Synthesis and Characterization of the Fluorescent Products Derived from Malonaldehyde and Amino Acids*

K. S. Chio and A. L. Tappel

ABSTRACT: Amino acids or their esters and *n*-hexylamine react with malonaldehyde to yield conjugated Schiff bases. The Schiff bases possess characteristic absorption in the ultraviolet and visible regions. In solutions, Schiff bases derived from amino acids show spectral shifts on standing at room temperature, and isosbestic points are found at 401–404 mμ. *cis–trans* isomerization about the C=C bond or C=N bond may account for the observed spectral shift. The electronic absorption and fluorescence properties have been attributed to the chromophoric system NC=CC=N which contains six π electrons. Infrared spectra contain a band in the 1650-cm^{-1} region indicating the presence of a C=N bond. Mass spectral analyses are carried out after the Schiff bases are reduced with sodium borohydride to confirm that 1 mole of malonaldehyde reacts with 2 moles of amino acid ester or *n*-hexylamine to yield *N,N'*-disubstituted 1-amino-3-iminopropenes.

Dialdehydes, such as glutaraldehyde, can react bifunctionally with proteins to give inter- and intramolecular cross-linking. Crystals of carboxypeptidase A, when reacted with glutaraldehyde, become completely insoluble in 1 M NaCl and show a marked increase in mechanical strength (Quiocho and Richards, 1964). It was assumed that the reaction of the aldehyde and the amino groups of the protein resulted in Schiff base formation. A review of bifunctional protein reagents was recently given by Wold (1967). Other aliphatic dialdehydes, like glyoxal and malonaldehyde, have been shown to react with DNA (Brooks and Klamerth, 1968); malonaldehyde also reacts with proteins (Kwon and Brown, 1965) and with amino acids (Crawford *et al.*, 1966). The mechanism of the reaction between malonaldehyde and glycine has been investigated by Crawford *et al.* (1966). It involves a 1,4 addition of the nucleophilic nitrogen atom of glycine to the enolic carbon atom of the α,β-unsaturated carbonyl system of the enol of malonaldehyde to form the enamine, *N*-prop-2-enolaminoacetic acid. By condensation with malonaldehyde in 10 N HCl at 25°, arginine was converted into δ-*N*-(2-pyrimidinyl)ornithine (King, 1966). Aromatic, but not aliphatic, primary amines react with malonaldehyde to yield *N,N'*-disubstituted 1-amino-3-iminopropenes, which are fluorescent conjugated Schiff bases (Sawicki *et al.*, 1963).

This investigation further examines the interaction of aliphatic primary amines, such as amino acids and *n*-hexylamine, with malonaldehyde to produce *N,N'*-disubstituted 1-amino-3-iminopropenes and includes study of their spectroscopic properties.

Methods and Materials

Materials. Malonaldehyde bis(dimethyl acetal), that is, 1,1,3,3-tetramethoxypropane, was obtained from Aldrich Chemical Co. Glycine, L-valine, L-leucine, L-valine methyl ester hydrochloride, and L-leucine ethyl ester hydrochloride were products of Nutritional Biochemicals Corp. Triglycine and pentaglycine were from Mann Research Laboratories. *n*-Hexylamine and Spectrograde KBr were from Matheson Coleman and Bell. Sephadex G-10 and Sephadex LH-20 were purchased from Pharmacia Fine Chemicals Inc. Bio-Gel P-2 (200–400 mesh) is a product of Bio-Rad Laboratories. Sodium borohydride was obtained from Sigma Chemical Co.

Synthesis of N,N'-Disubstituted 1-Amino-3-iminopropenes from Amino Acids. Two types of synthesis of amino acid with malonaldehyde were carried out. Firstly, an amino acid was reacted with malonaldehyde to obtain solid derivatives for spectroscopic studies. Secondly, the derivative of the methyl or ethyl ester of the amino acid and the aldehyde was prepared, so that after reduction by sodium borohydride, the product, *N,N'*-disubstituted 1,3-propanediamine, could be used for mass spectrometric studies and elemental analysis. Elemental analyses were performed at the Microanalytical Laboratory, University of California, Berkeley, Calif.

A. GLYCINE. An amino acid reacts with malonaldehyde to form an enamine, with an amino acid to aldehyde ratio of 1:1. These reactants also form a fluorescent *N,N'*-disubstituted 1-amino-3-iminopropene; a product with an amino acid to aldehyde ratio of 2:1. The fluorescent product obtained from glycine and malonaldehyde is prepared by the same method as for preparation of the 1:1 product (Crawford *et al.*, 1966). A solution of malonaldehyde acetal (24.6 g, 0.15 mole) in 13.5 ml of 1 N HCl was allowed to stand at 40° with occcasional shaking until miscible. After cooling to room temperature, the solution was added to 7.5 g (0.1 mole) of glycine in 20 ml of water. After stirring for 1 hr and then standing at 4° for 1 hr, the solid product (amino acid:aldehyde, 1:1) was removed by filtration. The yellow filtrate containing the product (amino acid: aldehyde, 2:1), which fluoresced under ultraviolet light (366 mμ), was extracted four times with equal volumes of ether to remove excess malonaldehyde and then lyophilized. After lyophilization, 3.4 g (36% yield) of crude fluorescent product was obtained; 200 mg of the crude product was dissolved in 3 ml of water and applied to a Bio-Gel P-2

* From the Department of Food Science and Technology, University of California, Davis, California 95616. *Received December 27, 1968.* This investigation was supported by U. S. Public Health Service Research Grant AM 09993 from the National Institutes of Arthritis and Metabolic Diseases.

