# The Chemistry of Lipid Peroxidation Metabolites: Crosslinking Reactions of Malondialdehyde

**Vasu Nair*, Curt S. Cooper, David E. Vietti** and **Gregory A. Turner**
*Department of Chemistry, University of Iowa, Iowa City, IA 52242*

**Malondialdehyde reacts readily with amino acids to form adducts containing vinylogous amidine linkages. Crosslinking reactions between nucleic acid bases and amino acids induced by malondialdehyde also have been investigated. The physical data obtained for the adducts provide structural information on the possible mode of crosslinking of proteins and nucleic acids induced by this lipid metabolite.**
***Lipids 21,* 6-10 (1986).**

The ubiquitous natural compound malondialdehyde (MDA) is produced in substantial quantity in mammalian tissues both as an end product of unsaturated lipid peroxidation and as a side product of prostaglandin and thromboxane biosynthesis (1-4). It also is produced in the $\gamma$-irradiation of carbohydrates (5). The measurement of MDA by the thiobarbituric acid test has been used commonly as a method for the detection of peroxidation of unsaturated fatty acids and in the estimation of oxidative rancidity in foods (2,3,6). We have reported recently on a detailed and unambiguous assignment of the structure of this adduct (7). MDA has been reported to be toxic (8,9), carcinogenic (8,10) and mutagenic (10-12). This reported degenerative chemistry of MDA may be the result of its ability to covalently bond and to crosslink a variety of biological macromolecules. For example, MDA is reactive towards nucleic acids, resulting in the loss of their template activity (13,14). Also, it has been suggested that MDA-induced modification of lipoproteins may play a role in atherosclerosis (15-17). Valuable information on both the reactive sites and the structural nature of modification can be obtained through investigation of model systems that represent the vulnerable components. We have shown previously that MDA reacts rapidly at the $\alpha$-amino group of amino acids to form 1:1 adducts (18), and that reaction occurs with adenine and cytosine bases to form hypermodified products (19). This paper reports some model crosslinking reactions of MDA.

## MATERIALS AND METHODS

Melting points are uncorrected and were determined on a Thomas-Hoover melting point apparatus fitted with a microscope. The $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker WM-360 or on a JEOL FX-90Q pulse Fourier transform nuclear magnetic resonance (NMR) spectrometer. Mass spectra were determined on a Hewlett-Packard 5985 gas chromatography/mass spectroscopy (GC/MS) instrument. Ultraviolet data were obtained on a Varian-Cary Model 219 UV-Visible spectrophotometer. Fluorescence spectra were recorded on an SLM-Aminco SPF-500C instrument. Elemental analyses were performed by Galbraith Laboratories. Amino acid derivatives and adenine were purchased from Sigma Chemical Co. (St. Louis, Missouri). MDA *bis*-dimethylacetal was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin) and 2-methyl-3-ethoxyprop-2-enal from Fluka Chemical Co. (Hauppauge, New York). 9-Ethyladenine was prepared as described previously (20).

Sodium MDA was prepared from MDA *bis*-dimethylacetal as described previously (18), except that after hydrolysis the reaction mixture was basified to pH 10 before work up.

Sodium methylmalondialdehyde (sodium MMDA) was prepared from commercial 2-methyl-3-ethoxyprop-2-enal (18).

*General procedure for formation of 2:1 adducts from amino acids and MDA and MMDA.* In an oven-dried flask with a condenser and a nitrogen bubbler were placed the amino acid methyl ester hydrochloride (2.0 mmol) and sodium MDA or sodium MMDA (1.0 mmol) in dry methanol (10 ml). The reaction mixture was heated under reflux in a nitrogen atmosphere and the progress of reaction was followed by UV spectroscopy. The solvent was removed under reduced pressure and the residue was triturated with 20 ml of 20% $CH_3OH/CH_2Cl_2$. The precipitated NaCl was removed by filtration and the filtrate was concentrated. The resulting residue was crystallized from $CH_3OH/CH_2Cl_2$. All adducts gave satisfactory elemental analyses.

