Amine-Malonaldehyde Condensation Products and Their Relative Color Contribution in the Thiobarbituric Acid Test

H. BUTTKUS and **R.J. BOSE**, Fisheries Research Board of Canada,
6640 N.W. Marine Drive, Vancouver 8, B.C.

ABSTRACT

Different malonaldehyde-amine condensation products were tested for their relative color contribution to the thiobarbituric acid (TBA) test, an indicator for oxidative rancidity of polyunsaturated lipids. The open chain mono- and disubstituted malonaldehyde (M) addition products (R-N=CH-CH=CHOH and R-N=CH-CH=CH-NH-R) gave complete (100%) recovery of M on a mole basis. When the M residue was incorporated into cyclic products which formed between the ureido- or guanidino- substituents of α-amino acids such as citrulline or arginine and M, recovery of M by the TBA color test was 30% and 6%, respectively. Products, from imine-amine type interactions, containing the M residue as in pyrazoline or pyrazole ring systems, released from 4% to no M.

INTRODUCTION

The thiobarbituric acid (TBA) test is based on a color reaction between thiobarbituric acid and malonaldehyde which is produced during the oxidation of polyunsaturated fatty acids (1,2). Although the TBA method has been described as one of the most useful, in complex biological systems it has at times shown poor correlation with other oxidative parameters. For instance, the TBA values leveled off or decreased after a certain time of incubation when oxygen absorption was still progressing (3). Similar results have also been observed in this laboratory during storage experiments with frozen fish. In freeze-dried meats the TBA values were low and could not be correlated with the appearance of off-odors or the absorption of oxygen (4), and recoveries of malonaldehyde (M) by distillation methods ranged between 70-95% (5). Reasons for the low recoveries of M have occasionally been advanced. However no experimental data are available to indicate the extent to which competing reactions between M and different functional groups of cellular constituents can diminish the color yield in the TBA test.

EXPERIMENTAL PROCEDURES

The experimental details for obtaining melting points, NMR and mass spectra were described previously (10). Measurements in the visible and UV regions were made with a Beckman DB-G spectrophotometer. Fluorescence spectra were taken with a Baird Atomic Inc. Fluorispec SF-1 instrument and a Moseley Autograph X-Y recorder.

The reagents used in these experiments were all commercially available. 1,1,3,3-Tetraethoxypropane, bp 105-112 C, was obtained from Eastman Organic Chemicals and 1,1,3,3-tetramethoxypropane, bp 178-179 C, was a J.T. Baker Chemical Co. product. L-Citrulline and L-arginine were purchased from Pierce Chemical Co.

Thiobarbituric Acid Assay

Malonaldehyde determinations were made by the 2-thiobarbituric acid (TBA) method (1). The color reaction was carried out by first pipetting 0.10 ml sample, 0.80 ml TBA reagent and 0.20 ml citrate buffer into test tubes and by heating the mixture for 10 min in a water bath at 100 C. After cooling the tubes, 3.60 ml water was added and the optical density was read at 535 nm. The TBA reagent consisted of 0.50 g 2-thiobarbituric acid, 96 ml water and 1.65 ml 2N NaOH. The citrate buffer consisted of 14.75 g $Na_3C_6H_5O_7.2H_2O$ and 12.5 ml HCl (conc.) diluted to 100 ml.

Stock solution of M was prepared from 1,1,3,3-tetramethoxypropane (TMP), bp 178-179 C, by adding 0.0164 ml TMP, ca. 10 ml water and 2 ml N HCl to a 100 ml volumetric flask. The flask was stoppered and held at 50 C for 1 hr, and was then diluted to 100 ml with water. The standard curve for M was prepared from 0.01-0.05 ml of the 1.0 mM stock solution, diluted to 0.10 ml with water and by following the procedure as described for samples above.

Amine-M condensation products, i.e., Cit-M, Arg-M, gly-M, etc., were dissolved in water so that 0.10 ml solution contained 0.05 μmoles, the amount which was subjected to the TBA test. The resulting TBA color was then converted to the amount of M released from the amine-M addition products. When only a faint or no TBA color was obtained, the concentration of compound used was increased up to 1000 times.

