TECHNICAL REPORT
FD-7

THE BASIS OF STABILITY IN LYSINE AND ARGININE SALTS OF UNSATURATED FATTY ACIDS

by

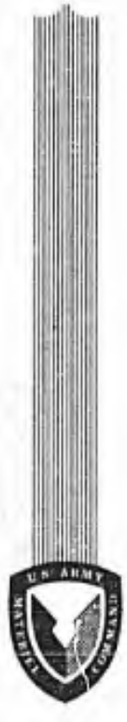
Stanley D. Koch

Asher A. Hyatt Dolores V. Lopiekes

MONSANTO RESEARCH CORPORATION
Everett, Massachusetts

Contract No. DA 19-129-QM-1922(N)

April 1965



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Everett, Massachusetts

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Natick, Massachusetts

FOREWORD

A major military objective in the design of packaged operational rations is the highest caloric density consistent with the requirements of good nutrition and stability of the ration items. Increase in fat content, the obvious way to increase caloric density, results in inherent incompatabilities. To insure stability the fat must be fully saturated, which is undesirable nutritionally, at least to the extent that essential polyunsaturated fatty acids are required in the diet. A series of compounds representing salts of basic amino acids and linoleic acid were reported to resist autoxidation of the linoleic acid. If this phenomenon could be utilized to protect the unsaturated fatty acids in dehydrated foods, it would be of importance in achieving greater caloric density in packaged operational rations.

The work covered in this report, performed by the Monsanto Research Corporation under Contract No. DA19-129-QM-1992(N) (May 1962 - May 1964) represents a preliminary investigation of the generality of the antioxidative effect of amine-linoleate salts and of the physico-chemical factors involved in the effect. The investigator was Dr. Stanley D. Koch. His collaborators were Dr. Asher A. Hyatt and Miss Dolores Lopiekes. They were assisted by Dr. Horace F. Martin, Dr. Steven Price, and Dr. Richard H. Nealey. Analytical work was done by Mr. William R. Smith, Mrs. Jeanette C. Alm, and Miss Mildred J. Landon. Research assistance was provided by Miss Dorothy A. Kibildis, Mr. Peter S. Simone, and Mr. Conrad A. Cenerizio.

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SUMMARY

The mechanism and generality of the known stabilization against autoxidation conferred on linoleic acid by certain basic amino acids, such as lysine and arginine, was investigated.

Basic amino acids were the only class of compounds found to confer the effect. However, the smallest basic amino acid, 2,3-diamino-propionic acid was not effective, nor was a β,ω-diamino acid, 3,6-diaminohexanoic acid although a simple isomer of lysine. The stabilization was observed only in the solid phase. Inclusion of sodium chloride in the solid matrix was deleterious to the effect.

A large number of physical and chemical observations were made and correlated but it has not been found possible to draw detailed conclusions about the mechanism of stabilization. On the basis of the reported observations no detailed structure of the stabilized complex can be suggested.

The cause of the phenomenon appears to be closely associated with the physical arrangement of the ions in the crystal lattice.

I. INTRODUCTION

A. STATEMENT OF THE PROBLEM

Salts of basic amino acids and linoleic acids were described in a patent by Chang and Moyer (ref. 1). They reported that the salts were far more resistant to becoming rancid by autoxidation than the linoleic acid. This phenomenon would be of importance if it could be utilized so that meats and other foods containing unsaturated fatty acid derivatives could be protected against autoxidation by means of edible additives.

The objectives of this study, as stated in the contract, comprised two phases:

(1) Study of the structure of lysine and organic salts of unsaturated fatty acids, especially linoleic acid, and of the relationship of the structure to the reactivities of the unsaturated sites. These studies were to include, but not be limited to, X-ray diffraction, nuclear magnetic resonance, spectrophotometry, chemical degradation, chromatography, and accelerated storage tests.

(2) Apply the structural principles to the synthesis and stability testing of compounds of unsaturated fatty acids with nitrogeneous bases including, but not limited to basic amino acids, α-hydroxy analogs of basic amino acids, guanidyl and histidyl compounds and simple peptides in order to study the generality of the effect.

B. APPROACH

Our proposal considered three possibilities for the structure of lysinium linoleate*: (1) it could be a true ionized salt involving the carboxyl group of linoleic acid with the free amino group of lysine, (2) it could be an inclusion compound such as these of linoleic acid with dextrins (ref. 2), deoxycholic acid (ref. 2,3,4), urea (ref. 5), which are all stabilized against oxidation; (3) a true covalent bond could unite the two components, involving perhaps an amide link or reaction across the double bonds of linoleic acid.

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*Early in the work the compounds of basic amino acids and linoleic acid or some of its analogs were shown to be equimolar salts and names were coined to emphasize this. The salt of lysine and linoleic acid was named "lysinium linoleate", etc.

Lysinium linoleate, in particular, was chosen for thorough examination. Its stoichiometry was checked and its electrical conductivity in solution investigated. A very thorough infrared spectroscopic examination was carried out and nuclear magnetic resonance spectroscopy, Raman spectroscopy and thin layer chromatography were also employed in this examination.

The generality of the oxidation protection phenomenon was studied thoroughly using the Warburg apparatus, and employing a variety of natural and synthetic basic amino acids, simple amino acids, mono amines, diamines, and derivatives of lysine with linoleic acid. Other unsaturated acids and derivatives of them were examined in the Warburg apparatus with lysine.

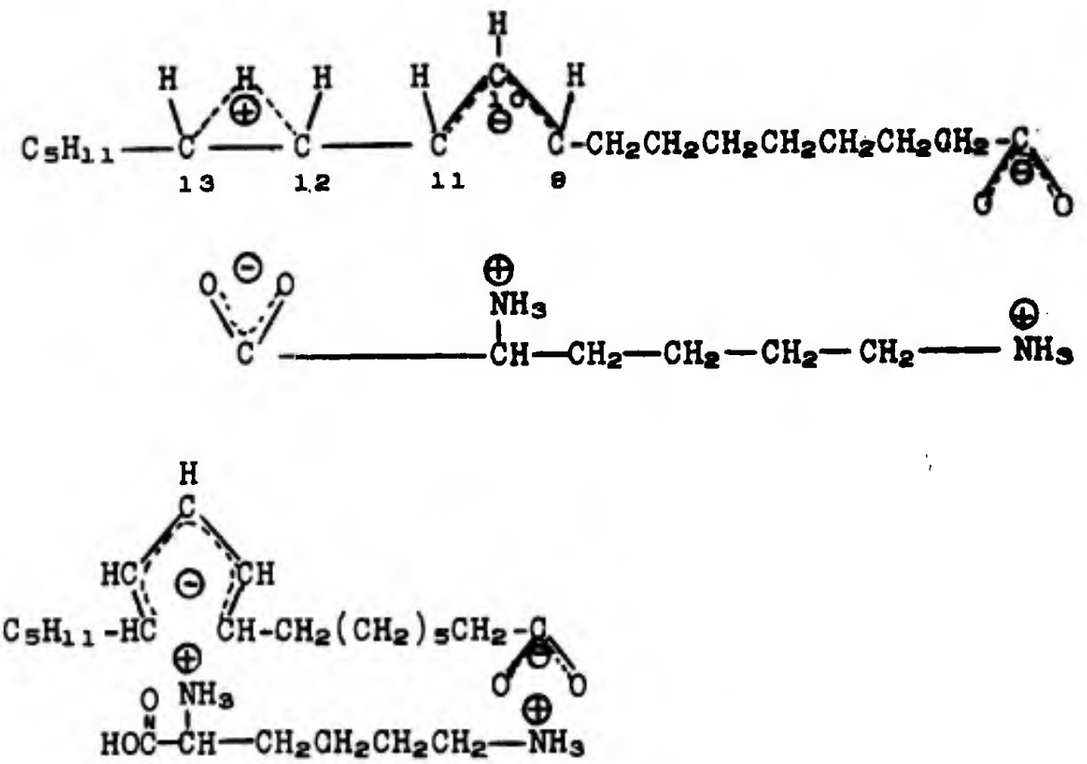
This apparatus was also used to study the effect of physical state on the autoxidation of linoleic acid and lysinium linoleate, and also the effect on the latter of contamination with trace metals or with sodium chloride, and to study the phenomenon of "gas release" during autoxidation.

II. CONCLUSIONS

Experiments early in the project showed gravimetrically that lysinium linoleate was a 1:1 compound from which the components could be recovered unchanged by appropriate chemical treatment. Use of linolelaidic acid (the trans,trans-isomer of linoleic acid) showed that the original stereochemical configurations were retained in the compounds.

The electrical conductivity work indicated that lysinium linoleate was dissociated in 90% methanol solution and behaved as a strong electrolyte of the uni-univalent type. Thin layer chromatography showed that lysinium linoleate was not dissociated in chloroform and contained no free lysine or linoleic acid.

These initial results indicated that lysinium linoleate was indeed a true ionized salt, rather than an inclusion complex or a covalently linked compound. Model structures such as the following were considered as possibly accounting for the reduced reactivity of the double bond region.



The first scheme shown involved a proton transfer from C-11 to the cation system at C-12/C-13 and showed a formal charge separation at both unsaturated sites. The second scheme makes a point of portraying C-9 to C-13 as approaching the cyclopentadieneide system. This is shown again in Figure 1, photograph of Fisher-Taylor-Hirschfelder models of linoleate ion with C-9 to C-13 in the cyclopentadieneide configuration. Figure 1 also shows the zwitterion-cations of two basic amino acids: 2,3-diaminopropionate and 2,4-diaminobutyrate. It is not necessarily clear from Figure 1, but these ions are capable of existing in many conformations based on carbon-carbon single bond rotations alone. The large numbers of conformations make it impossible to settle the question of structure from models alone. Many of the possible conformations do, however, discriminate between the two basic amino acid ions in Figure 1, accommodating the 2,4-diaminobutyrate and not allowing room for bond-formation with 2,3-diaminopropionate. The problem in these cases is that the linoleate carboxyl group cannot be brought close enough to the ω-amino group of the 2,3-diaminopropionate.

It was hoped that these models could be tested by qualitative examination of the double-bond system of lysinium linoleate by nuclear magnetic resonance, but this proved not to be practical. The nmr spectrum could only be determined in the solution phase, where the oxidation-protection phenomenon did not occur.

Infrared spectroscopy was carried out on Nujol mulls in which the protective effect was retained. The initial examination confirmed that lysinium linoleate was a salt; no free amino or carboxyl group vibrations were found, while those due to ammonium and carboxylate ions were strong. The intensity of the absorptions due to the latter groups were shown to be consistent with a composite of that of the lysinium and linoleate ions only. A more sophisticated infrared analysis involving deuteration and high-resolution work was used to examine the unsaturation vibrations. It was concluded that there was no anomaly in the number of double bonds in lysinium linoleate, effectively eliminating both model structures.

At this stage it seemed possible that the effect was specifically associated with the solid state, the rigid crystalline lattice of lysinium linoleate acting as a barrier to the diffusion of oxygen and oxidized intermediates and to the propagation of chain reactions. Studies of the oxidation in solution suggested that ionization was not the only phenomenon necessary for protection. It seemed that the ions had to be closely associated in the solid state. The fact that simple solid salts such as ammonium linoleate and frozen solid linoleic acid both oxidized, showed that the solid state alone was not sufficient to cause inhibition. Thus ionization and solid state are both necessary but neither is sufficient to explain the oxidation inhibition effect, which was found to persist in lysinium linoleate even when it was deliberately contaminated with a copper pro-oxidant.

A series of experiments in which sodium chloride was used to contaminate lysinium linoleate on a mole-to-mole basis was highly significant. The results showed that the more intimately foreign ions were included in the crystal lattice of lysinium linoleate, the greater was the damage to the autoxidation-inhibition phenomenon. The ions in lysinium linoleate prepared by the patented procedure were thus shown to be associated other than by just electrostatic attraction in the crystalline state. This association appeared to persist to a degree in solution.

The experiments to delineate the generality of the effect showed that compounds that were not basic amino acids did not protect linoleic acid from autoxidation. Some amines and diamines formed salts with linoleic acid that were found to decrease the rate of autoxidation, but these did not provide the total protection of the patented compound. This might have been a viscosity effect, which would have shown a maximum if a solid salt could have been found, but all these salts were liquid.

On the other hand, all the salts with basic amino acids were solids. At first it appeared that they all showed the oxidation-inhibition effect as claimed in the patent, but two were found that did not. They were 2,3-diaminopropionic acid, the smallest basic amino acid possible; and 3,6-diaminohexanoic acid, the only basic amino acid tested that was not α,ω-diamino. This particular one is a β,ω-diamino acid. The only basic amino acids available commercially or much studied are the α,ω-diamino examples, which apparently give the patent effect.

It was thus clear that the chain length and stereochemical fit were of some importance, although it is important in this context that lysine was found to protect linolelaidic acid (the trans,trans-isomer of linoleic acid), which has a totally different shape from linoleic acid.

To summarize, the following situations were found to be necessary for protection of linoleic acid by a basic amino acid:

a) Salt formation must occur, for it is the linoleate ion that is protected.

b) The salt must be solid and dry.

c) The ions in the solid state must be closely associated.

d) There must be an undefined stereochemical fit (which cannot be met by 2,3-diaminopropionic or 3,6-diaminohexanoic acids).

III. DISCUSSION AND RESULTS

A. STRUCTURAL BASIS FOR STABILITY OF LYSINIUM LINOLEATE

1. Stoichiometry

Linoleic acid was reacted with lysine as described in the patent (ref. 1). In the early phases of the work, linoleic acid was obtained from Sigma Chemical Co. who certified it to be 97% pure. That obtained from Fisher Scientific Corp. was only 92% pure, but lysinium linoleate samples made from linoleic acid from either source and washed on a Buchner funnel with excess methanol were found to have identical infrared spectra. It seems probable that the impurities in the Fisher acid were other fatty acids such as oleic and stearic. Since any unreacted materials were removed from the product by the methanol-wash procedure, the commercial 92% linoleic acid and methanol washes were used. In later phases of the work, linoleic acid was purchased from the Hormel Institute and had a purity of greater than 99%. The lysine and other materials used were of the highest purity commercially available.

The salts were made by the interaction of equimolar quantities of the two ingredients, but since they were sometimes made by precipitation from solution and were always purified by a solvent wash, the method of synthesis did not constitute proof that the starting materials reacted in an equimolar ratio. To prove this unequivocally, lysinium linoleate was treated with calcium chloride:

$$\text{lysinium linoleate} + CaCl_2 \longrightarrow \text{calcium linoleate} + \text{lysine}\cdot HCl$$

To 0.005 mole of lysinium linoleate dissolved in water was added with stirring an equivalent amount of calcium chloride also dissolved in water. The white precipitate was filtered off and dried. The calculated yield for calcium linoleate, the expected product, was 1.4980 g. The actual yield obtained was 1.4944 g. The infrared spectrum of the calcium linoleate obtained in this manner agreed with the spectrum for calcium linoleate made directly from calcium hydroxide and linoleic acid.

The identity and purity of the precipitated calcium linoleate was confirmed by ashing. The weight of calcium oxide formed after heating was 9.8% (calculated, 9.3%). The ashing procedure was checked by ashing a known sample of calcium carbonate: calculated, 56.0%; found, 55.9%.

The filtrate from the reaction mixture was evaporated to dryness and constant weight to give a white solid. The theoretical yield expected for lysine monohydrochloride was 0.9135 g. The actual yield obtained was 0.9110 g. The melting point of this crude white solid was 234-8°C. That of the recrystallized compound was 234-5°C on the Fisher-Johns hot stage apparatus [reported (ref. 6), 235-6°C]. When mixed with an

authentic sample of lysine monohydrochloride there was no melting point depression. Infrared spectra of the samples of crude, recrystallized, and known lysine monohydrochloride were identical.

For further confirmation, 0.36 g of the lysine hydrochloride obtained from this experiment was converted to lysine monopicrate. The yellow needles obtained melted at 264-6°C on the Fisher-Johns apparatus [reported (ref. 7), 266°C].

Summarizing, lysinium linoleate was converted to calcium linoleate and lysine monohydrochloride in a 1:1 ratio. This was established gravimetrically, and the identification of the derivatives was corroborated spectroscopically, in the case of calcium linoleate, and by the picrate derivative in the case of lysine hydrochloride. This settles beyond doubt the stoichiometry of lysinium linoleate as a 1:1 compound.

2. Recovery of Components

The lysine salts of linoleic acid and linolelaidic acid (the trans,trans-isomer of linoleic acid) were prepared by the method described in the patent (ref. 1). Lysinium linoleate and lysinium linolelaidate were treated with hydrochloric acid in aqueous methanol solution and extracted with ether. Infrared spectroscopy of the extracts showed them to be pure methyl linoleate and methyl linolelaidate. Neither sample showed any contamination with the other isomer. The acids were presumably esterified by the acidic methanol solution, but it was clear that the original stereochemical configurations had been retained in the lysinium salts.

3. Electrical Conductivity

a. Conductance at Infinite Dilution of Lysinium Linoleate

Electrolytes may be divided into two classes: strong electrolytes with a high conductance that increases slightly on dilution, and weak electrolytes with a low conductance that shows larger increases on dilution. A great many electrolytes, of course, fall between these two extremes. Lysinium linoleate solutions conducted a current and gave a straight line plot characteristic of a strong electrolyte. The equivalent conductances obtained were low for strong electrolytes in all the salts examined, but this is probably due to the fact that the solvent was 90% methanol rather than water (ref. 8).

Electrical conductivity was determined on an Industrial Instruments, Inc. conductivity bridge, model RC-16B2, at 1000 cps. The first two columns of Table 1 give the equivalent conductance of the pertinent compounds from 0.005M extrapolated to zero conductance. These points have been plotted against the square root of concentration (Figure 2).

Table 1

EQUIVALENT CONDUCTANCE AND IONIZATION CONSTANTS AT 25°C

	90% Methanol Solvent			
	M	Equiv. Cond. Λ	Degree of Dissoc. α	pK
Sodium chloride	0.0000	(87)	-	-
	0.0001	86	0.989	2.1
	0.0005	84	0.966	1.9
	0.0010	74	0.851	2.3
	0.0025	67	0.770	2.2
	0.0050	63	0.724	2.0
Sodium linoleate	0.0000	(63.5)	-	-
	0.0001	62.9	0.975	2.4
	0.0005	60	0.945	2.1
	0.0010	60	0.945	1.8
	0.0025	54.8	0.863	2.9
	0.0050	54	0.850	1.6
L-Lysine hydrochloride	0.0000	(60.5)	-	-
	0.0001	59.5	0.983	2.3
	0.0005	60	0.992	1.2
	0.0010	55	0.910	2.0
	0.0025	51	0.843	2.0
	0.0050	48	0.793	1.8
L-Lysinium linoleate	0.0000	(39)	-	-
	0.0001	37.9	0.972	2.5
	0.0005	34	0.872	2.5
	0.0010	35	0.897	2.1
	0.0025	31.8	0.815	2.0
	0.0050	29	0.744	1.9

For the strong electrolyte, sodium chloride, the equivalent conductance is seen to be a linear function of the square root of the concentration. For the salts sodium linoleate and lysine hydrochloride, straight lines were also obtained as anticipated. Lysinium linoleate also gave a straight-line plot, indicating that it was dissociated in solution and behaved as a strong electrolyte. Extrapolation of these lines to infinite dilution gave accurate values of Λ_o (the equivalent conductance at infinite dilution). A weak electrolyte would have given a steep curve that could have been extrapolated to infinite dilution only with great difficulty and inaccuracy.

Properties of the ions of strong electrolytes are known to be additive at infinite dilution. Thus, the equivalent conductance at infinite dilution of a given compound can be calculated if the equivalent conductances of the cations and anions are known. The equivalent conductance of lysinium linoleate can thus be calculated:

$$\Lambda_o(\text{Lys}\cdot\text{HCl}) + \Lambda_o(\text{NaLin}) - \Lambda_o(\text{NaCl}) = \Lambda_o(\text{LysLin})$$

$$60.5 + 63.5 - 87 = 37$$

The calculated Λ_o is 37 $\text{ohms}^{-1}\text{cm}^2$; the extrapolated experimental value for lysinium linoleate is 39 $\text{ohms}^{-1}\text{cm}^2$. This is considered very good agreement and hence confirmation of the normal behavior of lysinium linoleate as a strong electrolyte in solution.

b. Degree of Dissociation of Lysinium Linoleate

The extent to which an electrolyte is dissociated may be determined in many ways. The electrical conductance method is the most used because of its accuracy and simplicity. The degree of dissociation (α) can be calculated from the conductivity of the electrolyte and the equivalent conductance at infinite dilution.

$$\alpha = \frac{\Lambda}{\Lambda_o}$$

This conductance ratio (or degree of dissociation) is a useful quantity because it indicates the extent to which the equivalent conductance at any specified concentration differs from the limiting value. The change in conductance ratio with concentration gives a measure of the corresponding change of the equivalent conductance. In dilute solutions of strong electrolytes the conductance ratio is known to be almost independent of the nature of the salt and is determined almost entirely by its valence type. Table 2 gives the mean values of a number of electrolyte types in water at room temperature (ref. 9).

Table 2

CONDUCTANCE RATIO AND VALENCE TYPE OF SALT

Valence Type	0.001N	0.01N	0.1N
uni-uni	0.98	0.93	0.83
uni-bi bi-uni	0.95	0.87	0.75
bi-bi	0.85	0.65	0.40

The degrees of dissociation of lysinium linoleate at several concentrations are listed in column 3 of Table 1. Keeping in mind that the solvent was not water but 90% methanol, it appears that lysinium linoleate with a value of 0.897 at 0.001N is a uni-univalent type salt comparable to sodium chloride with an experimental value (in 90% methanol) of 0.851, lysine hydrochloride 0.902, and sodium linoleate 0.859.

When any electrolyte, MA, is dissolved in a suitable solvent, it yields M^+ and A^- ions in solution. If c moles of an electrolyte are dissolved in a liter of solvent and the degree of dissociation of the salt is α, then the concentration of cations is $c\alpha$, the concentration of anions is $c\alpha$, and the concentration of unionized material is $c(1-\alpha)$. The equilibrium or ionization constant (K) is given by the expression:

$$K = \frac{(c_{M^+})(c_{A^-})}{c_{MA}} = \frac{(c\alpha)(c\alpha)}{c(1-\alpha)} = \frac{\alpha^2 c}{1-\alpha}$$

This constant was calculated for lysinium linoleate at several concentrations. The activity coefficient was neglected and the degree of dissociation assumed to be equal to the conductance ratio. The results, listed in column 4 of Table 1, showed that in 90% methanol lysine hydrochloride, sodium chloride, and sodium linoleate had pK values of about 2, and the value for lysinium linoleate was 2 to 2.5. In water, values of pK of 2 to 3 are usually considered to show a moderately strong electrolyte, 5 weak, 9 very weak, and 12 extremely weak. Thus, lysinium linoleate was shown to be a moderately strong electrolyte of the uni-univalent type.

c. Conductivity of Lysinium Linoleate Analogs

Ornithinium linoleate, 3-amino-3-carboxypropylammonium linoleate, lysinium oleate, ornithinium oleate, lysinium stearate and ornithinium stearate were all made by the patented procedure (ref. 1).

All conducted a current in solution. For the linoleates a straight-line plot was obtained when equivalent conductance was plotted against the square root of concentration, indicative of a strong electrolyte. The oleates, however, gave a steep curve (Figure 3) typical of a weak electrolyte.

The two stearates were not as soluble as the linoleates and oleates, and another technique was necessary to obtain their conductivities. A separate graph has been made to show their conductivities (Figure 4). The stearates were suspended in the 90% aqueous methanol solution used in all previous conductivity measurements. Some insoluble material was filtered off on a "Millipore" filter funnel (0.45-micron pore) leaving a clear filtrate that conducted current. A curve for each stearate was obtained by making successive dilutions of this filtrate. Steep curves were obtained characteristic of weak electrolytes. The solubilities of the stearates were determined by making up saturated solutions in 90% aqueous methanol and filtering off the insoluble material as before. The filtrate was then evaporated to dryness and the residue, dried to constant weight, was identified by infared as the original compound. Both stearates showed solubilities of 0.40 g/liter.

4. Infrared Spectrum of Lysinium Linoleate

a. Gross Structural Features

A preliminary evaluation of the infrared spectrum of Nujol mull preparations of lysinium linoleate was made on a Perkin-Elmer Infracord Model 137 Spectrophotometer with sodium chloride optics.

A comparison of the spectra of the free base of L-lysine (Figure 5) with its monohydrochloride (Figure 6) showed the absence of the N-H stretching vibration in the monohydrochloride. Comparison of this region in lysine free base with lysinium linoleate (Figure 7) showed a complete absence of this band at 3 microns in the latter. It was concluded from these observations that the free amine was no longer present after reaction with linoleic acid.

A comparison of the spectrum of linoleic acid (Figure 8) with that of lysinium linoleate (Figure 7) showed the disappearance of the 5.8-micron carbonyl absorption band in the latter. This indicated formation of the carboxylate anion.

The 6.0 to 6.8-micron region of lysinium linoleate was reconstructed from the absorption intensities of lysine monohydrochloride and calcium linoleate, as indicated in Table 3. This table shows that the spectrum of lysinium linoleate is a composite of that of the lysinium ion and the linoleate ion. Small deviations from perfect additivity were to be expected because of displacement of bands by hydrogen-bonding, physical state, and other masking effects. The correlation is considered satisfactory.

Table 3

RECONSTRUCTED 6.0- TO 6.8-MICRON REGION FOR LYSINIUM LINOLEATE

	Absorption contribution, absorption units			
	1610-1635 cm^{-1}	1560 cm^{-1}	1510 cm^{-1}	1580 cm^{-1}
Linoleate ion	0.017	0.33	0.034	0.34
Lysine monohydrochloride	0.269	0.056	0.35	0.0525
Sum, calculated	0.286	0.386	0.384	0.3925
Lysinium linoleate, observed	0.24	0.31	0.38	0.39

b. The Double-Bond Region

The infrared spectra in the double-bond region were examined on Perkin-Elmer Model 21 and Beckman IR-8 instruments. The spectrum of lysinium linoleate in a KBr pellet or a Nujol mull showed a shoulder at the expected frequency of a double bond stretch at 3020 cm^{-1}. Because of the interfering absorption of the NH_3^+ ion, it was not possible to state definitely that unsaturation was present. The 1660 cm^{-1} vibration associated with C=C was also lost in the C=O vibrations. The out-of-plane CH of the cis-olefin was very weak and thus of little value in detecting unsaturation.

In order to eliminate the interfering NH_3^+ absorption between 3300 and 2600 cm^{-1}, the lysinium linoleate was deuterated, converting NH to ND.

The deuteration was carried out as follows. Approximately 100 mg of lysinium linoleate was added to 2 ml of deuterium oxide in a stoppered vial. After gentle heating and shaking for an hour, the salt dissolved in the deuterium oxide. The solution was frozen in liquid air and the deuterium oxide and water were removed by freeze-drying for four hours. The deuterated lysinium linoleate was obtained as a fluffy white solid.

Deuteration was accompanied by a shift of the NH stretch from 3300-2630 cm^{-1} to 2500-2000 cm^{-1} for the ND stretch. When most of the interfering NH absorptions were removed, only the CH stretching vibration showed in this region. By running the infrared spectrum of deuterated lysinium linoleate in a Kel-F mull (no CH present), the presence of aliphatic CH as well as olefinic CH at 3020 cm^{-1} was seen. This is summarized in Table 4 and can be seen in the infrared spectra (Figures 9,10) of the salt before and after deuteration, which clearly show that unsaturation was present in deuterated lysinium linoleate.

Table 4

INFRARED ASSIGNMENTS

Lysinium Linoleate (KBr)		Deuterated Lysinium Linoleate (Nujol Mull)	
Frequency cm^{-1}	Assignment	Frequency cm^{-1}	Assignment
3300-2630	NH_3^+	2500-2000	ND_3^+
3020	CH=	3020	CH=
2120	NH_3^+	1550*	ND_3^+
1563	NH_3^+ def., COO^-	1589 and/or 1560	COO^-
		1170	ND_3^+ def.

*Estimated (observed by COO^- vibration)

R. G. Sinclair, et al. (ref. 10) have shown that in the C-H stretching region the band at 3020 cm^{-1} increases with the number of cis-double bonds present, while the relative intensity of the methylene peak at 2920 cm^{-1} diminishes. If d_A is the optical density at 3020 cm^{-1}, and d_B at 2920 cm^{-1}, then a plot of d_B/d_B-d_A against the number of double bonds should be approximately linear for oleic, linoleic, and linolenic acids, which contain one, two and three cis double bonds, respectively. Curves similar to the one for cis-unsaturated acids were established for the methyl esters of these acids, and for their deuterated lysine salts. By this extrapolation, it was shown that there was no anomaly in the number of double bonds in solid lysinium linoleate.

Deuteration of the salts was carried out as described earlier and appeared to be about 80% complete as indicated by the infrared spectra. Nuclear magnetic resonance spectra were also run to check the degree of deuteration. The purity of the acids was checked by thin layer chromatography and the percentages of foreign acids (including trans-stereoisomers) were found to be negligible. If there had been appreciable quantities of trans-isomers, a correction would have been necessary because the optical density of the 3020 cm^{-1} band is weaker for trans-olefins than that for cis-olefins. However, the salts used were isomerically pure. A Perkin-Elmer 150-foot capillary (0.01 in. internal diameter) vapor phase chromatography column packed with diethylene succinate was available for the separation of isomers had this been found to be necessary. Once the factors had been standardized, the infrared spectra of the acids, their methyl esters and deuterated lysine salts were run on the high resolution double grating Perkin-Elmer 421 instrument at Monsanto Research Corporation's Dayton (Ohio) Laboratory.

The sodium chloride prism on this instrument gave a resolution of 0.3 cm^{-1} at 2200 cm^{-1}, which was sufficient sensitivity for the problem and far better than that of the Perkin-Elmer 21 and Beckman IR-8 used in the initial phases of this study.

Samples were prepared for spectroscopy as thin films between two rock salt crystals. The methyl esters and acids were run as liquid films while the lysinium salts were run as hexachloro-1,3-butadiene mulls.

Calibration of the spectrometer with a polystyrene reference film indicated that the band positions near 3000 cm^{-1} were recorded 1 cm^{-1} higher than the actual value, i.e., actual value was 3003 cm^{-1} rather than 3004 cm^{-1} for polystyrene. This resulted in a slight displacement in the spectral data from those recorded by Sinclair, et al. (ref. 10), i.e., 3019 instead of 3020 cm^{-1} for the olefinic CH stretch.

The absorbance (optical density) measurements were made by the base line technique shown below. The results are summarized in Table 5.

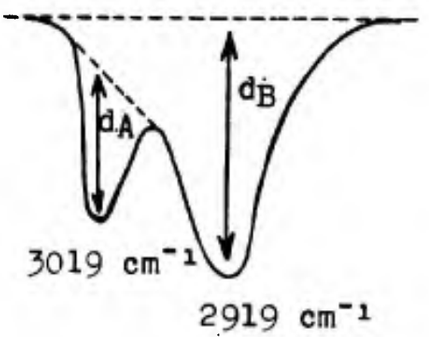


Table 5

INFRARED ABSORBANCE MEASUREMENTS

Compound	Absorbance at 3019 cm^{-1} (d_A)	Absorbance at 2919 cm^{-1} (d_B)	$\frac{d_B}{d_B - d_A}$	Number of Double Bonds
Oleic acid	0.047	0.852	1.06	1
Linoleic acid	0.119	0.645	1.23	2
Linolenic acid (thick film)	0.387	1.389	1.39	3
Linolenic acid	0.210	0.714	1.42	3
Methyl oleate	0.057	0.791	1.08	1
Methyl linoleate	0.150	0.848	1.21	2
Methyl linolenate	0.256	0.795	1.47	3
Deuterated lysinium oleate	0.065	0.789	1.09	(1)
Deuterated lysinium linoleate	0.165	1.111	1.17	(2)
Deuterated lysinium linolenate	0.124	0.660	1.23	(3)

It can be seen from the table that the results confirmed those in the literature (ref. 10) in that d_B/d_B-d_A plotted against the number of double bonds was approximately linear for the acids and their methyl esters. As the same plot for the deuterated salts also gave a straight line, it was concluded that there was no anomaly in the number of double bonds in lysinium linoleate, i.e., there were two double bonds in the solid salt.

Iodine titration was also considered as a method of determining the number of double bonds, but the determination would have had to be carried out in solution and the protective phenomenon being studied did not exist in solution.

5. Nuclear Magnetic Resonance Spectroscopy

It was readily possible to obtain nmr spectra of lysinium linoleate in solution. Useful solvents were 5 to 10% solutions in deuterochloroform, deuterium oxide and pyridine, and deuterium oxide and methanol. Since the autoxidation-inhibition effect is not exhibited by solutions of lysinium linoleate, such nmr data throw no useful light on the problem.

The nuclear magnetic resonance of organic solids cannot usually be determined with sufficient resolution to be meaningful (ref. 11).

This was confirmed for this problem. Even at high spinning rates, no signals were observed from solid lysinium linoleate, nor from a mull of the salt in hexachlorobutadiene. Broad signals with no definitive resolution were observed with the Varian DP 40 Mc nmr Spectrometer by applying the dispersion mode rather than the absorption mode. This technique minimized line-broadening arising from relaxation phenomena. It was clear that line-broadening in the solid state was several powers of ten greater than the separation between proton resonance signals of individual environments, so no useful information was obtained.

6. Raman Spectroscopy

Although some attempts were made to use the Raman technique on this problem, this determination also must be done on a solution phase where the autoxidation-inhibition effect was not exhibited. Thus, for the same reason that nmr was unsatisfactory, this method could not be applied either.

7. Thin Layer Chromatography

Chromatography was carried out on silica gel G (250 μ). Lysine, linoleic acid, and lysinium linoleate were spotted onto a plate in chloroform suspensions. Chloroform was used as the development solvent, and iodine vapor as the stain. Linoleic acid was found to have $R_f \approx 0.4$, while lysine and lysinium linoleate were not moved by chloroform. Nothing appeared at $R_f \approx 0.4$ from the lysinium linoleate.

Both the linoleic acid and lysine spots were irreversibly stained by iodine vapor, but on atmospheric exposure the brown spot at the lysinium linoleate origin faded rapidly, indicating no free lysine in that spot.

Hence, lysinium linoleate in chloroform was not dissociated and contained no free lysine or linoleic acid. Experiments with methanol as the solvent were less conclusive. The results indicated dissociation and probable oxidation during development. These results were consistent with the differences between oxidation experiments in polar and non-polar media reported later in this report.

8. X-Ray Crystallography

A consultation was arranged with Prof. David P. Shoemaker of the Massachusetts Institute of Technology at no cost to the contract. He confirmed an original impression that a complete X-ray crystallographic study of lysinium linoleate was not economically feasible. With a 0.2-mm single crystal (if one could be prepared), the large number of carbon atoms involved would have led to an extremely complex analysis, lasting about a year. There was no guarantee of a solution and there would have been no preliminary indications; success or failure would have come all at once at the end of the analysis. If successful, a molecular orientation pattern in the crystal would have been obtained, but even then relevance to the problem would have been doubtful unless it had been found, for example, that the crystal had an extremely tight hydrogen-bonded structure in which each molecule was rigidly fixed, so that there was no room for oxygen to penetrate.

Powder photographs could have been obtained more quickly and these would have by-passed the need for a single crystal. Such experiments would have told only if there was a well-defined crystal structure or not and perhaps allowed a computation of the dimensions of the unit cell and its symmetry type. These, together with a density, would have allowed a determination of the number of molecules per unit cell. However, even for this work, a competent consultant would have been required for the interpretative work. Professor Shoemaker indicated that the chances were only 1% that the structure would have resembled some other structure, such as the urea inclusion complexes, to make analysis simpler.

Therefore, no further work or consultation was done on this approach to the problem.

B. GENERALITY OF THE OXIDATION-PROTECTION EFFECT

1. Basic Amino Acids

The stabilizing effect in these salts was investigated in comparison to the free fatty acid by oxidation studies in the Warburg apparatus. This apparatus is a useful tool and has served to show whether a given salt actually does or does not show protection for linoleic acid.

Warburg experiments were carried out on a "Precision" 20-unit Warburg manometricon using conventional procedures (ref. 12). Oxidations were conducted on samples containing 90 mg of fatty acid or the equivalent amount of salt (2.2×10^{-4} mole assuming a molecular weight of 400). The dimensions of the manometers limited the total oxygen uptake to 300 microliters (1.3×10^{-5} mole). Thus, the Warburg reading represents a ratio of moles of oxygen consumed to moles of compound charged of 0.06. A leveling off, therefore, of oxygen uptake below 300 microliters indicated a very stable system.

All salts of basic amino acids and fatty acids were prepared as described in the patent (ref. 1).

Infrared studies were carried out on all the salts. Comparison of the spectra of the free bases with those of the salts showed a complete absence of the N-H band at 3 microns in the spectra of the salts. Comparison of the spectra of the fatty acids with those of the salts showed the disappearance of the 5.8-micron carbonyl absorption band from the spectrum of the fatty acid. In such basic amino acids, one of the amino functions contributes to the zwitterion structure while the other is available for salt formation. The infrared spectra of the salts, acids, amino acids, and amino acid hydrochlorides are shown in Figures 5-29.

The oxidations in the Warburg manometer at 25°C are summarized in Table 6. Each compound was also run in the Warburg apparatus without linoleic acid. These blanks showed no oxygen uptake. The table lists the time required for an oxygen uptake of 300 microliters, which gives an index of whether or not the autoxidation was inhibited. The actual Warburg curves for each experiment are not reproduced in this report for simplicity and because the method should not be thought of as quantitative. The Warburg experiments were run in duplicate and found to be approximately reproducible. The observed lack of complete reproducibility was attributed partly to the great sensitivity of the method. Reproducibility was more than adequate to evaluate the protection phenomenon concerned. The problem of "gas evolution" is discussed below in section B.4.

The first experiments were conducted at 37°C. Although it appeared from the results that lysinium linoleate was stabilized after an initial oxidation stage, the rates were too high for convenient observation. Except for those experiments near the freezing point of linoleic acid, described below in section C.2, all of the succeeding Warburg experiments were conducted at 25°C.