*Preparation of 9-ethyladenine enaminal* **6**. 9-Ethyladenine hydrochloride (1.170 g, 5.85 mmol) in dry methanol (65 ml), was treated with sodium MDA (0.657 g, 5.87 mmol). The mixture was heated (60 C under $N_2$) and progress of reaction was monitored by UV spectroscopy (19). The reaction was terminated when the 322 nm to 260 nm peak ratio had maximized (about 15 hr). The solvent was removed under reduced pressure and the residue was chromatographed on silica gel plates using 8% $CH_3OH/CH_2Cl_2$ as the eluent. The band at $R_f$ 0.48 afforded 0.162 g (13%) of **6** as yellow crystals: mp 148-149 C; UV (EtOH) $\lambda$ max 322 nm ($\epsilon$ 42114), 243 nm ($\epsilon$ 8810), 223 nm ($\epsilon$ 10934); $^1H$ NMR ($Me_2SO$-$d_6$) $\delta$ 11.30 (d, 1H, J = 11.5 Hz), 9.41 (d, 1H, J = 8.7 Hz), 8.77 (dd, 1H, J = 11.5, 13.4 Hz), 8.55 (s, 1H), 8.52 (s, 1H), 5.98 (dd, 1H, J = 8.7, 13.4 Hz), 4.28 (q, 2H), 1.45 (t, 3H); $^{13}C$ NMR ($Me_2SO$-$d_6$) $\delta$ 191.2, 151.6, 151.3, 149.1, 148.9, 143.6, 120.2, 38.4, 15.0; mass spectrum, m/z (relative intensity) 217 ($M^+$, 11.3), 189 ($M^+$ - CHO, 20.6), 188 (100), 160 (29.4), 148 (4.8), 135 (10.3), 120 (purine, 4.2), 119 (10.7). Anal. Calcd. for $C_{10}H_{11}N_5O$: C, 55.29; H, 5.10; N, 32.24. Found: C, 55.64; H, 5.27; N, 31.97.

*Reaction of 9-ethyladenine enaminal* **6** *with glycine methyl ester hydrochloride.* In an oven-dried flask fitted with a condenser and nitrogen bubbler was placed glycine methyl ester hydrochloride (0.093 g, 0.74 mmol) in dry methanol (22 ml). To this was added 9-ethyladenine enaminal (0.160 g, 0.74 mmol) and 3Å molecular sieves. The reaction mixture was heated under reflux with stirring and under a nitrogen atmosphere. Aliquots were removed

*To whom correspondence should be addressed.

periodically for kinetic analysis by UV spectral methods. Isolation of **7** in a completely pure form was difficult because of its instability, but strong evidence for its formation came from UV, mass spectral and high-field $^1$H NMR data.

## RESULTS AND DISCUSSION

Crosslinking of proteins by MDA has been reported to occur within 24 hr of reaction (21-23). However, characterization of the products of such modification remains incomplete. The investigation of the structural details of crosslinking of amino acid residues induced by MDA therefore was undertaken as models of protein modification by this natural multifunctional compound. The choice of amino acids for this study was based on several considerations. Initially, the reaction of glycine methyl ester was examined, as its structural simplicity facilitated isolation and identification of the adduct. Lysine, tyrosine, histidine and arginine were chosen because they represent amino acids that were most consistently altered by MDA in studies involving enzymes and other proteins (21-23). These amino acids also have reactive sites at positions other than the $\alpha$-amino group. Studies with lysine were particularly important as the only primary amino group in protein structures apart from the N-terminal $\alpha$-amino groups is the $\epsilon$-amino group of lysine. For purposes of comparison, a substituted MDA, MMDA, also was utilized in these studies.

The reactions were carried out by combining solutions of the amino acid methyl ester hydrochloride (two molar equivalents [meq]) and the dialdehyde enolic sodium salt (one meq) in dry methanol (Scheme 1). The reactions were monitored by UV spectroscopy. The disappearance of MDA and the appearance of the vinylogous amidine were monitored at their absorption maxima of ca. 247 and 300 nm, respectively. MMDA reactions were monitored at ca. 252 and 310 nm. The yields, physical properties and UV data of the products are shown in Table 1.