Amine-Aldehyde Addition Reactions

α-Amino-δ-(N-2-oxopyrimidine)-n-valeric acid. (Cit-M). The condensation reaction was carried out with 0.350 g (0.0020 moles) of α-amino-δ-carbamido-*n*-valeric acid (common name citrulline), 0.485 g (0.0022 moles) of 1,1,3,3-tetraethoxypropane, and 8 ml HCl (conc.) which was allowed to stand for ca. 20 hr at room temperature. The solution was then flash-evaporated and the residue dried over NaOH, redissolved in water and chromatographed on Dowex-50W x 2 with a linear gradient of pyridine-acetate buffers rising from pH 4.2-4.6 (6).

The elution profile contained two major peaks absorbing light at 315 nm with elution volumes from 60-200 ml (fraction I) and 390-500 ml (fraction II), which were individually pooled, flash-evaporated and dried over NaOH. To the residues were added 1 ml N HCl and 9 ml ethanol. On the addition of 0.09 ml pyridine, a crystalline product was obtained only from fraction I, which upon recrystallization from 90% ethanol formed needle-like crystals, mp 236-238 C. Yield 45%; UV max. (H_2O) at pH 4.0, 308 nm (ε 4860) and 220 nm (ε 4410); ^1H NMR (D_2O, 25 C), from TMS, δ 9.04 ppm (qt, 1H, $J_{6,5}$ = 4.1, $J_{6,4}$ = 3.0 Hz, H-6 of the 2-oxopyrimidine portion) 8.63 (qt, 1H, $J_{4,5}$ = 6.0, $J_{4,6}$ = 3.0 Hz, H-4), 7.16 (qt, 1H, $J_{5,4}$ = 6.0, $J_{5,6}$ = 4.1 Hz, H-5), 5.18 (*s*, 3H, DOH from $-CO_2H$ and $-NH_2$), 4.42 (*t*, 2H, $J_{\delta,\gamma}$ = 6.0 Hz, H-δ of α-amino-*n*-valeric acid portion of molecule), 4.18 (*t*, 1H, $J_{\alpha,\beta}$ = 6.0 Hz, H-α), 2.30 (multiplet, 4H, H-β and H-γ).

For mass spectrometric analysis 10 mg of the crystalline substance was silylated with 1.0 ml of N,0-bis-(trimethylsilyl)-acetamide reagent in pyridine (Pierce Chemical Co., Rockford, Ill.) by heating at 70 C for 20 min. Mass spectrum (70eV) m/e relative intensity) 355 M^+(5), 340 (3), 283 (3), 266 (3), 238 (6), 210 (3), 211 (2), 186 (5), 185 (4), 173 (5), 172 (16), 169 (8), 167 (6), 166 (100), 164 (5), 155 (6), 153 (13), 149 (20), 148 (30), 147 (61), 144 (5), 142 (10), 137 (8), 133 (5), 132 (5), 131 (19), 130 (7), 121 (6), 116 (7), 115 (23), 111 (6), 110 (7), 109 (5), 105 (5), 103 (8), 101 (7), 100 (9), 99 (6), 97 (19), 96 (9), 95 (6), 87 (5), 85 (10), 84 (6), 83 (10), 82 (8), 81 (8), 77

(7), 76 (10), 75 (95), 74 (12), 73 (105), 72 (8), 71 (15), 70 (104), 69 (14), 68 (17), 67 (7), 66 (18), 61 (9), 60 (6), 59 (28), 58 (7), 57 (38), 56 (22), 55 (15), 54 (10), 53 (8), 52 (13), 51 (6), 47 (18), 46 (6), 45 (52), 44 (30), 43 (37), 42 (20), 41 (5).

Analysis: Calculated for $C_9H_{13}N_3O_3$: C, 51.15; H, 6.20; N, 19.89. Found: C, 51.06; H, 6.25; N, 19.67.