Table 6

WARBURG OXIDATIONS, BASIC AMINO ACIDS

Time required for uptake of 300 microliters of oxygen; charge equivalent to 90 mg of linoleic acid; temperature 25°C.

Name	Uptake Time, hours	Notes
Lysinium linoleate	1440	a,c
	1336	a,c
	1007	
	888	a
	391	a,b
2,3-Diaminopropionic acid/linoleic acid	24	
2,4-Diaminobutyric acid/linoleic acid	384	a,b
	330	a,c
Ornithinium linoleate	791	a,c
L-Argininium linoleate	552	a,c
D-Argininium linoleate	552	a,c
3,6-Diaminohexanoic acid/linoleic acid	456	
N_ϵ-Benzoyllysine/linoleic acid	24	
N,N,N',N'-Tetramethyllysine/linoleic acid	120	
Linoleic acid + aqueous ethanol	18	
	24	
	<18	
	<18	
	<24	
	<24	
Lysinium linoleate + aqueous ethanol	<17	
	<17	
	<24	
	<24	
Lysinium linoleate (0.13 g) + 30% aqueous ethanol	<20	
Lysinium linoleate (0.13 g) + 0.5 g water	288	
Lysinium linoleate (0.13 g) + 1 g water	264	
Lysinium linoleate (0.13 g) + 1 g water + 0.001 g EDTA	384	
50% Mull of lysinium linoleate and linoleic acid	<48	
	<48	
	45	
	30	

Table 6
(continued)

Name	Uptake Time, hours	Notes
20% Mull of lysinium linoleate + linoleic acid	124	
	<24	
	<20	
50% Mull of lysine free base + linoleic acid	598	
	598	
20% Mull of lysine free base + linoleic acid	100	
	240	
	240	
50% Mull of 2,4-diaminobutyric acid linoleate + linoleic acid	264	a,b
	264	a,b
20% Mull of 2,4-diaminobutyric acid linoleate + linoleic acid	168	
	192	
	192	
	216	
Deuterated lysinium linoleate	528	
Lysinium linoleate freeze-dried	960	a
Lysinium linoleate + copper acetate	1032	a,b
Linoleic acid/butylamine/L-norleucine	<72	
Linoleic acid/butylamine/glycine	456	

Notes: a Deliberately terminated
b Slight oxidation
c No oxidation at all

Solutions of linoleic acid in butanol, aqueous ethanol, and Nujol also oxidized very rapidly.

In the early phases of the work it was found that 20% and 50% mulls of lysinium linoleate in linoleic acid autoxidized rapidly as did various mulls of linoleic acid with lysine/2,4-diaminobutyric acid, lysinium oleate, and lysinium stearate. These experiments emphasized that oxidation stability is associated with the dry salts made by the patented procedure (ref. 1) and that the phenomenon does not extend to the protection of greater-than-stoichiometric ratios of linoleic acid to basic amino acids.

The simple analogs of lysine studied were ornithine, 2,4-diaminobutyric acid, and 2,3-diaminopropionic acid. Ornithine and 2,4-diaminobutyric acid were found to behave like lysine and to protect linoleic acid from autoxidation. However, 2,3-diaminopropionic acid, the smallest basic amino acid, when reacted with linoleic acid, though it formed a salt, did not retard the autoxidation at all. It was thus clear that the chain length, and presumably stereochemical fit, of the amino acid were important factors affecting oxidation protection ability. The 3-carbon acid was apparently too small, while the 4-, 5- and 6-carbon acids had sufficient chain length to afford protection. It would have been very interesting to have examined higher molecular weight basic amino acids to see if an upper limit existed to the chain length required for protection. Such materials were not commercially available and the expense of synthesis precluded their trial. It is of interest in connection with the possibility that the effect being studied is a solid-state phenomenon, that the salts of linoleic acid with basic amino acids were always solids. Full protection against autoxidation was obtained only with basic amino acids. Salts of linoleic acid with other amino compounds were, in general, not solid. However, the fact that the salts with 2,3-diaminopropionic and 3,6-diaminohexanoic acids are solids, and yet oxidize, shows that the solid state alone is not the basis for the stability.

One basic amino acid (arginine) was examined for salt formation and oxidation protective ability in both the D- and L-forms. Although this was a valid experiment, no difference of behavior was expected, and no difference was found in ability to confer resistance to oxidation to linoleic acid.

To ascertain whether free amino groups were necessary for the protective ability of a basic amino acid, N,N,N',N'-tetramethyllysine was synthesized (as described in the Appendix) and reacted with linoleic acid. The product was not a normal salt, nor was it a physical mixture of the two components. The infrared spectrum (Figure 27) showed that the carboxyl group of linoleic acid was ionized as anticipated, but the expected $-NH^+$ bands were extremely weak, while an unexplained free -OH or -NH band appeared as a full-scale peak at 3390 cm^{-1}. Warburg experiments showed that the material oxidized rapidly.

One basic amino acid without an α-amino group was examined for salt formation and oxidation inhibition of linoleic acid to indicate whether the inhibition phenomenon was limited to close analogs of lysine or is a characteristic of more varied basic amino acids. 3,6-Diaminohexanoic acid, $NH_2CH_2CH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCH_2COOH$, was chosen as the most desirable candidate to study. The compound was lysine in which the α-amino group had simply been moved to the β-position. The compound was synthesized (as described

in the Appendix) and reacted with linoleic acid to give a solid salt that was normal as judged by the infrared spectrum (Figure 23). It was slightly impure, showing some absorption attributed to -OH or -NH. Warburg experiments showed that the salt did not offer total inhibition of autoxidation; the salt gradually oxidized, absorbing 300 microliters of oxygen in 19 days. This acid, which did not protect, was the only β,ω-diamino acid tested. The lengthy synthesis involved precluded testing other examples.

The rate of autoxidation of deuterated lysinium linoleate (prepared for the infrared work described earlier in this report) was measured in the Warburg apparatus. The samples oxidized, although very slowly. The method of preparation differed from the usual procedure for lysinium linoleate in that freeze-drying was involved. This caused a change in physical appearance. Since changes in physical state caused changes in oxidation behavior (described later in this report), a sample of freeze-dried lysinium linoleate was tested. The protonated freeze-dried sample did resist autoxidation. This leads to the conclusion that deuteration had a real effect. In the absence of corroborating experiments, however, we prefer to regard this result as an artifact.

Attempts were made to see whether lysine could protect the preformed linoleate ion, but when an attempt was made to prepare a sample of lysine and ammonium linoleate for Warburg experiments, ammonia was displaced and only lysinium linoleate was formed. Ammonium linoleate was also treated with two simple amino acids, which are monoamino analogs of lysine. In this way, lysinium linoleate analogs would have been obtained in which the lysine skeleton had been broken. The amino acids were norleucine $CH_3CH_2CH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH$ and 6-aminohexanoic acid $NH_2CH_2CH_2CH_2CH_2CH_2COOH$

However, these experiments also failed when ammonia was again displaced and only mixtures of the amino acids and linoleic acid were obtained. In order to get a similar effect, a less volatile amine than ammonia was used. Linoleic acid was successfully complexed with butylamine and norleucine. The combination did not show inhibition of autoxidation. In a similar experiment, linoleic acid was successfully complexed with butylamine in the presence of glycine to give lysinium linoleate in which the lysine skeleton was broken along the carbon chain. This combination did not show the protection effect, but was only slowly autoxidized, taking up 300 microliters of oxygen in 456 hours. Infrared spectra for this series of combinations and their constituents are shown in Figures 30-35.

To see whether trace metals were responsible for initiating oxidation, a Warburg oxidation study was made of lysinium linoleate deliberately contaminated with a copper pro-oxidant (copper acetate). Cupric acetate, 0.2 mg, was dissolved in 1000 ml of methanol. A 100-ml portion of this solution was added to 0.3 g of lysinium linoleate dissolved in 10 ml of

methanol. The reaction mixture was evaporated to dryness and the recovered solid subjected to Warburg oxidation. It was found that the stability of the salt had been retained. The infrared spectrum of the lysinium linoleate, Figure 36, was unchanged by the addition of the copper acetate.

2. Lysine Analogs Other Than Basic Amino Acids

In order to study the generality of the autoxidation inhibition effects, salts and mixtures of linoleic acid with a variety of lysine analogs were prepared for Warburg oxidation studies. The analogs were derived from lysine by systematically varying the chain length and the presence or absence of each of the functional groups. The data collected showed that compounds that were not basic amino acids did not protect linoleic acid from autoxidation. In some cases, the materials prepared for Warburg experiments were shown to be salts by infrared spectroscopy; in other cases, they were physical mixtures or solutions. Some of the materials were solids, while others were liquids. Physical state, salt formation, and behavior in the Warburg apparatus could not be correlated. The Warburg experiments on these lysine analogs are summarized in Table 7.

The salts and mixtures of lysine analogs with linoleic acid were made by the patented procedure. The physical states of the products are listed in Table 7; the infrared spectra appear as Figures 31, 32, 35, 37-72. Each material was also run in the Warburg apparatus without linoleic acid. These blanks showed no oxygen uptake. All the Warburg experiments were run in duplicate and found to be reproducible.

Modification of lysine by elimination of the carboxyl group and one amino group leaves a straight chain primary amine. The simple primary amines investigated gave liquid complexes with linoleic acid. Comparison of the infrared spectra of the free amines with those of the alkylammonium linoleates showed an absence of the band at 3 microns in the spectrum of the salt. This primary amine stretching frequency was replaced by the RNH_3^+ band. A comparison of the infrared spectrum of linoleic acid with that of the salts showed the disappearance of the 5.8-micron carbonyl absorption band and its replacement by the vibration associated with the carboxylate anion. These infrared spectra are shown in Figures 35, 38-44. Since these simple alkylammonium linoleates were all liquids, the experiments did not answer the key question of whether the inhibition of autoxidation was due to the solid physical state or to a chemical effect peculiar to basic amino acids. An attempt was made to find an alkylammonium linoleate that was itself a solid, but none was found. To this end, experiments with a few higher molecular weight amines were carried out. The salts were viscous liquids. Although they did not fully protect linoleic acid from autoxidation, such amines decreased the rate of autoxidation, and the magnitude of the effect apparently increased with the molecular weight of the amine and the viscosity of the salt.

Table 7

WARBURG OXIDATIONS, LYSINE ANALOGS OTHER THAN BASIC AMINO ACIDS
(Average time required for uptake of 300 microliters of oxygen; charge equivalent to 90 mg of linoleic acid; temperature 25°C)

Name and Structure of Compound Mixed with Linoleic Acid		Physical State	Salt or Mixture	Average Uptake Time, hr
ε-Aminocaproic acid	$NH_2-(CH_2)_4-CH_2-COOH$	semi-solid	M	<24
L-Norleucine	$CH_3-(CH_2)_3-\underset{\displaystyle NH_2}{\underset{\vert}{CH}}-COOH$	semi-solid	M	<48
Valeric acid	$CH_3-(CH_2)_3-COOH$	liquid	M	<48
Adipic acid	$HOOC-(CH_2)_4-COOH$	semi-solid	M	<36
Glutamic acid	$HOOC-(CH_2)_2-\underset{\displaystyle NH_2}{\underset{\vert}{CH}}-COOH$	semi-solid	S	<24
Heptadecylamine	$C_{17}H_{35}-NH_2$	semi-solid	S	<240
Dodecylamine	$C_{12}H_{25}-NH_2$	liquid	S	240
Butylamine	$C_4H_9-NH_2$	liquid	S	72
Putrescine	$NH_2-(CH_2)_4-NH_2$	viscous liquid	S	576
Cadaverine	$NH_2-(CH_2)_5-NH_2$ (1 mole)	viscous liquid	S	792
Cadaverine	$NH_2-(CH_2)_5-NH_2$ (1/2 mole)	viscous liquid	S	552
1,6-Hexanediamine	$NH_2-(CH_2)_6-NH_2$	viscous liquid	S	600
1,12-Diaminodecane	$NH_2-(CH_2)_{10}-NH_2$	viscous liquid	S	360
Piperazine		viscous liquid	S	336
Ammonia	NH_3	viscous liquid	S	28
Ethylenediaminetetraacetic acid	$HOOC-CH_2$ $HOOC-CH_2$ $\rangle N-CH_2-CH_2-N\langle$ CH_2-COOH CH_2-COOH	semi-solid	M	<24
Ethylenediamine	$NH_2-(CH_2)_2-NH_2$	liquid	S	24
Diethylenetriamine	$NH_2-(CH_2)_2-NH-(CH_2)_2-NH_2$	liquid	S	144
Lysine hydrochloride	$\left[NH_2-(CH_2)_4-\underset{\displaystyle NH_2}{\underset{\vert}{CH}}-COOH\right] HCl$	semi-solid	M	24
1,4-Diazabicyclo[2.2.2]-octane		liquid	M	48
3-Azabicyclo[3.2.2]nonane		liquid	M	48

Ammonium linoleate itself was prepared and subjected to Warburg oxidation. It was found to autoxidize very rapidly.

Modification of lysine by elimination of only the carboxyl group leaves a diamine. The simple straight chain diamines formed normal salts with an equimolar amount of linoleic acid. These salts were liquids of differing viscosities.

The infrared spectra of preparations of linoleic acid with the diamines showed the disappearance of the 5.8-micron carbonyl absorption of linoleic acid and its replacement by the vibration associated with the carboxylate anion. The free $-NH_2$ bands were complemented by $-NH_3^+$ bands showing that salt-formation had occurred (see Figures 45-63). It was not possible to determine whether the products consisted of all monoamine-monosalt or a mixture of disalt and unchanged diamine. Thin layer chromatography was attempted to settled this point in the case of cadaverine, but the results were inconclusive. This variable was obviated in the case of cadaverine by also preparing the salt with two molar equivalents of linoleic acid. It was found that autoxidation proceeded at the same slow rate for the 1:2 salt as for the 1:1 salt. This is of interest because after 1:2 salt formation has taken place, each NH_3^+ group of the diamine is associated with a linoleate COO^- and there are no functional groups left to associate with the unsaturated region. These salts with diamines did not fully protect linoleic acid from autoxidation, but exhibited the same retardation phenomenon as the simple amines, the effect again being most noticeable with higher molecular weight diamines. Thus, the observation that autoxidation is retarded in these cases fits with the retardation observed earlier with long-chain monoamines. The weights of the samples used in the Warburg experiments were chosen to allow for the molecular weight of the amine so that the same amount of linoleic acid was present in each case. Thus, the retardation effect is real and not caused by dilution. However, the effect could be caused by the increase in viscosity of the salt with increase of molecular weight observed with both the monoamines and diamines. Experiments with a few branched and cyclic mono-, di- and tri-amines were carried out. In general, these offered no protection to linoleic acid. Infrared spectroscopy on the preparations with 1,4-diazabicyclo[2.2.2]-octane and 3-azabicyclo[3.2.2]-nonane (Figures 44 and 59) showed the 5.8-micron carbonyl band of unionized linoleic acid indicating that salt formation had not occurred. However, the spectra were not those of physical mixtures of the amine and acid, but rather suggest that strong hydrogen bonding had occurred. Possibly, quaternization of the amino group was sterically unfavorable.

The methyl ester of lysine can also be regarded as a lysine analog from which the free carboxyl group had been eliminated by blocking. However, attempts to prepare the free base of the ester for salt formation with linoleic acid were abandoned when it was found by instrumental methods that the synthetic procedures reported in the literature (refs. 13,14) did not, in fact, give the desired product. Details of the synthetic work are given in the Appendix.

The structural modification made by eliminating one or both amino functions gives rise to simple amino acids and carboxylic acid, respectively. Salts were not formed with linoleic acid and no protection at all against autoxidation was found. The infrared spectra in these cases were essentially the sums of the spectra of the components (Figures 64-72).

Related to these experiments with simple amino acids and carboxylic acids was a Warburg experiment with linoleic acid and ethylenediaminetetraacetic acid. This was found not to protect linoleic acid from autoxidation. Salt formation could not occur as EDTA has four carboxyl groups but only two basic nitrogen atoms and so probably exists as a di-zwitterion with two free carboxyl groups. However, if trace metals had been responsible for initiating oxidation, the EDTA would effectively have removed them from solution and so protected the linoleic acid. That this did not, in fact, occur, undermined this trace-metal hypothesis.

All of these experiments suggested that salt formation was essential for protection and that it was the linoleate ion that was protected. That these conditions were necessary but not sufficient was emphasized by the fact that calcium linoleate autoxidized rapidly in the Warburg apparatus.

A mixture of lysine hydrochloride and linoleic acid also autoxidized rapidly. The spectrum of the preparation (Figure 68) was the sum of the spectra of the components. These facts were true also for a mixture of N_ϵ-benzoyllysine and linoleic acid (Figure 25). These two experiments confirmed the need for an unionized amino group in the protective agent. Salt formation was essential and it was clearly the linoleate ion that was protected rather than linoleic acid itself.

b. Linoleic Acid Analogs

Lysine was studied with oleic and stearic acids. These acids were found to oxidize at much slower rates than linoleic acid, so the question of whether or not they are stabilized as lysinium salts is less meaningful. Protection of these two acids is of less practical interest than protection of linoleic acid. It would have been more significant if a protective effect for linoleic acid had been found with lysinium oleate or stearate.

Warburg oxidation results with all linoleic acid analogs are accumulated in Table 8.

Pure linolelaidic acid (trans, trans-isomer of linoleic acid) was purchased from the Hormel Institute. This acid was found to oxidize quite rapidly, although not as fast as its stereoisomer, linoleic acid. It formed a normal salt with lysine, and the salt was resistant to autoxidation. Thus, the stereochemical configuration of the carboxylic acid does not seem to be as important a factor in the phenomenon of stabilization to autoxidation as the geometry of the basic amino acid.

Table 8

WARBURG OXIDATIONS OF LYSINE WITH LINOLEIC ACID DERIVATIVES
(Time required for uptake of 300 microliters of oxygen charge equivalent to 90 mg of linoleic acid; temperature 25°C)

Name and Structure of Compound Mixed with Lysine		Physical State	Salt(S) or Mixture (M)	Average Uptake Time, hr
Linoleyl alcohol	$C_5H_{11}-CH=CH-CH_2-CH=CH-(CH_2)_7-CH_2OH$	Viscous liquid	M	<72
Methyl linoleate	$C_5H_{11}-CH=CH-CH_2-CH=CH-(CH_2)_7-COOCH_3$	Viscous liquid	M	120
Trilinolein	$CH_2OOC-(CH_2)_7-CH=CH-CH_2-CH=CH-C_5H_{11}$ $CHOOC-(CH_2)_7-CH=CH-CH_2-CH=CH-C_5H_{11}$ $CH_2OOC-(CH_2)_7-CH=CH-CH_2-CH=CH-C_5H_{11}$ (1 mole)	Viscous liquid	M	52
Trilinolein	$CH_2OOC-(CH_2)_7-CH=CH-CH_2-CH=CH-C_5H_{11}$ $CHOOC-(CH_2)_7-CH=CH-CH_2-CH=CH-C_5H_{11}$ $CH_2OOC-(CH_2)_7-CH=CH-CH_2-CH=CH-C_5H_{11}$ (1/3 mole)	Viscous liquid	M	48
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	Solid	S	791
Stearic acid	$CH_3(CH_2)_{16}COOH$	Solid	S	791[a,c]
Oleic acid (no lysine)		Liquid	-	792[a,c]
Stearic acid (no lysine)		Solid	-	792[a,c]
50% Mull of lysinium oleate + linoleic acid		Liquid	-	576[a,b] 576[a,b]
20% Mull of lysinium oleate + linoleic acid		Liquid	-	120 120
50% Mull of lysinium stearate + linoleic acid		Liquid	-	<68 <68
20% Mull of lysinium stearate + linoleic acid		Liquid	-	28 28
Linolelaidic acid	trans,trans-$C_5H_{11}CH=CHCH_2CH=CH(CH_2)_7COOH$	Solid	S	1416[a]
Linolelaidic acid (no lysine)		Liquid	-	168

Notes: [a] deliberately terminated
[b] slight oxidation
[c] no oxidation at all

That salt formation was essential for protection and that it was the linoleate ion that was protected was confirmed by studying the autoxidation of mixtures of lysine with linoleyl alcohol, methyl linoleate, and trilinolein. In each case, autoxidation was very rapid; the lysine clearly did not protect. No protection was found for trilinolein in admixture with three molar equivalents of lysine, either. The linoleyl alcohol, methyl linoleate, and trilinolein were purchased from the Hormel Institute and had purities of 99%+. The infrared spectra of the preparations of lysine with linoleyl alcohol, methyl linoleate, and trilinolein showed that salt formation did not occur. The spectrum in each case was the sum of the spectra of the components. Infrared spectra for all linoleic acid analogs and their salts or mixtures with lysine are shown in Figures 73-86.

4. The "Evolved Gas Problem"

Warburg autoxidations of unsaturated substrates occasionally showed a "negative uptake" of gas, sometimes referred to in the literature as "gas release" (refs. 2, 15, 16, 17, 18, and 19) (for an example, see Figure 87). These apparent negative values for oxygen absorption are troublesome to explain. They were apparently caused by the evolution of a gas. In operation of the Warburg apparatus, readings were taken at atmospheric (i.e., constant total) pressure, but if the composition of the gas changed, the partial pressure of oxygen would not have been constant, as had been generally assumed. Thus, the evolution of a gas complicated an analysis of the kinetics of oxygen uptake in the Warburg apparatus both because of the changing concentration and by masking the evolution of any gas. In French, Olcott, and Mattill's work (ref. 19), evidence was presented that the positive pressure was due, at least in part, to the evolution of hydrogen by the oxidized substrate. On this contract, great care was taken to check for uniformity of bath temperature [corrected for in any event by a thermobarometer (ref. 20)] and for absence of adsorbed or trapped gases. The possibility that carbon dioxide might have been evolved prompted an experiment using 0.28 ml of 20% potassium hydroxide solution in the well of a flask containing oxidizing lysinium linoleate. This sample showed oxygen uptake and positive pressure identical with the control over a 14-day period. This ruled out carbon dioxide evolution as a cause of the positive pressure readings.

When a reducing gas is brought into contact with palladium chloride solution, reduction to metallic palladium occurs:

$$CO + PdCl_2 + H_2O \longrightarrow Pd + CO_2 + 2HCl$$

The reduced acidic palladium chloride in turn reduces phosphomolybdic acid to molybdenum blue. This reaction (ref. 21) was applied to the detection of reducing gases in the Warburg system. The palladium chloride-phosphomolybdic acid solution was prepared according to the literature (ref. 21) and, following Warburg oxidation experiments in which the released gas phenomenon was noted, 3 ml of the reagent was added to the side arm of the Warburg flask. Care was taken to keep the

system closed. Over a period of 2 days, the pale yellow reagent turned green, indicative of the presence of a reducing gas such as hydrogen, carbon monoxide, ethylene, or acetylene.

The possibility that the evolved gas was carbon monoxide, derived from formyl radical or glyoxal intermediates, could not be excluded. A mechanism can be written for the autoxidation of linoleic acid involving these intermediates. A test series was run to determine whether the gas was carbon monoxide, using reagents that were specific for that gas (ref. 22).

Initially, a hemoglobin solution was prepared at a concentration such that the maximum optical density in the visible region was equal to one. Air was bubbled through 5 ml of this solution while carbon monoxide was bubbled through another 5 ml sample for 10 minutes. To 2 ml of each of the hemoglobin solutions was added 1 ml of 2% tannic acid and 1 ml of 2% pyrogallol. The mixtures were shaken gently and allowed to stand at room temperature for 30 minutes. Visible spectra with water in the reference cell were run (400-700μ). There was only a slight spectral difference between the O_2-hemoglobin and the CO-hemoglobin.

The experiment was repeated using 1.0 ml of citrated whole blood (human). The control blood sample was gray-brown while the blood in which the hemoglobin had combined with carbon monoxide turned carmine.

The side arms of Warburg flasks containing samples exhibiting "gas release" were used for the addition of 2 ml of blood. The flasks were then closed and shaken in the bath for 5 days before a solution of 1.0 ml of 2% pyrogallol and 1.0 ml of 2% tannic acid was added to the blood. The experiments were carried out on lysinium linoleate, lysinium linoleate/copper acetate, and lysinium linolelaidate (2 samples). In no case was a positive test for carbon monoxide obtained. The controls with carbon monoxide described above would have detected that compound if it had made up at least half of the "evolved gas".

More sophisticated experiments on this aspect of the problem could not be carried out with the funds available. There apparently was a reducing gas evolved in quantities greater than experimental error. It was not carbon monoxide. Other possibilities were hydrogen or an unsaturated hydrocarbon.

C. THE EFFECT OF THE PHYSICAL STATE

1. Lysinium Linoleate in Solution

Although it was shown that lysinium linoleate did not oxidize in Nujol mulls, it was found that when lysinium linoleate was dissolved in aqueous ethanol there was no inhibition of autoxidation. It was also found that the inhibitory effect was considerably reduced when the salt was in aqueous solution. These experiments are summarized in Table 6. In each case 0.13 g of lysinium linoleate was used and the runs were duplicated.

A small amount of ethylenediaminetetraacetic acid was found not to protect an aqueous solution of lysinium linoleate from the slow autoxidation. If trace metals had been responsible for initiating oxidation, the EDTA would effectively have removed them by chelation. Lysinium linoleate was found to be insoluble in anhydrous ethanol and in polar non-hydroxylic solvents such as dimethylsulfoxide, dimethylformamide, methylene chloride, the sulfolanes and glymes, solvents which might have placed the salt in the liquid phase without breaking up the complex. If such a non-hydroxylic solvent had been found, Warburg experiments would have been run on the solutions (if necessary replacing the aqueous Brodie's solution in the manometers with silicone oil) to check whether oxidation was still inhibited in the liquid phase. These experiments would have tested the apparent need for close association of the ions if oxidation was to be inhibited.

2. Oxidations Near the Freezing Point of Linoleic Acid

The part played in the inhibition of autoxidation v the solid state was investigated by running Warburg oxidations of linoleic acid just above (-9°C) and just below (-12°C) the freezing point of linoleic acid and compared with lysinium linoleate at the same temperatures.

Warburg oxidations were run in a methanol bath cooled by a mechanical refrigeration unit and kept at a constant temperature with electric heating and a conventional mercury-to-mercury thermoregulator. The bath was large enough for the thermostatting machinery, a stirrer, a stationary thermobarometer (ref. 20), and two rocking Warburg manometers. Special bridges were constructed so that the rocking action of the "Precision" 20-unit Warburg manometricon could be used to rock the two low-temperature manometers at the same time that the other positions were in use at +25°C. This arrangement is shown in Figure 88.

The results of the experiments are summarized in Figure 89 and Table 9. The data showed that the solid state alone was not the cause of the inhibition of autoxidation of linoleic acid. The induction period was prolonged the lower the temperature, but once oxidation began, it continued in the characteristic manner of uninhibited linoleic acid.

Table 9

WARBURG OXIDATIONS AT VARIOUS TEMPERATURES,
LYSINE AND LYSINIUM LINOLEATE

(Time required for uptake of 300 microliters of oxygen; charge equivalent to 90 mg of linoleic acid.)

System	Average uptake time, hr
Linoleic acid at 37°C	<24 <24
Lysinium linoleate at 37°C	72*
Linoleic acid at 25°C	48 48
Lysinium linoleate at 25°C	1336* 391*
Linoleic acid at -9°C	144 240
Lysinium linoleate at -9°C	168* 312*
Linoleic acid at -12°C	384 408
Lysinium linoleate at -12°C	384* 480*

*No oxidation; deliberately terminated

3. Concentration Studies

An experiment was devised to determine whether free linoleic acid or lysinium linoleate was the species oxidized, by studying the effect of concentration in solutions. The experiment could not be carried out due to the unsuitability of the oxygen uptake data from both the Warburg procedure and the oxygen galvanic cell.

The conductivity work reported above showed that for the equilibrium

$$Lys + Lin \rightleftharpoons [Lys^+Lin^-] \overset{K}{\rightleftharpoons} Lys^+ + Lin^-$$

the equilibrium constant $K = \frac{[Lys^+Lin^-]}{[Lys]^+[Lin]^-}$ was approximately 0.01 (in aqueous methanol).

Thus, by adjusting concentrations, it was intended to vary the percentage of the total linoleic acid in solution that exists as free linoleic acid and as the lysinium salt. If only free linoleic acid oxidized, the rate of oxygen uptake would have been directly proportional to the free linoleic acid concentration. If the lysinium salt were oxidizable, the rate would have been proportional to the sum of the free linoleic acid and lysinium linoleate concentrations. This discussion assumed the concentration of oxygen to be rate-limiting. Since the solubility of oxygen is of the order of 10^{-4} to 10^{-3} molar, and the linoleic acid concentrations were usually of the order of 10^{-1} molar, the assumption was probably valid.

A 0.138-g (3.3×10^{-4} mole) sample of lysinium linoleate was dissolved in 10 ml of 90% methanol (equivalent to 90 mg of linoleic acid). Further dilutions with 90% methanol were made, halving the concentration successively and making solutions containing 45, 22.5, and 11.25 mg of linoleic acid, respectively.

The Warburg autoxidations were carried out on each sample in the usual manner. Readings were taken every 10 minutes over a period of six hours. Instead of seeing oxygen uptake, positive readings were obtained, indicating gas evolution. Thus, rates of oxygen uptake could not be obtained for the initial stages of these oxidations and no conclusions could be drawn from the experiment. The experiments were therefore repeated using a Beckman Instruments oxygen galvanic cell which measured only the change in its environmental oxygen concentration. However, as described below, it was not possible to get meaningful readings on the oxygen galvanic cell in this system.

A 1.62-g (3.8×10^{-2} mole) sample of lysinium linoleate was dissolved in 100 ml of 90% methanol. Further dilutions with 90% methanol were made changing the concentrations of the solutions to 1.21 g, 0.91 g, and 0.46 g lysinium linoleate per 100 ml, respectively. In addition, a sample of lineoleic acid (1.06 g) dissolved in 100 ml of 90% methanol was used.

The autoxidation measurements were carried out on each sample in the same general manner. Fifty ml of solution was placed in a stoppered Erlenmeyer flask. The electrode of the Beckman Oxygen Analyzer was fitted tightly through the stopper. The solution was magnetically stirred at 27°C and the time was noted for every 0.2 ppm change in oxygen concentration.

The results of the initial experiments, expressed graphically in Figure 90, are typical of data obtained throughout the study. These experiments were performed with the electrode in the solution. It was found that the zero point reading of the instrument wandered from the normal 7.6 ppm (equivalent to 159 mm partial pressure of oxygen in air at 28°C) at the beginning of an experiment to about 6.3 ppm (142 mm) at its end.

From the graphs it can be seen that this drift was of the same order of magnitude as the effect being measured. Hence, no conclusions could be drawn from these experiments. The error appeared to be due to coating of the cell membrane and could not be eliminated in later experiments in which the electrode was cleaned and the membrane changed after each solution, nor in experiments run at 50°C.

Further sets of experiments were run at 34°C with the electrode above the solution, monitoring the change in oxygen concentration in the gas rather than the liquid. Although the membrane did not appear to become coated, the zero point of the instrument still wandered. The change in oxygen concentration was found to be very slow and liquid condensed on the electrode.

Finally, an experiment was tried using an electrode that had been saturated with linoleic acid; the experiment had to be abandoned because readings could not be obtained on scale, and calibration was impossible.

4. The Effect of Sodium Chloride

The hypothesis that it was the linoleate ion in a specific crystalline matrix whose autoxidation was inhibited was tested by studying the autoxidation of lysinium linoleate prepared in the presence of sodium chloride by several routes. When equimolar amounts of lysinium linoleate and sodium chloride were ground together in the solid state, the product was as completely resistant to oxidation as if the sodium chloride were not present. But, when this mixture was prepared by evaporating a solution containing lysinium linoleate and sodium chloride, the product was no longer completely resistant. Oxidation occurred, although it was very slow. This slow oxidation was also observed when lysinium linoleate was dispersed in potassium bromide and pressed at 20,000 psi into a pellet in the manner used for infrared spectroscopy. (It should be noted that this last experiment did not bear on the validity of the infrared work on lysinium linoleate, for those spectra were taken in Nujol mulls, a state in which the oxidation inhibition phenomenon had been shown to be retained.) The infrared spectrum of lysinium linoleate-sodium chloride ground dry was identical to that of lysinium linoleate in a Nujol mull or potassium bromide pellet. However, the spectrum of the evaporation co-solution, although grossly similar, differed in several details characteristic of a change of state (Figures 7, 9, 91, 92). These results suggested that when the crystal lattice of lysinium linoleate was altered by the inclusion of foreign ions, the autoxidation inhibition phenomenon was damaged.

The effect was even more pronounced when lysinium linoleate-sodium chloride was prepared from equimolar amounts of sodium linoleate and lysine hydrochloride, either ground together or recovered by evaporation of their co-solution. The oxidation rates were then comparatively fast. The infrared spectra (Figures 93, 94) differed in several details from that of lysinium linoleate prepared by the patented procedure (ref. 1), again suggesting a change of state. [The spectra of sodium linoleate and lysine hydrochloride should be compared for reference (Figures 6 and 95).] The fact that the material recovered by evaporating a co-solution

of lysinium linoleate-sodium chloride was more resistant to oxidation than that recovered from the sodium linoleate-lysine hydrochloride co-solution suggested that the ions in lysinium linoleate, prepared by the patented procedure (ref. 1), were associated other than by electrostatic attraction in the crystalline state.

The Warburg oxidation results for this series are shown in Table 10.

Table 10

WARBURG OXIDATIONS, EFFECT OF SODIUM CHLORIDE

(Time required for uptake of 300 microliters of oxygen; charge equivalent to 90 mg of linoleic acid; temperature 25°C.)

Mixture	Physical State	Average Uptake Time, hr
Lysinium linoleate and sodium chloride (from solution)	Solid	576
Lysinium linoleate and sodium chloride (solid mix)	Solid	696[a]
Sodium linoleate and lysine hydrochloride (from solution)	Solid	236
Sodium linoleate and lysine hydrochloride (solid mix)	Solid	216
KBr pellet of lysinium linoleate	Solid	720
(Control) Lysinium linoleate	Solid	888[a]

[a] No oxidation; deliberately terminated

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APPENDIX. SYNTHESIS

A. METHYL ESTER OF LYSINE (FREE BASE)

$$H_2NCH_2CH_2CH_2CH(NH_2)COOCH_3$$

Initially an attempt was made to prepare the free base of lysine methyl ester from the hydrochloride by the ion exchange method used for making other free bases from hydrochlorides. No product was obtained. An alternate procedure to the desired product was tried using sodium methoxide as described in the literature (refs. 13,14). Although the reaction proceeded as reported and the product obtained had the reported physical appearance, instrumental analysis could not confirm the desired structure. The literature reported no analytical data.

L-Lysine methyl ester dihydrochloride (20 g, 0.086 mole) was dissolved in 100 ml of methanol and treated slowly with stirring with a solution of sodium (4 g, 0.172 mole) in 100 ml of methanol. The turbid solution was evaporated under reduced pressure with periodic removal of sodium chloride by filtration. The gummy residue was dissolved in chloroform/methanol and filtered to remove a little more sodium chloride (recovered 9.5 g, 98%). Evaporation was completed (0.1 mm) to yield 12 g (87%) of a very viscous, colorless, slightly turbid gum.

The infrared spectrum showed the main carbonyl band at 1653 cm^{-1}, which is consistent with an amide or lactam. The expected ester carbonyl band at 1742 cm^{-1} was missing. Similarly, the NH- bands are more consistent with an amide than an amine. The nuclear magnetic resonance spectrum was not consistent with the presence of a $-CH_3$ group in the product.

Analysis Calc'd for $C_7H_{16}N_2O_2$: C, 52.2; H, 10.1; N, 17.5.
Found: C, 51.3; H, 9.8; N, 17.4; residue 2.8.

Corrected (assuming residue is NaCl): C, 52.7; H, 10.1; N, 17.9.

It was felt that the amino acid ester free base underwent autocondensation to the diketopiperazine derivative and lysine peptides as reported elsewhere in the literature (refs. 23,24).

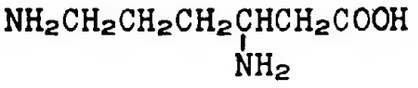
B. N,N,N',N'-TETRAMETHYLLYSINE

$$(CH_3)_2NCH_2CH_2CH_2CH_2CH(N(CH_3)_2)-COOH$$

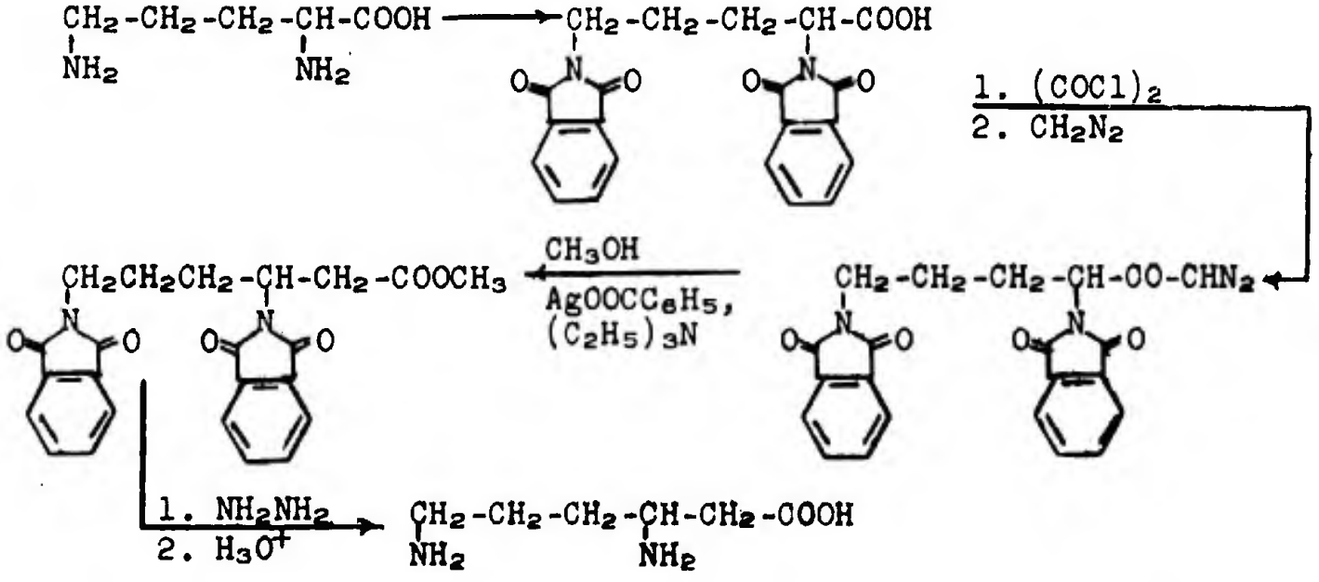
The hydrochloride of this compound was synthesized by the literature procedure (ref. 25). L-Lysine hydrochloride (9.1 g, 0.05 mole) was dissolved in 200 ml of water to which was added 16.6 g (0.2 mole) of 36% formaldehyde solution and 5 g of 10% palladium-on-charcoal. The suspension was treated with hydrogen at an initial pressure of 25 psig

in a Parr hydrogenation apparatus. Uptake of 0.2 mole of hydrogen took three hours. The catalyst was filtered off and the solution evaporated to dryness under reduced pressure. A quantitative yield of product was obtained as a hygroscopic cream solid (mp 202.5°C - 204°C; reported mp 203-204°C). The nuclear magnetic resonance and infrared spectra were consistent with the desired structure. The hydrochloride was converted to the desired free base by passage through the ion exchange column. Infrared studies indicated the yellow viscous oil to be the desired product.