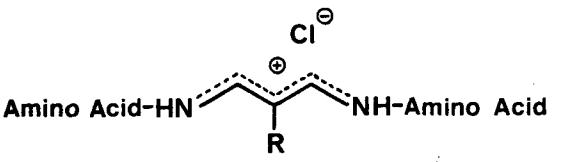
Use of methanol as solvent under acid catalysis provided optimum conditions for conversion to crosslinked adducts in terms of reaction times, product yields and ease of purification. The rates of formation of adducts containing vinylogous amidine linkages were much slower in acetate buffer at pH 4.2, the reported optimum conditions for the reaction of MDA with proteins (18). Considerably faster protein modification by MDA in aqueous buffered systems at pH 4.2 than under the same conditions in these model studies may be due to the presence of a more favorable environment in proteins, not only for crosslinking, but also for survival of the vinylogous amidine linkage. With respect to the latter, our model studies suggest that, once formed, the 1:2 adducts of MDA and amino acids are relatively stable even in aqueous acidic solutions.

Unambiguous evidence for the formation of the adducts **1-5** came from spectral data and elemental analysis. The mass spectral data showed parent ions (minus HCl) for each product. The $^1$H and $^{13}$C NMR spectra of the products provided insight into their stereochemistry and are summarized in Tables 2 and 3. In all cases for the MDA adducts, the coupling constants ($J \geq 11.0$ Hz) for the vinylogous amidine moiety and the single resonance for $H_\alpha$ and $C_\alpha$ in the $^1$H and $^{13}$C NMR spectra suggests an all-*trans* or W form for the stereochemistry of these crosslinked adducts. Although unequivocal assignment with respect to the stereochemistry of the vinylogous amidine moiety cannot be made for the MMDA adducts, it is reasonable to assume that the *trans* form predominates for these compounds as well (24).

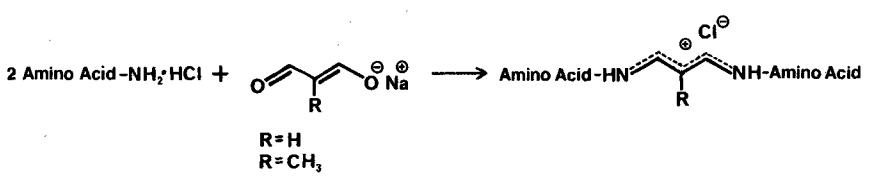
Tappel and coworkers have reported that UV absorp-

**TABLE 1**

**Yields and Physical and Spectral Properties of Vinylogous Amidinium Salts from the Reaction of MDA and MMDA with Amino Acid Derivatives**




| Compound (amino acid, R) | Yield, % | mp, °C | $UV_{max}(H_2O)(\log \epsilon)$ |
|---|---|---|---|
| **1a** Glycine methyl ester R=H | 47 | 160 | 298 nm (4.55) |
| **1b** Glycine methyl ester R=$CH_3$ | 60 | 98-100 | 307 nm (4.54) |
| **2a** $\alpha$-N-Acetyllysine, methyl ester R=H | 58 | Low melting solid | 300 nm (4.52) |
| **2b** $\alpha$-N-Acetyllysine, methyl ester R=$CH_3$ | 65 | Low melting solid | 307 nm (4.53) |
| **3a** Tyrosine methyl ester R=H | 76 | 92-94 | 300 nm (4.58) |
| **3b** Tyrosine methyl ester R=$CH_3$ | 67 | 93-95 | 310 nm (4.55) |
| **4a** Histidine methyl ester (2HCl) R=H | 54 | 113-115 | 301 nm (4.55) |
| **4b** Histidine methyl ester (2HCl) R=$CH_3$ | 60 | 133-135 | 310 nm (4.58) |
| **5a** Arginine methyl ester (2HCl) R=H | 76 | 85-88 | 302 nm (4.54) |
| **5b** Arginine methyl ester (2HCl) R=$CH_3$ | 51 | 96-98 | 306 nm (4.57) |