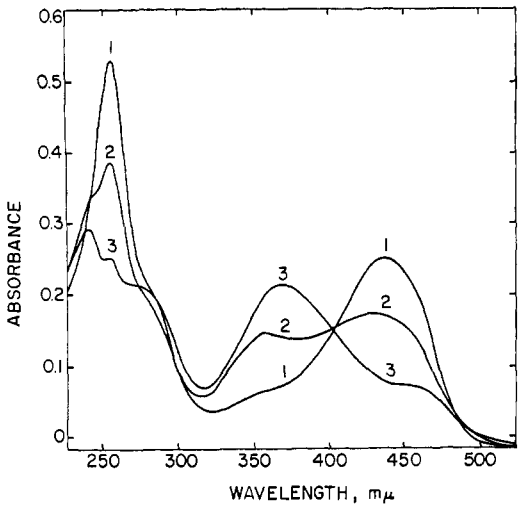


FIGURE 1: Absorption spectrum of the 1-amino-3-iminopropene derivative of glycine in water (10 μg/ml; 1-cm cuvet) at (1) 0, (2) 65, and (3) 240 min.

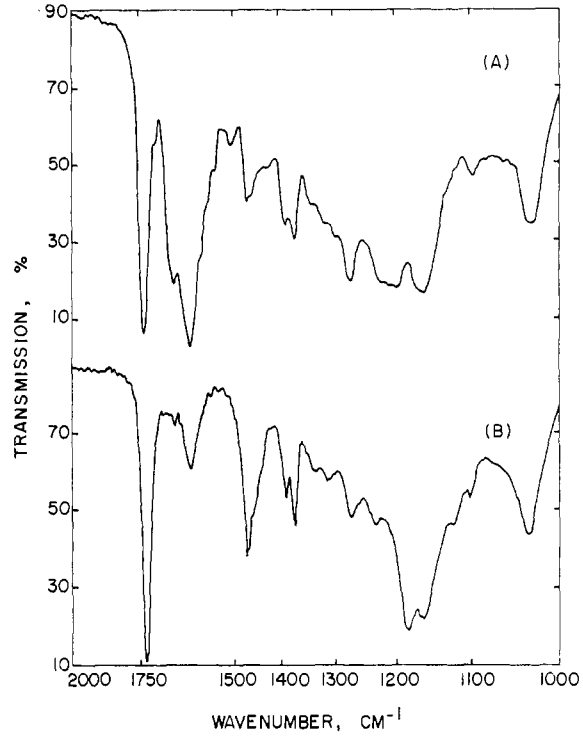


FIGURE 2: Infrared spectra of the 1-amino-3-iminopropene derivative of L-leucine ethyl ester. (A) Before reduction with sodium borohydride (KBr disk) and (B) after reduction with sodium borohydride (liquid, neat).

column (100 × 2.5 cm). The column was eluted with distilled water at a flow rate of 68 ml/hr using a Technicon proportioning pump. The 6.8-ml fractions were analyzed by absorbance at 400 mμ or by fluorescence at 450 mμ when excited at 370 mμ. The major fluorescent fractions peak at fraction 66. The effluent, about 80 ml, was pooled and lyophilized (80 mg, 40% yield), and the process of chromatography and lyophilization was repeated. The yellow, fluorescent compound was hygroscopic, turned brown on heating to 136–140°, and the yellow color faded on standing at room temperature or when exposed to light.

The molecular weight of this glycine–malonaldehyde derivative was determined by its elution position from a calibrated Sephadex G-10 column by the method of Whitaker (1963). The column was calibrated with glycine, triglycine, and pentaglycine. From this linear relationship between V_e/V_0 and the logarithm of the molecular weight, the 1-amino-3-iminopropene derivative of glycine gave a molecular weight of 182 (theoretical mol wt 186).

B. L-Leucine. The L-leucine and malonaldehyde derivatives were prepared by the same method as for glycine. A solution which contained 2.6 g (0.02 mole) of leucine in 100 ml of H_2O was added to a solution of malonaldehyde acetal (4.9 g, 0.03 mole) in 2.7 ml of 1 N HCl and allowed to stir for 1 hr. The suspension was cooled, filtered, and excess malonaldehyde was extracted with ether. The aqueous solution was lyophilized, acetone was added, and the unreacted leucine was removed by filtration. The filtrate containing the fluorescent product was evaporated to dryness (0.7 g, 23% yield) and purified by chromatography as before. Maximum absorbance was found at fraction 46. The leucine derivative has the same characteristics as those of the glycine derivative, and decomposes at 170–173°.

C. L-Valine. The L-valine derivative was prepared in the same way, with 4.5 g of valine in 50 ml of H_2O and 10 ml of malonaldehyde acetal in 5.5 ml of 1 N HCl. However, after lyophilization the product was dissolved in absolute ethanol, the unreacted valine was removed by filtration, and the ethanol from the filtrate was evaporated under vacuum (1.6 g, 30% yield) and purified as before, with highest absorbance being found at fraction 50. The compound decomposes at 155–160°.