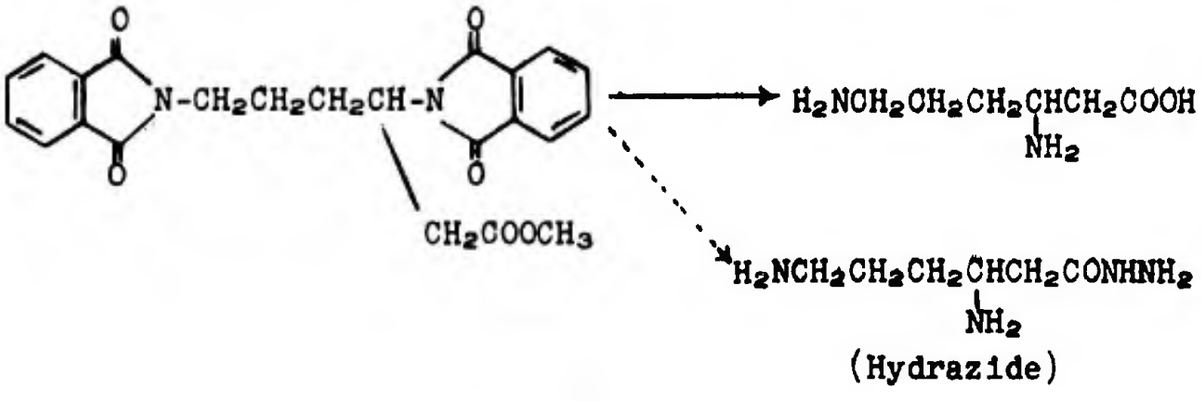
C. 3,6-DIAMINOHEXANOIC ACID



This material was synthesized by a 5-step literature procedure (ref. 26) from ornithine. The amino functions of the starting material were protected by reaction with phthalic anhydride and the chain was then lengthened by an Arndt-Eistert procedure. Removal of the protective groups yielded the desired product.



The first four steps were performed successfully but the final hydrazinolysis to saponify the ester and remove the phthaloyl protective groups simultaneously could not be reproduced. The authors had found this step troublesome, hydrazides (amides) being formed as by-products.



An alternative procedure (ref. 27) was therefore tried in which the ester protective grouping was removed first by dilute hydrochloric acid. Subsequent hydrazinolysis would then have been successful as the troublesome hydrazide would not form from the carboxylic acid, as it did from the ester. However, this dilute acid hydrolysis could be carried out only in very poor yield. The method finally used (ref. 28) and described below was a concentrated hydrochloric acid hydrolysis of all the protective groups simultaneously.

DL-Ornithine monohydrochloride (25 g, 0.15 mole) was heated with anhydrous sodium acetate (12.3 g, 0.15 mole) and phthalic anhydride (44.4 g, 0.3 mole) in an oil bath at 135-140°C for one hour and at 155°C for a further hour. The product (39 g) was isolated in 66% yield from ethanol/water as a white solid [mp 192-193°C; reported (ref. 29) mp 192-194°C]. The infrared spectrum and elemental analysis were consistent with the desired structure.

This DL-di-(N-phthaloyl)ornithine was then converted to its acid chloride in 91% yield by refluxing for 2 hours with an excess of oxalyl chloride in dry 1,2-dimethoxyethane, followed by solvent removal.

The acid chloride was converted to DL-1-diazo-3,6-diphthalimidohexanone-2 by reaction with diazomethane in ether at 0°C. Solvent removal and trituration with methanol yielded the product (17 g) in 75% yield as a yellow solid [mp 132-135°C (dec.); reported (ref. 26) mp 138.5-139.0°C (dec.)].

The diazoketone was catalytically decomposed to methyl DL-3,6-diphthalimidohexanoate by treatment with silver benzoate and triethylamine in methanol, the reaction being followed by collecting the evolved nitrogen in a gas burette. The product (10.1 g) was isolated in 60% yield from methanol after treatment with decolorizing charcoal. It was obtained as a white solid [mp 144-146°C; reported (ref. 26) mp 147-148°C]. The infrared spectrum was consistent with the desired structure.

The final step of the synthesis involved heating the ester for 24 hours at 100°C with excess concentrated hydrochloric acid. Evaporation to dryness yielded 3.7 g of the crude amino acid dihydrochloride in 84% yield as a sticky, off-white solid. Purification was effected via the picrate salt. This was prepared by dissolving the crude hydrochloride in boiling 80% methanol (60 ml) and treating it with sodium hydroxide (1.36 g) and picric acid (7.78 g). The picrate was removed by filtration and recrystallized from water to give 5.5 g of a yellow solid [mp 200-202°C; reported mp 200-201°C (ref. 29)].

The picrate was suspended in 200 ml of water containing 10 ml of concentrated hydrochloric acid and heated to boiling for an hour. The picric acid was removed from the cooled solution by filtration, followed by ether extraction (6 x 100 ml) and treatment with decolorizing charcoal. Evaporation to dryness yielded 3,6-diaminohexanoic acid dihydrochloride as a white solid, mp 158-160°C. The yield was 1.65 g (76%

based on the picrate; 45% based on the methyl DL-3,6-diphthalimide-hexanoate). Reported mp 153-155°C (ref. 30). The infrared spectrum was consistent with the desired structure.

Analysis Calc'd for $C_8H_{16}Cl_2N_2O_2$: C, 32.9; H, 7.4; N, 12.8.
Found: C, 32.6; H, 7.2; N, 12.5.

The dihydrochloride was converted to the desired free base as follows. A chromatography column three feet long and one inch in diameter was packed with Amberlite IR-120, and conditioned with 10% ammonium hydroxide. Distilled water was added until the eluant was neutral to pHydrion paper. A solution of an amino acid hydrochloride was added slowly. Distilled water was added until chloride ion was no longer present in the eluant. The product was washed off with 10% ammonium hydroxide solution. The desired free base was obtained upon concentration of the solution.

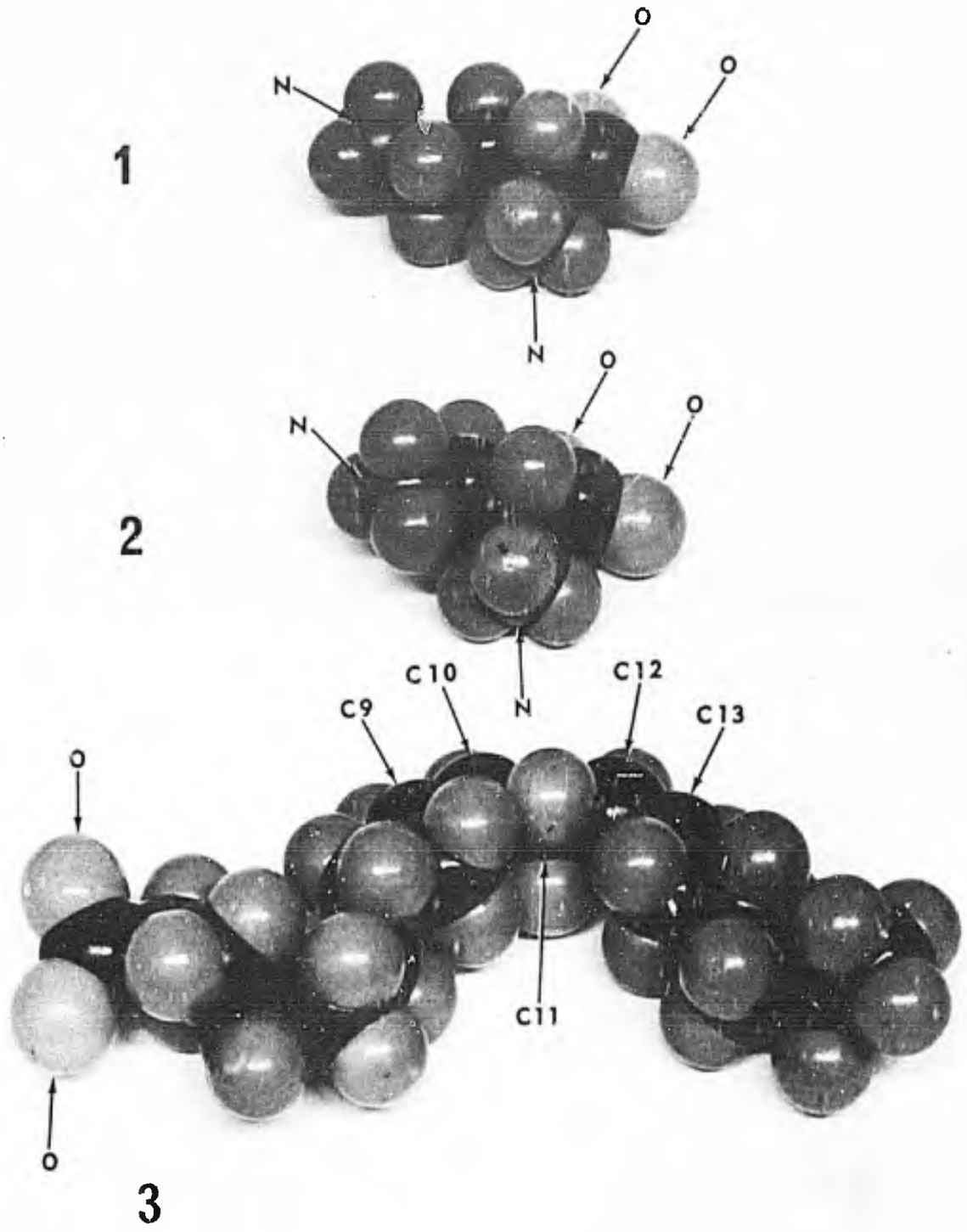


Figure 1. Models of 2,3-diaminopropionate and 2,4-diaminobutyrate zwitterion-cations; and linoleate ion.

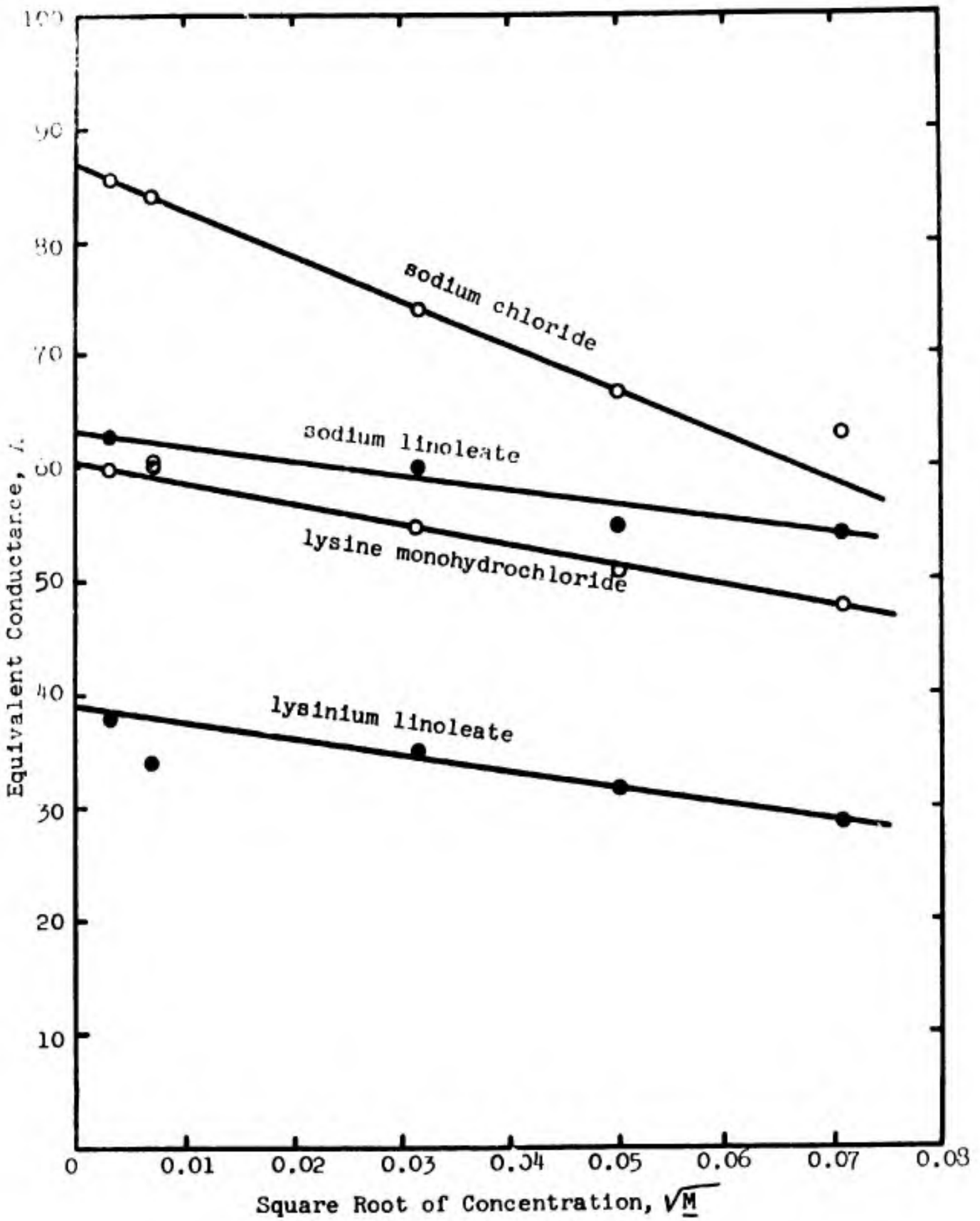


Figure 2. Equivalent conductance as a function of the square root of concentration for lysinium linoleate and related compounds.

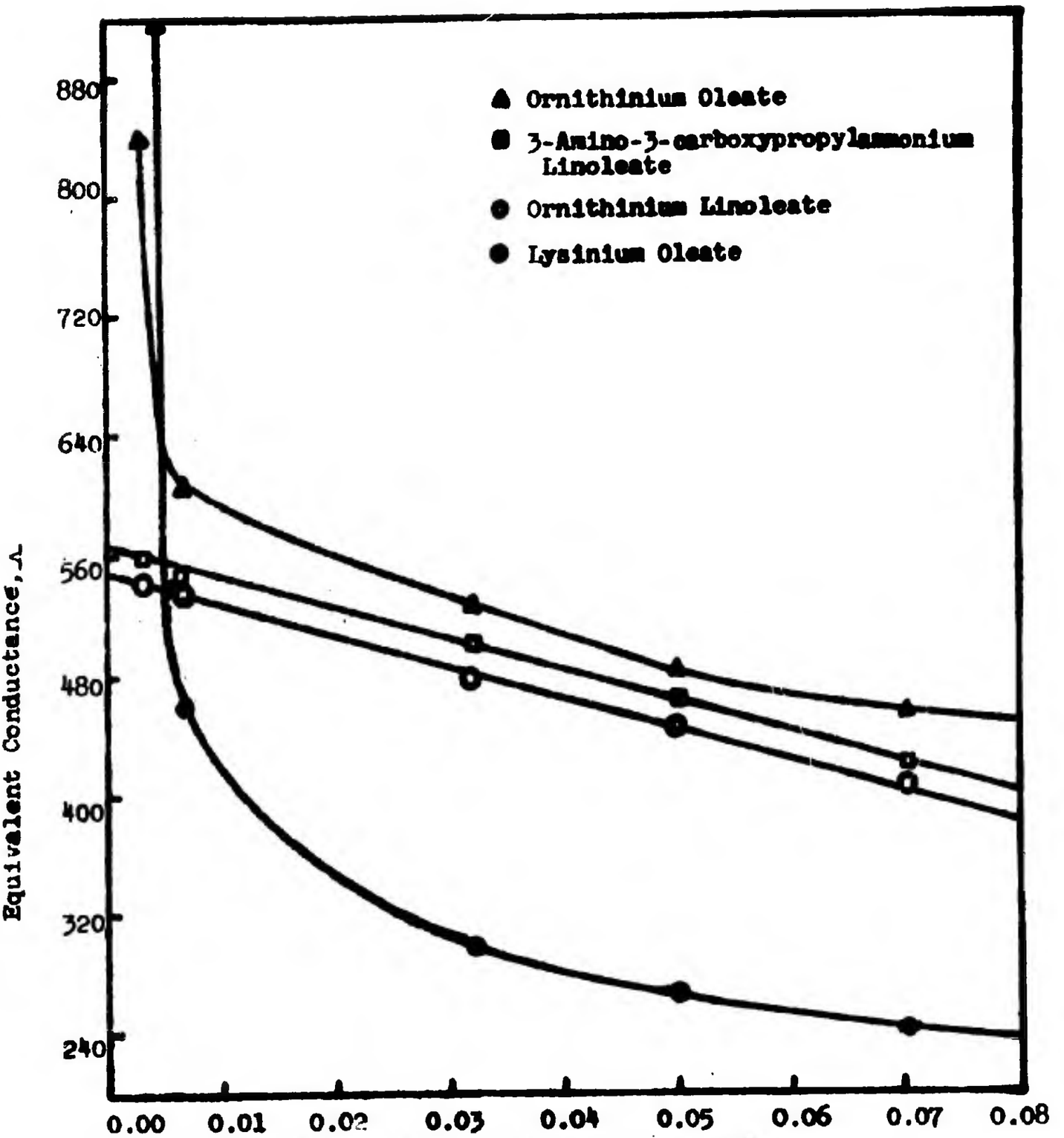


Figure 3. Electrical Conductivity of Oleates and Linoleates

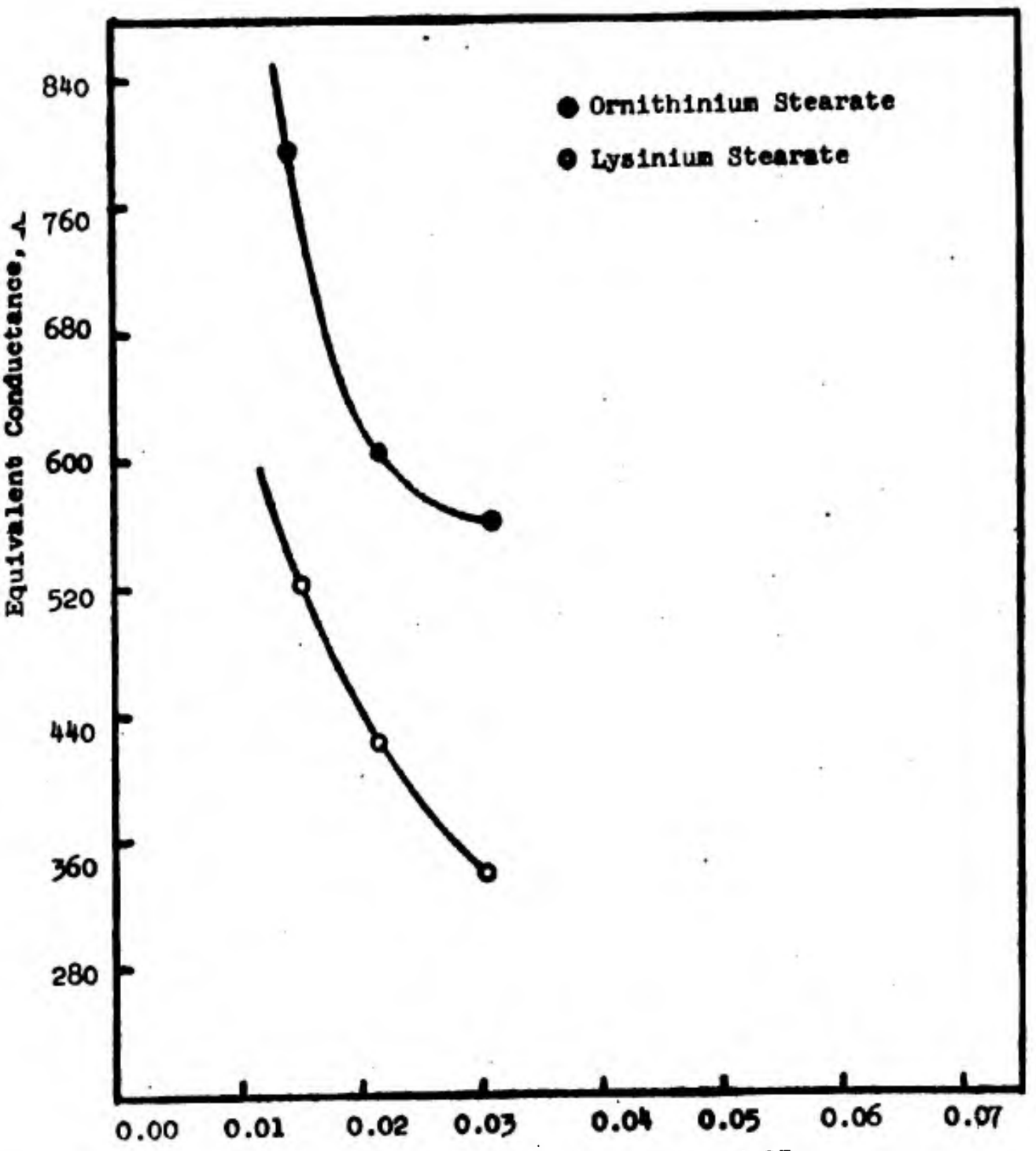


Figure 4. Electrical Conductivity of Stearates

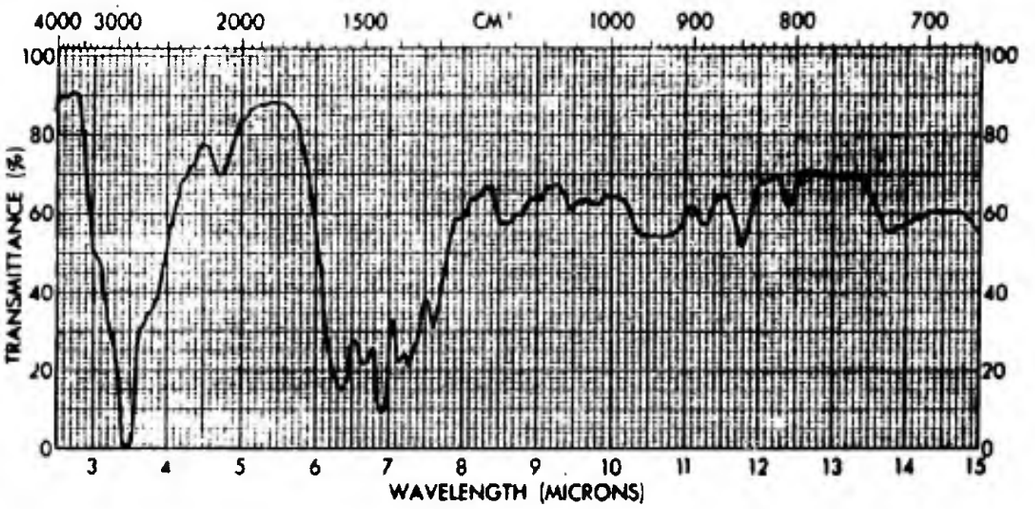


Figure 5. Infrared spectrum of L-lysine, Nujol mull.

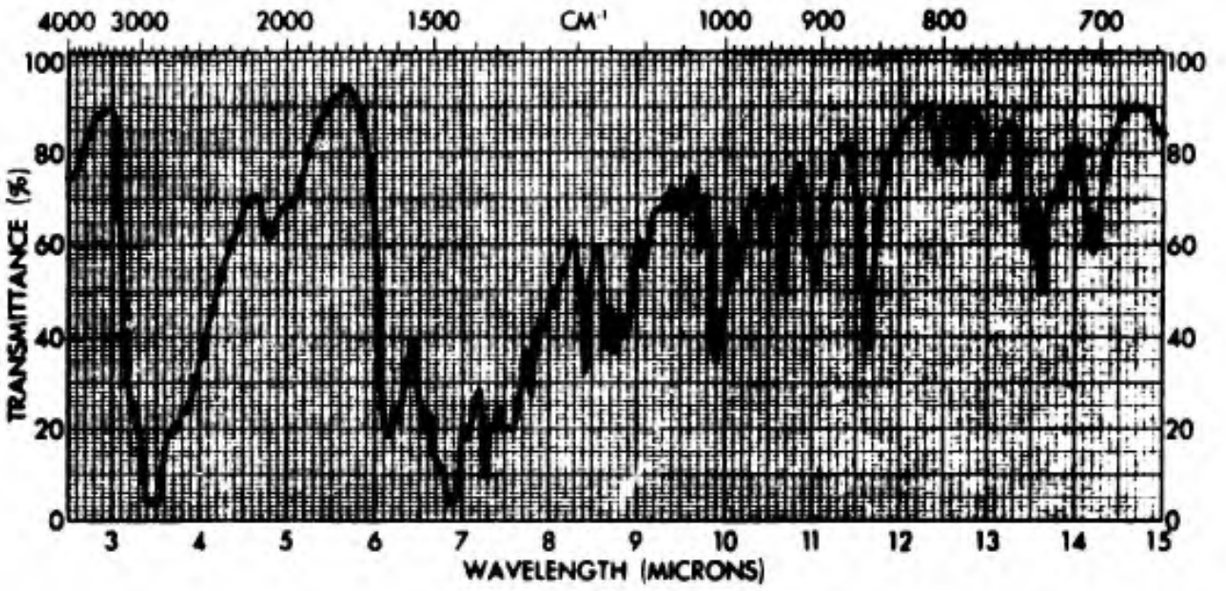


Figure 6. Infrared Spectrum of Lysine Hydrochloride, Nujol Mull.

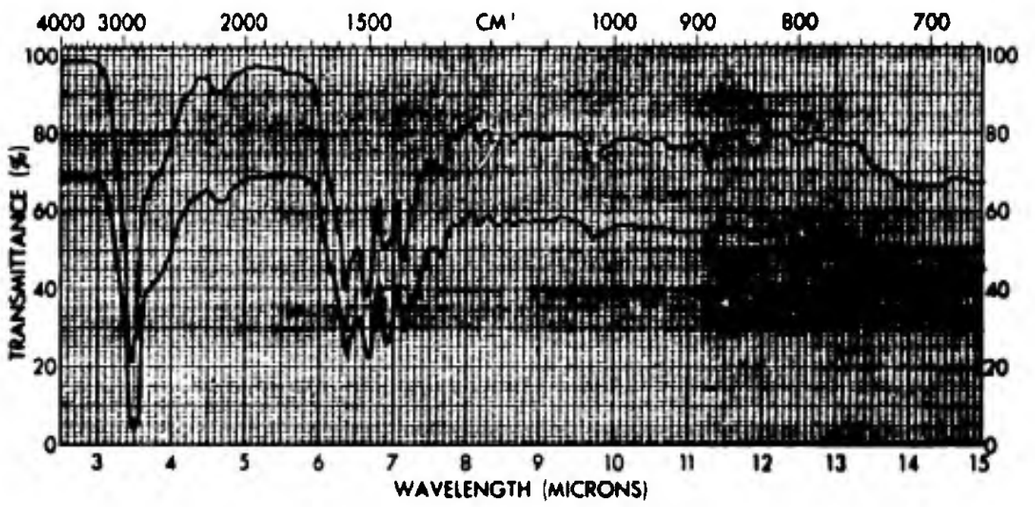


Figure 7. Infrared spectrum of L-lysinium linoleate, Nujol mull.

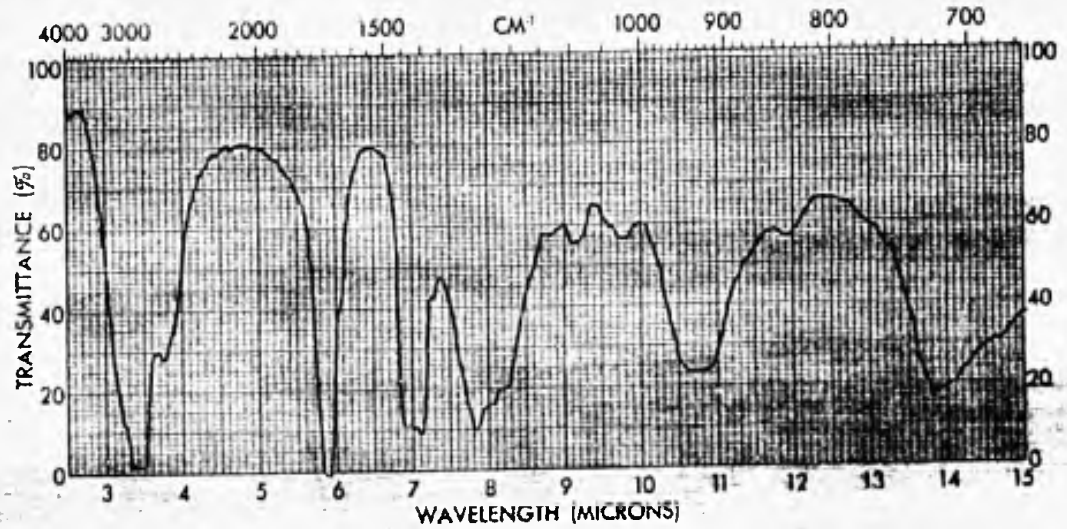


Figure 8. Infrared spectrum of linoleic acid, liquid film.

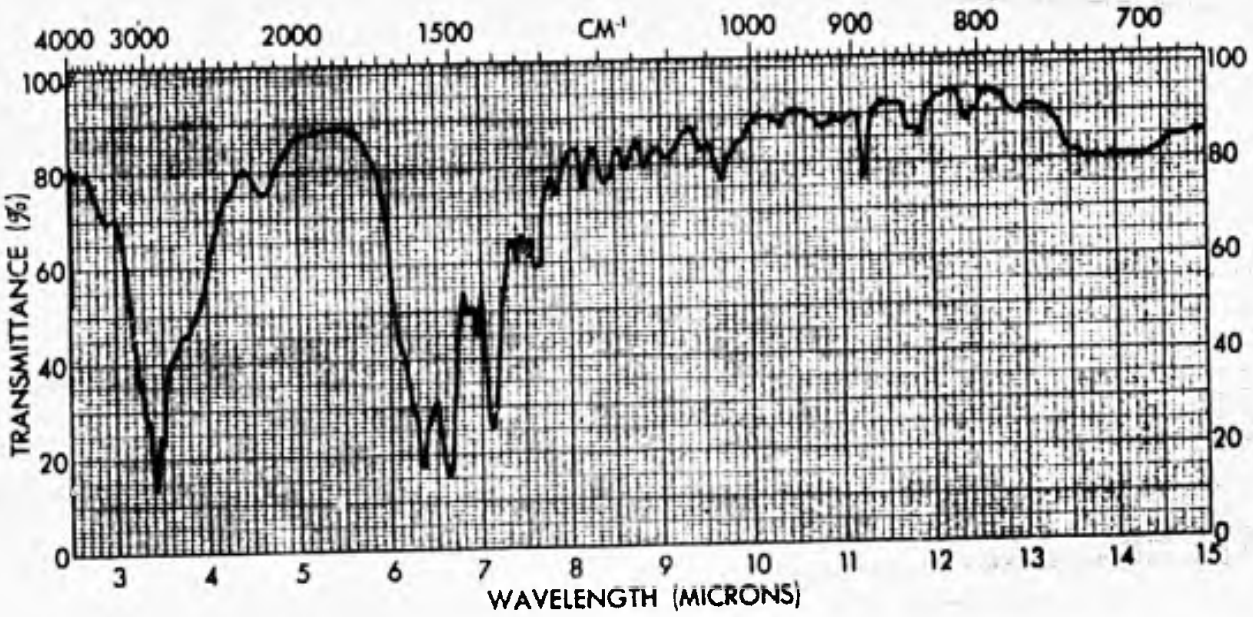


Figure 10. Infrared spectrum of deuterated lysinium linoleate, Nujol mull.

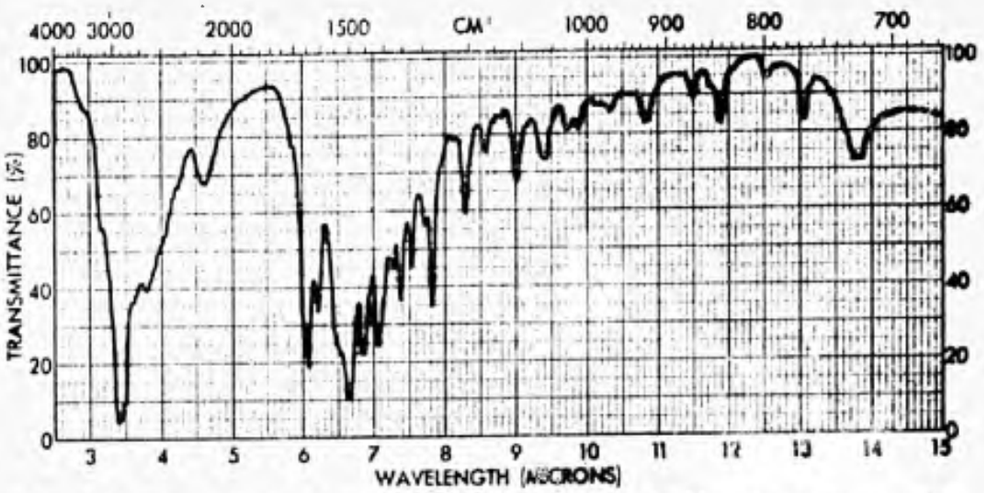


Figure 11. Infrared spectrum of 2,3-diaminopropionic acid/linoleic acid, Nujol mull

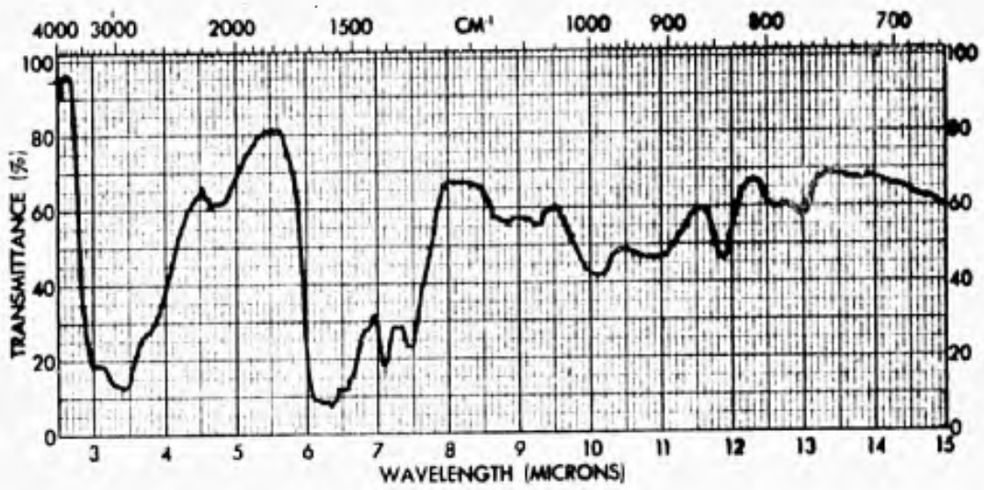


Figure 12. Infrared spectrum of 2,3-diaminopropionic acid, liquid film

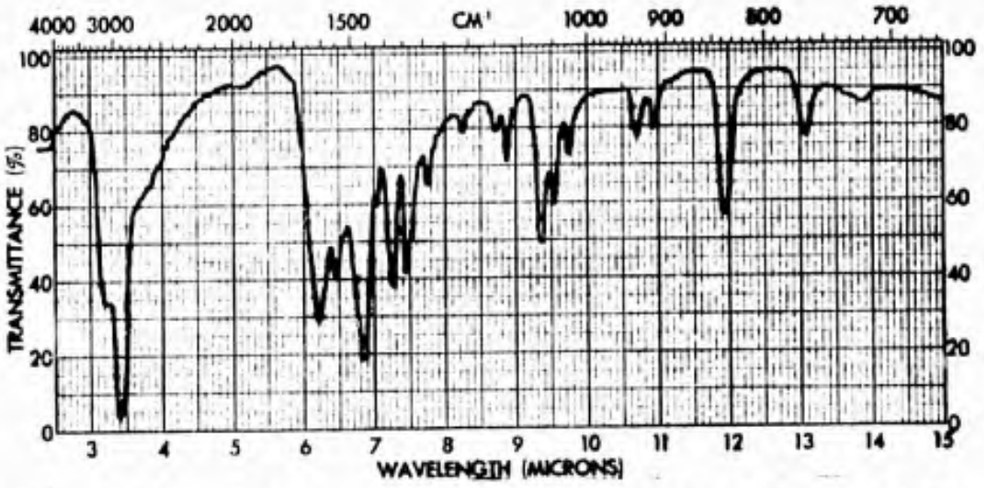


Figure 13. Infrared spectrum of 2,3-diaminopropionic acid monohydrobromide, Nujol mull

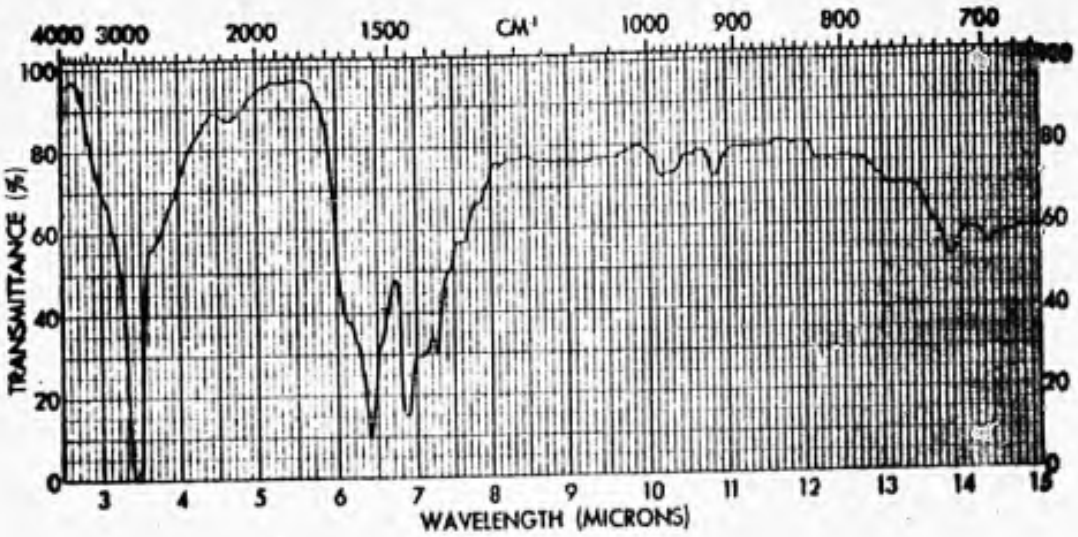


Figure 14. Infrared spectrum of 2,4-diaminobutyric acid/linoleic acid, Nujol mull.