α-amino-δ-(2-aminopyrimidine)-n-valeric acid. (Arg-M) or (δ-N-(2-pyrimidinyl)-L-ornithine). The compound was prepared as described by King (6), except that Dowex 50W x 2 was used, for reasons of availability, instead of the x 8 resin. The elution profile contained two major peaks which absorbed light at 315 nm. The fractions with elution volumes between 70-200 ml and 295-432 ml were flash-evaporated and dried over NaOH; however only from the first fraction was a crystalline product obtained, which upon recrystallization from 80% ethanol had a melting point at 260-262 C. ^{1}H NMR (D_2O, 33 C), from TMS δ 8.61 ppm (*d*, 2H, $J_{4,5}$ = 5.0, $J_{6,5}$ = 5.0 Hz, H-4 and H-6 of the 2-aminopyrimidine portion), 7.05 (*t*, 1H, $J_{5,4}$ = 5.0, $J_{5,6}$ = 5.0 Hz, H-5), 4.18 (*t*, 1H, $J_{\alpha,\beta}$ = 6.0 Hz, H-α of α-amino-*n*-valeric acid portion of molecule), 3.74 (*t*, 2 H, $J_{\delta,\gamma}$= 6.0 Hz, H-δ), 2.23 (multiplet, 4 H, H-β and H-γ).

The substituted α-amino acid was silylated as described above for the Cit-M product and the fragmentation pattern was determined. Mass spectrum (70eV) m/e (relative intensity) 354 M^+ (9), 339 (2), 259 (5), 258 (14), 249 (15), 237 (6), 236 (4), 232 (4), 218 (9), 175 (10), 169 (12), 152 (8), 149 (48), 148 (100), 147 (21), 146 (6), 142 (16), 136 (10), 133 (10), 122 (10), 121 (6), 120 (12), 115 (12), 109 (12), 108 (31), 100 (14), 97 (29), 96 (14), 95 (13), 81 (10), 80 (6), 79 (10), 77 (6), 76 (6), 75 (56), 74 (19), 73 (113), 70 (8), 68 (11), 61 (4), 59 (12), 57 (6), 56 (9), 55 (8), 54 (8), 53 (8), 47 (12), 45 (33), 44 (15), 43 (11), 42 (8), 41 (12).

N-Methyl-2-oxopyrimidine. The compound was prepared according to a known method for the preparation of 2-hydroxypyrimidine (7) by using N-methylurea instead of urea. N-Methyl urea, 3.76 g (0.0507 moles), 55 ml ethanol and 11.1 g (0.0505 moles) of 1,1,3,3-tetraethoxypropane were mixed with stirring, and 10 ml HCl (conc.) was added. Pale yellow needles precipitated out within minutes. The crystals were filtered off by suction and washed with ethanol. On the basis of NMR it appeared to be substantially the open chain N-methylurea-malonaldehyde adduct mp 149-151 C, although no consistent elemental analysis could be obtained. The mother liquor from this preparation was then cooled to 0 C and left overnight. Crystals of the N-methyl-2-oxopyrimidine HCl had the appearance of white, flat needles mp 205 C (decomp.). This hydrochloride salt (0.42 g or 5.7%) was neutralized with an equivalent of NaOH solution and flash-evaporated to dryness. The dried material was extracted with 30 ml hot ethylacetate from which fine needles or plates crystallized mp 132-133 C. ^{1}H NMR ($CDCl_3$, 30 C) from TMS, δ 8.55 ppm (qt, 1H, $J_{6,5}$ = 4.0, $J_{6,4}$ = 3.0 Hz, H-6), 7.89 (qt, 1H, $J_{4,5}$ = 6.0, $J_{4,6}$ = 3.0 Hz, H-4), 6.33 (qt, 1H, $J_{5,4}$ = 6.0, $J_{5,6}$ = 4.0 Hz, H-5), 3.60 (*s*, 3H, N-CH_3).

Analysis: Calculated for $C_5H_6N_2O$: C, 54.33; H, 5.49; N, 25.44. Found: C, 54.32; H, 5.60; N, 25.33.

