry No. KTBA, 583-92-6; ascorbic acid, 50-81-7; cysteine, 52-90-4; iron, 7439-89-6; ethylene, 74-85-1; hydroxyl, 3352-57-6.

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Determination of Fluoride in Foods

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With the development of modern analytical methods and techniques for fluoride analysis the acid-diffusible (ionic plus acid-labile) and total fluorine content of a food can be reliably determined. The difference between these values represents nonionic form(s) not released under conditions of acid diffusion. Higher amounts of fluorine were generally found with the fluoride ion specific electrode when ashed foods were analyzed employing the open-ashing–heat-facilitated diffusion method or the oxygen bomb–reverse extraction technique for isolation of the fluoride than when acid-labile fluoride was isolated from an unashed sample by heat- or silicone-facilitated diffusion. Similar results were obtained by both diffusion techniques. The simplest method for isolation of acid labile fluoride is by silicone-facilitated diffusion. Recovery of added fluoride by all methods and techniques was, in most instances, excellent.

The development of modern reliable analytical methods and techniques for fluoride analyses has made the determination of the acid-diffusible (ionic plus acid-labile) fluoride and total fluorine in foods possible. Nonionic fluorine is defined as the difference between the total fluorine and acid-diffusible fluoride. The reliability of some results previously obtained with routine fluoride analytical methods for foods has been questioned (Dabeka et al., 1979; Singer and Ophaug, 1979; Singer et al., 1980; Taves, 1979). There is evidence that methods based on the separation of fluoride from an unashed sample by acidic diffusion and determination of the isolated fluoride with some colorimetric methods may yield erroneous results. The use of a colorimetric reagent (American Conference of Governmental Industrial Hygienists, 1969) for the analysis of fluoride in diffusates from unashed commercially prepared infant foods by Dabeka et al. (1979) suggested that the fluoride concentration found was significantly lower when the same diffusates were analyzed with the fluoride ion specific electrode. Much higher results have been obtained for the analyses of fluoride in some foods with the eriochrome cyanine R colorimetric method (Megregian, 1954) than when the fluoride was determined with the fluoride ion specific electrode (Singer and Ophaug, 1979; Singer et al., 1980; Taves, 1979). The difference was not attributable to fluoride in any form.

Recognizing the problems encountered in determination of fluoride in foods, Dabeka et al. (1979) attempted to develop a simple, accurate, routine method for fluoride determination in unashed infant foods based on the isolation of the fluoride from the sample by microdiffusion from perchloric acid (47 °C for 16 h) and the subsequent measurement of the ion in the diffusate with the fluoride ion specific electrode. Their relative standard deviations varied from 4 to 20% from day to day. A collaborative study of 12 laboratories using the method described by Dabeka et al. (1979) with an added freeze-drying step demonstrated an interlaboratory mean coefficient of variation of 21.6% (Dabeka and McKenzie, 1981). They suggested the elimination of this added step to overcome some of the variations. Attempts to apply a routine physical method based on the $^{18}F(P,P'\partial)^{19}F$ reaction have not been promising (Shroy et al., 1982).

There obviously is need for documentation of the reliability of analytical procedures for the determination of fluoride and total fluorine in foods. The present investigation was undertaken to determine whether simple rapid methods not requiring specialized equipment could be recommended for the accurate determination of various forms of fluoride in foods and for determining how much of the total fluorine is nonionic.

PROCEDURES

The U.S. Food and Drug Administration (FDA) has maintained a program of Market Basket food collections of young males 15–19 years of age in order to estimate the dietary intake of certain metals and to monitor pesticide residues in the food chain. Between 1975 and 1982 these collections were comprised of 117 food items placed into composite food groups (Tables II and III) by the FDA based on the relative quantities of each item found in the average diet of the young male living in the United States (U.S. Food and Drug Administration Compliance Program Guidance Manual, 1977). Appropriate samples of 11 composites from six Market Baskets collected prior to 1982 were analyzed by four fluoride techniques. These techniques are contrasted in Table I. Foods may be ashed prior to analysis for fluoride to convert some or all nonionic fluorine to the ionic form, which is detected with the fluoride ion specific electrode. Some fluorocarbons may be lost during open ashing because of their volatility (Venkateswarlu, 1975A), and total fluorine in the sample, for this reason, may be underestimated with these procedures. There is some evidence that such fluorocarbons are

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