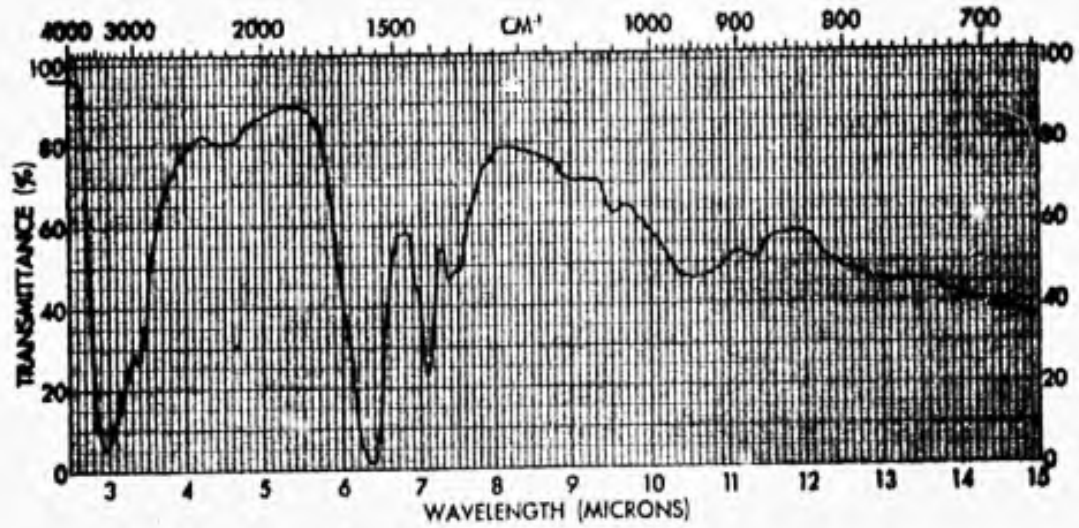


Figure 15. Infrared spectrum of 2,4-diaminobutyric acid free base, Nujol mull.

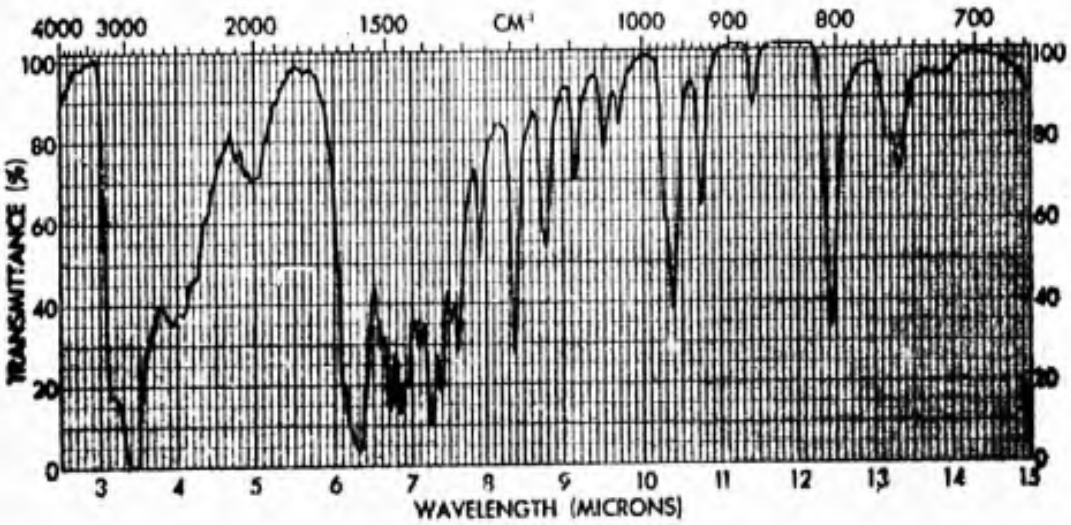


Figure 16. Infrared spectrum of 2,4-diaminobutyric acid mono-hydrochloride, Nujol mull.

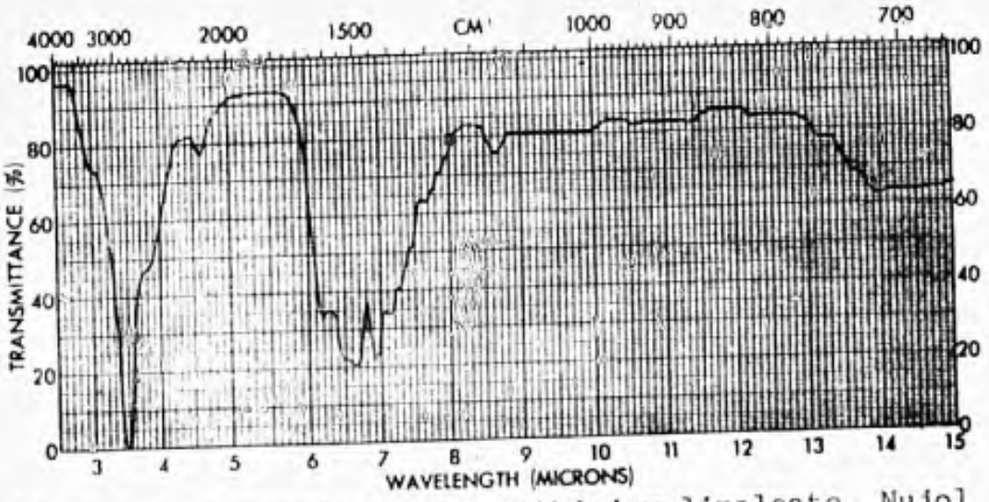


Figure 17. Infrared spectrum of ornithinium linoleate, Nujol mull.

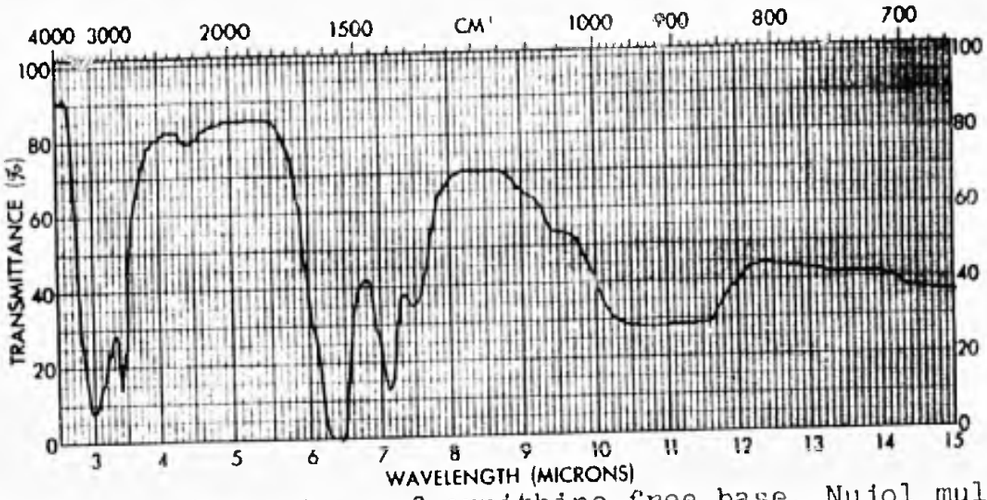


Figure 18. Infared spectrum of ornithine free base, Nujol mull.

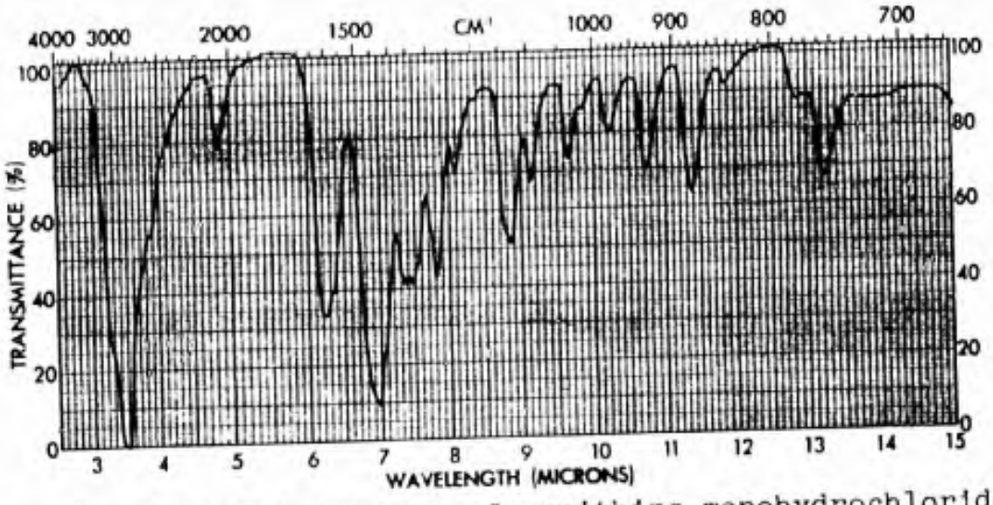


Figure 19. Infrared spectrum of ornithine monohydrochloride, Nujol mull.

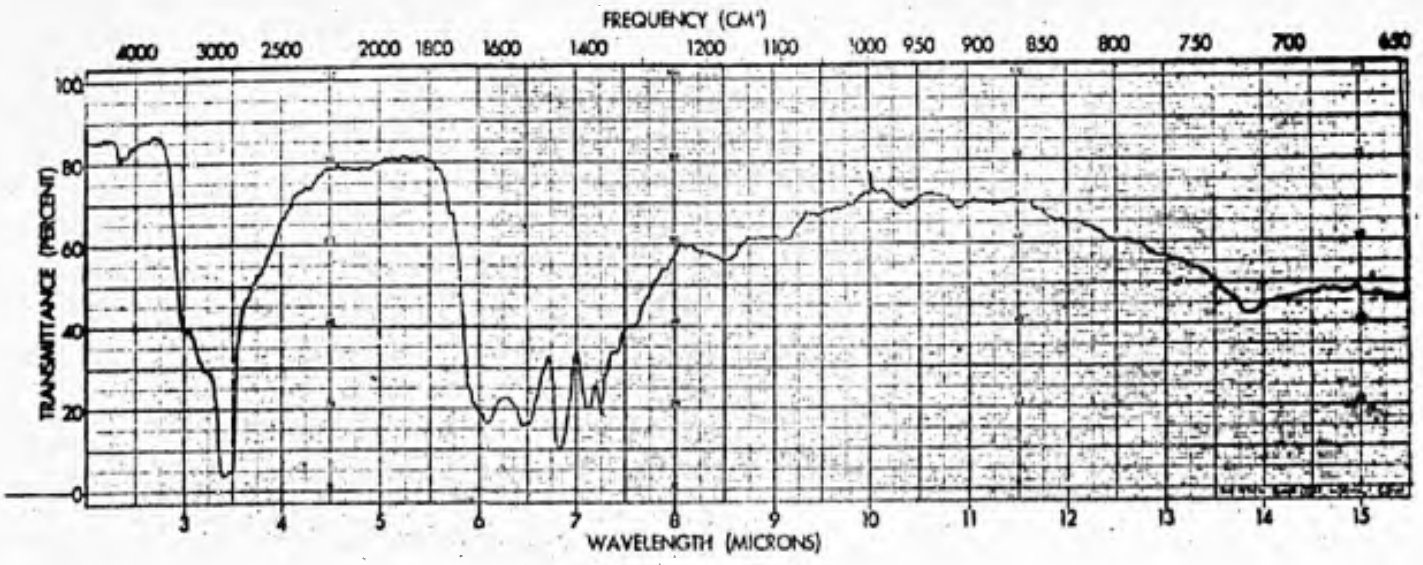


Figure 20. Infrared Spectrum of L-Arginine and Linoleic Acid, Nujol Mull.

Note: The infrared spectra of D-Arginine and its Linoleate are identical with those for the L-isomer.

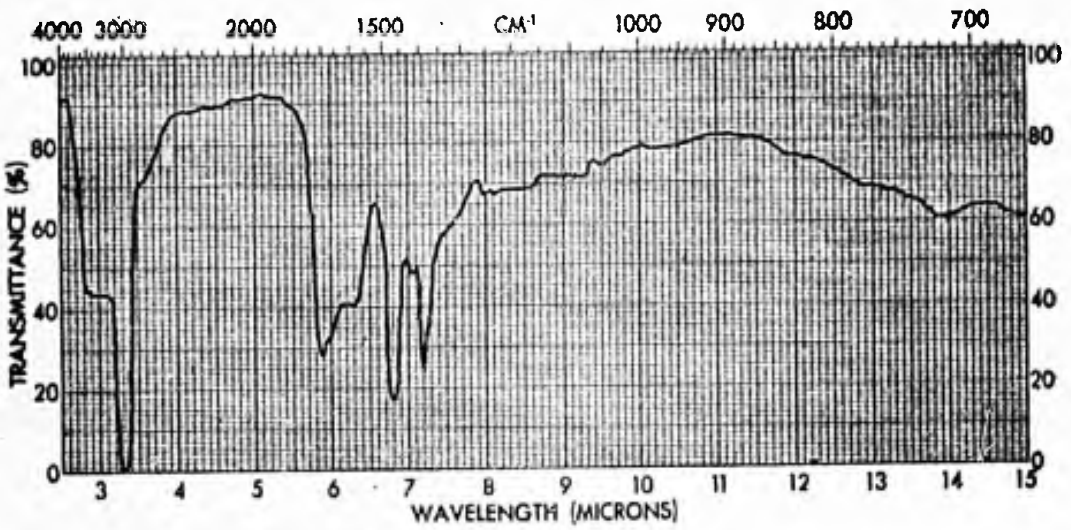


Figure 21. Infrared spectrum of L-arginine, Nujol mull.

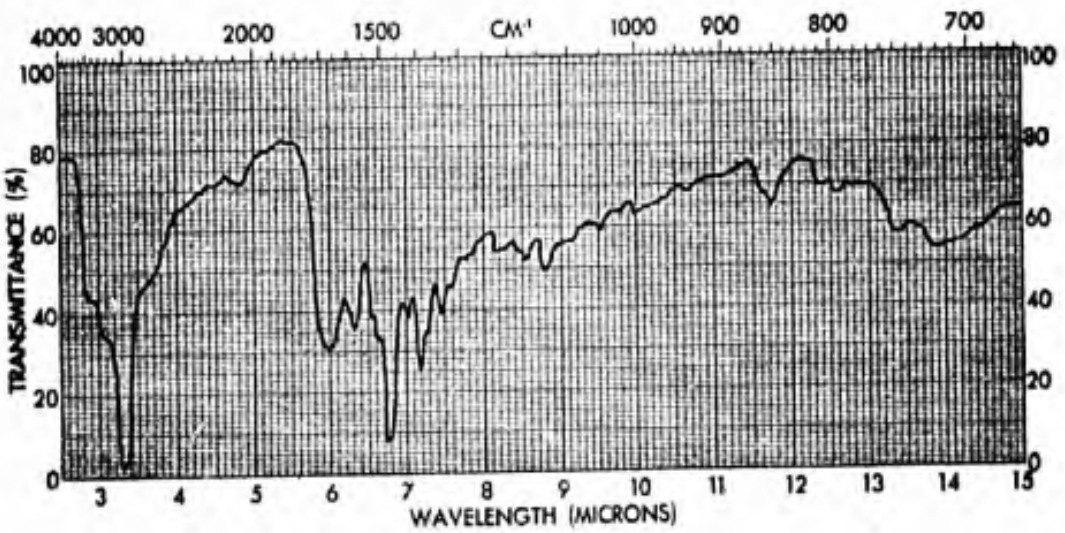


Figure 22. Infrared spectrum of L-arginine monohydrochloride, Nujol mull.

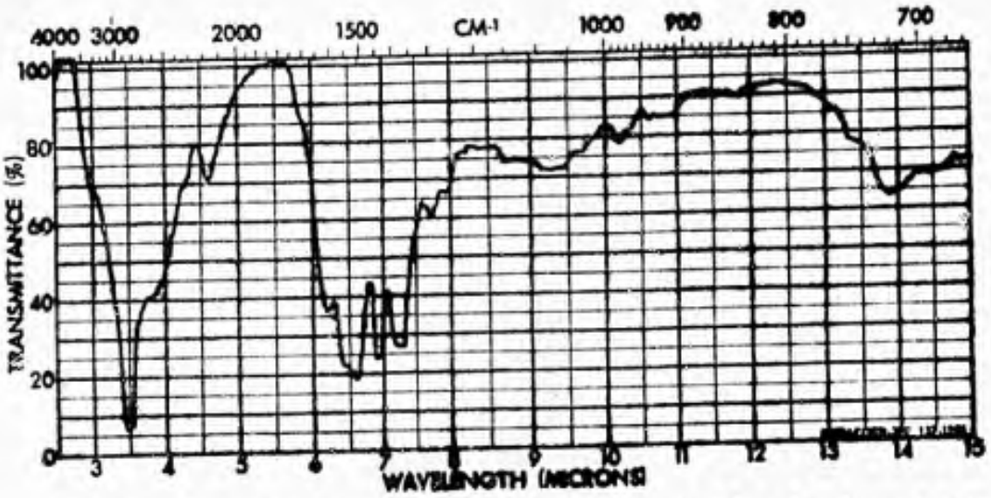


Figure 23. Infrared spectrum of 3,6-diaminohexanoic acid/linoleic acid, Nujol mull.

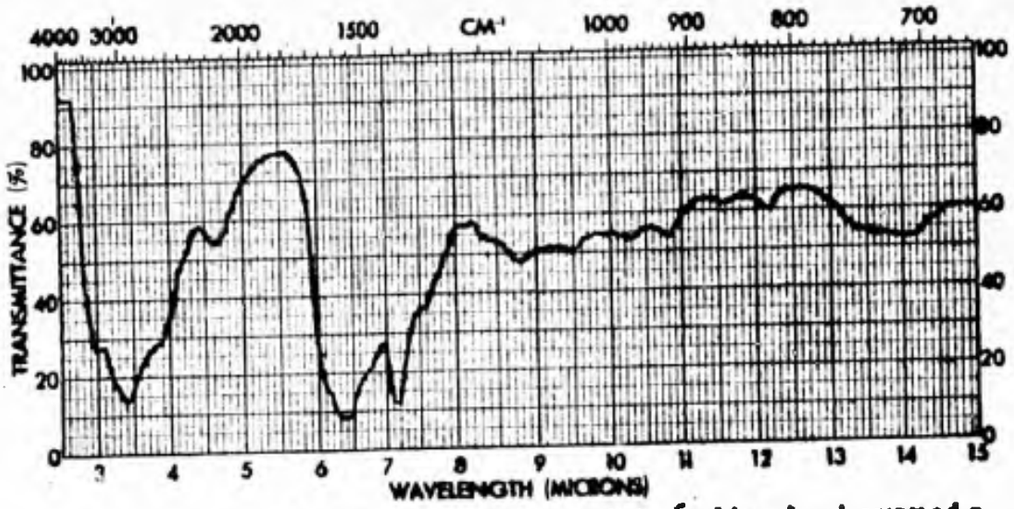


Figure 24. Infrared spectrum of 3,6-diaminohexanoic acid, semi-solid film.

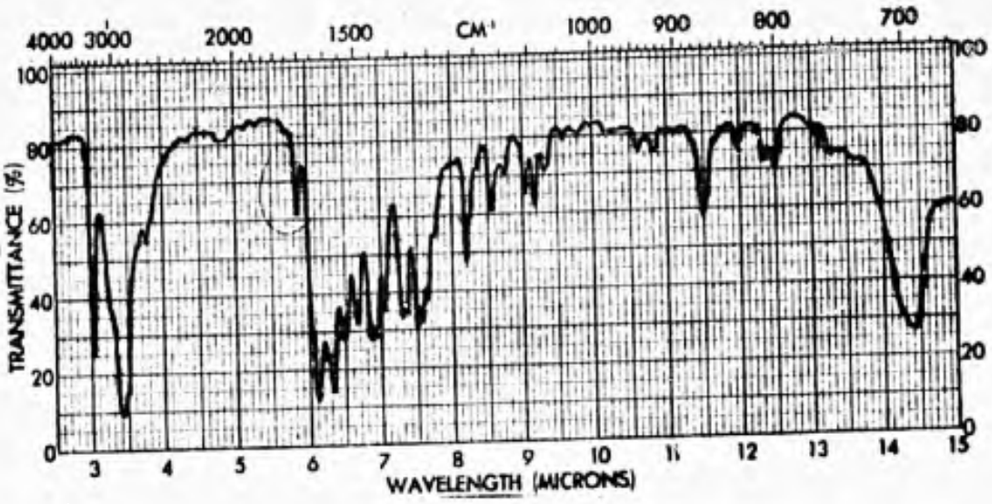


Figure 25. Infrared spectrum of N_ϵ-benzoyllysinium linoleate, Nujol mull.

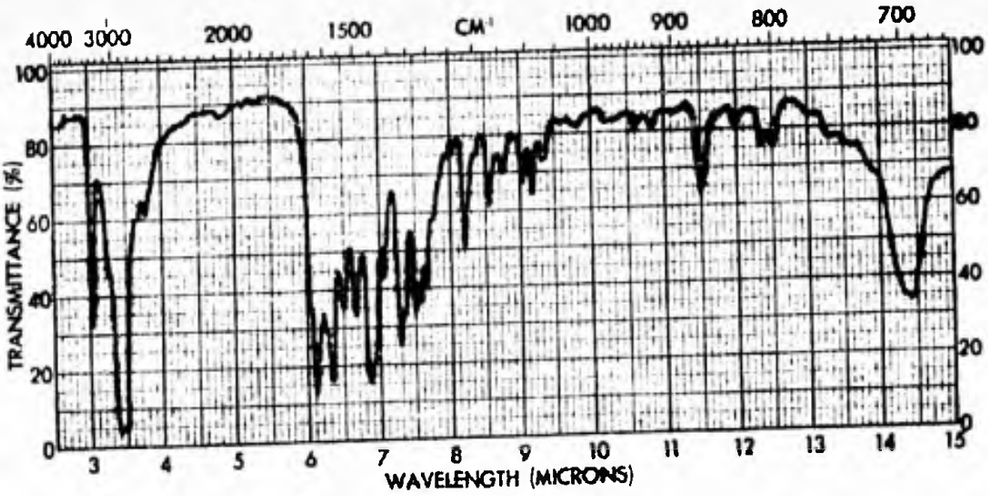


Figure 26. Infrared spectrum of N_ϵ-benzoyllysine, Nujol mull.

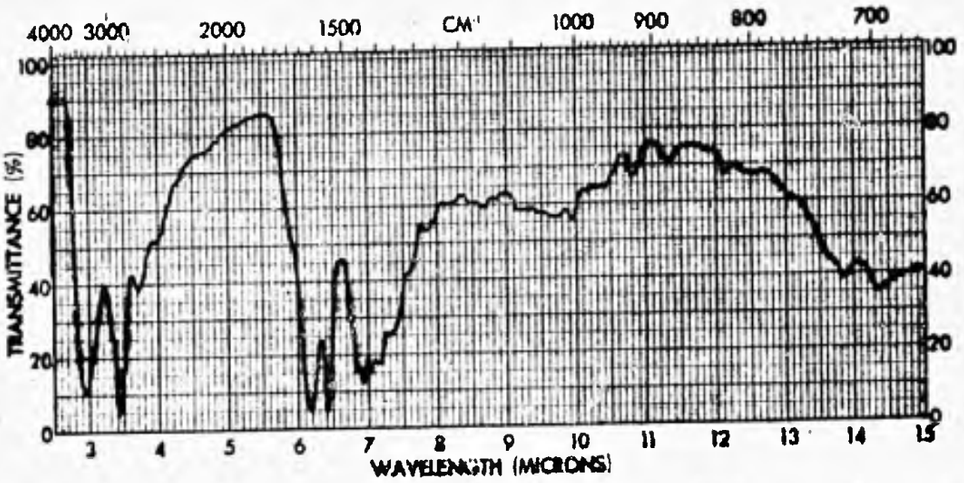


Figure 27. Infrared spectrum of tetramethyllysinium linoleate, film.

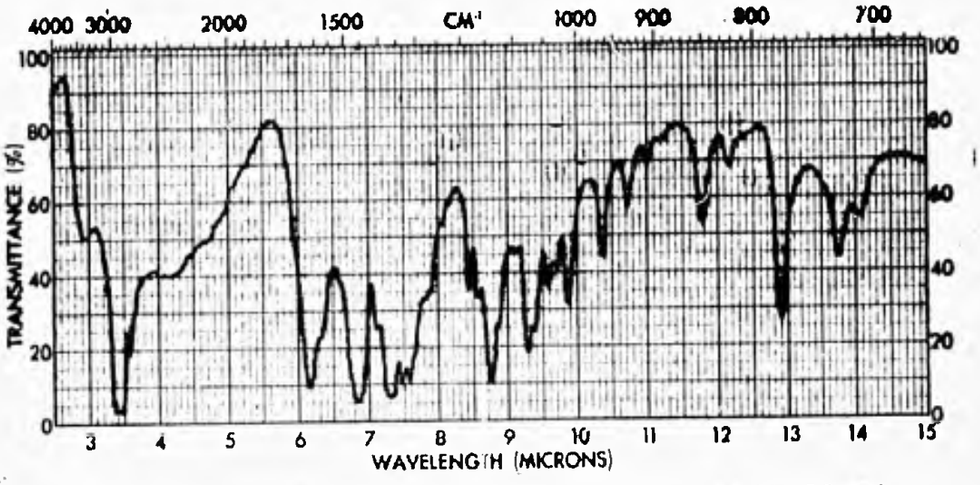


Figure 28. Infrared spectrum of tetramethyllysine, Nujol mull.

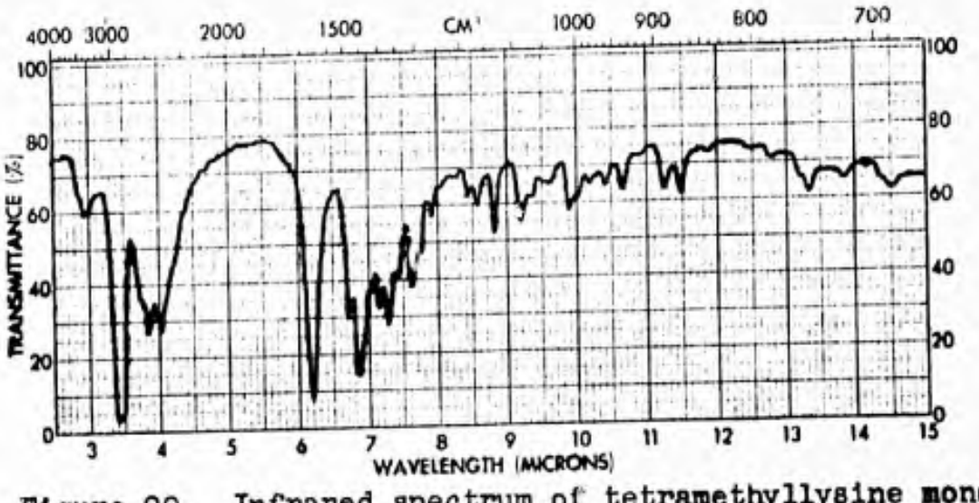


Figure 29. Infrared spectrum of tetramethyllysine monohydrochloride, Nujol mull.

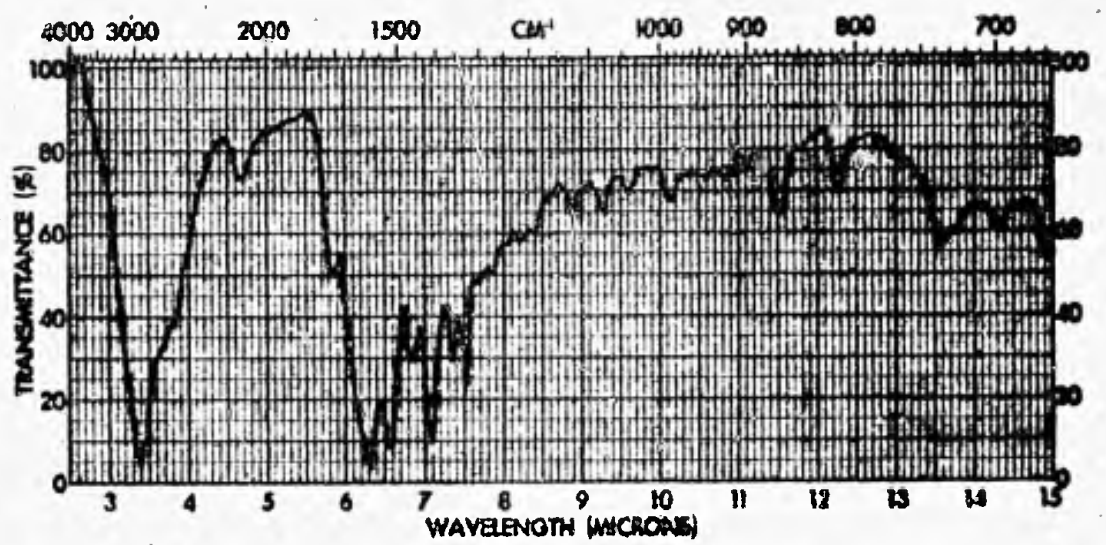


Figure 30. Infrared spectrum of linoleic acid/butyl amine/norleucine, film.

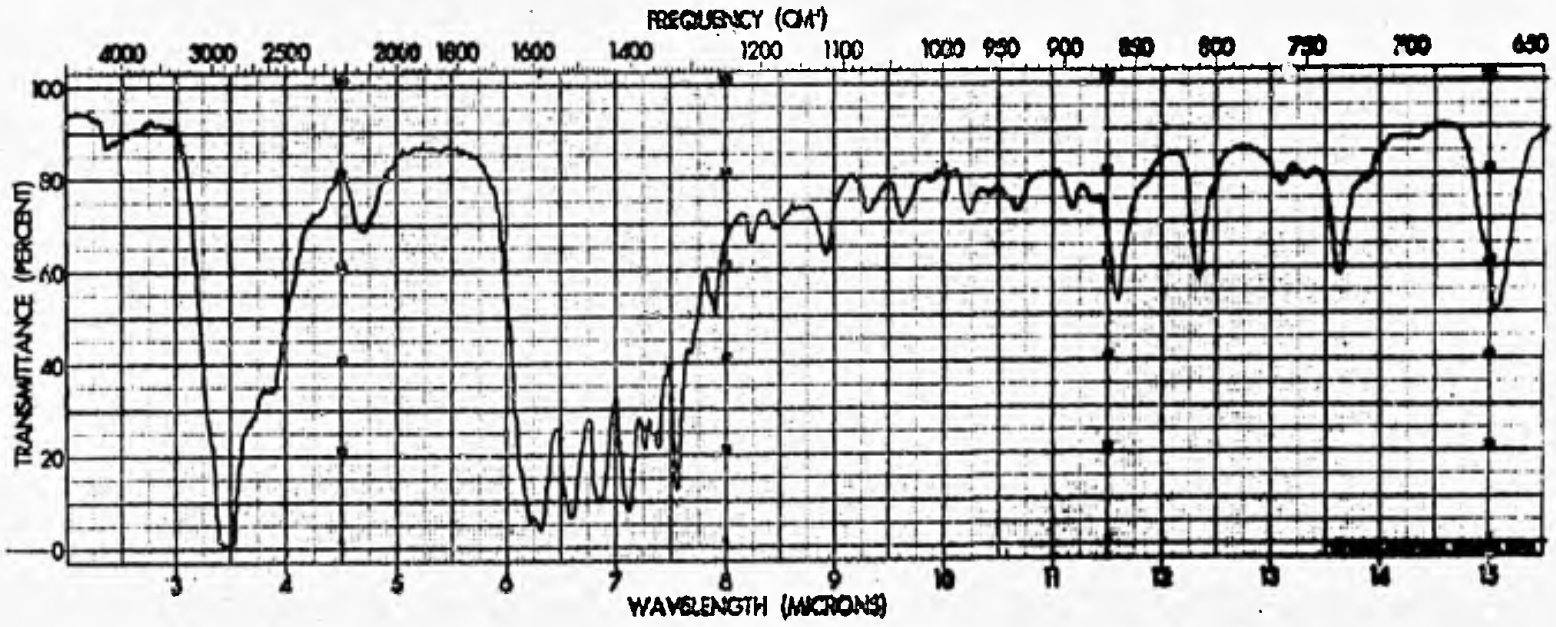


Figure 31. Infrared spectrum of L-norleucine, Nujol mull.

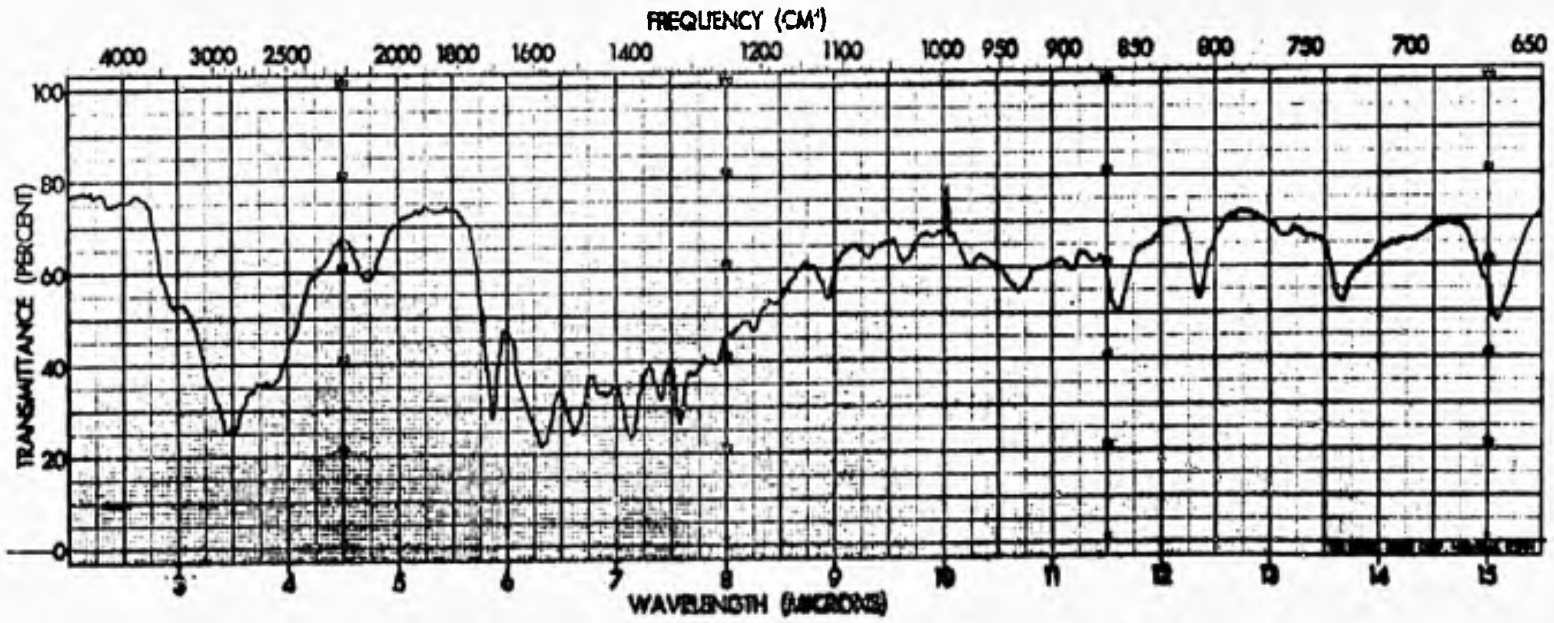


Figure 32. Infrared spectrum of L-norleucine and linoleic acid, Nujol mull.