Malonaldehydedianil was prepared according to the method of Huttel (8) by using 0.052 moles of malonaldehyde prepared from 11.44 g of 1,1,3,3-tetraethoxypropane, instead of propargylaldehyde, and 15.0 g (0.161 moles) aniline in 50 ml water and 15.0 ml acetic acid. The precipitated acetate salt was treated with carbonate and the free base of the malonaldehydedianil was extracted with diethyl ether and recrystallized from ethanol. The yellow-brown needles at times aggregated to plates mp 118-120 C. ^{1}H NMR ($CDCl_3$, 30 C) from TMS, δ 11.6 ppm (broad *s*, 1H of N-H), 7.59 (*d*, 2H $J_{\alpha,\beta}$ = 6.4, $J_{\alpha',\beta}$ = 6.4 Hz, H-α, H-α'), 7.10 (*m* 10H, aromatic protons), 5.08 (low amplitude, broad *t*, 1H, $J_{\beta,\alpha}$ = 6.4, $J_{\beta,\alpha'}$ = 6.4 Hz, H-β. In (DMSO-d_6, 30 C) from TMS, no downfield N-H signal was observed, but, after addition of a drop of D_2O a DOH signal appeared and integrated for approximately one proton, δ 8.17 ppm (*d*, 2H, $J_{\alpha,\beta}$ = 10.5, $J_{\alpha',\beta}$ = 10.5 Hz, H-α, H-α'), 7.37 (*m*, 10H, aromatic protons), 6.08 (low amplitude, broad *t*, 1H, $J_{\beta,\alpha}$ = 10.5, $J_{\beta,\alpha'}$ = 10.5 Hz, H-β); mass spectrum (70eV) m/e (relative intensity) 222 M^+ (100), 221 (100), 223 (16), 219 (18), 206 (11), 194 (18), 180 (5), 145 (13), 144 (5), 143 (5), 131 (5), 130 (32), 129 (18), 128 (18), 118 (18), 117 (10), 116 (5), 111 (18), 104 (13), 103 (11), 93 (18), 91 (10), 78 (5), 77 (40), 66 (5), 65 (18), 51 (18), 39 (5).

M-Bis-bisulfite sodium salt was prepared from M and sodium metabisulfite (9). The pyrazoline and pyrazole compounds (10) and the open chain mono- addition products glycine- and methionine-M (11,12), and the diglycine-M (13) diaddition products were prepared as reported previously. Cysteine-M (12), 2-amino- and 2-oxo-pyrimidine (7) were synthesized according to previously published procedures.

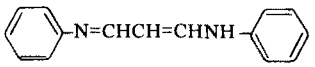
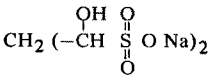
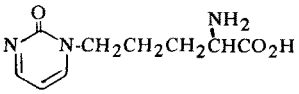
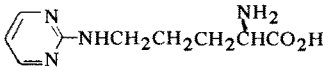
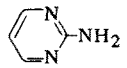
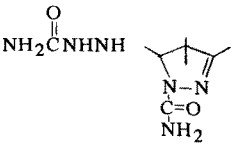
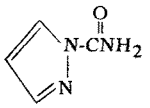
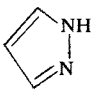
RESULTS AND DISCUSSION

The experimental results are compiled in Table I and show that the open chain mono- addition products glycine-M and methionine-M when assayed by the TBA method yield one mole of M per mole of addition product. The structure of these products has not been fully elucidated, although by analogy with the preferred conformations of M or preferably β-hydroxyacrolein (14) and the results of the structural studies on M-dianil in the present work, it can be inferred that the predominant tautomer of the mono-amine-M-addition products, in polar solvents, will probably exist in the *s-trans* conformation. The cysteine-M-product (12) can be expected to be more complex due to the reaction of M with the sulfhydryl in addition to the amino groups, and the total M recovery with the TBA procedure was only 70%. The disubstituted M products tested, such as the M-dianil, the M-sodium bisulfite and the M-diglycine, all gave quantitative recoveries in the assay for M, showing that the M-amine or bisulfite addition products are readily dissociated under conditions at which the TBA color reaction was carried out, and that the released M reacted quantitatively with the TBA. Similar observations were made earlier when it was shown that semicarbazide catalyzed hydrolysis of the amine addition products and formed the semicarbazone of M (12). Reaction of the amino acid citrulline with M resulted in a new α-amino acid containing an oxypyrimidine ring at the δ position of the alkyl chain. When this compound was subjected to the TBA test, only 30% of the expected theoretical color was obtained. For comparative purposes the unsubstituted pyrimidine ring system, 2-oxypyrimidine, containing the three carbon M-residue, when subjected to the test, gave only 22% of the total theoretical TBA color.