Synthesis of N,N'-Disubstituted 1-Amino-3-iminopropenes and Their Sodium Borohydride Reduced Compounds from Amino Acid Esters and n-Hexylamine. A. L-Leucine ethyl ester. To 2 g (0.01 mole) of L-leucine ethyl ester hydrochloride dissolved in 2 ml of H_2O was added 2.46 g (0.015 mole) of malonaldehyde acetal in 0.5 ml of 1 N HCl. The solution was stirred for 1 hr, and excess aldehyde was removed by extracting four times with equal volumes of ether. The aqueous layer was neutralized with 6 N NaOH, and the neutral fluorescent ester derivative was extracted with ether. After evaporating the ether, the yield of the orange ester derivative was 1 g (60% yield). The fluorescent ester derivative was purified by passing it through a Sephadex LH-20 column (45 × 2.5 cm) with absolute ethanol as eluent at a flow rate of 60 ml/hr. The peak was at an elution volume of 117 ml. For reduction, 100 mg of the semisolid ester derivative was dissolved in 5 ml of absolute ethanol and four batches, 40 mg each, of sodium borohydride were added. The reduction was completed when the solution did not fluoresce under ultraviolet light (366 mμ). After the ethanol was removed by vacuum evaporation, 10 ml of H_2O was added to the residue and then the derivative was extracted with ether. The ether solution was evaporated to dryness (70 mg, 70% yield) and the residue which was *N,N'*-(1,3-trimethylene)bis(L-leucine ethyl ester) was purified twice by passing it through the Sephadex LH-20 column. The purified compound eluted as a single peak and was a pale yellow liquid.

Anal. Calcd for $C_{19}H_{38}N_2O_4$: C, 63.69; H, 10.61; N, 7.82. Found: C, 63.57; H, 10.64; N, 7.98.

TABLE I: Electronic Absorption Spectral Characteristics[a] of 1-Amino-3-iminopropene Derivatives of Amino Acids and *n*-Hexylamine.

Compound	Solvent	Absorption Maxima and (Molar Extinction Coefficients) 0 min	240 min	Isosbestic point
RNHCH=CHCH=NR,	H_2O		240 (5,400)	403 (2,810)
R = CH_2COOH		256 (9,850)	256 (4,650)	
		280 (3,650)[b]	280 (3,620)[b]	
		370 (1,360)[b]	370 (3,960)	
		435 (4,650)	435 (1,450)[b]	
R = $CH(COOH)CH(CH_3)_2$	H_2O		240 (4,510)	404 (2,600)
		256 (6,310)	256 (4,570)	
		290 (5,070)[b]	290 (5,450)	
		373 (2,960)	373 (4,460)	
		435 (2,890)	435 (1,120)[b]	
R = $CH(COOH)CH_2CH(CH_3)_2$	H_2O		240 (4,840)	401 (2,770)
		256 (6,580)	256 (4,620)	
		282 (4,140)[b]	282 (4,620)	
		373 (2,700)	373 (3,990)	
		435 (2,810)	435 (1,050)[b]	
R = $(CH_2)_5CH_3$	Ethanol	303 (27,800)	303 (27,800)	
		393 (2,370)	393 (2,370)	

[a] Wavelengths are given in millimicrons; the molar extinction coefficients are in parentheses. [b] Shoulder.

B. L-VALINE METHYL ESTER. The reduced derivative of the product of L-valine methyl ester (0.01 mole) and malonaldehyde (0.015 mole) was prepared and purified in the same way as for L-leucine ethyl ester. The product, *N,N'*-(1,3-trimethylene)-bis(L-valine methyl ester), obtained was 820 mg (55% yield) of a pale yellow liquid.

Anal. Calcd for $C_{15}H_{30}N_2O_4$: C, 59.60; H, 9.93; N, 9.27. Found: C, 60.53; H, 10.13; N, 8.31.

C. *n*-HEXYLAMINE. Similarly, instead of amino acid, *n*-hexylamine (0.01 mole) was reacted with malonaldehyde (0.015 mole) and after sodium borohydride reduction and purification by Sephadex LH-20 column chromatography, yellow-white solids (410 mg, 34% yield) appeared upon evaporation of the ethanol. Further purification was carried out by dissolving the solids in 1 N HCl and filtering. The filtrate was neutralized with 4 N NaOH and the white precipitate was extracted into ether. After evaporation of ether, the precipitate was recrystallized in ethanol and white crystals of *N,N'*-di-*n*-hexyl-1,3-propanediamine with a mp 53–54° were obtained.

Anal. Calcd for $C_{15}H_{34}N_2$: C, 74.38; H, 14.05; N, 11.57. Found: C, 74.10; H, 13.80; N, 11.50.

Absorption Spectroscopy. The electronic absorption spectra in the visible and ultraviolet regions were recorded at room temperature with a Beckman DB-G spectrophotometer on a Sargent recorder (Model SRL). The spectrophotometer was previously calibrated using a holmium oxide standard.