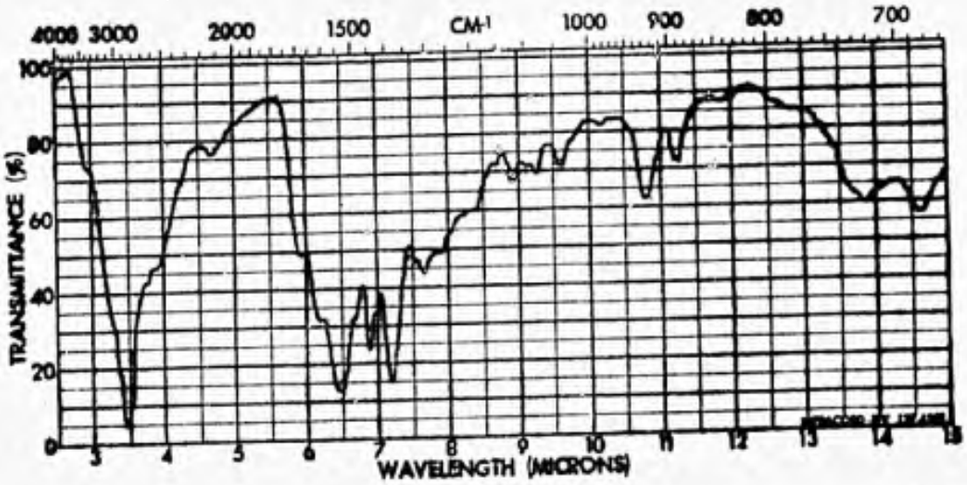


Figure 33. Infrared spectrum of linoleic acid/butyl amine/ glycine, liquid film.

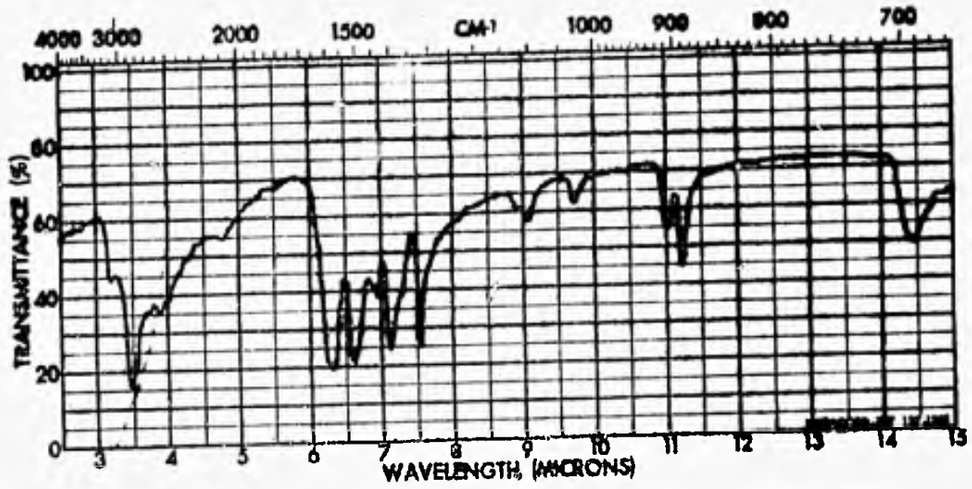


Figure 34. Infrared spectrum of glycine, Nujol mull.

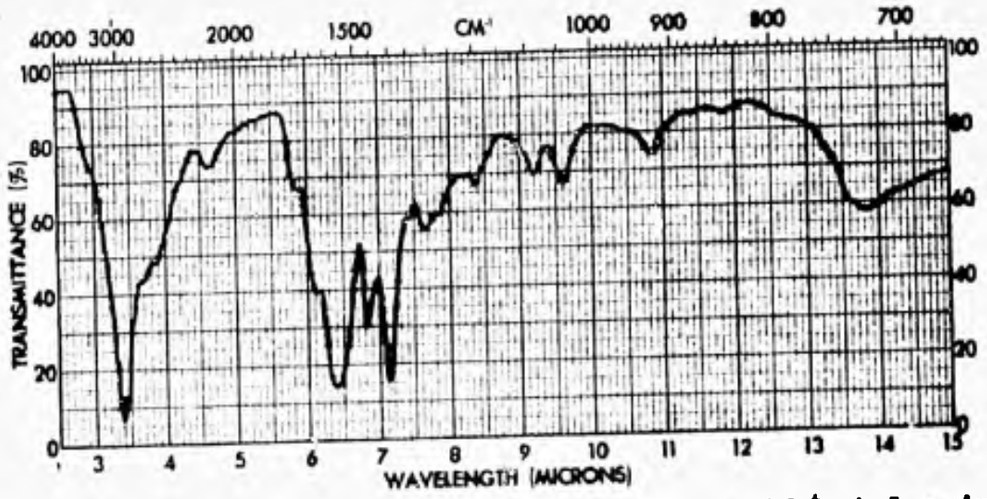


Figure 35. Infrared spectrum of linoleic acid/butyl amine, liquid film.

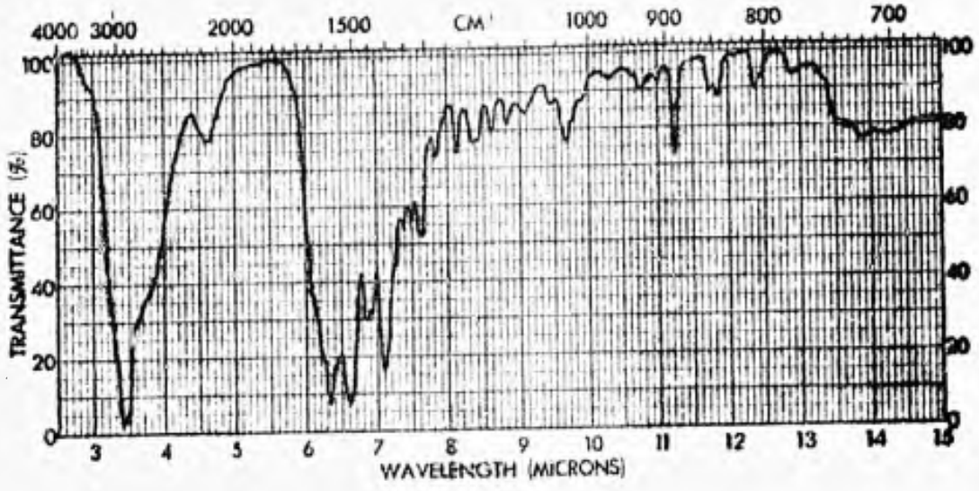


Figure 36. Infrared spectrum of lysinium linoleate/ copper acetate, Nujol mull.

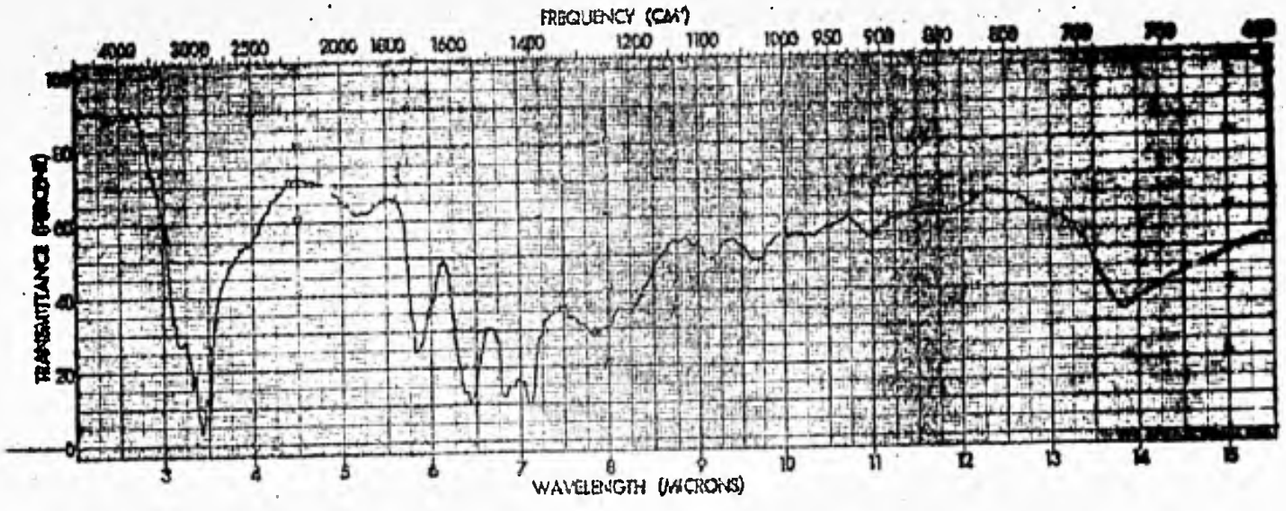


Figure 37. Infrared Spectrum of Ammonium Linoleate, Liquid Film.

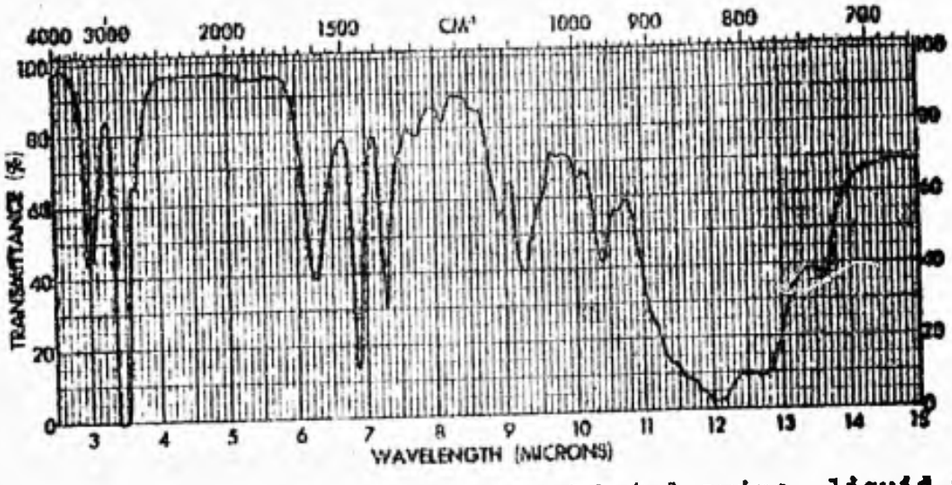


Figure 38. Infrared spectrum of butyl amine, liquid film.

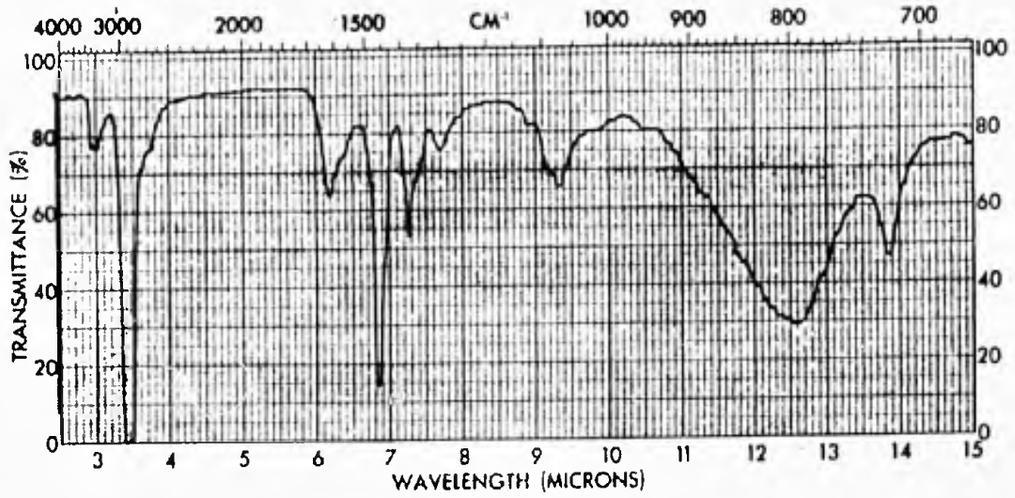


Figure 39. Infrared spectrum of dodecyl amine, liquid film.

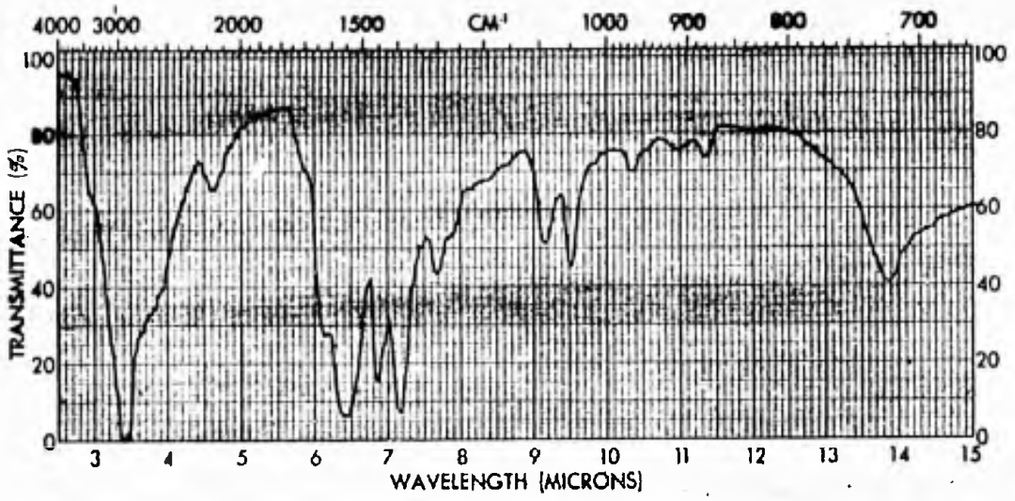


Figure 40. Infrared spectrum of dodecylammonium linoleate, liquid film.

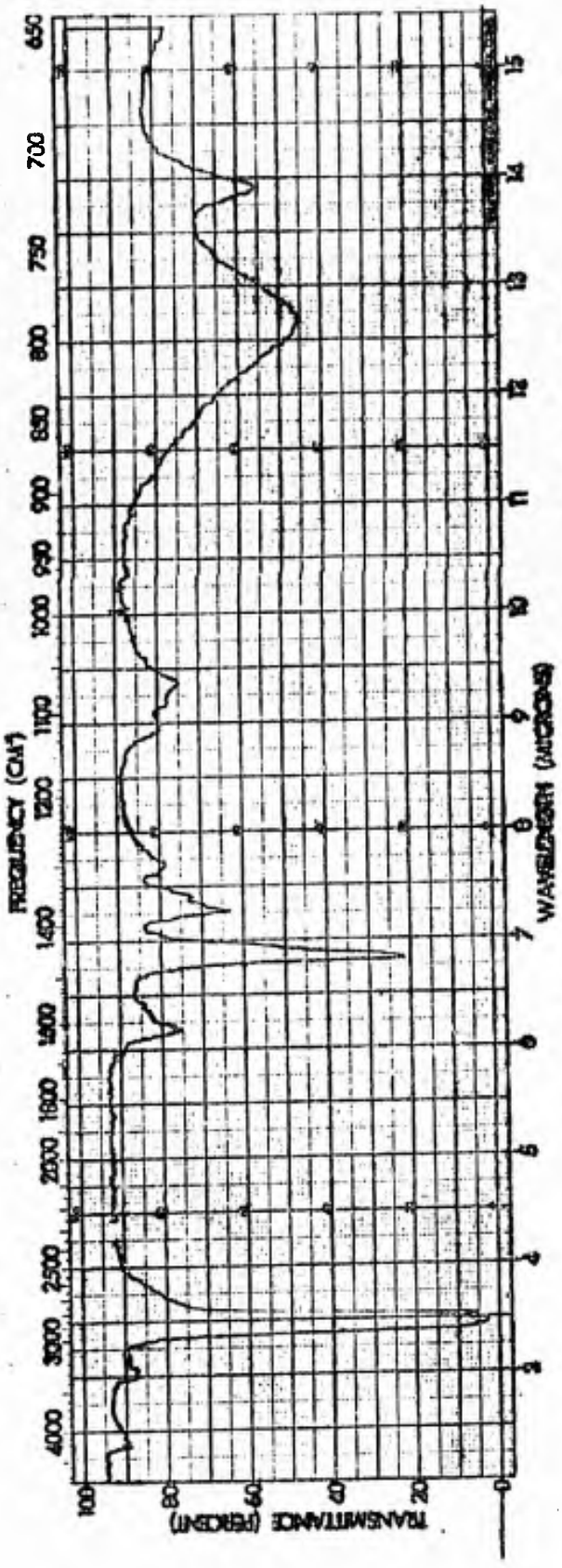


Figure 41. Infrared spectrum of n-hept
heptadecylamine, film.

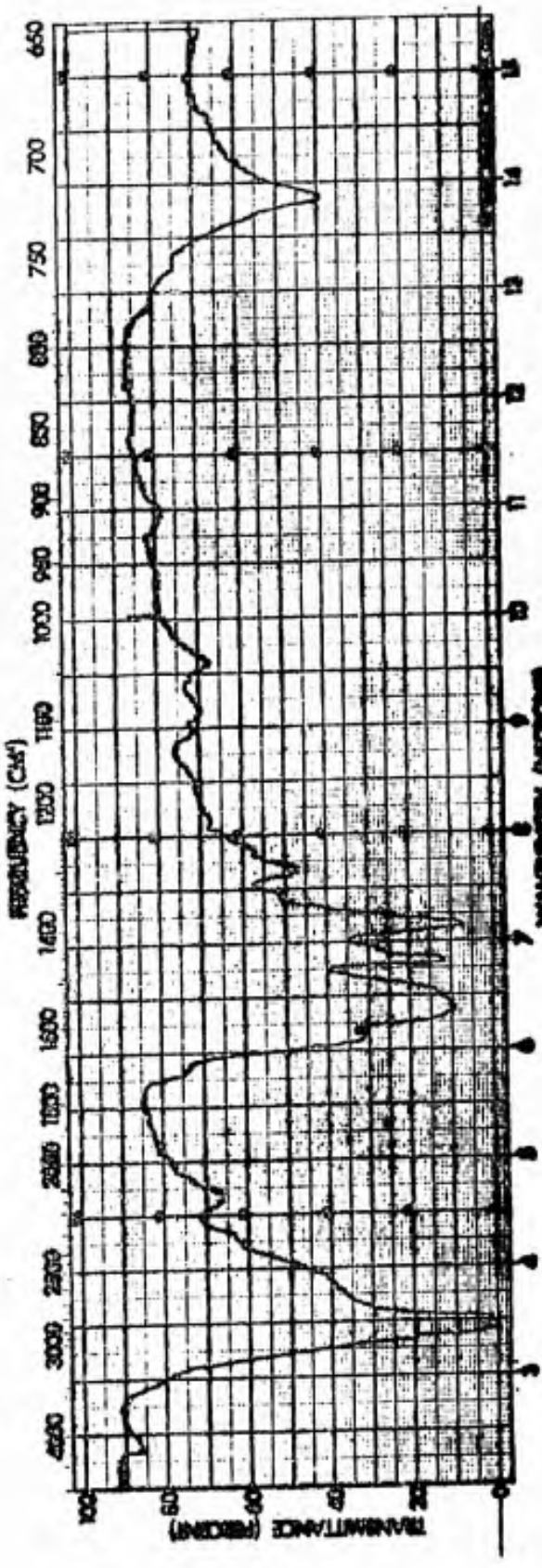


Figure 42. Infrared spectrum of n-hep
heptadecylamine and linoleic
acid, liquid film.

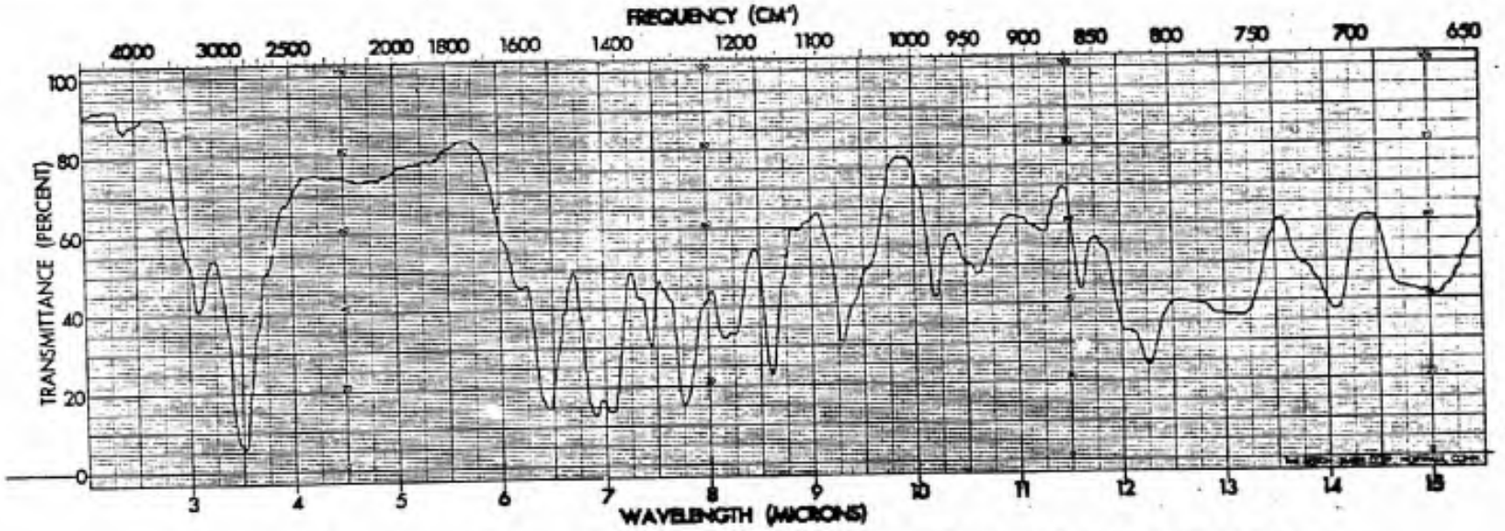


Figure 43. Infrared Spectrum of 3-Azabicyclo[3.2.2]nonane, Liquid Film

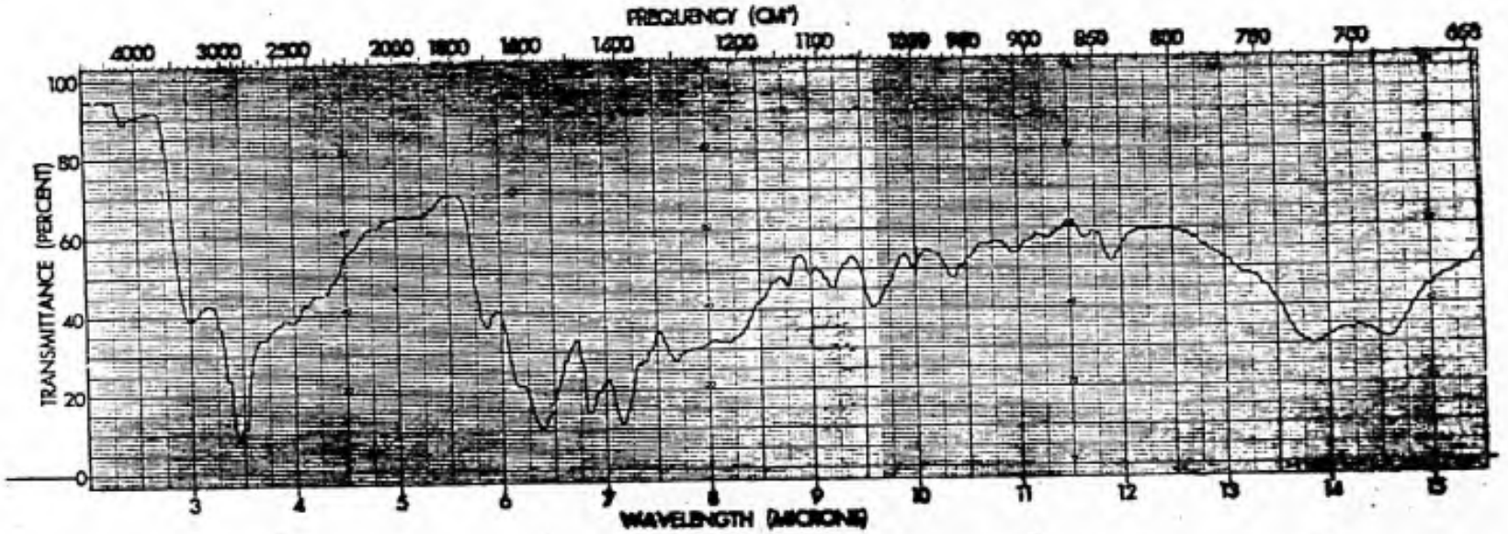


Figure 44. Infrared Spectrum of 3-Azabicyclo[3.2.2]nonane and Linoleic Acid, Liquid Film

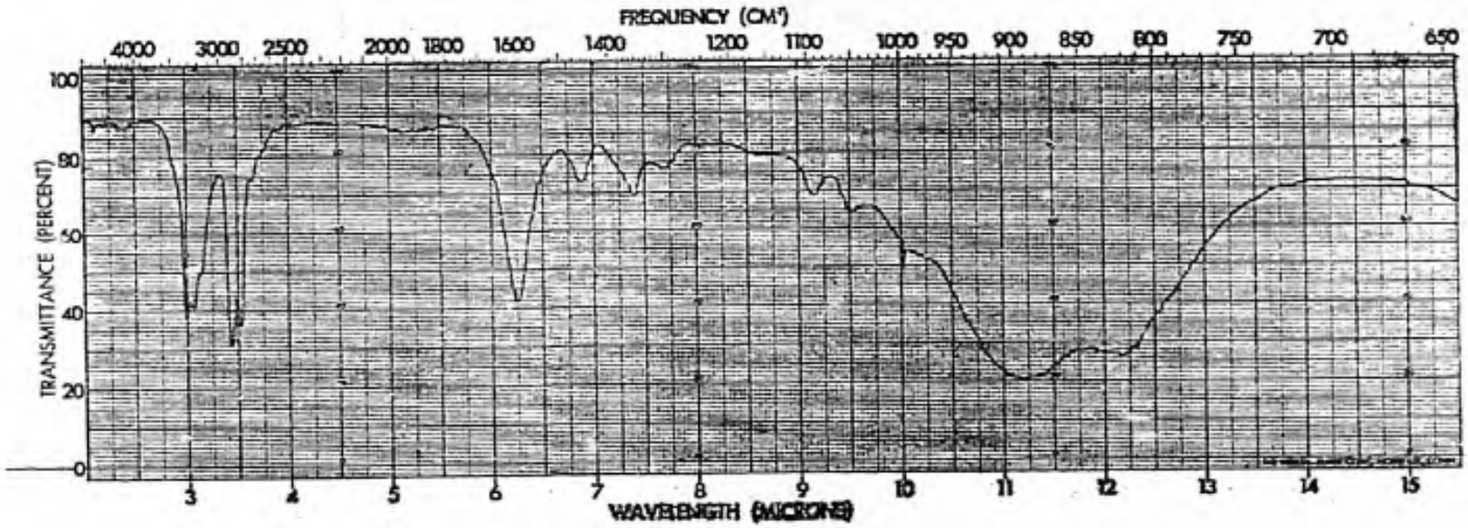


Figure 45. Infrared Spectrum of Ethylenediamine, Liquid Film

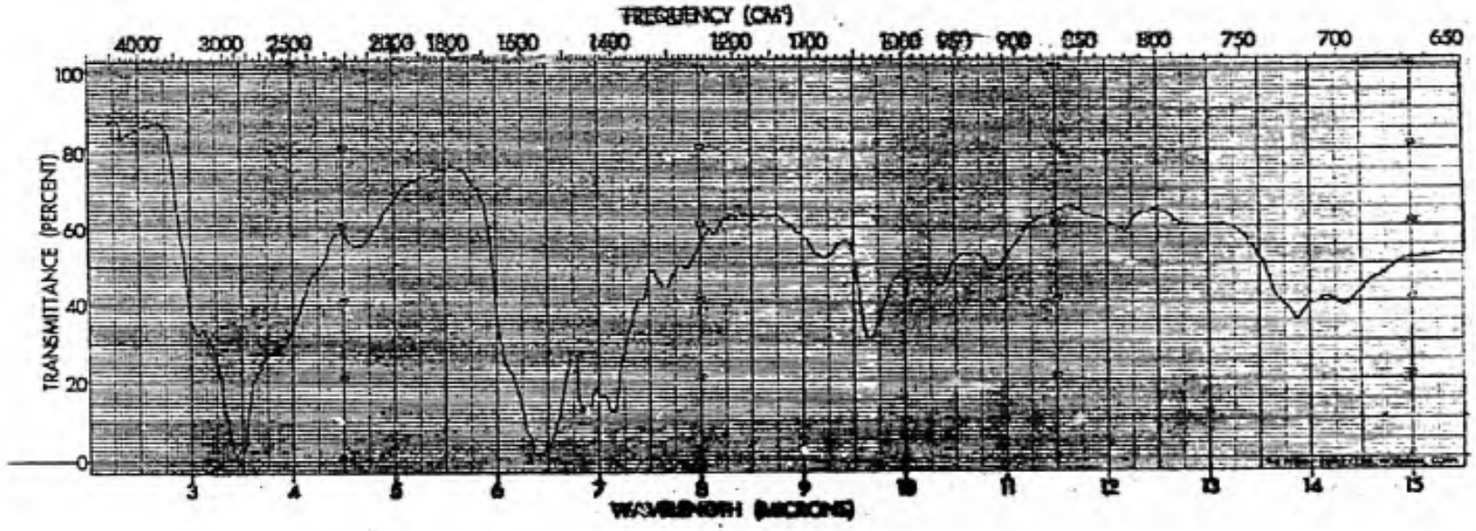


Figure 46. Infrared Spectrum of Ethylenediamine and Linoleic Acid, Liquid Film

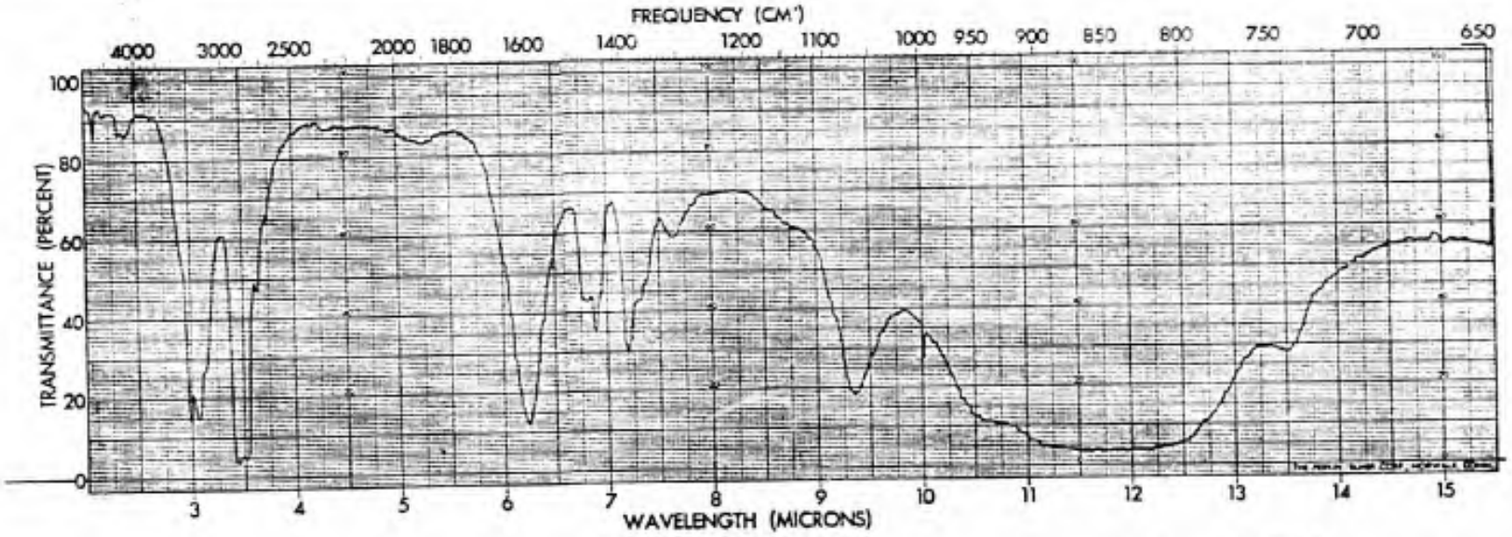


Figure 47. Infrared Spectrum of Putrescine, Liquid Film

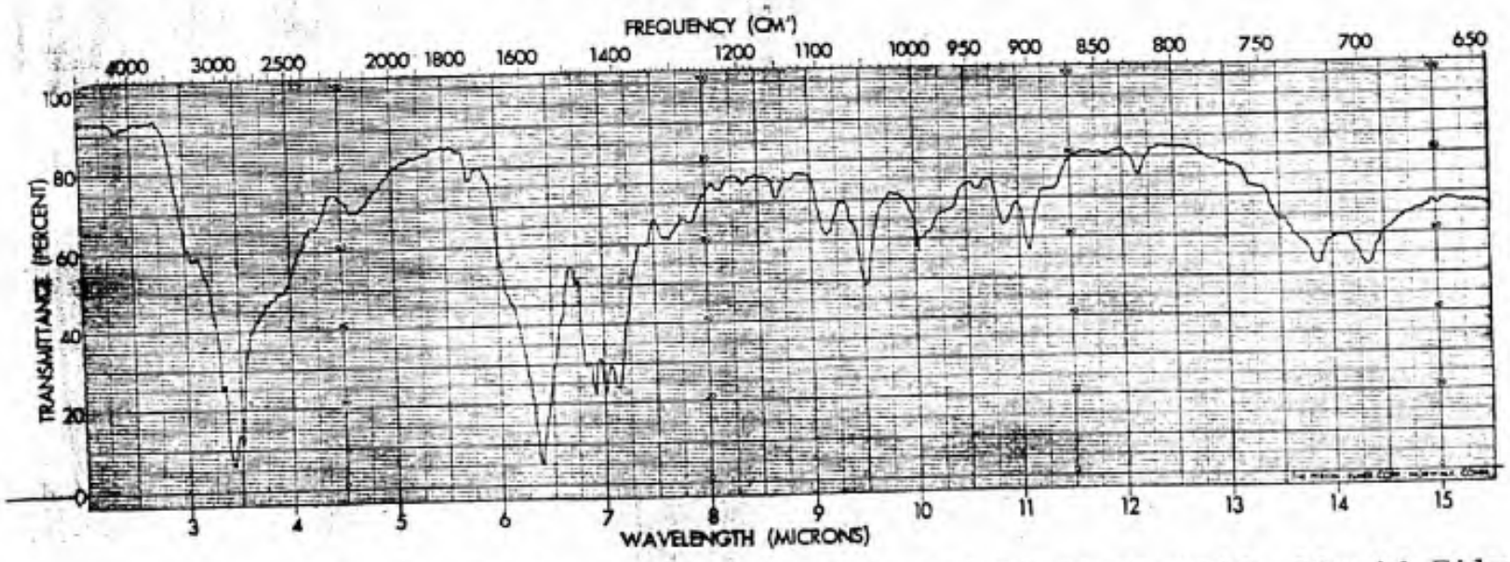


Figure 48. Infrared Spectrum of Putrescine and Linoleic Acid, Liquid Film

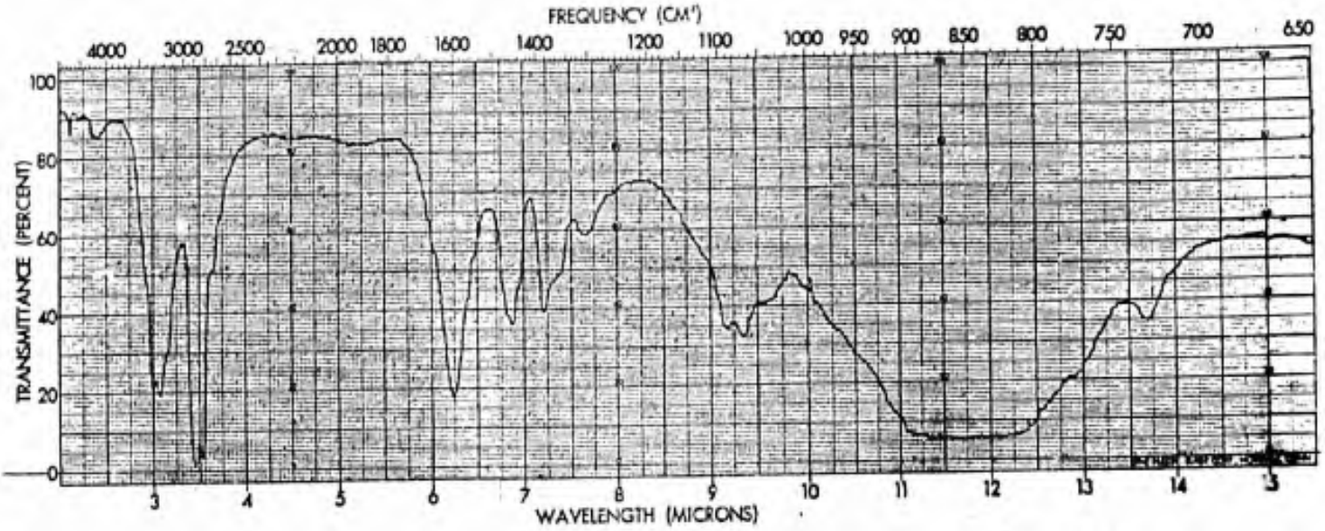


Figure 49. Infrared Spectrum of Cadaverine, Liquid Film

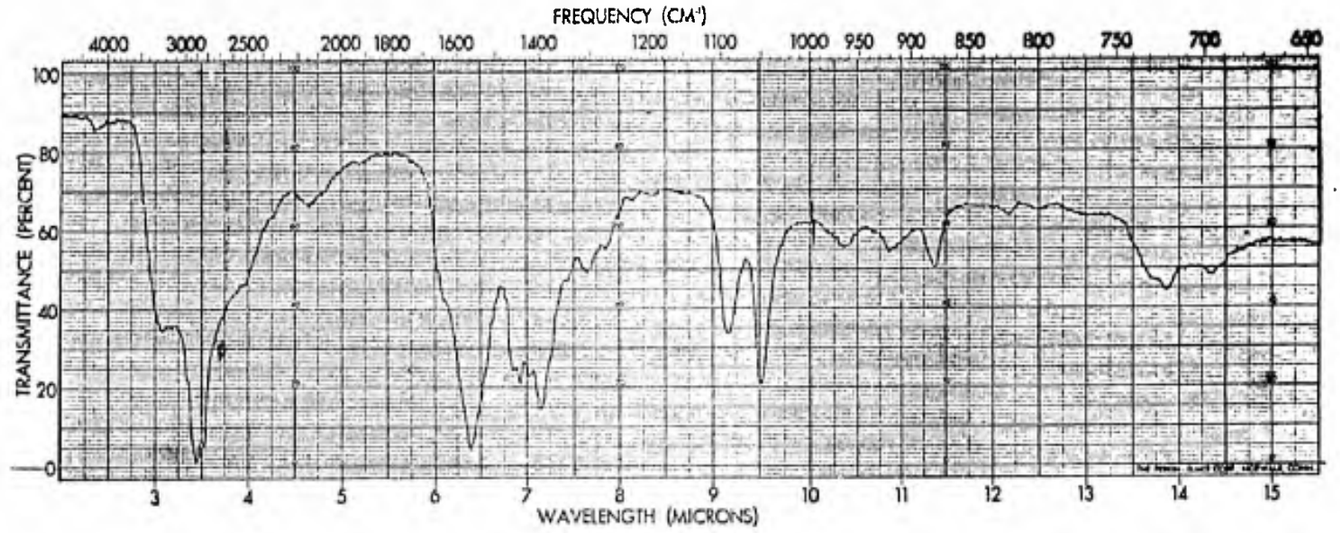


Figure 50. Infrared Spectrum of Cadaverine and Linoleic Acid, Equimolar Mixture, Liquid Film

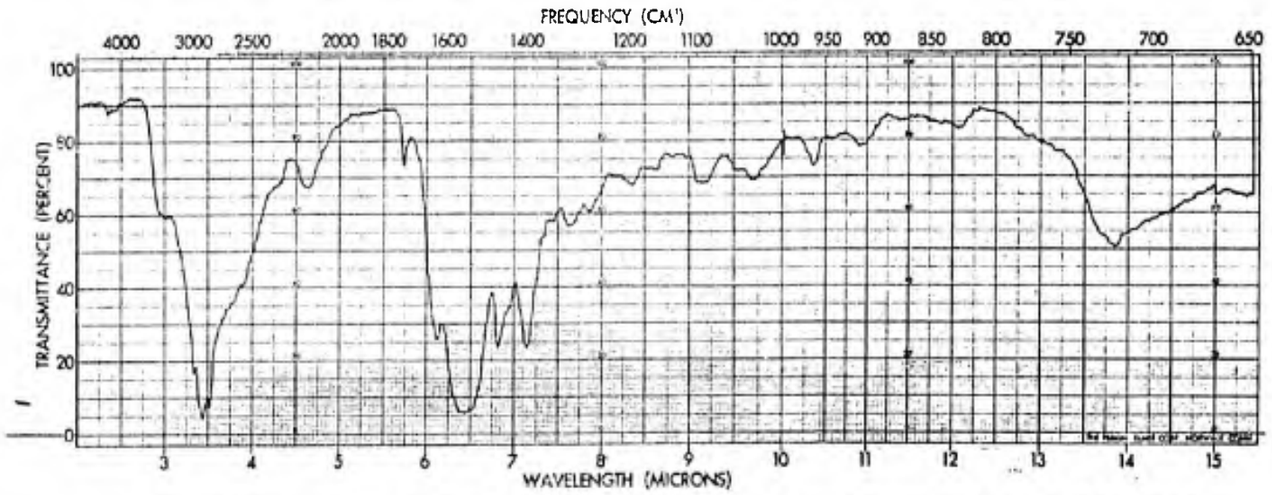


Figure 51. Infrared Spectrum of Cadaverine (1 mole) and Linoleic Acid (2 moles), Liquid Film.