Upon reacting M with arginine, the guanidino group of this α-amino acid was cyclized to form an aminopyrimidine substituent at the δ position of its alkyl chain. NMR and mass spectra provided unequivocal proof for the structure of this compound, and when it was subjected to the colorimetric TBA test only 6% of the theoretical color was produced. The unsubstituted 2-aminopyrimidine compound containing the three carbon M residue gave 0.2% of the expected TBA color. A substituted pyrazoline, also containing the three carbon skeleton of M, was prepared recently (10) and can be looked upon as a model system representing a product from intramolecular imine-amine type addition. This parent pyrazoline gave 4.0% TBA color, and the degradation products pyrazole-1-carboxamide and pyrazole

TABLE I

Relative Color Contribution in the Thiobarbituric Acid (TBA) Test by Compounds Containing the Malonaldehyde Residue

Compound		Moles M[a] per mole of compound	Fluorescence
Malonaldehyde[b] (M)	HOCH=CHCHO	1.00	–
Glycine-M	R-N=CHCH=CHOH	1.00	+
Methionine-M	R'-N=CHCH=CHOH	1.00	+
$(Glycine)_2$-M	R-N=CHCH=CHNH-R	1.00	+
M-Dianil		1.00	–
Cysteine-M		0.70	+
M-Na bisulfite		1.00	–
Citrulline-M		0.30	–
2-Oxypyrimidine		0.22	–
Arginine-M		0.06	+
2-Aminopyrimidine		0.002	+
M-Bissemicarbazone (pyrazoline)		0.040	–
Pyrazole-1-carboxamide		0.000	–
Pyrazole		0.000	–

[a]"Moles M per mole of compound" expresses the ratio of the number of moles of M determined by the TBA method as related to the number of moles of the compound used in the analysis.

[b]Prepared from 1,1,3,3-tetramethoxypropane, bp 178-179 C.

obtained from it, but still containing the M backbone of carbon atoms, gave no TBA color even in concentrated solution. While the cyclic products into which M is incorporated are therefore relatively stable under the conditions at which the TBA test is carried out, the straight chain mono- or diaddition M-amino products were readily hydrolyzed. These results, in addition to evidence for a Dimroth-type rearrangement of 2-imino-1-methyl-pyrimidine to 2-methylaminopyrimidine via an open chain intermediate (19), then also explain the formation of the red TBA pigment from sulfadiazine:2-sulfanilamidopyrimidine (1).

In proteins the properties of the imine-enamine type of bonds formed are not expected to be very much different from those of the model compounds disussed above. However the reaction of M with sulfhydryl groups leading to the formation of mercaptals can also result in more stable linkages, and the lower color yield (70%) of the cysteine-M preparation in the TBA test can be explained on the basis that only the M residues attached to the amino groups are hydrolyzed in the TBA test, while the M involved in the mercaptal formation with the sulhydryl group does not contribute to the TBA color. In sulfhydryl containing proteins similar conditions could exist where the mercaptals, stable toward the assay conditions of the TBA test, are formed along with the more labile azomethine bonds. Furthermore, addition reactions of thiols to carbon-nitrogen double bonds can also lead to stable products (15). In a similar way, stabilization of the amine-aldehyde addition products may also be achieved by addition of an amino group to the imino bond which, in the special case of the bissemicarbazone of M, formed a stable pyrazoline product. This type of secondary addition reaction is initiated by nucleophilic attack of an amino nitrogen on the carbon atom of the azomethine bond. The formation of other cyclic systems along the peptide backbone of proteins might also be possible, since M does react with substituted amides as in N-methyl urea or citrulline. However, in aqueous systems, water will always be a competing reactant. In the formation of of most of the carbonyl-amine condensation products, as well as in the cyclization steps, the rate of the second order reactions can therefore be expected to proceed more rapidly in concentrated solutions such as would be present in frozen or dehydrated food systems, or possibly in hydrophobic regions of a native protein where the structured water present has diminished activity.