Infrared Spectroscopy. Infrared spectra of the derivatives were obtained with a Beckman IR-5 infrared spectrophotometer. For a solid compound, a pellet was prepared from a mixture obtained by grinding 100 mg of oven-dried Spectrograde KBr with 0.5 mg of the compound. Liquid samples were run neat with NaCl disks.

Fluorescence Measurements. Fluorescence spectra were obtained using an Aminco-Bowman spectrophotofluorometer attached to a Moseley Autograf X-Y recorder. The following settings were used: sensitivity 40, meter multiplier 0.03, slit arrangement no. 3, and 1P28 photomultiplier tube.

Mass Spectroscopy. Mass spectra were determined on a Varian M66 mass spectrometer operating at an ionization potential of 70 eV and the electron current was 30 μA. Samples were introduced through the solid probe at 30°.

Results

Absorption and Fluorescence Spectra. The electronic absorption spectra in aqueous solution of the conjugated Schiff bases obtained from glycine, valine, and leucine showed two main absorption peaks, at 256 and 435 mμ, with shoulders at 280 and 370 mμ. In absolute ethanol, the Schiff base of *n*-hexylamine has λ_{max} at 303 and 393 mμ. On standing at room temperature the maximum at 435 mμ decreased in absorbance and the shoulder at 370 mμ increased in absorbance, and at the same time the peak at 256 mμ decreased and a new peak at 240 mμ was formed. The spectral shift with time of 1-amino-3-iminopropene derivative of glycine is shown in Figure 1, and an isosbestic point at 403 mμ is observed. Table I shows the extinction coefficients of the derivatives before and after spectral shift. After the spectral shift, if the aqueous solution was lyophilized and redissolved in water, partial recovery of the original spectrum was obtained. Whether in 0.01 M so-

TABLE II: Relative Fluorescence Intensity of 1-Amino-3-iminopropene Derivatives of Amino Acids and *n*-Hexylamine.

Compound	Solvent	λ_{max} (mμ)		Relative Molar Intensity	
		Excitation	Emission	Excitation at λ_{max}[a]	Emissions at λ_{max}[b]
Quinine	0.1 N H_2SO_4	350	450	1	1
RNHCH=CHCH=NR, R = CH_2COOH	H_2O	370	450	0.250	0.250
R = $CH(COOH)CH(CH_3)_2$	H_2O	370	450	0.432	0.432
R = $CH(COOH)CH_2CH(CH_3)_2$	H_2O	370	450	0.285	0.290
R = $(CH_2)_5CH_3$	Ethanol	396	462	0.200	0.200

[a] Instrument set at emission wavelength maximum. [b] Instrument set at excitation wavelength maximum.

dium phosphate buffer (pH 7) or in absolute alcohol, the 1-amino-3-iminopropene derivative of glycine exhibited the same pattern of spectral shift as in aqueous solution.

The infrared spectra of the Schiff bases contain a band of the C=N bond at 1650–1655 cm^{-1}, and a band of the C=C bond in the 1610–1620-cm^{-1} region. The infrared spectrum of the Schiff base formed between leucine ethyl ester and malonaldehyde is shown in Figure 2A. After reduction of the Schiff base by sodium borohydride, the bands at 1650 and 1610 cm^{-1} are diminished as shown by the spectrum of the reduced compound in Figure 2B. In Figure 2B the band at 1600 cm^{-1} can be attributed to the weak asymmetric NH bending.

The Schiff bases derived from glycine, valine, and leucine have intense blue fluorescence in aqueous solution with an excitation maximum at 370 mμ and an emission maximum at 450 mμ (Figure 3); that of *n*-hexylamine in absolute ethanol has an excitation maximum at 396 mμ and an emission maximum at 462 mμ (Figure 4).

The results of the relative fluorescence intensity are tabulated in Table II. The fluorescence spectra of the amino acid derivatives were taken at a concentration of 1 μg/ml or less. At a higher concentration similar to that used for the electronic absorption spectrum, the excitation maximum shifted with time from 430 to 370 mμ, and the emission maximum shifted from 505 to 460 mμ, respectively.

Mass Spectra. The mass spectrum of *N,N'*-(1,3-trimethylene)bis(L-leucine ethyl ester) (Figure 5) shows a parent peak at *m/e* 358 with additional peaks at *m/e* 343 $(M - 15)^+$, *m/e* 315 $(M - 43)^+$, and *m/e* 301 $(M - 57)^+$. Accurate mass spectral analysis gave *m/e* 358.2771; the calculated value for $C_{19}H_{38}N_2O_4$ was 358.2831. The base peak at *m/e* 286 corresponds to a loss of $C(=O)OC_2H_5$ from the parent molecule to give the amine fragment plus one H. This amine fragmentation is typical of the ethyl esters of unsubstituted acids (Biemann *et al.*, 1961). The intensities (83%) of the peak at *m/e* 169 and (78%) of the peak at *m/e* 171 are derived from $[(CH_2)_3NHCH(HC=CH_2)COOCH_2CH_3]^+$ which is from another amine fragmentation together with loss of the two methyl groups from the *gem*-dimethyl group.