SCHEME 1

**TABLE 2**

**$^1$H NMR Data for 2:1 Adducts**

| Compound | Solvent[a] | $H_\alpha$ | $H_\beta$ | $-CH_3$ | $-\overset{O}{\overset{\|}{C}}OCH_3$ | H (others) |
|---|---|---|---|---|---|---|
| **1a** | $Me_2SO$-$d_6$ | 7.90 d, J = 11.7 | 5.75 t, J = 11.7 | — | 3.71 | 4.29 (s, 4H); 9.55 (bs, 2H) |
| **1b** | $Me_2SO$-$d_6$ | 7.70 s | — | 1.82 | 3.71 | 4.32 (s, 4H); 9.10 (bs, 2H) |
| **2a** | $CD_3OD$ | 7.74 d, J = 11.5 | 5.64 t, J = 11.5 | — | 3.71 | 1.40–1.75 (m, 12H); 2.00 (s, 6H); 2.84 (t, 4H); 4.38 (t, 2H) |
| **2b** | $CD_3OD$ | 7.62 s | — | 2.00 | 3.71 | 1.40–1.75 (m, 12H); 2.00 (s, 9H); 2.83 (t, 4H); 4.38 (t, 2H) |
| **3a** | $CD_3OD$ | 7.45 d, J = 11.3 | 5.49 t, J = 11.3 | — | 3.77 | 3.29 (m, 4H); 4.12 (t, 2H); 6.75 (m, 4H); 6.95 (m, 4H) |
| **3b** | $CD_3OD$ | 7.23 s | — | 1.61 | 3.82 | 3.10 (d, 4H); 4.10 (m, 2H); 6.82 (d, 4H); 6.85 (d, 4H) |
| **4a** | $D_2O$* | 7.47 d, J = 11.0 | 6.10 t, J = 11.0 | — | 3.43 | 3.51 (m, 4H); 4.52 (m, 2H); 7.38 (s, 2H); 8.68 (m, 2H) |
| **4b** | $CD_3OD$ | 7.07 s | — | 1.50 | 3.45 | 3.40 (m, 4H); 4.30 (t, 2H); 7.37 (s, 2H); 8.30 (s, 2H) |
| **5a** | $D_2O$* | 7.93 d, J = 11.0 | 6.20 t, J = 11.0 | — | 3.92 | 1.85–2.06 (m, 12H); 3.35 (m, 2H) |
| **5b** | $CD_3OD$ | 7.50 s | — | 1.57 | 3.72 | 1.75–2.90 (m, 12H); 3.10 (m, 2H) |
| **7** | $Me_2SO$-$d_6$ | 8.69 bd, J = 11.5 | 6.70 bt, J = 11.5 | — | 3.72 | 1.45 (m); 4.24 (m); 4.36 (m); 8.35 (s); 8.41 (s) |

[a]Chemical shifts given are with $Me_4Si$ as internal standard ($\delta$=0), except for *, where external $Me_4Si$ was used.