Because of the current interest in the detection of lipid

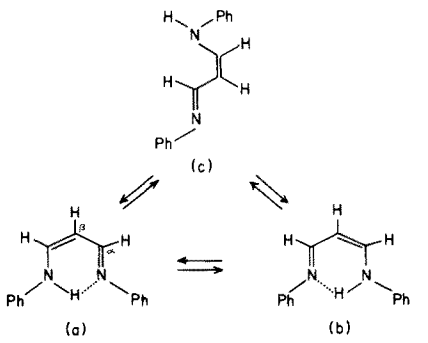
oxidation by a fluorescent method in which aldehyde- or ketone-amine condensation products form the fluorescent species (3), we also examined the fluorescent properties of the compounds used in the present studies. Condensation products, presumably of the general structure R-N=CHCH=CHOH and R-N=CHCH=CH-NH-R where the nitrogen-alkyl or hydrogen substituents originated from glycine, methionine, or ammonia, exhibited strong fluorescence with excitation and emission maxima at 395 and 470 nm, respectively, when the measurements were carried out in water solutions containing 1.0 mg/ml of the amorphous, uncrystallized products. However, when R in the diaddition product was a phenyl group (M-dianil), the compound could be readily purified by crystallization, but no significant fluorescence was detected in comparison to 0.5 μg of quinine per milliliter of 0.1 N H_2SO_4. It would therefore appear that the substituents on nitrogen have a determining influence on the fluorescence of the system, although additional fluorescent studies on more highly purified β-hydroxyacrolein-amino acid addition products, in which structures have been more rigorously assigned, would seem in order.

Of the products into which M was incorporated into a ring system, 2-oxopyrimidine or α-amino-δ-(N-2-oxopyrimidine)-*n*-valeric acid prepared from urea or citrulline and M, respectively, did not display any prominent fluorescence; however, α-amino-δ-(2-aminopyrimidine)-*n*-valeric acid prepared from arginine and M, as well as the 2-aminopyrimidine, a guanidine-M addition product, both showed a comparatively strong fluroescence at a concentration of 0.05 mg/ml of water, with excitation and emission maxima at 335 and 395 nm. The remaining compounds in Table I, which are marked with a negative sign, did not display any significant fluorescent properties.

The mass spectra of the pyrimidine-substituted amino acid derivatives had to be obtained from their silylated derivatives and were consequently dominated by ions characteristic of the fragmentation of N- and O-trimethylsilyl groups. Presence of the significant ions m/e 109 ($C_4H_3ONN^{+}=CH_2$) and m/e 108 ($C_4H_3N_2$-$N^{+}H=CH_2$), derived by fragmentation of the alkyl pyrimidine moiety from the molecular ions of α-amino-δ-(N-2-oxopyrimidine)-*n*-valeric acid, m/e 355 and α-amino-δ-(2-aminopyrimidine)-*n*-valeric acid, m/e 354, respectively, confirmed assignment of the heterocyclic portions of these molecules on the basis of their NMR spectra.

In the mass spectrum of M-dianil an intense molecular ion (M^+), m/e 222, and M^+-1 were observed relative to a low abundance for the ions m/e 118 (Ar-$\underline{N^{+}HCH=CH}$) and m/e 104 (Ar-$N^{+}\equiv CH$), findings which are consistent with the existence of a strong contribution of mesoionic resonance form to the structure of M-dianil and the consequent stability of its molecular ion.