Figure 6 shows the mass spectrum of *N,N'*-(1,3-trimethylene)bis(L-valine methyl ester) with a molecular weight of 302, and peaks at *m/e* 273 $(M - 29)^+$, *m/e* 259 $(M - 43)^+$, and *m/e* 243 $(M - 59)^+$. Accurate mass spectral analysis gave *m/e*

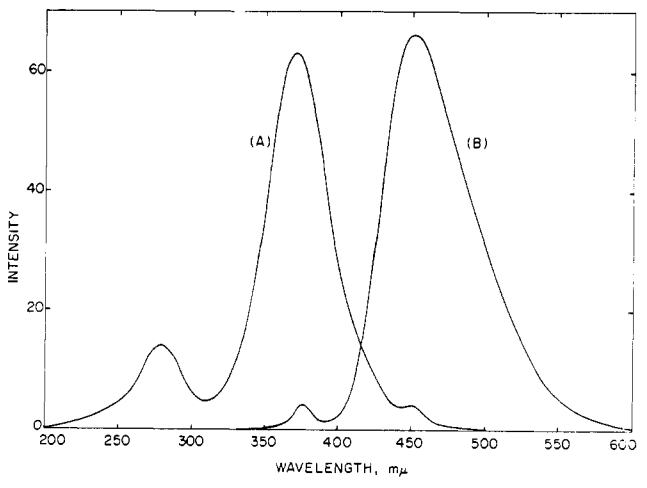


FIGURE 3: Excitation (curve A) and fluorescence (curve B) spectra of 1-amino-3-iminopropene derivatives of L-leucine in aqueous solution (1 μg/ml).

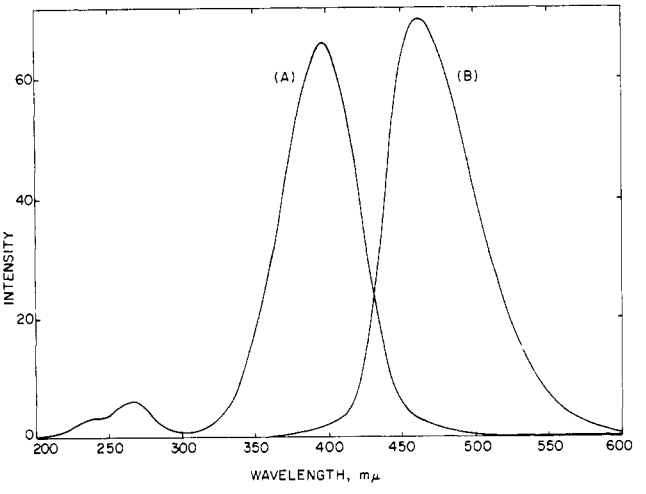


FIGURE 4: Excitation (curve A) and fluorescence (curve B) spectra of 1-amino-3-iminopropene derivatives of *n*-hexylamine in ethanol solution (1.25 μg/ml).

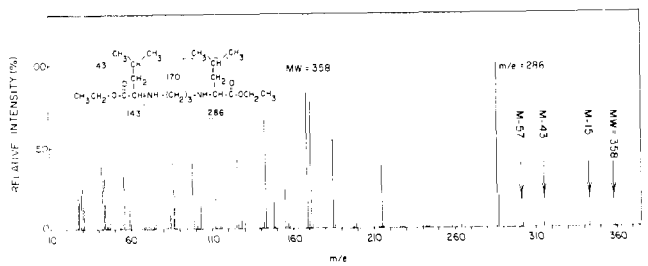


FIGURE 5: Mass spectrum of *N,N'*-(1,3-trimethylene)bis(L-leucine ethyl ester).

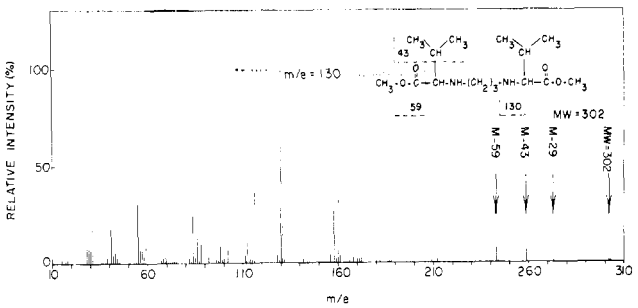


FIGURE 6: Mass spectrum of *N,N'*-(1,3-trimethylene)bis(L-valine methyl ester).

302.2242; the calculated value for $C_{15}H_{30}N_2O_4$ was 302.2205. The base peak at *m/e* 130 for $[NHCH(CH(CH_3)_2)C(=O)OCH_3]^+$ is a result of cleavage at the C–N bond.

The mass spectrum of *N,N'*-di-*n*-hexyl-1,3-propanediamine is shown in Figure 7, with a parent peak at *m/e* 242 and additional peaks at *m/e* 227 $(M - 15)^+$, *m/e* 213 $(M - 29)^+$, and *m/e* 199 $(M - 43)^+$. Accurate mass gave 242.2669; the calculated value for $C_{15}H_{32}N_2$ was 242.2722. The base peak at *m/e* 112 is probably the result of α and β cleavage of the secondary amine and rearrangement to give $[CH_2NH(CH_2)_3NHCH_2CH_2]^+$ less 2 H. α Cleavage gave *m/e* 114, which is $[CH_3(CH_2)_5NHCH_2]^+$, and α cleavage together with the formation of olefin gave *m/e* 70, that is $CH_3(CH_2)_2CH{=}CH_2$ (Budzikiewicz *et al.*, 1964).