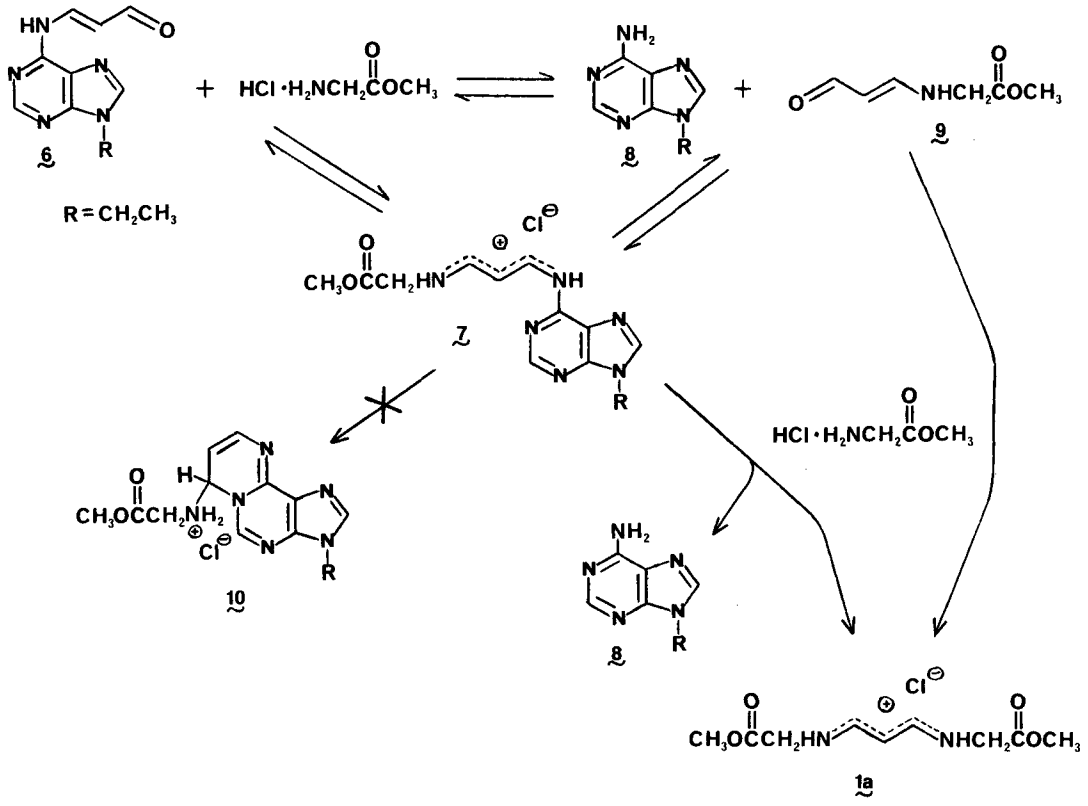
**TABLE 3**

**$^{13}$C NMR Data for 2:1 Adducts**

| Compound | Solvent[a] | $C_\alpha$ | $C_\beta$ | $-CH_3$ | $-\overset{O}{\overset{\|}{C}}OCH_3$ | C (others) |
|---|---|---|---|---|---|---|
| **1a** | $Me_2SO$-$d_6$ | 163.3 | 90.7 | — | 52.2 | 44.7; 168.1 |
| **1b** | $Me_2SO$-$d_6$ | 164.9 | 101.5 | 8.3 | 52.3 | 48.6; 169.3 |
| **2a** | $CD_3OD$ | 163.4 | 90.0 | — | 53.7 | 22.5; 23.9; 29.9; 32.0; 41.1; 52.6; 173.1; 174.0 |
| **2b** | $CD_3OD$ | 164.3 | 101.8 | 7.9 | 53.7 | 22.4; 24.0; 29.9; 32.1; 41.2; 52.7; 173.3; 174.1 |
| **3a** | $CD_3OD$ | 158.1 | 95.5 | — | 53.1 | 29.3; 61.7; 115.5; 127.3; 131.2; 157.6; 173.2 |
| **3b** | $CD_3OD$ | 157.6 | 116.1 | 7.0 | 52.6 | 28.2; 61.0; 116.4; 127.8; 131.2; 157.4; 171.1 |
| **4a** | $D_2O$* | 166.5 | 93.4 | — | 54.5 | 28.2; 61.8; 118.6; 128.5; 134.8; 171.3 |
| **4b** | $CD_3OD$ | 165.6 | 104.2 | 8.3 | 54.5 | 27.9; 62.0; 118.6; 129.0; 134.8; 171.4 |
| **5a** | $D_2O$* | 166.1 | 93.1 | — | 54.2 | 24.9; 28.7; 41.2; 62.3; 157.6; 173.1 |
| **5b** | $CD_3OD$ | 165.1 | 103.5 | 8.3 | 54.2 | 25.2; 29.1; 41.2; 62.4; 157.5; 173.1 |