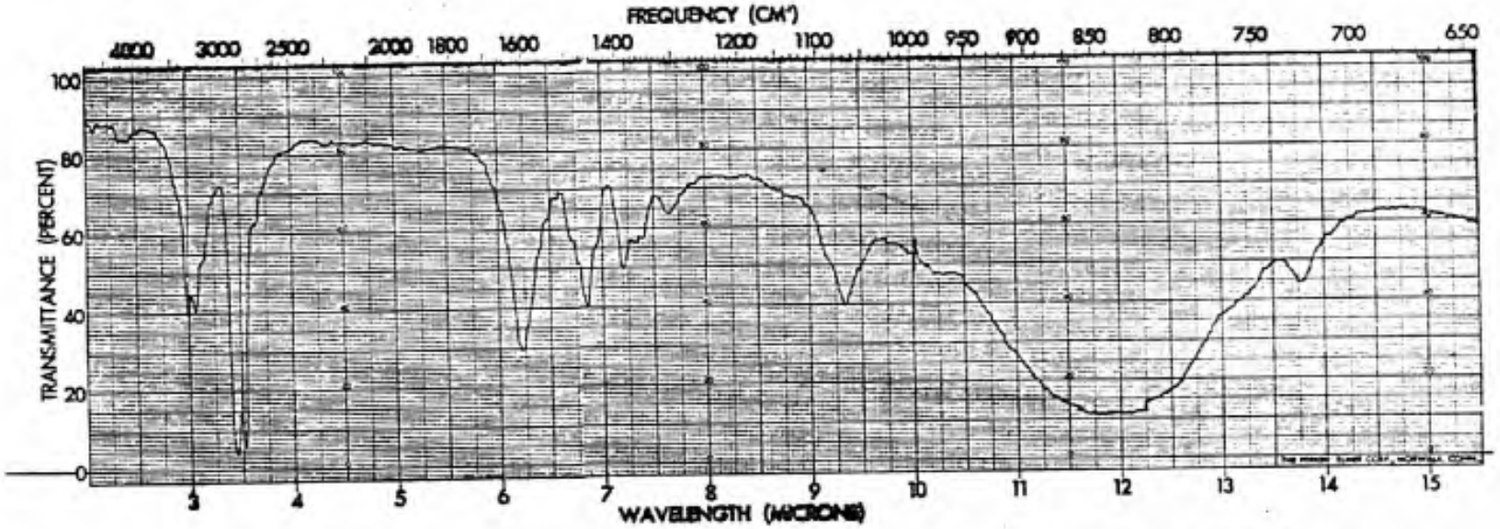


Figure 52. Infrared Spectrum of 1,6-Hexanediamine, Liquid Film

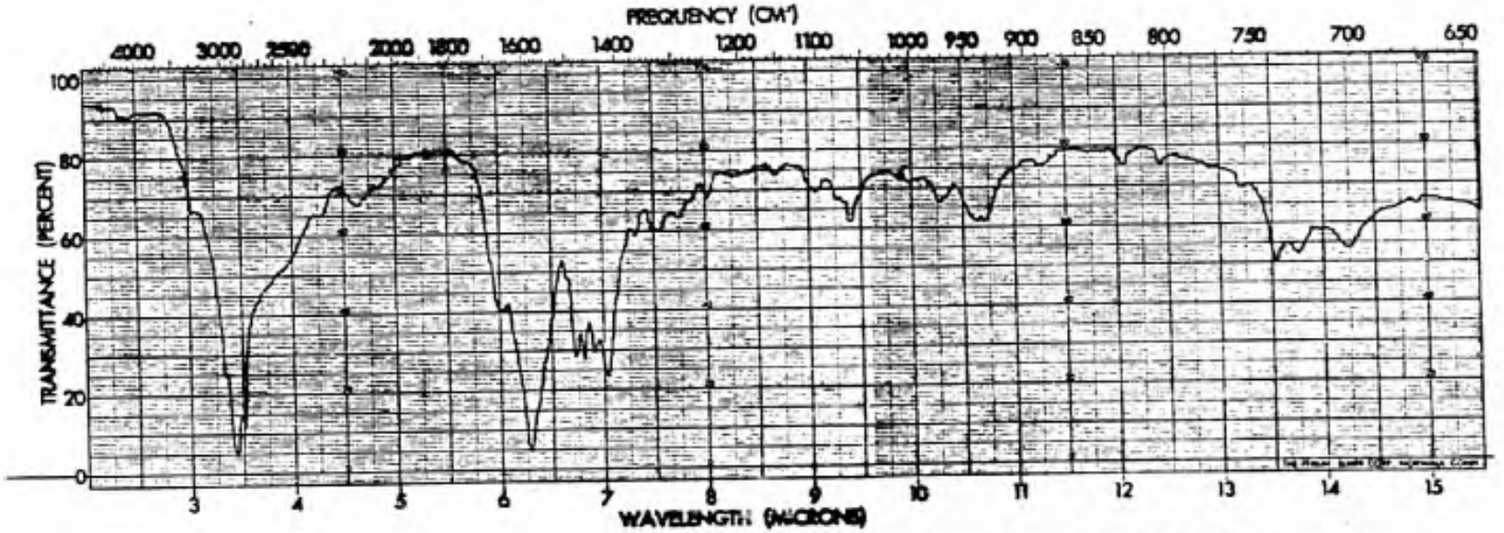


Figure 53. Infrared Spectrum of 1,6-Hexanediamine and Linoleic Acid, Liquid Film

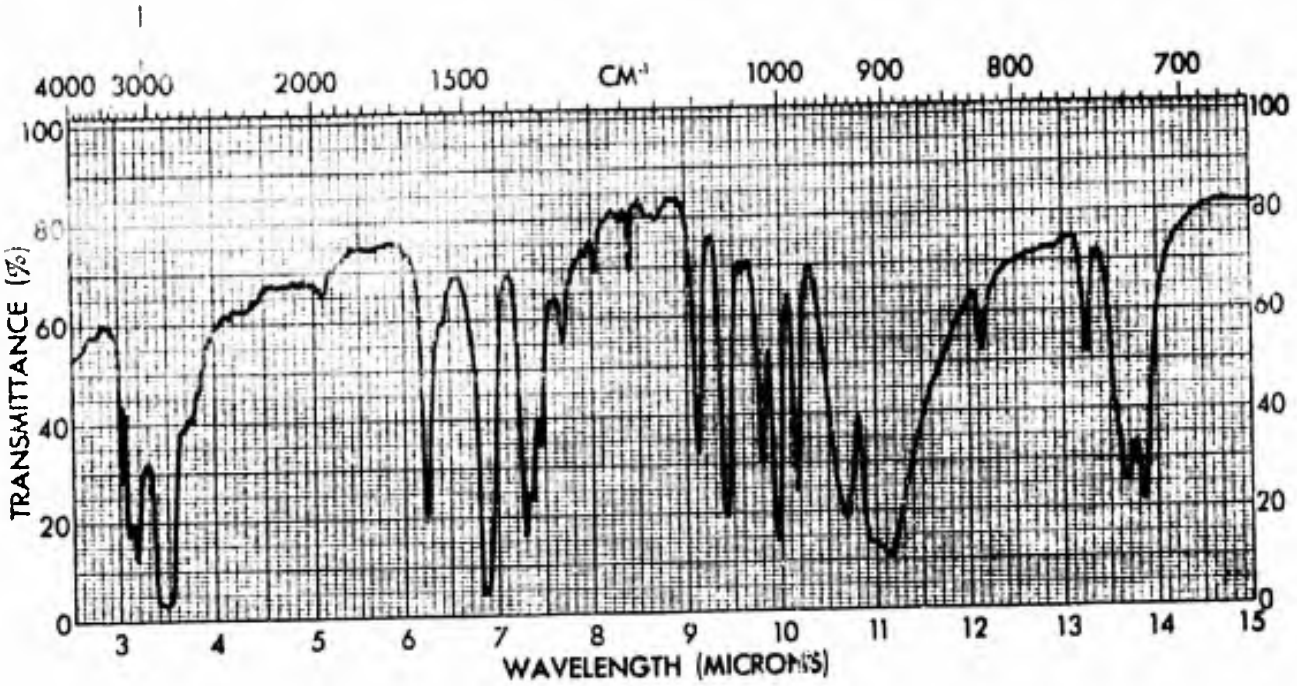


Figure 54. Infrared Spectrum of 1,12-Diaminododecane, Nujol Mull.

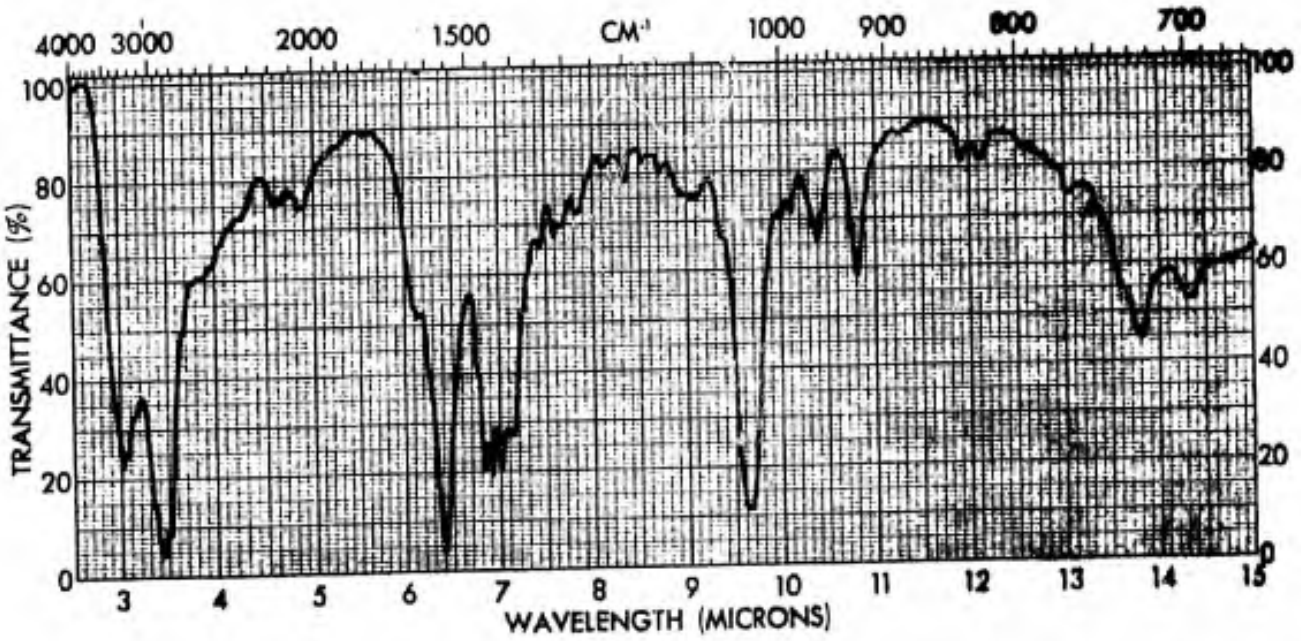


Figure 55. Infrared Spectrum of 1,12-Diaminododecane (1 mole) and Linoleic Acid (1 mole), Liquid Film.

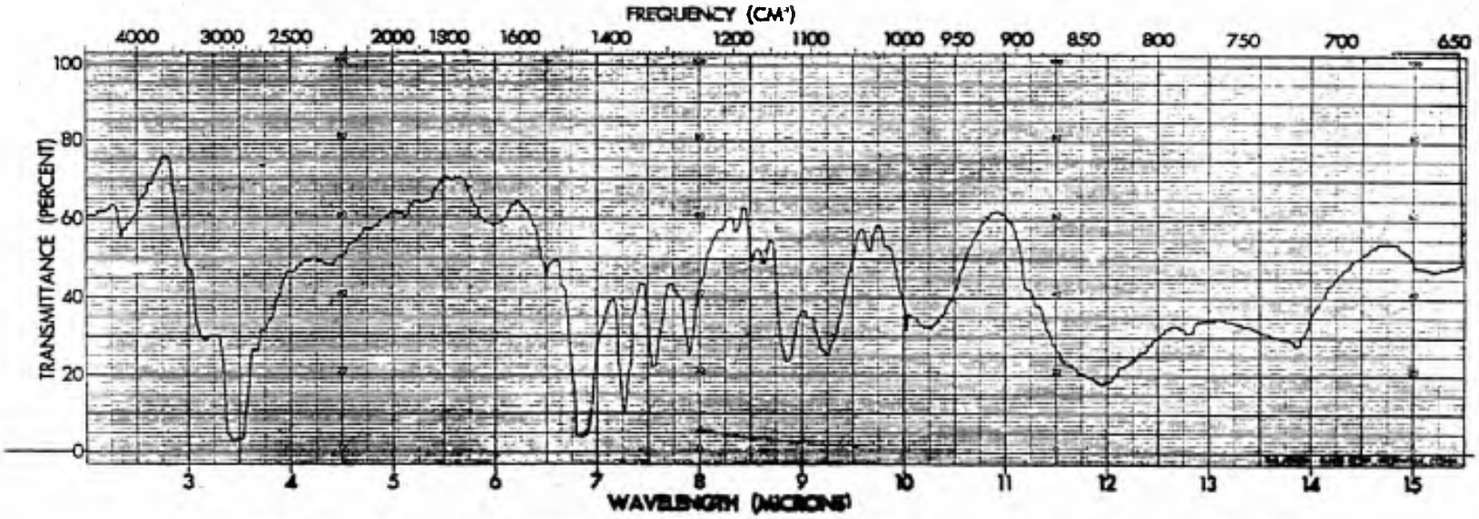


Figure 56. Infrared Spectrum of Piperazine, Nujol Mull

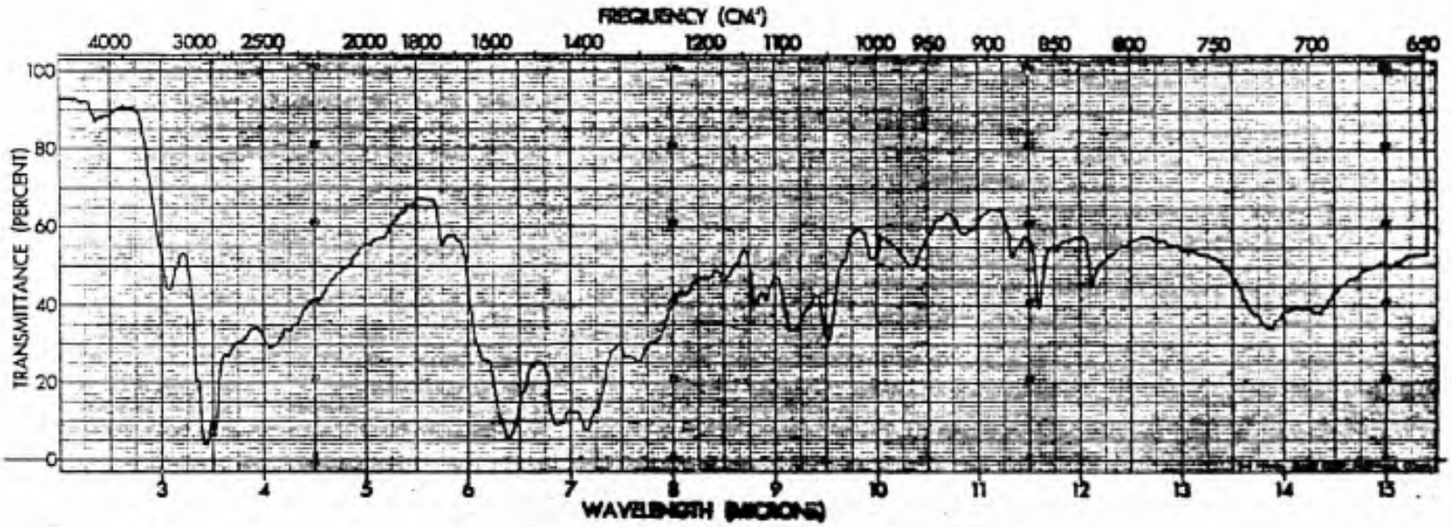


Figure 57. Infrared Spectrum of Piperazine and Linoleic Acid, Liquid Film

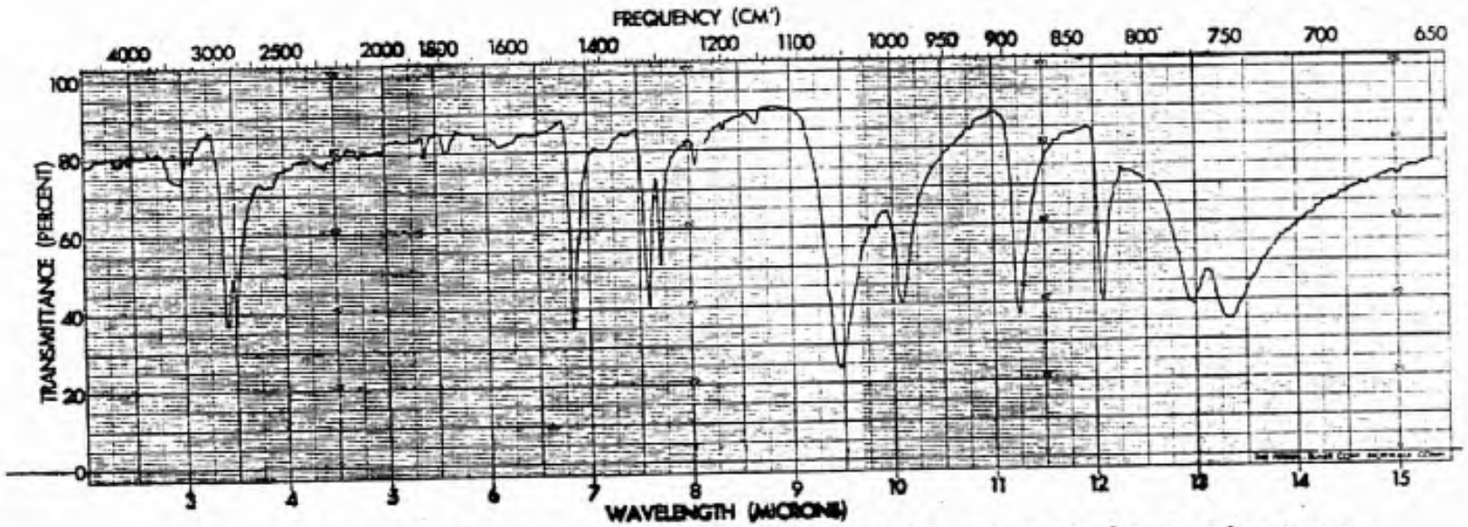


Figure 58. Infrared Spectrum of 1,4-Diazabicyclo[2.2.2]octane, Potassium Bromide Pellet

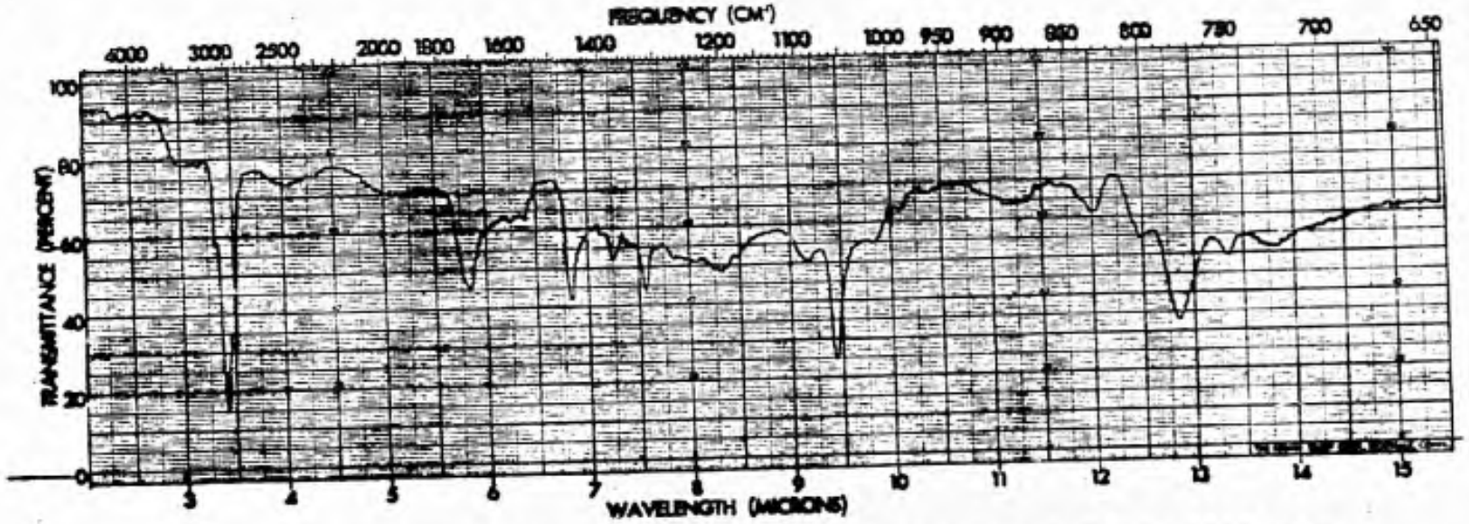


Figure 59. Infrared Spectrum of 1,4-Diazabicyclo[2.2.2]octane and Linoleic Acid, Liquid Film

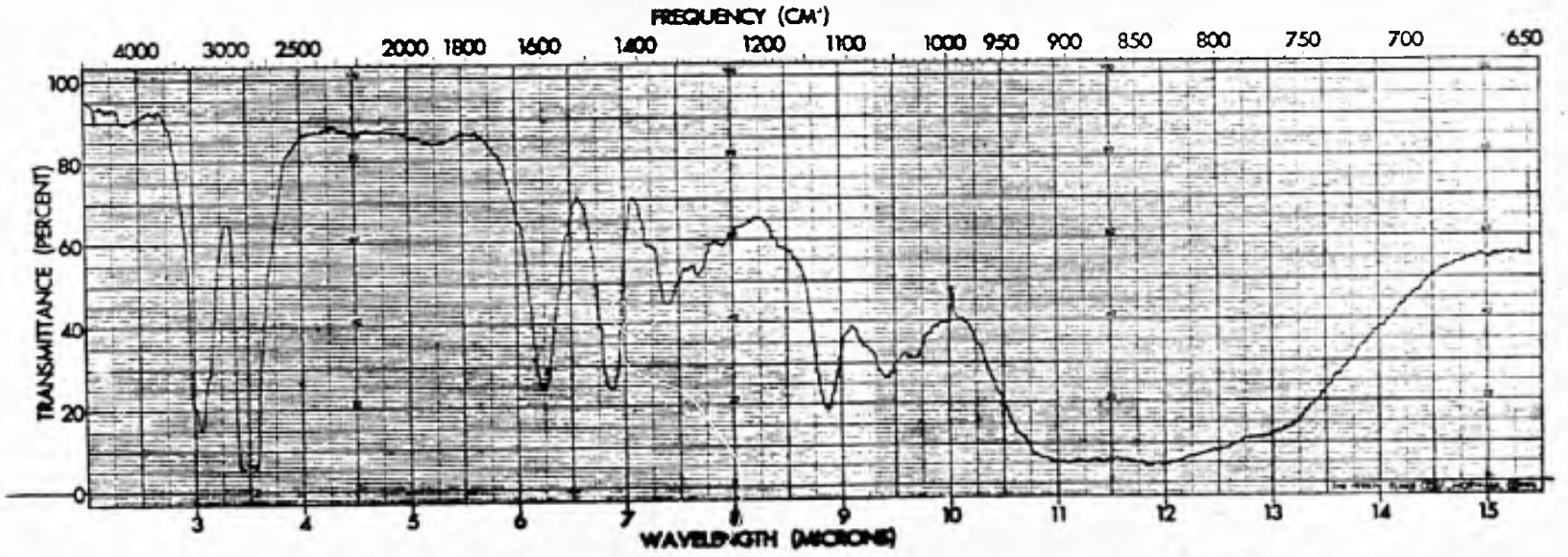


Figure 60. Infrared Spectrum of Diethylenetriamine, Liquid Film

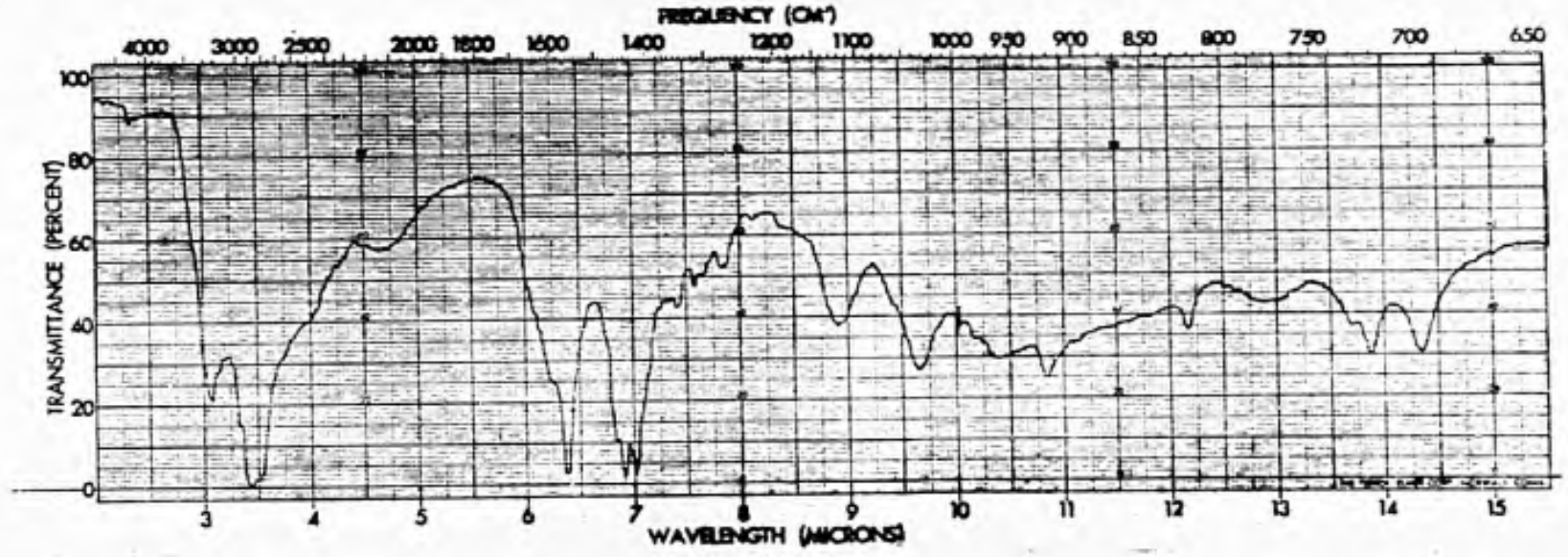


Figure 61. Infrared Spectrum of Diethylenetriamine and Linoleic Acid, Liquid Film

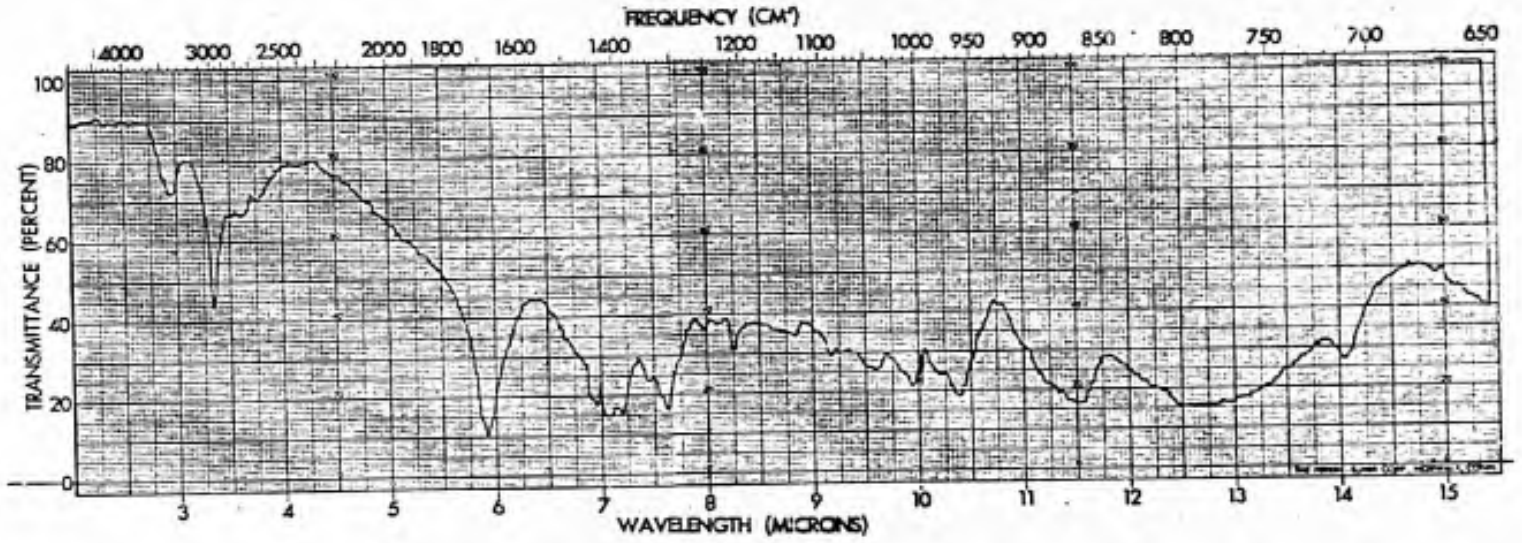


Figure 62. Infrared Spectrum of Ethylenediaminetetraacetic Acid, Potassium Bromide Pellet.

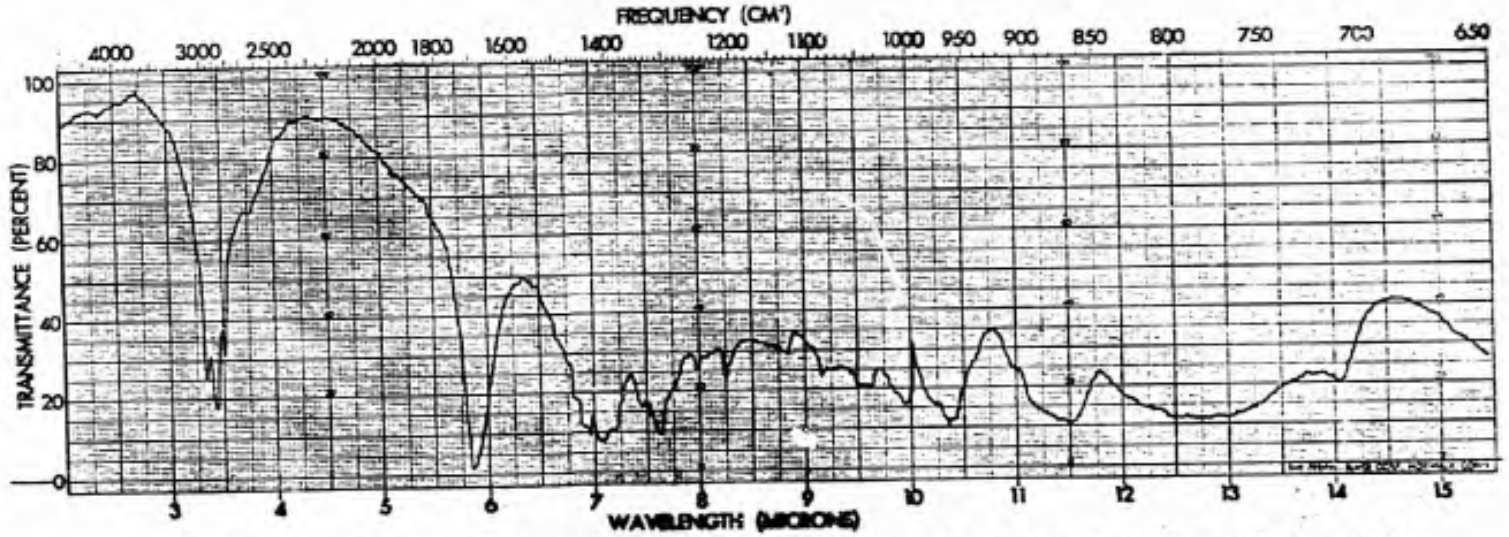


Figure 63. Infrared Spectrum of Ethylenediaminetetraacetic Acid and Linoleic Acid, Liquid Film.

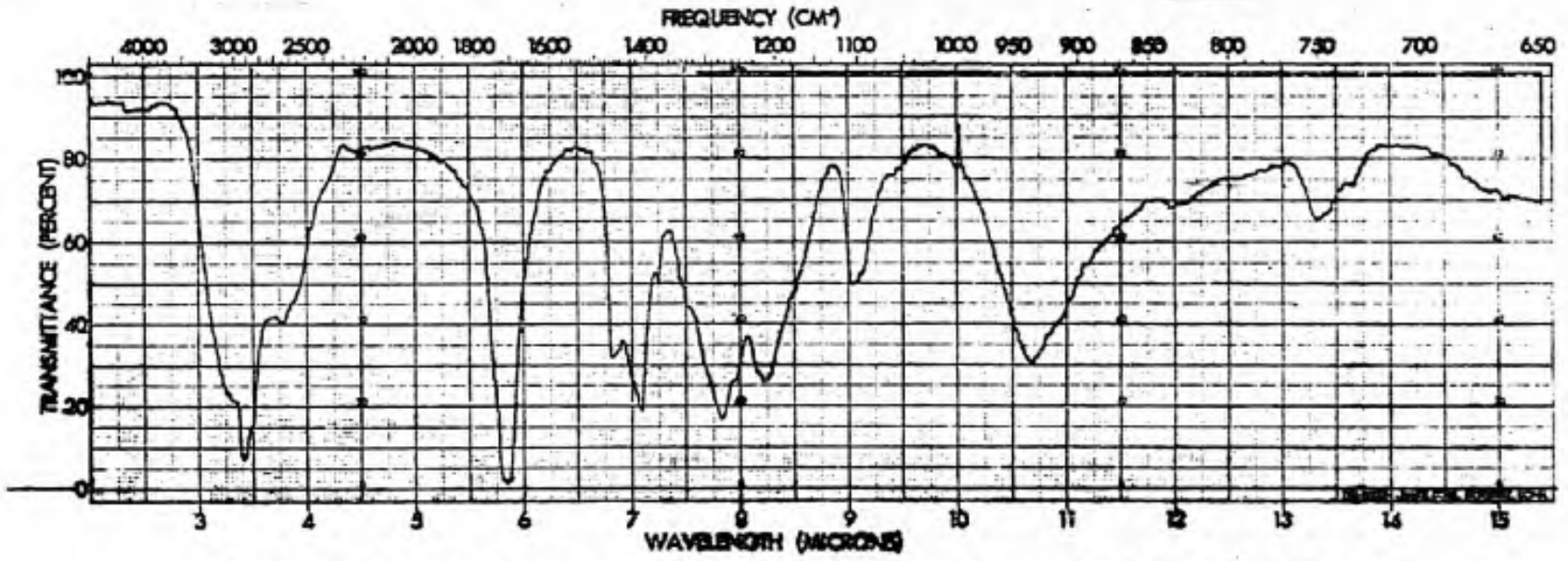


Figure 64. Infrared spectrum of valeric acid, liquid film.