The NMR spectra of the M-dianil were studied in detail and, of the acyclic compounds under consideration, permitted the most definitive assignment of structure. The NMR spectra gave a doublet for the α-protons which integrated for two hydrogens and had a solvent-dependent coupling constant of 6.45 Hz in $CDCl_3$ (10.5 Hz in d_6-DMSO). The β-proton appeared as a broadened triplet and did not exchange with D_2O. The absence of any evidence for the methylene protons of a 1,3-diimine structure in the spectrum therefore allowed for the existence of only the amine-enimine tautomeric form Ph-NH-CH=CH-CH=N-Ph of the dianil. This finding is consistent with the structures assigned to the anils of β-diketones previously reported (16). The *s-cis* conformation of the *syn-cis* configuration would give a mesoionic



structure represented by the rapid tautomeric exchange between (a) and (b). Rapid exchange between the *s-cis* conformation (a and b) and the *s-trans* conformation (c) could also take place, although need not be invoked to explain the coupling $J_{\alpha,\beta}$ 6.45 Hz which is in the range of J_{cis} and J_{trans} couplings in olefins (17). Buttkus, H. and R.J. Bose amine-malonaldehyde condensation products and their relative color contribution in the thiobarbituric acid test.

Increasing the temperature of the $CDCl_3$ solution to 60 C gave a sharp triplet for the β-proton with unaltered spacing. Sharpening of the triplet peak at δ 5.08 ppm could be ascribed to loss of long range ^{14}N quadrupole-induced relaxation due to increased symmetry about the nitrogen from rotation and inversion. On lowering the temperature, the α-proton doublet broadened and finally coalesced at about -60 C. Similar broadening effects were observed at ambient temperature in d_6-DMSO reflecting strong solute-solvent interaction, larger barriers to rotation and solvent dependent distribution of rotamers favoring the *s-trans* conformer, similar to the effects of solvent on conformation observed for M (14). The N-H proton was not observed in d_6-DMSO because of suppressed exchange (18) and "washing out" by ^{14}N quadrupole relaxation. Further work is in progress to determine if the broadening observed for the β-proton is due to ^{14}N quadrupole relaxation effects, as expected, or to yet undetected slow chemical exchange.

REFERENCES

1. Sinnhuber, R.O., T.C. Yu and T.E. Chang Yu, Food Res. 23: 626 (1958).
2. Kwon, T.W., and H.S. Olcott, Nature 210: 214 (1966).
3. Dillard, C.J., and A.L. Tappel, Lipids 6: 715 (1971).
4. Chipault, J.R., and J.M. Hawkins, J. Agr. Food Chem. 19: 495 (1971).
5. Holland, D.C. J. Ass. Offic. Anal. Chem. 54: 1024 (1971).
6. King, T.P., Biochemistry 5: 3454 (1966).
7. Protopopova, T.V., and A.P. Skoldinov, J. Gen. Chem. USSR 28: 1360 (1958).
8. Hüttel, R., Ber. 74: 1825 (1941).
9. Saslaw, L.D., and V.S. Waravdekar, J. Org. Chem. 22: 843 (1957).
10. Buttkus, H., and R.J. Bose, Ibid. 36: 3895 (1971).
11. Crawford, D.L., T.C. Yu and R.O. Sinnhuber, J. Agr. Food Chem. 14: 182 (1966).
12. Buttkus, H., JAOCS 46: 88 (1969).
13. Chio, K.S., and A.L. Tappel, Biochemistry 8: 2821 (1969).
14. George, W.O., and V.G. Mansell, J. Chem. Soc. B. 132 (1968).
15. Harada, K., in "The Chemistry of the Carbon-Nitrogen Double Bond," Interscience Publishers, London, 1970, p. 255.
16. Dudek, G.O., and E.P. Dudek, J. Amer. Chem. Soc. 88: 2407 (1966).
17. Bovey, F.A., "Nuclear Magnetic Resonance," Academic Press, New York, 1969.
18. Perlin, A.S., Can. J. Chem. 44: 539 (1966).
19. Perrin, D.D., and I.H. Pitman, Aust. J. Chem. 18: 763 (1965).

[Received February 28, 1972]