[a]Chemical shifts given are with $Me_4Si$ as internal standard ($\delta$=0), except for *, where internal dioxane ($\delta$=67.4) was used.

tion maxima at approximately 256, 285, 370 and 435 nm result from the products of MDA interaction with proteins (21,25). The model crosslinked compounds isolated in our work gave UV data inconsistent with these observations. The UV data reported previously were very likely for a mixture of products including those containing vinylogous amidine linkages. It also should be explained that MDA generated in situ from the acid-catalyzed hydrolysis of the corresponding *bis*-acetals is contaminated significantly by reactive side products such as $\beta$-methoxyacrolein and 3,3-dimethoxypropionaldehyde. We have employed highly purified sodium MDA in this work. Fluorescence spectra of MDA-modified proteins exhibit emission at 440-470 nm with excitation at 370-400 nm (22,25), also indicative of the formation of vinylogous amidines and other linkages. The lysine crosslinked compound **2a** showed a fluorescence emission maximum at 440 nm on excitation at 360 nm, with the other adducts giving similar



SCHEME 2

spectra. These data lend further support for the formation of vinylogous amidine linkages in the modification of proteins by MDA.

MDA is reactive towards nucleic acids, resulting in the loss of their template activity (13,14). The modification of nucleic acids may involve direct modification of the bases, crosslinking between the bases or crosslinking between the bases and proteins (26,27). The direct modification of nucleic acid bases by MDA has been reported by us (19). To provide further basic chemical information on the nature and stability of MDA-induced protein-nucleic acid crosslinks, we studied the reactivity of a 9-substituted adenine with MDA in the presence of glycine methyl ester. No adenine-amino acid crosslinking was observed under these conditions and the only product formed was the glycine adduct **1a**. However, in the absence of the amino acid, 9-ethyladenine was converted slowly to its enaminal **6** by reaction with MDA. No crosslinked base pairs could be detected under a wide variety of conditions. However, if the enaminal were allowed to react with an equimolar amount of glycine methyl ester hydrochloride in scrupulously dry methanol, formation of the base-amino acid crosslinked adduct **7** could be detected at 348 nm in the UV spectrum. Quantitative monitoring of the reaction by UV and high-field $^1$H NMR spectroscopy showed an initial buildup of **7** to a maximum value of 21% after about one hr. At this point no enaminal **6** remained, and the reaction mixture also contained the glycine adduct **1a** (26.5%) and 9-ethyladenine, **8** (52.5%). After three hr, however, the level of **7** had fallen to 3%, the amount of **1a** had maximized at 35.5% and that of **8** had stabilized at 61.5%. The 2:1 adduct **1a** very likely is formed through the intermediacy of **9**, the initial product of a reaction involving the transfer of the MDA moiety from **6** to glycine. Adduct **1a** also may be produced from the reaction of glycine with **7**, followed by elimination of 9-ethyladenine (Scheme 2).

The crosslinked adduct **7** is much less stable in aqueous solutions than the corresponding amino acid adducts containing vinylogous amidine linkages. However, although adduct **7** could not be isolated in a pure state because of its instability, its structure could be established unambiguously by its UV, high-field NMR and mass spectral data. The mass spectrum showed a parent ion at m/z 288 ($M^+$-HCl) and appropriate peaks for product fragmentation. The 360 MHz $^1$H NMR data showed a doublet at $\delta$8.69 (J = 11.5 Hz) and a triplet at $\delta$6.70 (J = 11.5 Hz) corresponding to $H_\alpha$ and $H_\beta$ of the diazapentadienium moiety. Resonances due to the amino acid components also were observed (see Table 2). The possibility of cyclization of the crosslinked adduct **7** to give **10** (cf ref. 19) may be ruled out by the UV and NMR data.