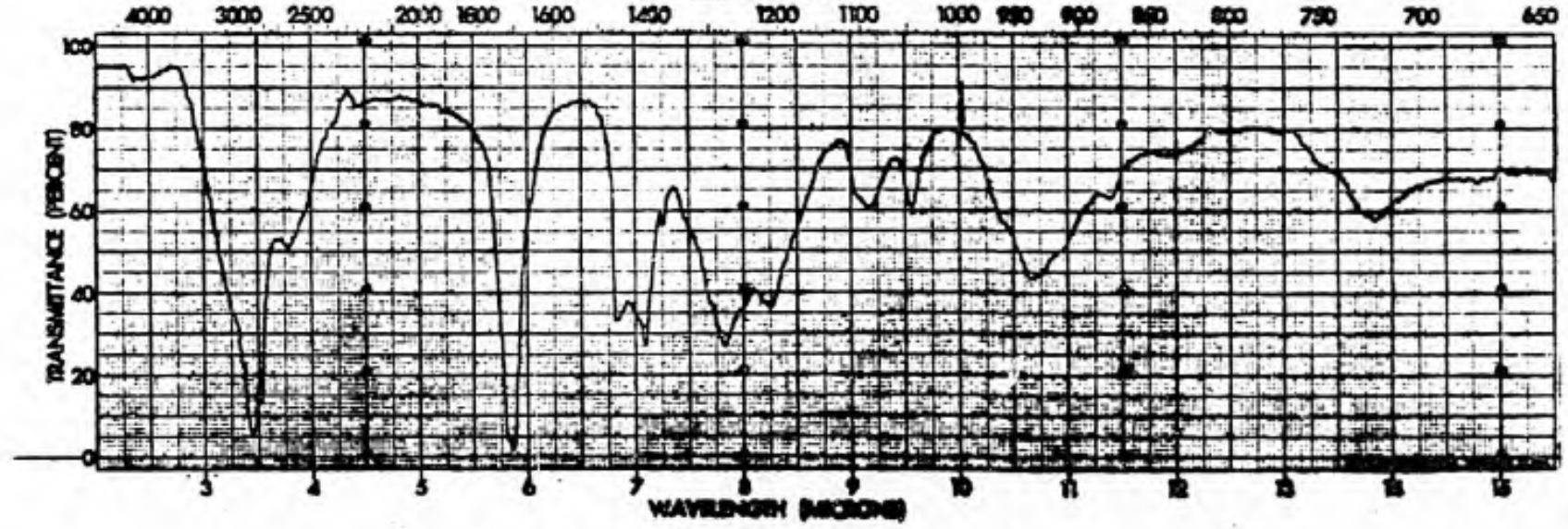


Figure 65. Infrared spectrum of valeric acid and linoleic acid, liquid film.

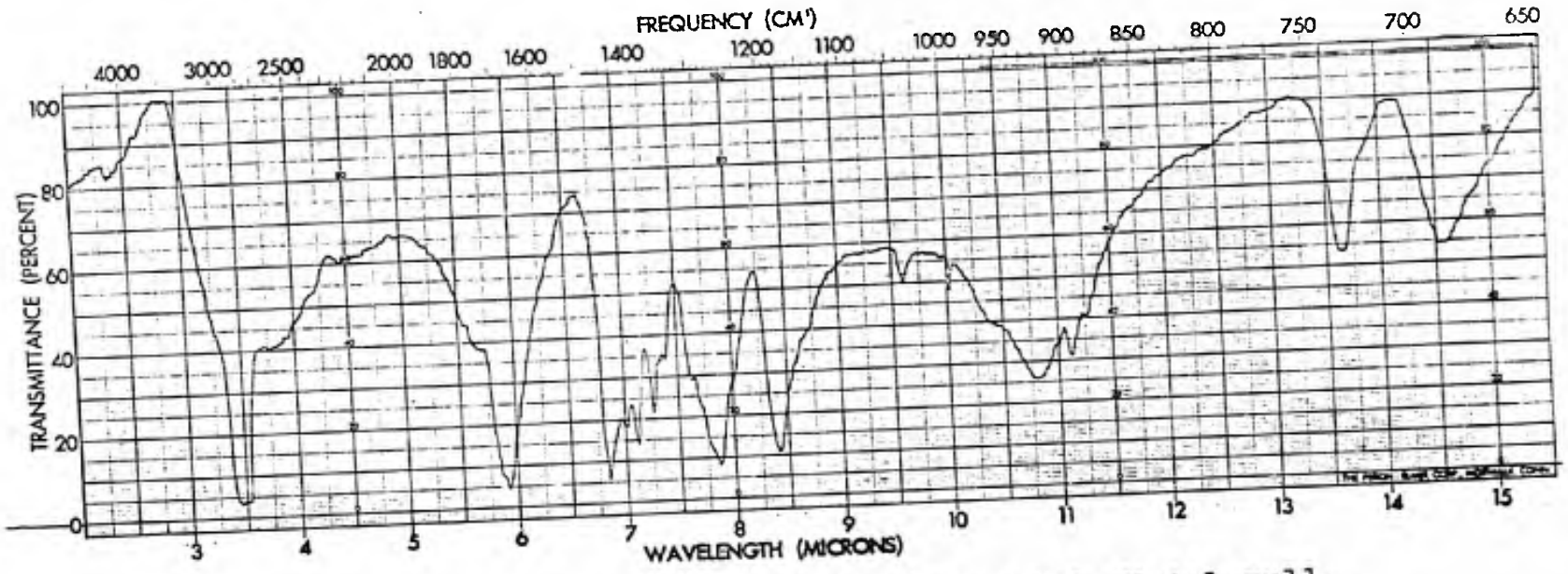


Figure 66. Infrared spectrum of adipic acid, Nujol mull.

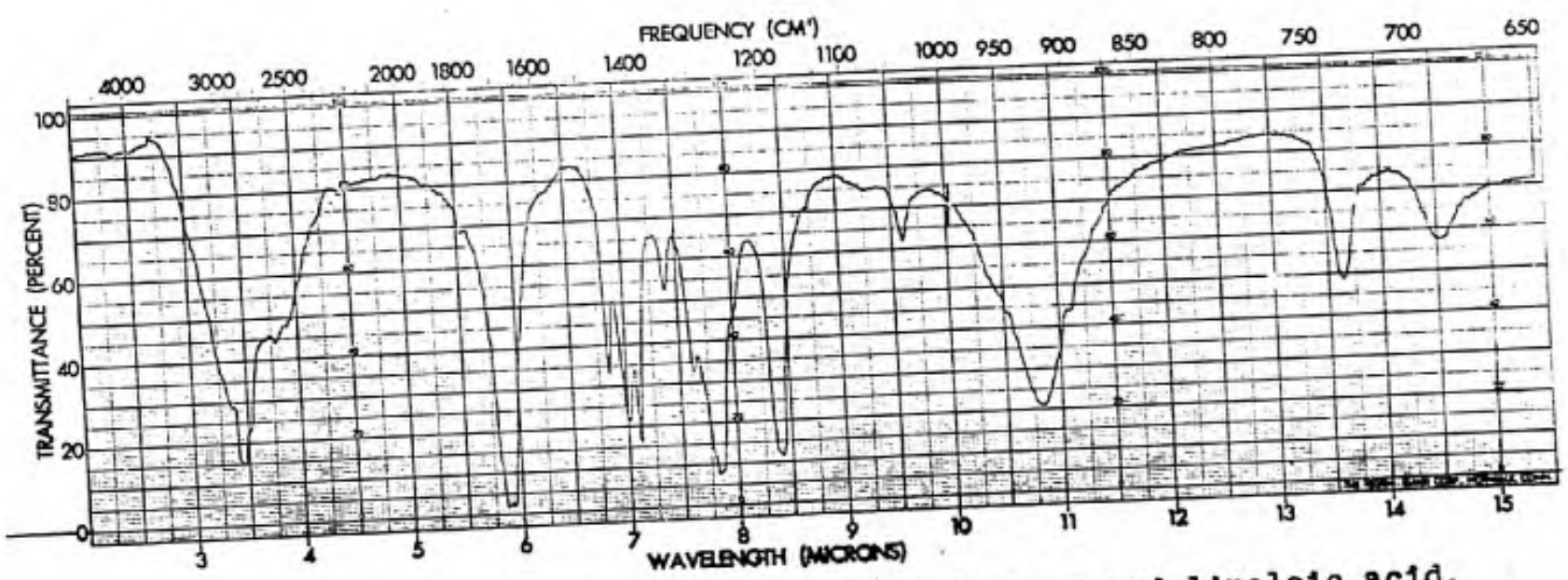
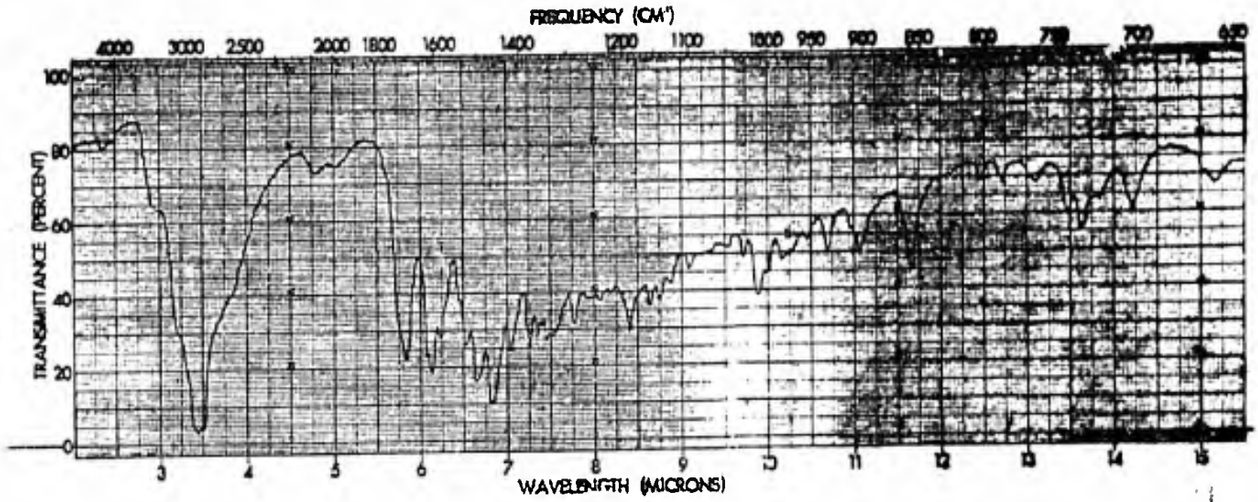


Figure 67. Infrared spectrum of adipic acid and linoleic acid, mull.



68. Infrared Spectrum of L-Lysine Hydrochloride and Linoleic Acid, Nujol Mull.

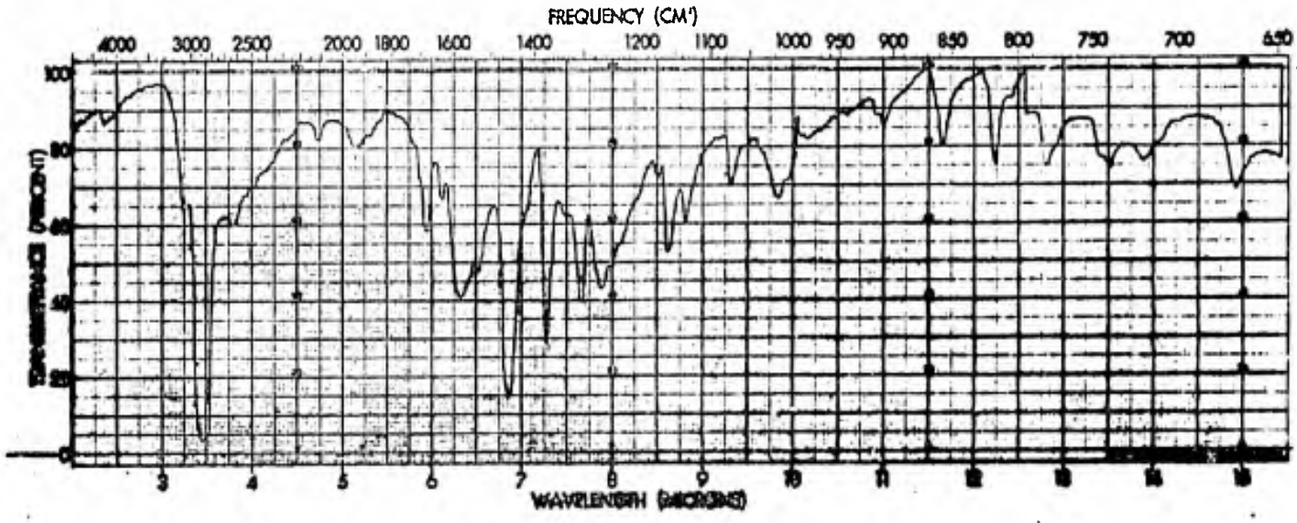


Figure 69. Infrared spectrum of glutamic acid, Nujol mull.

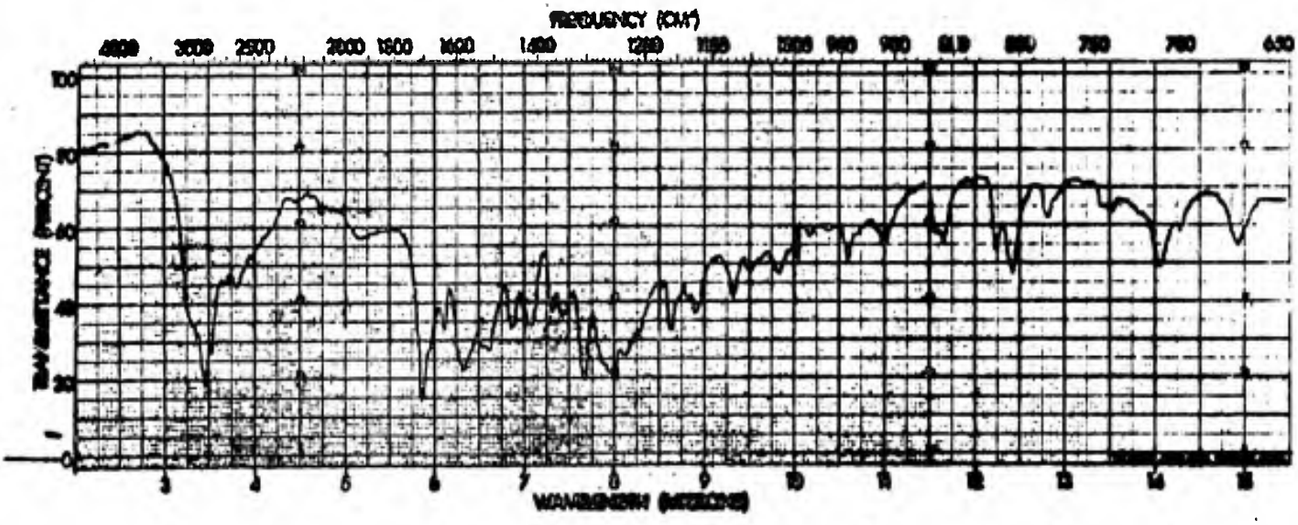


Figure 70. Infrared spectrum of glutamic acid and linoleic acid, mull.

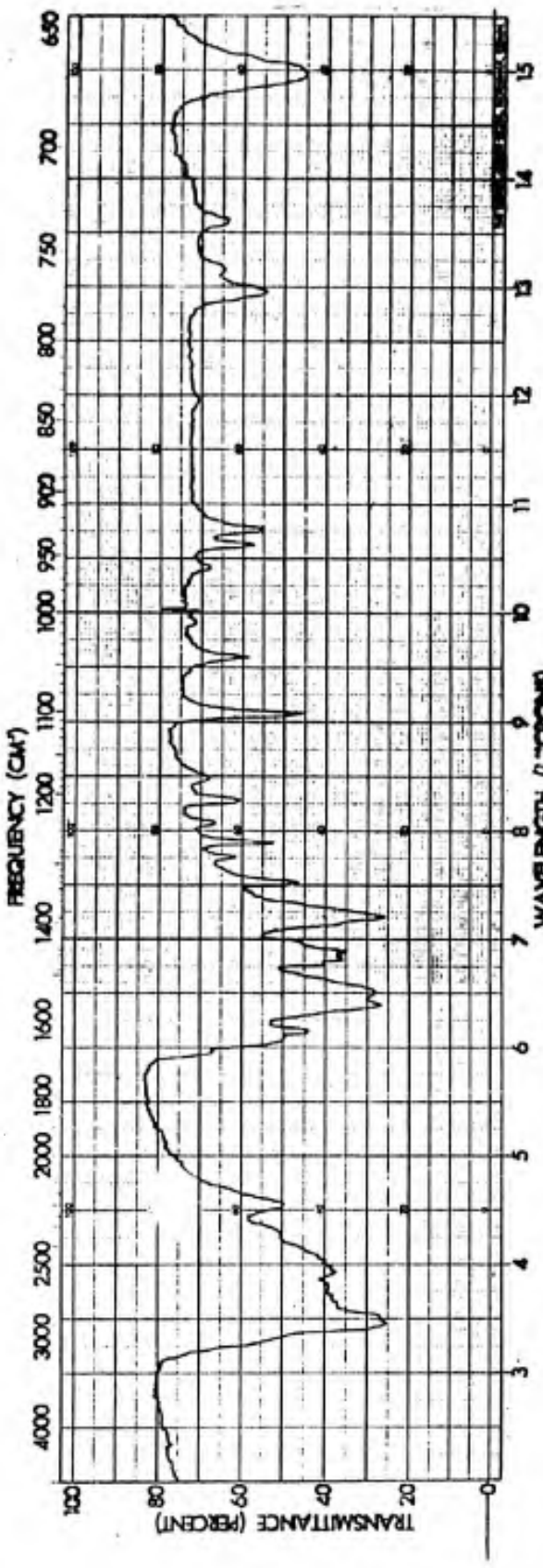


Figure 71. Infrared spectrum of ϵ-aminocaproic acid, Nujol mull.

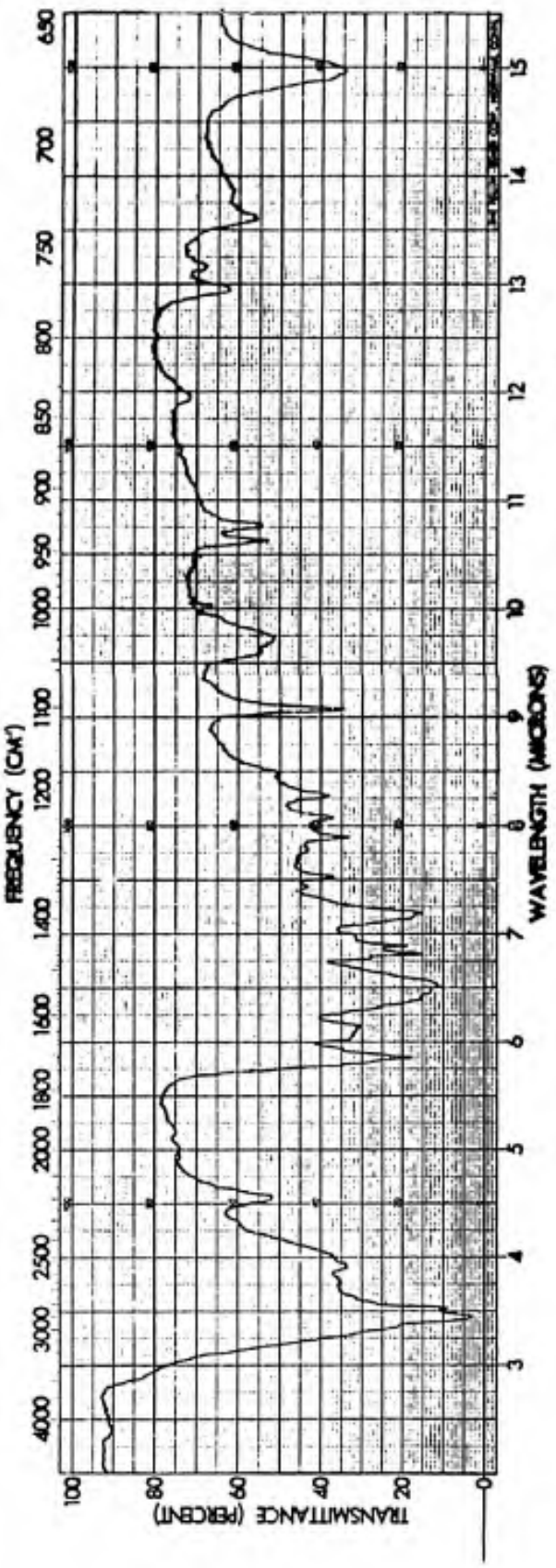


Figure 72. Infrared spectrum of ϵ-aminocaproic acid, liquid film.

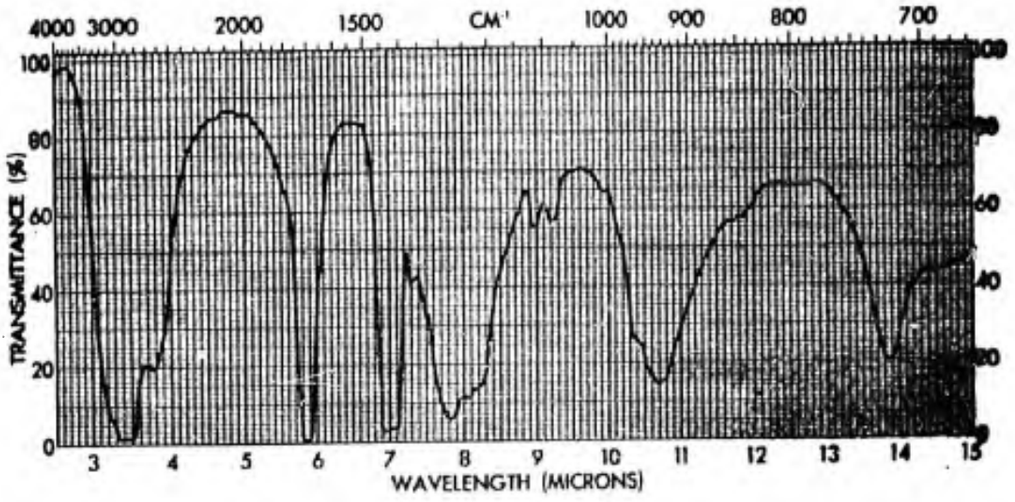


Figure 73. Infrared Spectrum of Oleic Acid, Liquid Film

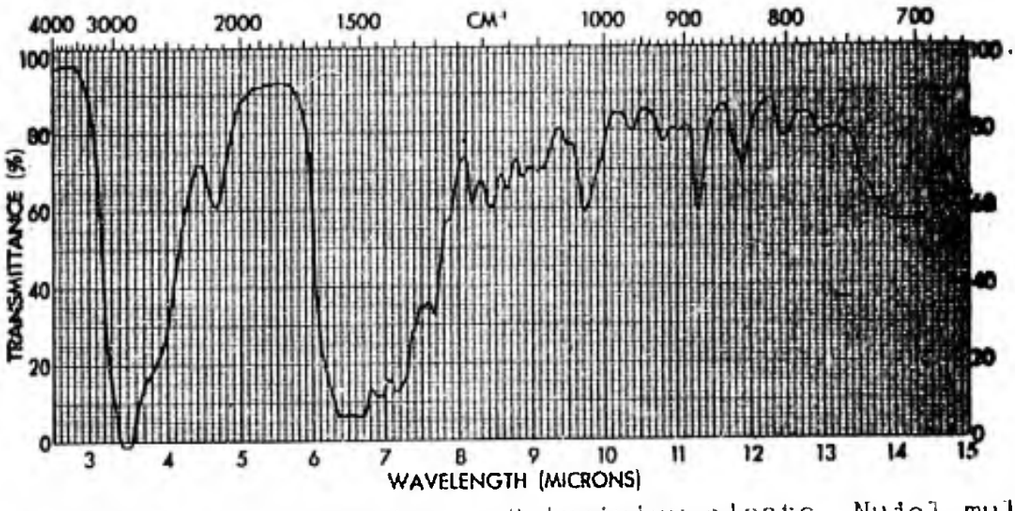


Figure 74. Infrared spectrum of lysinium oleate, Nujol mull.

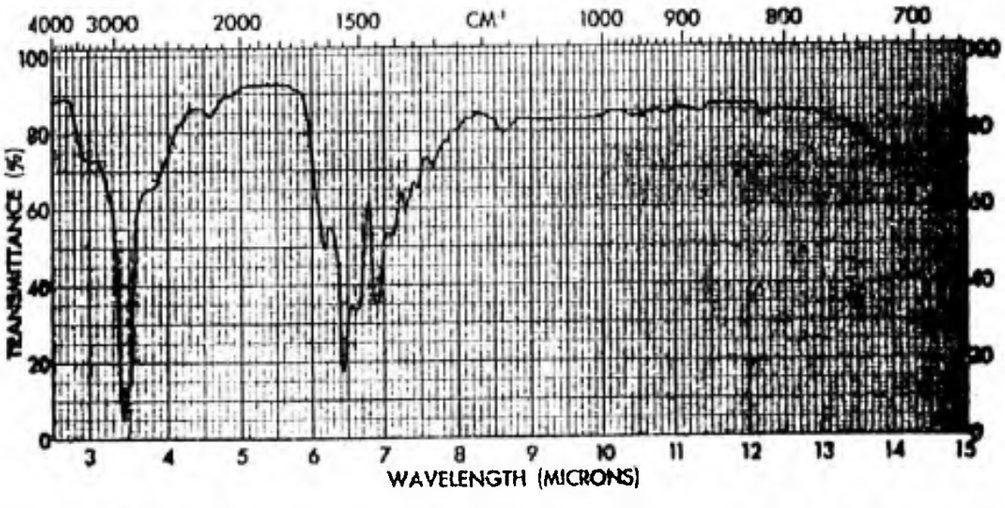


Figure 75. Infrared spectrum of ornithinium oleate, Nujol mull.

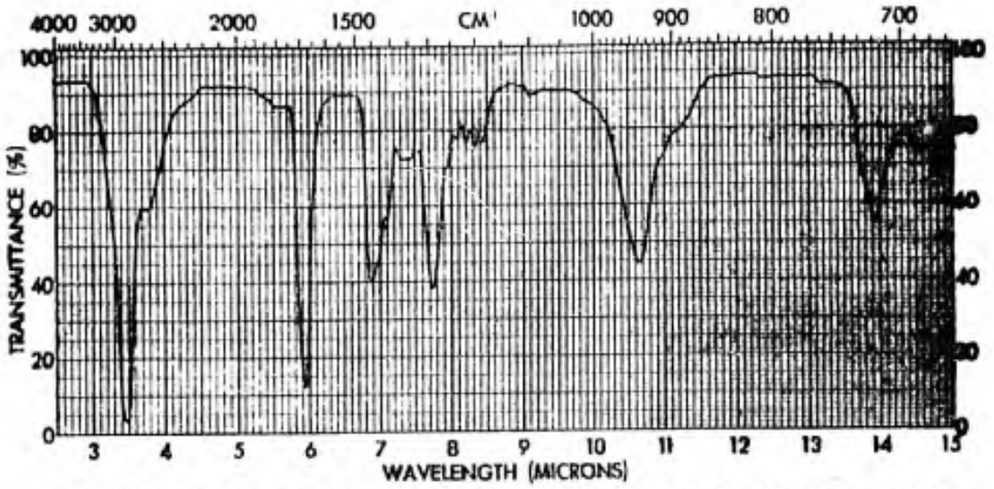


Figure 76. Infrared spectrum of stearic acid, Nujol mull.

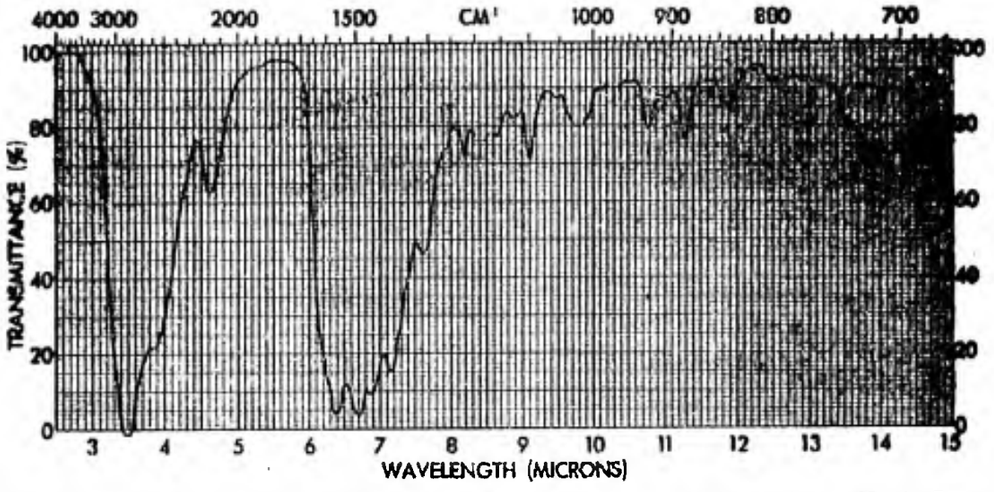


Figure 77. Infrared spectrum of lysinium stearate, Nujol mull.

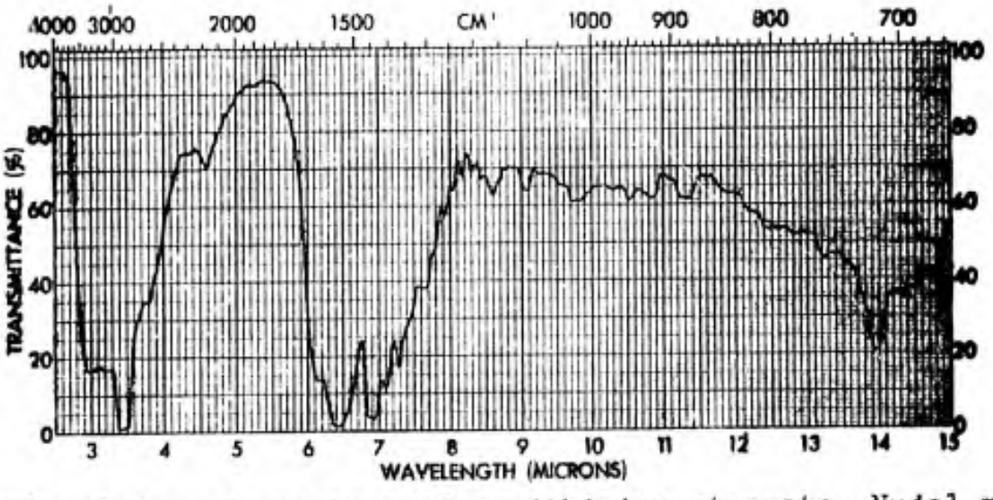


Figure 78. Infrared spectrum of ornithinium stearate, Nujol mull.

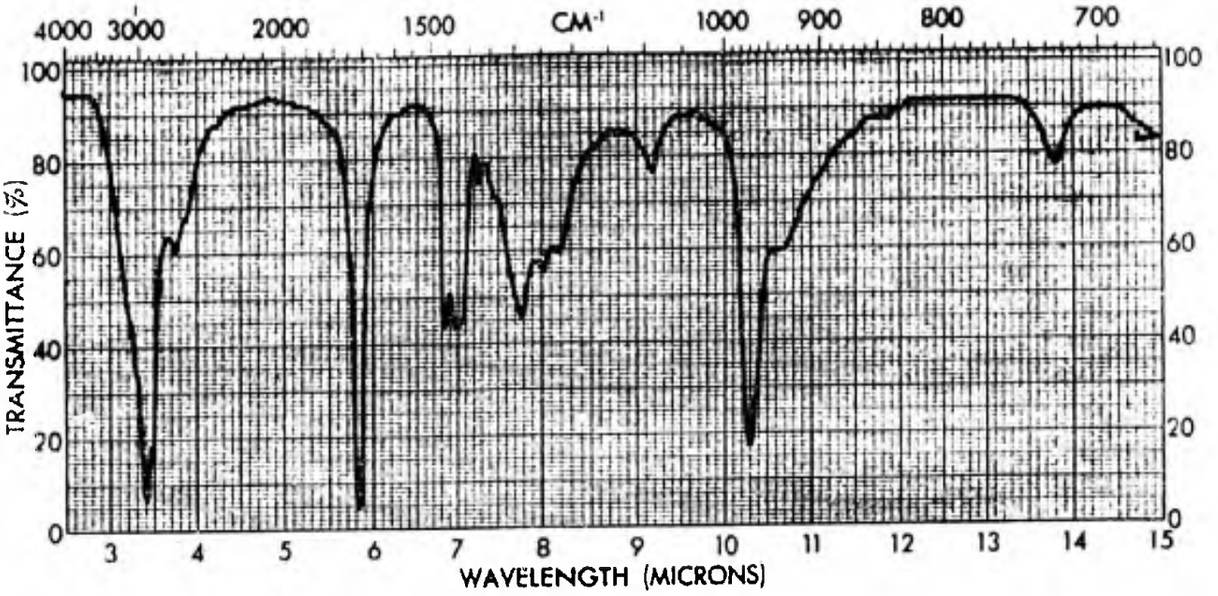


Figure 79. Infrared Spectrum of Linolelaidic Acid, Liquid Film.

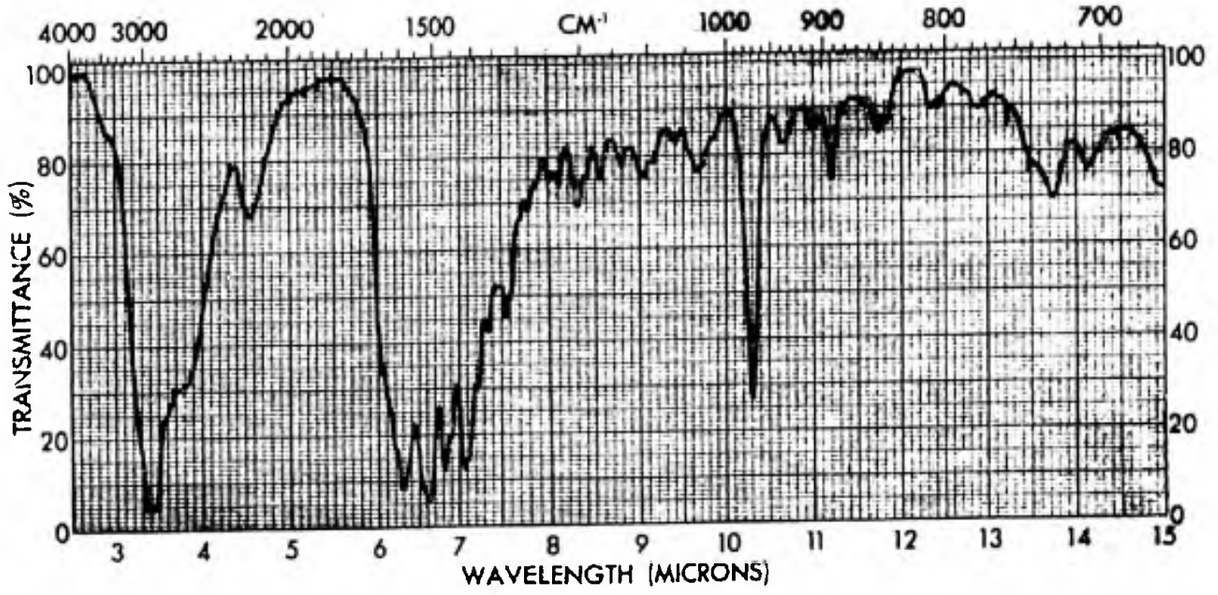


Figure 80. Infrared Spectrum of Lysinium Linolelaidate, Nujol Mull.

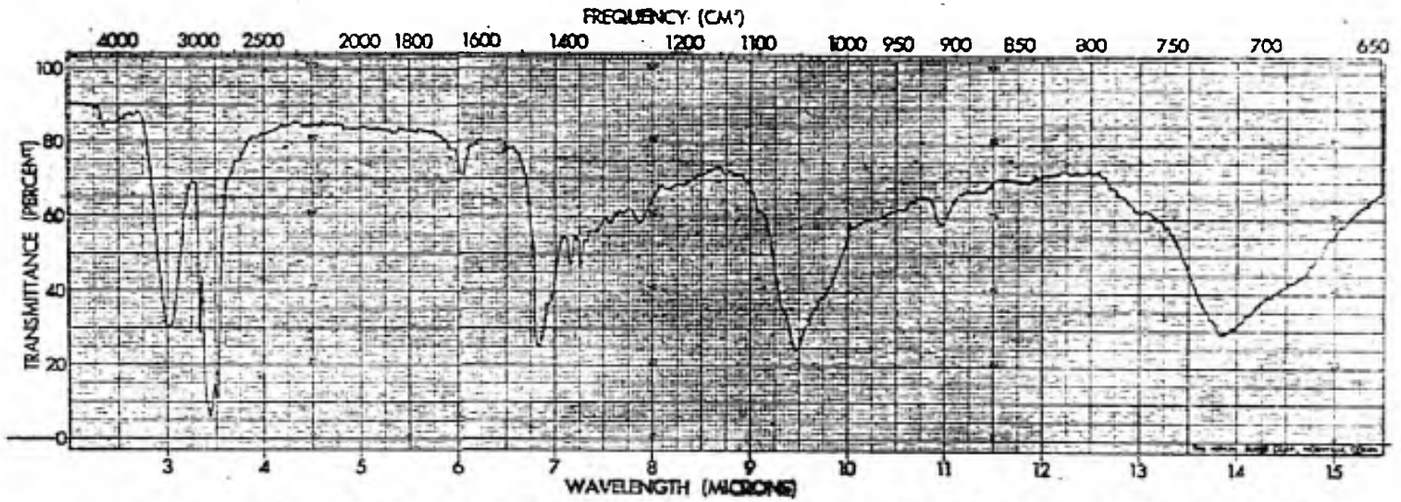


Figure 81. Infrared Spectrum of Linoleyl Alcohol, Liquid Film.