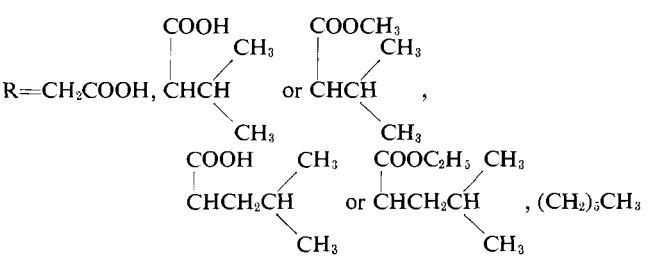
Discussion

The *N,N'*-disubstituted 1-amino-3-iminopropene derivatives have been synthesized with a stoichiometry of 2 moles of an amino acid or its ester or *n*-hexylamine and 1 mole of malonaldehyde. The reaction may proceed as shown in Scheme I.

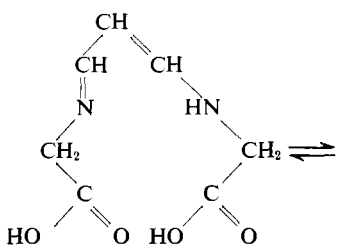
SCHEME I

$$\underset{\text{malonaldehyde}}{O{=}CHCH{=}CHOH} + \underset{\substack{\text{amino acid}\\(\text{ester}) \text{ or}\\ n\text{-hexylamine}}}{RNH_2} \xrightarrow{-H_2O} \underset{\text{enamine}}{O{=}CHCH{=}CHNHR}$$

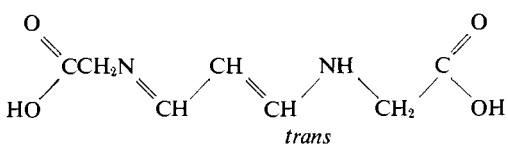
$$O{=}CHCH{=}CHNHR + RNH_2 \xrightarrow{-H_2O} \underset{\substack{N,N'\text{-disubstituted}\\ 1\text{-amino-3-iminopropene}}}{RN{=}CHCH{=}CHNHR}$$



The electronic absorption is due to the conjugated imine system. The *N,N'*-disubstituted 1-amino-3-iminopropenes of the amino acids with absorption maxima at 256 and 435 mμ and shoulders at about 280 and 370 mμ can be compared with *N*-salicylidene valine which has absorption maxima at 257, 278, 318, and 411 mμ (Heinert and Martell, 1963). The spectrum has been correlated with the presence of enolimine and the ketoenamine species of the Schiff base. The spectral shift observed for the aminoiminopropene of glycine can be explained in terms of a *cis–trans* equilibrium as described for malonaldehyde dianils (Feldmann *et al.*, 1967). The *cis–trans* equilibrium was solvent and temperature dependent. In polar solvent (*cis*) malonaldehyde dianil had an absorption maximum at about 373 mμ, and in nonpolar solvent (*trans*) it had its maximum at 343.5 mμ. As the temperature was increased from -42 to $+20°$, the absorption maximum of malonaldehyde dianil of benzylamine at 281.6 mμ decreased and that at 317.4 mμ increased. Therefore, the glycine derivative can be represented as



cis



trans

The fact that lyophilization of dilute aqueous solutions of the glycine, leucine, or valine derivatives, which have been standing at room temperature for a few hours, yields only partial recovery of the original spectrum, indicates that the solution can undergo thermal or photoisomerization. Further investigation is necessary to confirm this phenomenon.

The isosbestic points in the absorption spectra of the amino acid derivatives have about the same extinction coefficients although the extinction coefficients at absorption maxima are different as shown in Table I. This could be due to the difference in the methods used to purify the amino acid derivatives. The presence of isosbestic points further supports the existence of *cis–trans* isomerization either about the C=C bond or with regard to the C=N bond. Isosbestic points were found in the spectra of diacetylindigo subjected to irradiation and the spectral changes were attributed to *cis–trans* isomerization about the C=C bond (Brode *et al.*, 1954). Fischer and Frei (1957) reported the effect of irradiation on spectral shift

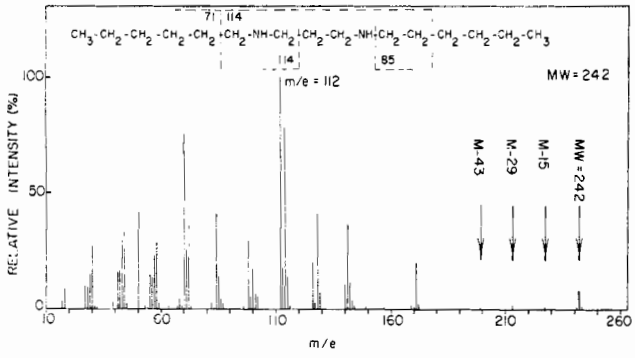


FIGURE 7: Mass spectrum of *N,N'*-di-*n*-hexyl-1,3-propanediamine.

on anils as caused by *cis–trans* isomerism about the C=N bond.