In summary, we conclude that MDA is capable of crosslinking amino acids through vinylogous amidine linkages. Additionally, MDA may crosslink amino acids with nucleic acid bases or may transfer the MDA moiety from one to the other. The detrimental biological effects of MDA may be mediated by such modifications.

## ACKNOWLEDGMENTS

These investigations were supported by a grant from the National Science Foundation. The high-field NMR spectrometer used was purchased in part by another NSF grant.

## REFERENCES

1. Pryor, W.A., ed. (1976-84) *Free Radicals in Biology*, Vol. I-VI, Academic Press, New York.
2. Simic, M.G., and Karel, M., eds. (1980) *Autoxidation in Food and Biological Systems*, Plenum Press, New York.
3. Bhatnager, R.S., ed. (1980) *Molecular Basis of Environmental Toxicity*, Ann Arbor Science, Ann Arbor.
4. Esterbauer, H. (1982) in *Free Radicals, Lipid Peroxidation, and Cancer* (McBrien, D., and Slater, T., eds.) pp. 101–128, Academic Press, London.
5. Bucknall, T., Edwards, H.E., Kemsley, K.G., Moore, J.S., and Phillips, G.O. (1978) *Carbohydrate Res. 62*, 4959.
6. Gray, J.I. (1978) *J. Amer. Oil Chem. Soc. 55*, 539–546.
7. Nair, V., and Turner, G.A. (1984) *Lipids 19*, 804–805.
8. Shamberger, R.J., Andreone, T.L., and Willis, C.E. (1974) *J. Natl. Cancer Inst. (U.S.) 53*, 1771–1773.
9. Munkres, K.D. (1979) *Mech. Ageing Dev. 10*, 249–260.
10. Basu, A.K., and Marnett, L.J. (1983) *Carcinogenesis (London) 4*, 331–333.
11. Mukai, F.H., and Goldstein, D.B. (1976) *Science (Washington, DC) 191*, 868–869.
12. Yau, T.M. (1979) *Mech. Ageing Dev. 11*, 137–144.
13. Brooks, B.R., and Klamerth, O.L. (1968) *Eur. J. Biochem. 5*, 178–182.
14. Summerfield, F.W., and Tappel, A.L. (1981) *Anal. Biochem. 111*, 77–82.
15. Mahley, R.W., Innerarity, T.L., Weisgraber, K.H., and Oh, S.Y. (1979) *J. Clin. Invest. 64*, 743–750.
16. Fogelman, A.L., Schecter, I., Seager, J., Hokom, M., Child, J.S., and Edwards, P.A. (1980) *Proc. Natl. Acad. Sci. USA 77*, 2214–2218.
17. Schecter, I., Fogelman, A.L., Haberland, M.E., Seager, J., Hokom, M., and Edwards, P.A. (1981) *J. Lipid Res. 22*, 63–71.
18. Nair, V., Vietti, D.E., and Cooper, C.S. (1981) *J. Am. Chem. Soc. 103*, 3030–3036.
19. Nair, V., Turner, G.A., and Offerman, R.J. (1984) *J. Am. Chem. Soc. 106*, 3370–3371.
20. Nair, V., and Richardson, S.G. (1980) *J. Org. Chem. 45*, 3969–3974.
21. Chio, K.S., and Tappel, A.L. (1969) *Biochemistry 8*, 2821–2826.
22. Chio, K.S., and Tappel, A.L. (1969) *Biochemistry 8*, 2827–2832.
23. Shin, B.C., Huggins, J.W., and Carraway, K.L. (1972) *Lipids 7*, 229–233.
24. Nair, V., and Cooper, C.S. (1981) *J. Org. Chem. 46*, 4759–4765.
25. Bidlack, W.R., and Tappel, A.L. (1973) *Lipids 8*, 203–207.
26. Smith, K.C., ed. (1976) *Aging, Carcinogenesis, and Radiation Biology: The Role of Nucleic Acid Addition Reactions*, Plenum Press, New York.
27. Bjorkstein, J. (1977) in *Protein Crosslinking* (Friedman, M., ed.) Plenum Press, New York.

[Received May 2, 1985]