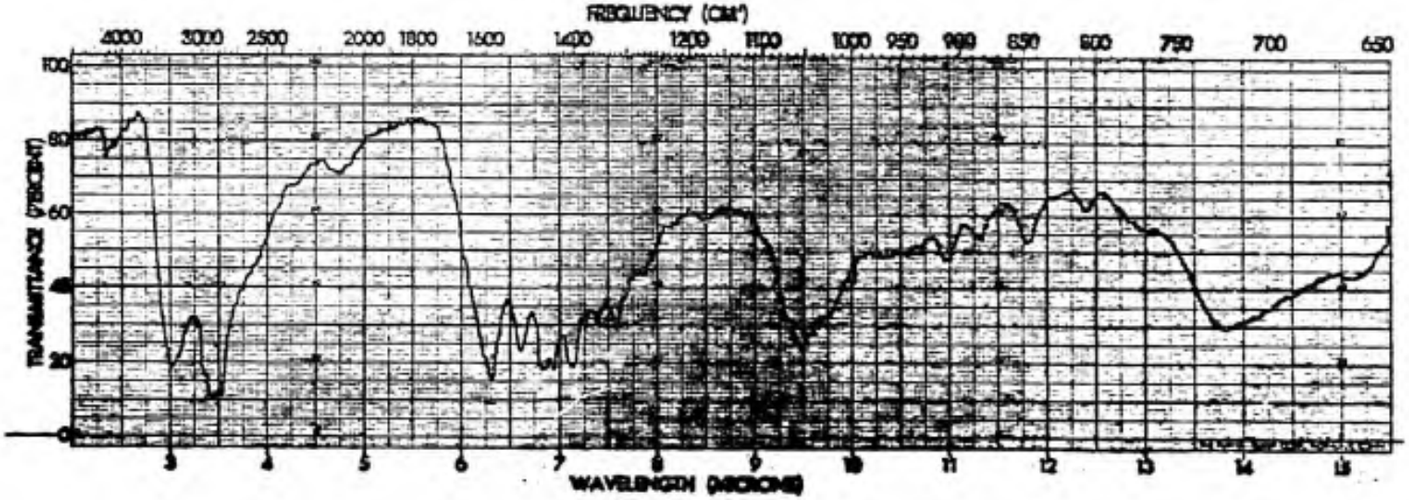


Figure 82. Infrared Spectrum of Linoleyl Alcohol and L-Lysine, Liquid Film.

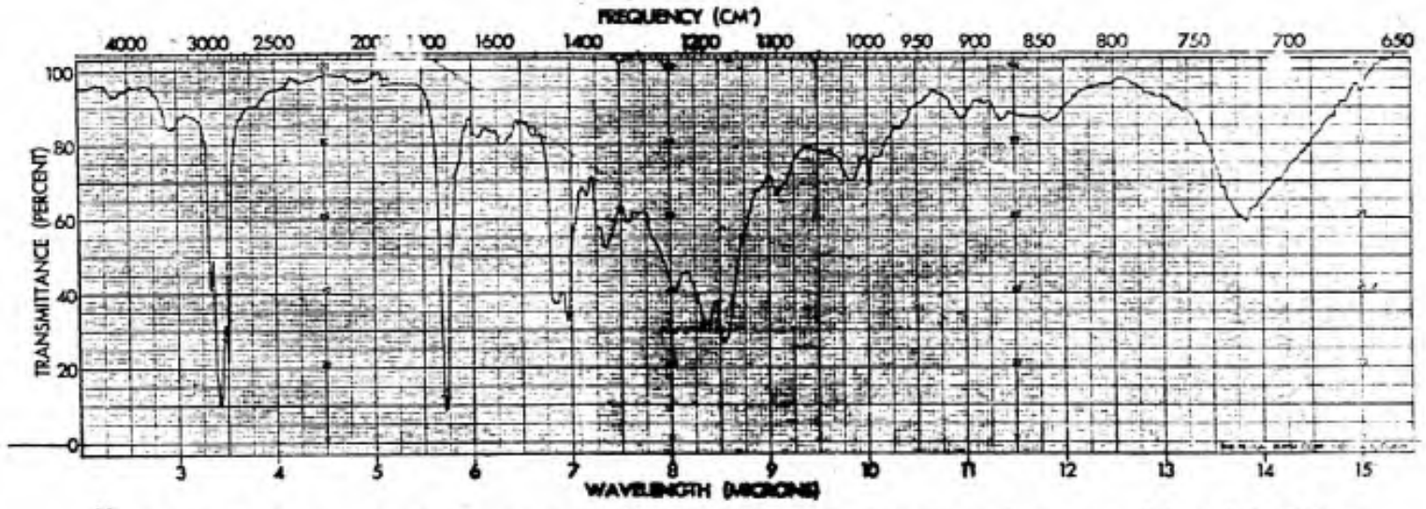


Figure 83. Infrared Spectrum of Methyl Linoleate, Liquid Film.

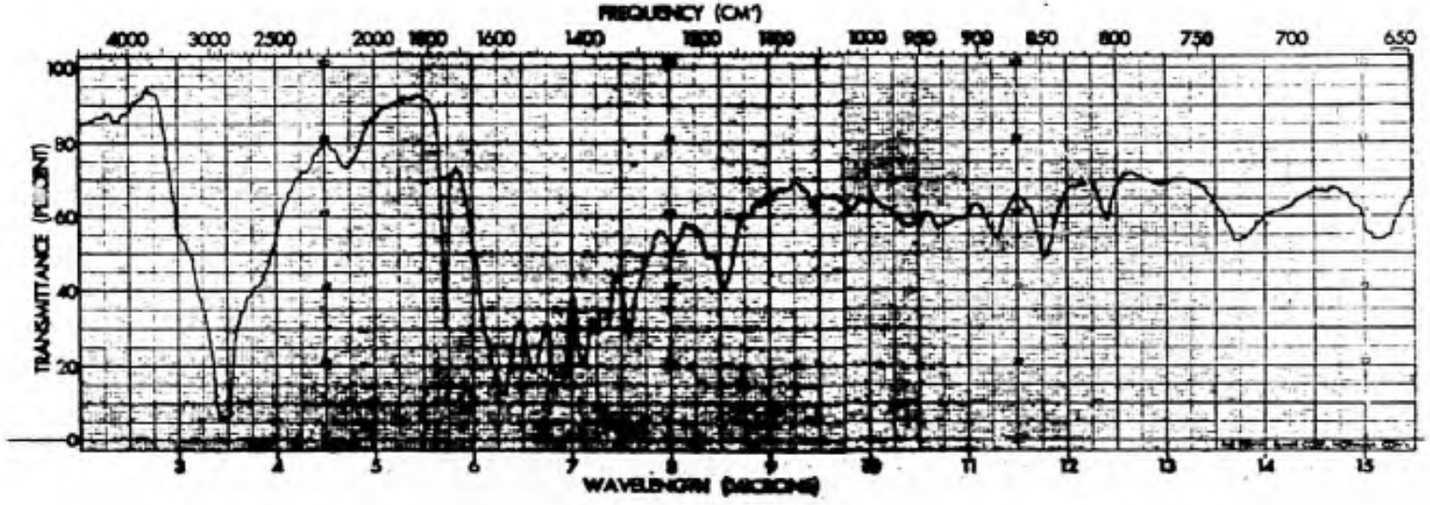


Figure 84. Infrared Spectrum of Methyl Linoleate and L-Lysine, Nujol Mull.

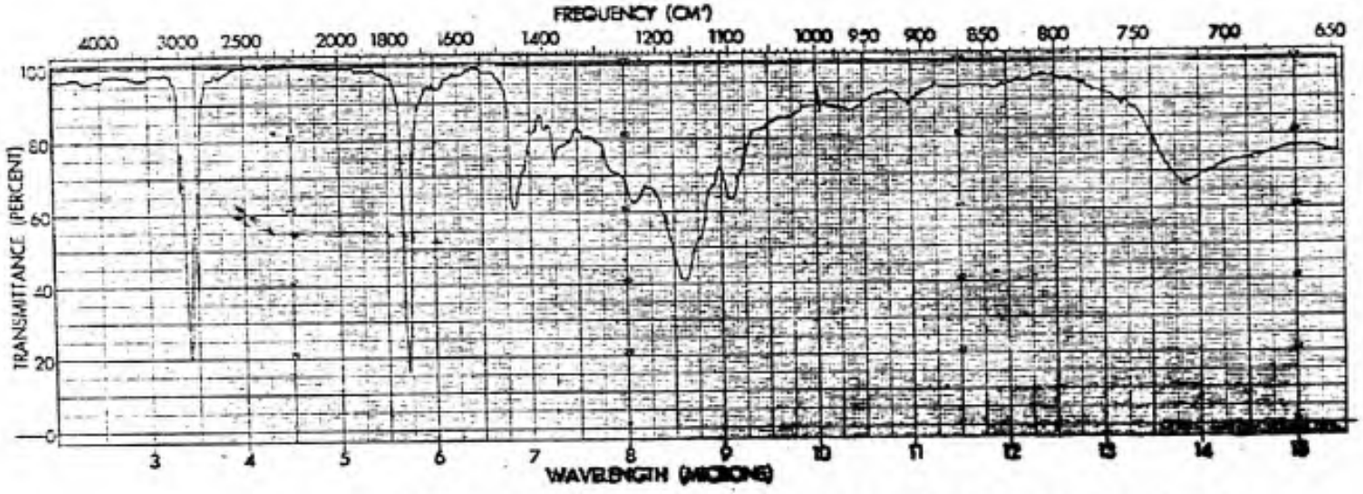


Figure 85. Infrared Spectrum of Trilinolein, Liquid Film.

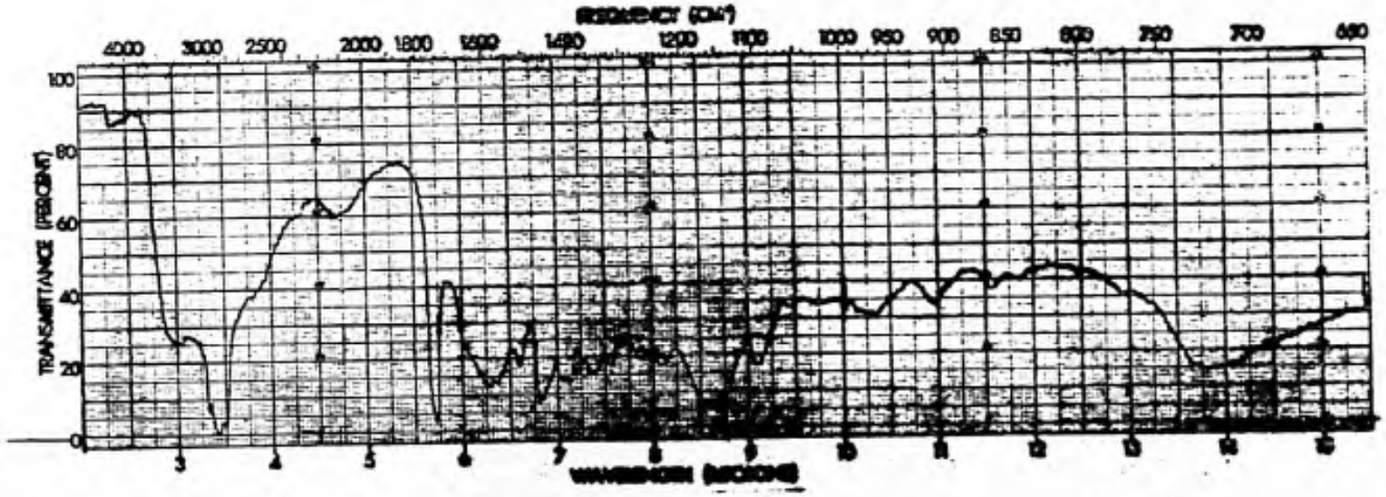


Figure 86. Infrared Spectrum of Trilinolein (1 mole) and L-Lysine (1 mole), Liquid Film.

Note: The infrared spectrum of the Trilinolein (1 mole) with L-Lysine (3 moles) is identical with the 1:1 mixture.

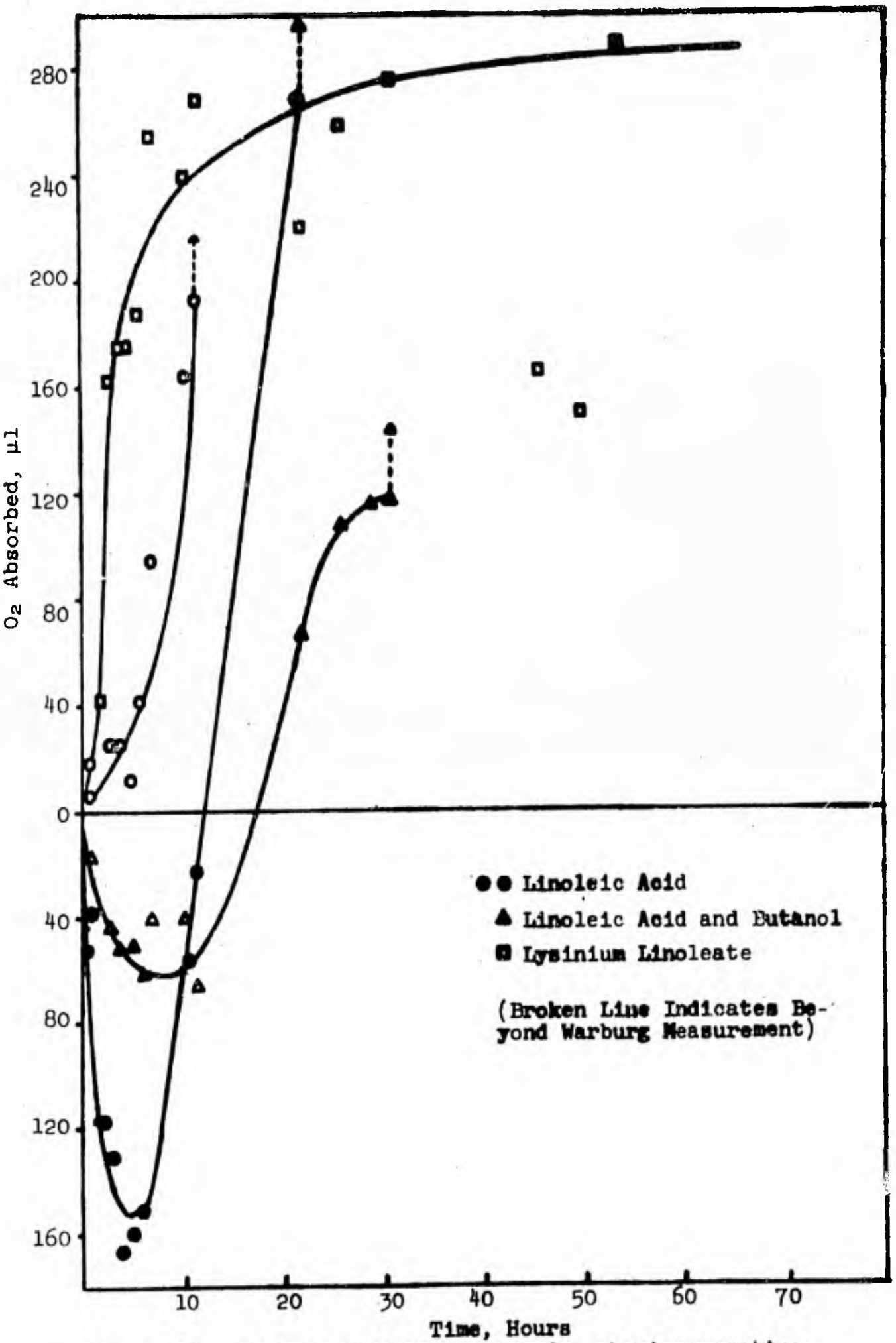
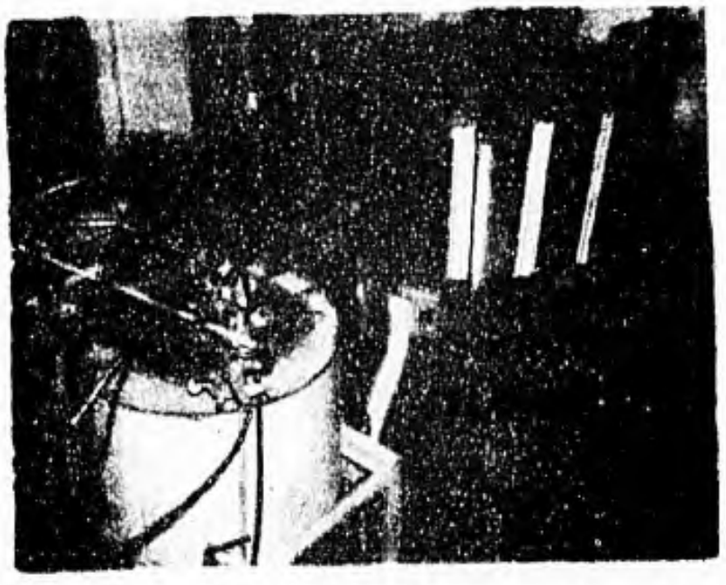


Figure 87. **Warburg Oxidations at 37°C, showing negative uptake of oxygen**



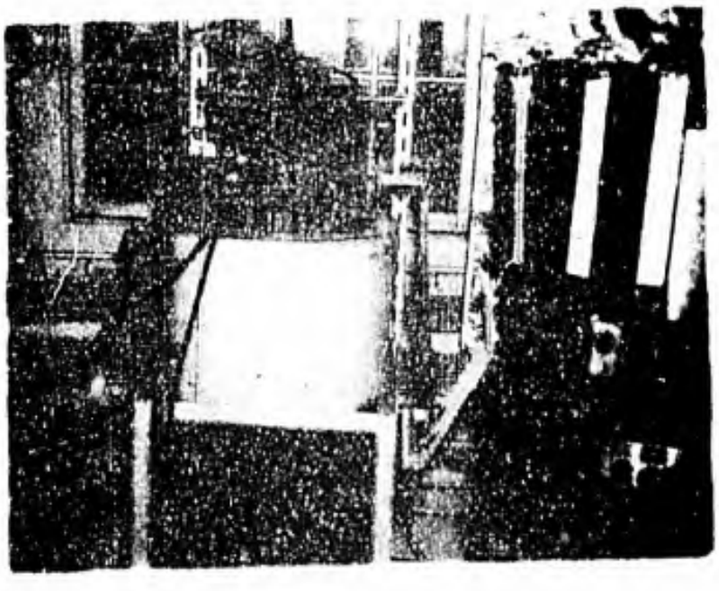


Figure 88. Two Views of the Warburg Manometricon, Showing Arrangement for Readings at -12°C.

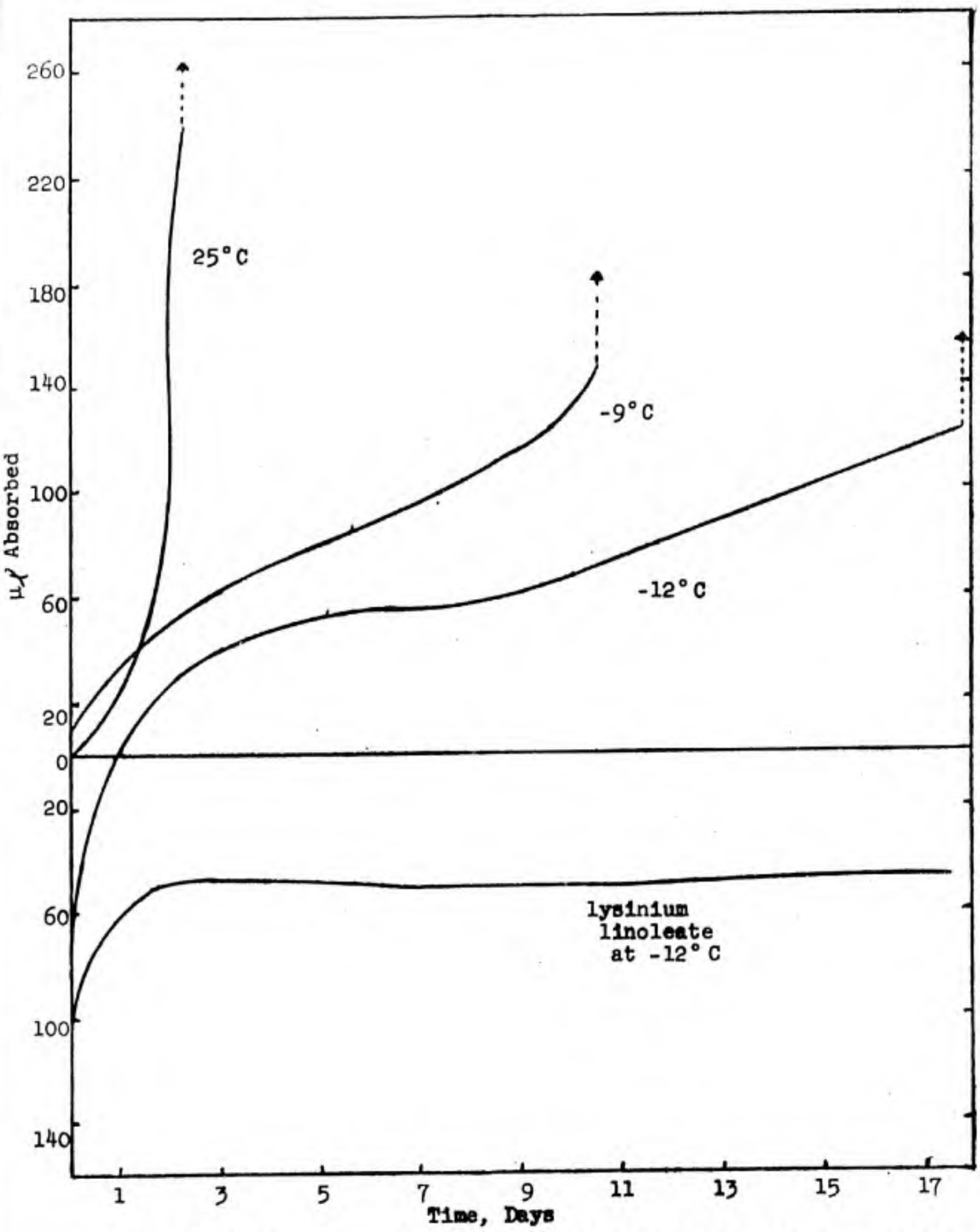
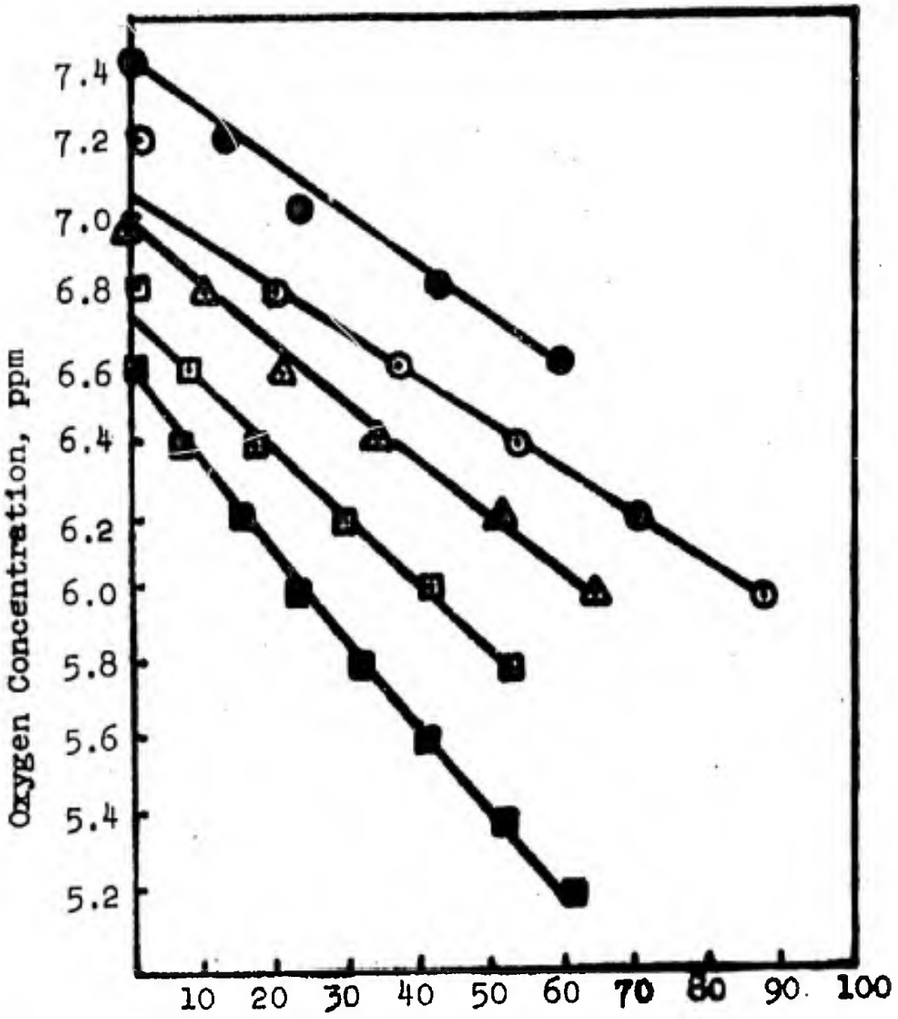


Figure 89. Warburg Oxidations of Linoleic Acid and Lysinium Linoleate near the freezing point of linoleic acid



	Concentration (g/100 ml aq. methanol)	Molarity	Change in Oxygen Concentration (ppm/hr)
■	1.62 g lysinium linoleate	3.8×10^{-2}	1.4
⊡	1.21 g lysinium linoleate	2.85×10^{-2}	1.1
△	0.91 g lysinium linoleate	2.14×10^{-2}	0.9
⊙	0.46 g lysinium linoleate	1.07×10^{-2}	0.7
●	1.06 g linoleic acid	3.8×10^{-2}	0.84

Figure 90. Apparent Oxidation Rates of Linoleic Acid and Lysinium Linoleate Solutions at 27°C, determined with oxygen galvanic cell.

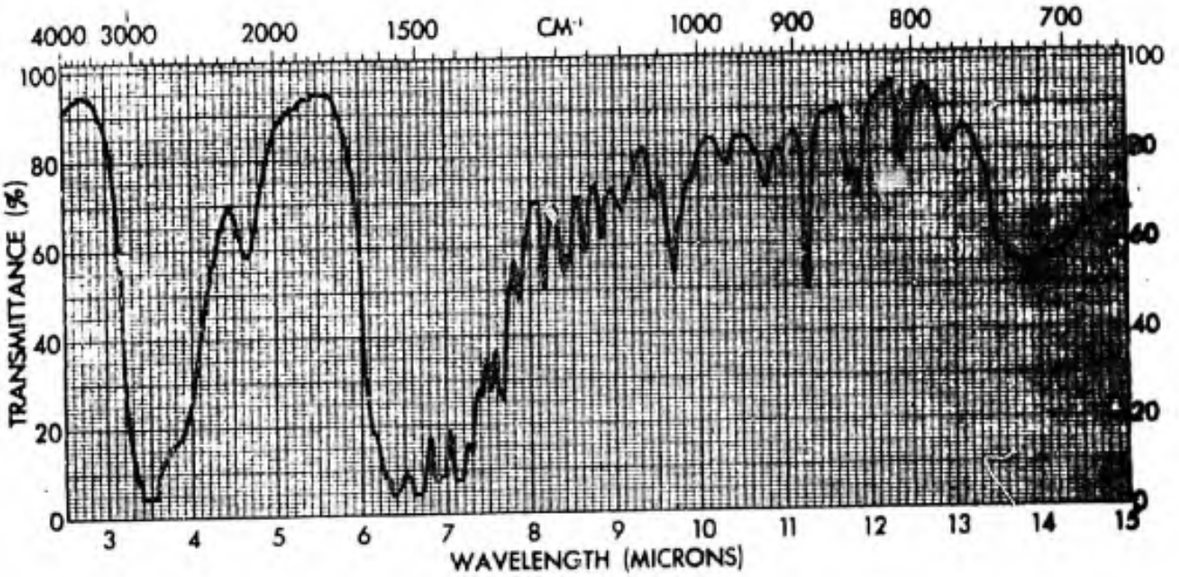


Figure 91. Infrared Spectrum of Lysinium Linoleate and Sodium Chloride (Ground Dry), Nujol Mull.

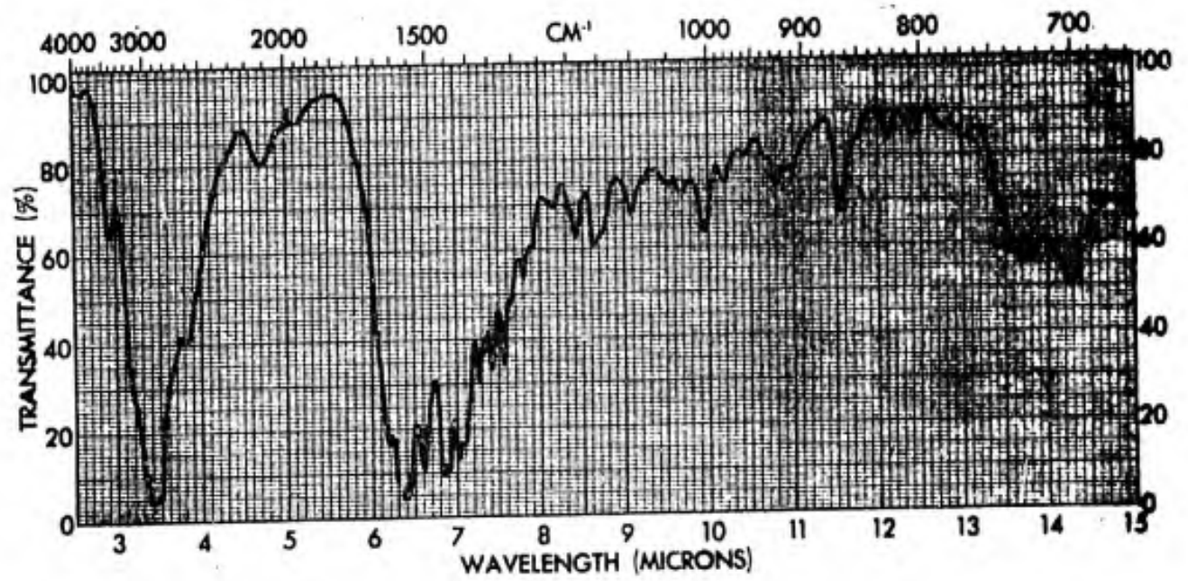


Figure 92. Infrared Spectrum of Lysinium Linoleate and Sodium Chloride (from co-solution), Nujol Mull.

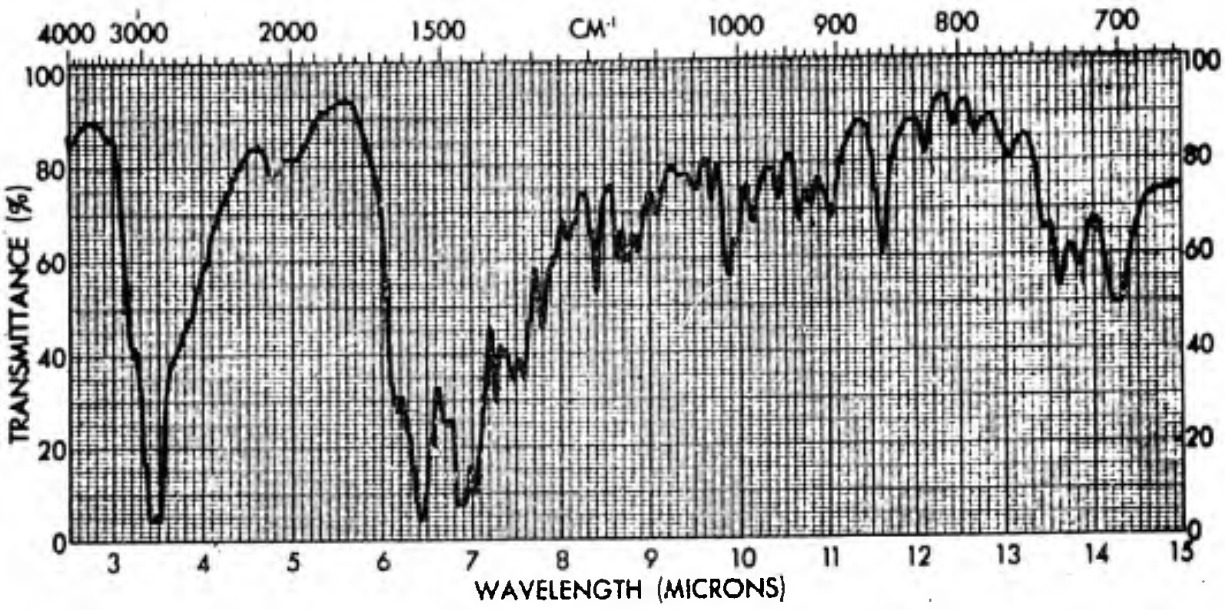


Figure 93. Infrared Spectrum of Sodium Linoleate and Lysine Hydrochloride (Ground Dry), Nujol Mull.

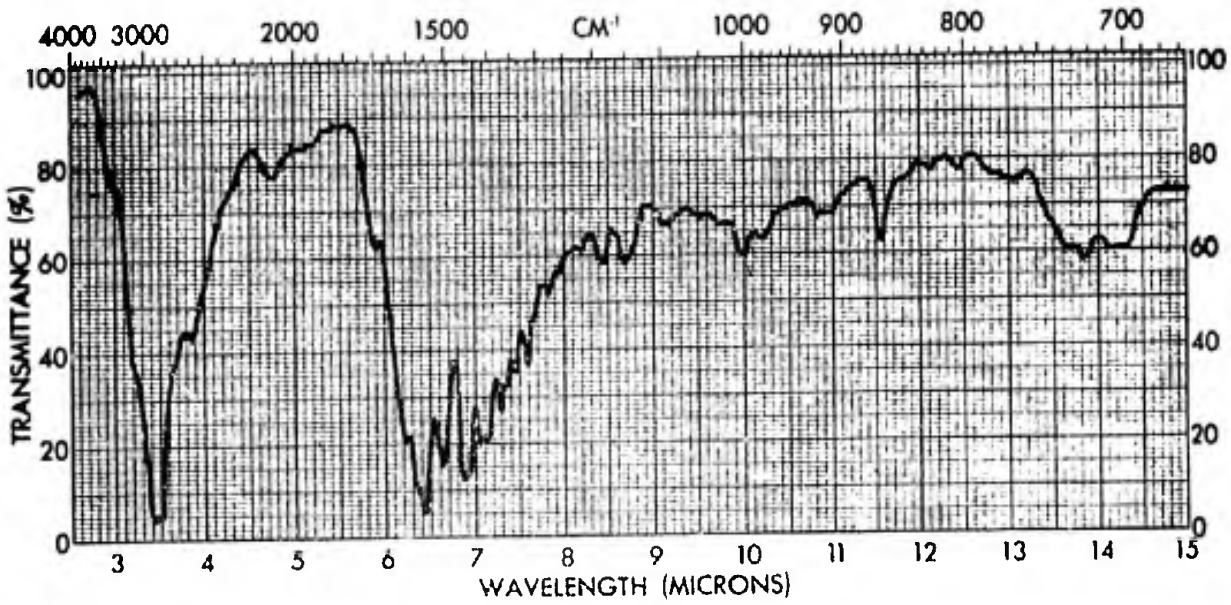


Figure 94. Infrared Spectrum of Sodium Linoleate and Lysine Hydrochloride (from co-solution), Nujol Mull.

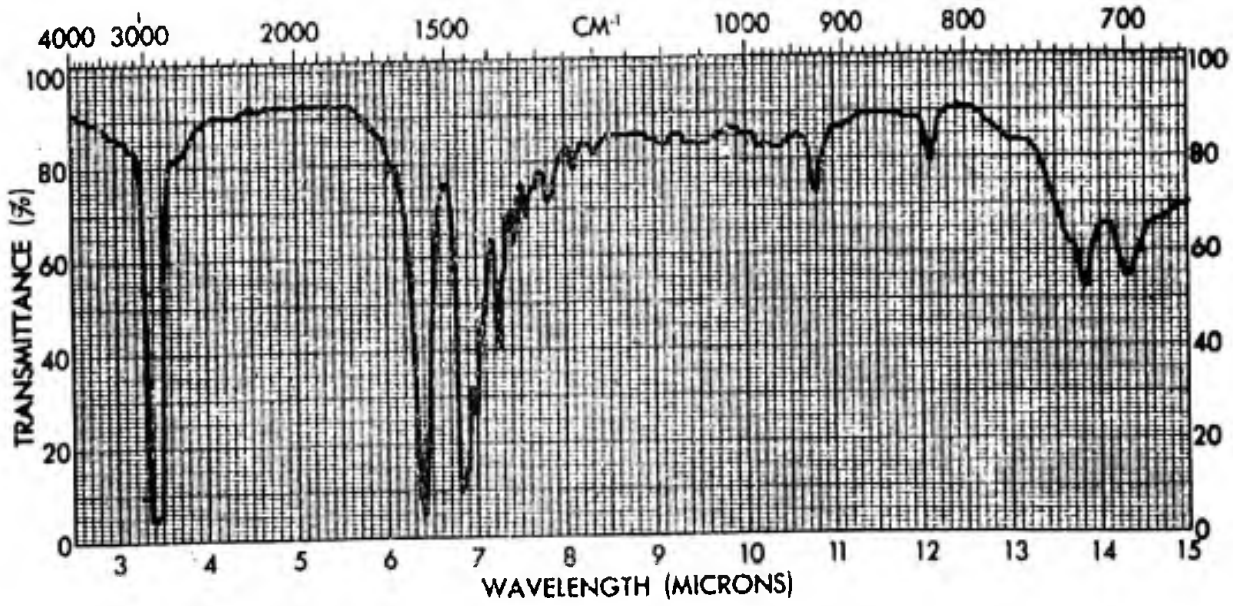


Figure 95. Infrared Spectrum of Sodium Linoleate, Nujol Mull.