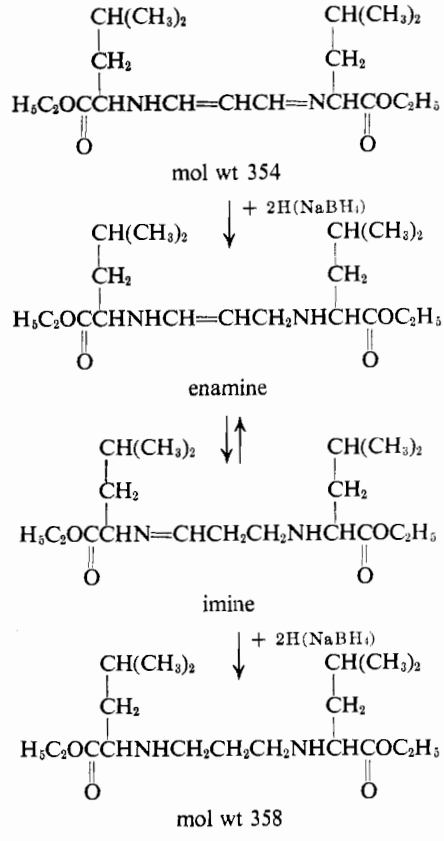
The chromophoric system NC=CC=N, which contains six π electrons, could explain the fluorescence properties of the Schiff base derivatives. Sawicki *et al.* (1963) used the reaction of aromatic amines with malonaldehyde to estimate malonaldehyde by fluorometric methods. The π electrons of the conjugated Schiff base were postulated to be delocalized with the aromatic rings.

The absorption at about 1650 cm^{-1} in the infrared spectra of the amino acid malonaldehyde derivatives suggests that these compounds are imines. Malonaldehyde dianil has its C=N band at about 1650 cm^{-1} (Tsybina *et al.*, 1966). Reduction of the C=N bond with sodium borohydride removes the imine adsorption band at 1650 cm^{-1} as shown in Figure 2B.

The presence of conjugated imine structure is confirmed when the 1-amino-3-iminopropene derivative of leucine ethyl ester is reduced with sodium borohydride. Purification through Sephadex LH-20 gives pure reduced compound whose mass spectrum is shown in Figure 5. The mechanism of the reduction process can be postulated to consist of two steps: (1) the reduction of the C=N bond by sodium borohydride to yield an enamine; and (2) the rearrangement of the enamine the corresponding imine, which is then reduced to amine by sodium borohydride. The reduction is represented in Scheme II. The reduction of the conjugated Schiff bases of valine methyl ester and *n*-hexylamine is assumed to take place by the same mechanism.

Malonaldehyde could be used in the modification of proteins to give fluorescent products. Enzymes with lysine residues, such as RNase A, can be inactivated and cross-linked by malonaldehyde (Chio and Tappel, 1969). The importance of malonaldehyde in biological systems must be emphasized. Malonaldehyde is produced during the γ-irradiation of amino acids such as arginine, glutamic acid, methionine, and homocystine (Ambe and Tappel, 1961). It has been shown to be the major constituent in water extracts of oxidized methyl arachidonate and methyl linolenate (Kwon and Olcott, 1966a,-b). It interacts with DNA *in vitro* and *in vivo* as demonstrated by thermal denaturation profiles, chromatographic behavior, and incomplete degradation of the reaction product by deoxyribonuclease (Brooks and Klamerth, 1968). Recently, malonaldehyde has been reported to be formed in the irradiation of glycerol solution (Scherz, 1968) and in the ^{60}Co γ-irradiation of glucose solution (Scherz and Stehlik, 1968). The aldehyde formed could react with primary amines, such

SCHEME II



as amino acids, and could produce adverse effects during the radiation preservation of food. Imines derived from aliphatic aldehydes, for example, acetaldehyde, show a greater tendency toward polymerization and subsequent reactions than those derived from aromatic aldehydes like benzaldehyde (Royals, 1959). The imines produced may play important roles in the browning of foodstuffs. In the nonenzymic browning systems of glucose–glycine and of sucrose–glycine, fluorescence and browning were observed to increase with concomitant decrease in the content of free α-amino groups (Burton *et al.*, 1963). Reaction products of malonaldehyde and amines appear to be responsible for the yellow color and the fluorescent characteristics of lipofuscin age pigments. Human neuronal lipofuscin has an absorption peak at 375 mμ and fluorescence peaks at 440–460 mμ (Hydén and Lindström, 1950). Hendley *et al.* (1963) found fluorescence peaks at 450–470 mμ when the lipid extract of human heart homogenate was excited at 365 mμ.

References

Ambe, K. S., and Tappel, A. L. (1961), *J. Food Sci. 26*, 448.

Biemann, K., Seibl, J., and Gapp, F. (1961), *J. Am. Chem. Soc. 83*, 3795.

Brode, W. R., Pearson, E. G., and Wyman, G. M. (1954), *J. Am. Chem. Soc. 76*, 1034.

Brooks, B. R., and Klamerth, O. L. (1968), *European J. Biochem. 5*, 178.

Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1964),

Interpretation of Mass Spectra of Organic Compounds, San Francisco, Calif., Holden-Day, p 63.
Burton, H. S., McWeeny, D. J., and Biltcliffe, D. O. (1963), *J. Food Sci. 28*, 631.
Chio, K. S., and Tappel, A. L. (1969), *Biochemistry 8*, 2827 (this issue, following paper).
Crawford, D. L., Yu, T. C., and Sinnhuber, R. O. (1966), *J. Agr. Food Chem. 14*, 182.
Feldmann, K., Daltrozzo, E., and Scheibe, G. (1967), *Z. Naturforsch. 22B*, 722.
Fischer, E., and Frei, Y. (1957), *J. Chem. Phys. 27*, 808.
Heinert, D., and Martell, A. E. (1963), *J. Am. Chem. Soc. 85*, 183.
Hendley, D. D., Mildvan, A. S., Reporter, M. C., and Strehler, B. L. (1963), *J. Gerontol. 18*, 144.
Hydén, H., and Lindström, B. (1950), *Discussions Faraday Soc. 9*, 436.
King, T. P. (1966), *Biochemistry 5*, 3454.
Kwon, T. W., and Brown, W. D. (1965), *Federation Proc. 24*, 592.
Kwon, T. W., and Olcott, H. S. (1966a), *J. Food Sci. 31*, 552.
Kwon, T. W., and Olcott, H. S. (1966b), *Nature 210*, 214.
Quiocho, F. A., and Richards, F. M. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 833.
Royals, E. E. (1959), Advanced Organic Chemistry, Englewood Cliffs, N. J., Prentice-Hall, p 649.
Sawicki, E., Stanley, T. W., and Johnson, H. (1963), *Anal. Chem. 35*, 199.
Scherz, H. (1968), *Experientia 24*, 420.
Scherz, H., and Stehlik, G. (1968), *Monatsh. Chem. 99*, 1143.
Tsybina, N. M., Vinokurov, V. G., Protopopova, T. V., and Skoldinov, A. P. (1966), *Zh. Obshch. Khim. 36*, 1372.
Whitaker, J. R. (1963), *Anal. Chem. 35*, 1950.
Wold, F. (1967), *Methods Enzymol. 11*, 617.

Inactivation of Ribonuclease and Other Enzymes by Peroxidizing Lipids and by Malonaldehyde*

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ABSTRACT: Quantitative enzyme inactivation by lipid peroxidation has been studied. Sulfhydryl enzymes are most susceptible to inactivation by lipid peroxidation intermediates. Oxidation products of polyunsaturated lipids also inactivate nonsulfhydryl enzymes, for example, ribonuclease A. Concomitant with the loss of ribonuclease A activity is the appearance of fluorescence in the enzyme–lipid reaction mixture. The inactivated RNase A shows fluorescent monomer, dimer, and higher molecular weight species in the Sephadex G-100 fractionation pattern. The fluorescence maximum is at 470 mμ; and the excitation maximum is at 395 mμ. Ribonuclease A, inactivated by malonaldehyde, has fluorescence and a gel filtration pattern similar to that of the enzyme inactivated by peroxidizing polyunsaturated lipids. Malonaldehyde is probably the agent responsible for the intra- and intermolecular cross-linking of ribonuclease A. The fluorescence produced from the cross-linking is attributed to the conjugated imine structure formed in protein between two ε-amino groups and malonaldehyde. There are marked similarities between the ribonuclease A–polyunsaturated lipid product and age pigment; cardiac age pigment is a protein–lipid complex and both have similar fluorescence characteristics.

Lipid peroxidation has been considered to be a damaging reaction in biological systems (Barber and Bernheim, 1967). Oxidation of polyunsaturated fatty acids is postulated as a mechanism of disruption of biological membranes and has been reviewed by Packer *et al.* (1967). Inactivation of sulfhydryl enzymes in mitochondria has been associated with lipid peroxidation (McKnight and Hunter, 1966). Tappel (1965) has summarized the properties of protein damage in peroxidation reaction systems and showed that there were considerable similarities between protein damage by lipid peroxidation and that caused by radiation. It is suggested that age pigments were derived from oxidized lipid constitutents of damaged membranes. In the study of the mechanism of damage of cytochrome *c*, a lipid peroxide–protein complex intermediate is reported (Desai and Tappel, 1963). Andrews *et al.* (1965) showed that in an autoxidized lipid–protein system, lipid intermediates reacted with the free amino groups of proteins. On the basis of trypsin hydrolysis and hydrogen fluoride solubility tests, they concluded that reactive lipid intermediates are produced which insolubilize proteins *via* a cross-linking reaction. By gel filtration and hydrogen fluoride solubility studies, Roubal and Tappel (1966) have shown that oxidized lipid–protein reaction products resulted in protein–protein cross-linked polymers which are formed by a free-radical chain polymerization mechanism.

Malonaldehyde, one of the many carbonyl compounds derived from oxidation of polyunsaturated lipids, was shown to react with bovine serum albumin (Kwon and Brown, 1965;

* From the Department of Food Science and Technology, University of California, Davis, California. *Received December 27, 1968.* This investigation was supported by U. S. Public Health Service Research Grant AM 09933 from the National Institute of Arthritis and Metabolic Diseases.