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Autoxidation of Fatty Materials in Emulsion. III. Application of GLC and TLC to Studies of the Histidine-Catalyzed Autoxidation of Methyl Oleate[1]

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Abstract

Methyl oleate, emulsified with sodium dodecylsulfate, was extensively oxidized, and the amount of oleate which was reacted was determined by GLC using methyl palmitate as an internal standard. The effects of histidine, iron, and histidine plus iron were compared with the uncatalyzed reaction in the presence and absence of ultraviolet light. Results in the absence of ultraviolet light confirmed previous findings that histidine and histidine plus iron are prooxidants at the concentration studied. In the presence of ultraviolet light the rate of oleate oxidation was about 100× faster than that of the nonirradiated reaction, and the effect of the catalysts was almost negligible. The principal products, determined by GLC and TLC, were epoxide and α,β-unsaturated carbonyl. Hydroperoxy, hydroxy, acid, and aldehyde compounds were also present. Epoxy stearate, determined by GLC, compared favorably with epoxy stearate as determined by use of the Durbetaki method.

Introduction

TWO RECENT REVIEW ARTICLES on metal coordination compounds as catalysts (1) and as biocatalyst models (2) prompted this study. It was thought that histidine and iron might possibly catalyze the formation of specific compounds.

Previous papers from this laboratory (3,4) demonstrated the prooxidative effect of histidine and trace metal ions on linoleate esters in emulsion. Some of the factors which influence this effect were also given. Buffers (especially phosphate) were shown to retard histidine's effect. A low or high pH and nonionic emulsifiers also reduced histidine's prooxidative action. Optimum conditions were pH 6.5 with the use of an ionic emulsifier. The purpose of this investigation is to confirm histidine's prooxidative action on a monounsaturated system and to determine by GLC and TLC whether or not any specific compounds are formed in substantial quantities.

Autoxidation of fatty materials is usually studied by oxygen uptake, peroxide values, iodine number, and diene conjugation, or combinations of these. But these methods fail to indicate the exact amount of substrate remaining or the exact amount of secondary products formed. Even functional group analysis reveals only that specific groups are present. For this reason it was decided to use GLC to determine the amount of oleate oxidation and to use TLC along with GLC for the identification of products. Privett (5,6) and Oette (7) used both GLC and TLC to study lipid autoxidation, but these studies did not include methyl oleate emulsions catalyzed by histidine and iron.

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Experimental Section

Materials. Methyl oleate, methyl palmitate, and reference compounds were laboratory preparations which were analyzed by GLC and TLC for purity. Other reagents (histidine, emulsifier, inorganic salts) have already been described (3).

Oxidation Procedure. An oil-in-water emulsion of methyl oleate-methyl palmitate was prepared in a Virtis 45 homogenizer by using sodium dodecylsulfate as the emulsifier. Aliquots of this emulsion were placed in a 500-ml Erlenmeyer flask, containing distilled water, so that the final volume of the reaction mixture was 25 ml. The concentration in the emulsified mixture was 0.08 M oleate, 0.009 M palmitate, and 0.002 M sodium dodecylsulfate. Flasks were placed on a Burrell wrist-action shaker at room temperature (about 26C), flushed with oxygen, and connected by means of a side-arm and Tygon tubing to a polyethylene bag which was also filled with oxygen. As oxygen was used up, the bag gradully collapsed and the system remained at atmospheric pressure. After shaking was carried on for various reaction times, the emulsion was broken and the oil was recovered by extraction and evaporation as previously described (3). The recovered oil, which was usually >90% of the starting weight, was then subjected to the following analyses.

GLC Analyses. All analyses were performed in an apparatus designed and built at this laboratory using polar and nonpolar columns and a thermal conductivity detector. The polar column was an 8-ft × 3/16-in. O.D. (I.D. = 0.118 in.) stainless steel tube, packed with 42–60 mesh acid and base-washed Chromosorb "W," coated with 25% ethylene glycol succinate polyester. Operating conditions were: injection temperature, 252C; column temperature, 200C (isothermal); detector temperature, 208C; helium flow, 35 ml/min. The nonpolar column was a 2-ft × 3/16-in. O.D. (I.D. = 0.118 in.) stainless steel tube, packed with 60–80 mesh Diatoport which was coated with 15% silicone polymer SE-30 (General Electric). Operating conditions were: injection temperature, 269C, column temperature, 175–265C (programmed 4C/min); detector temperature, 265C; helium flow, 50 ml/min. The internal standard method (8) was employed to follow the oleate disappearance. The peak areas were determined by means of an Infotronics CRS-1 integrator.

TLC Analyses. Procedures were similar to those described by Mangold (9) with the use of Silica Gel G according to Stahl. The solvent system was petroleum ether-diethyl ether (75/25). Lipids were visualized by spraying the plates with 50% sulfuric acid–0.1 M potassium permanganate (1/1) and then heating to char the individual components. Iodine vapor, 2,4-dinitrophenylhydrazine, potassium iodide in (3/2) acetic acid-chloroform, Schiff's reagent, and bromocresol purple (10) were also used to visualize spots.

After visualization the spots were copied by using Ozalid paper similar to procedures already reported (11). This copying technique was particularly useful for making records of plates containing carbonyls or peroxides because the yellow-brown spots formed by the developing reagents, 2,4-dinitrophenylhydrazine and potassium iodide, are good absorbers of light.

Spectrophotometric Analyses. A Beckman Model IR-7 was used to obtain infrared spectra. Measurements were made on thin films held between sodium chloride windows. Ultraviolet spectra were obtained with a Cary Model 14 spectrophotometer using methanol as the solvent.

Other Analyses. Peroxide values were determined by a modification of the Wheeler method (12), iodine numbers by the Wijs technique, and oxirane oxygen by the Durbetaki method (13).

Ultraviolet Lamp. This was a Pen-Ray Model 11SC-1 (Ultra-Violet Products Inc., San Gabriel, Calif.) with a 24/40 stopper of Vycor as an integral part. The stopper enabled the lamp to be inserted directly into the reaction flask, which minimized intensity losses. The primary wavelength was 2537 Å, and the average intensity at 1 in. was 2400 μ W/cm^2.

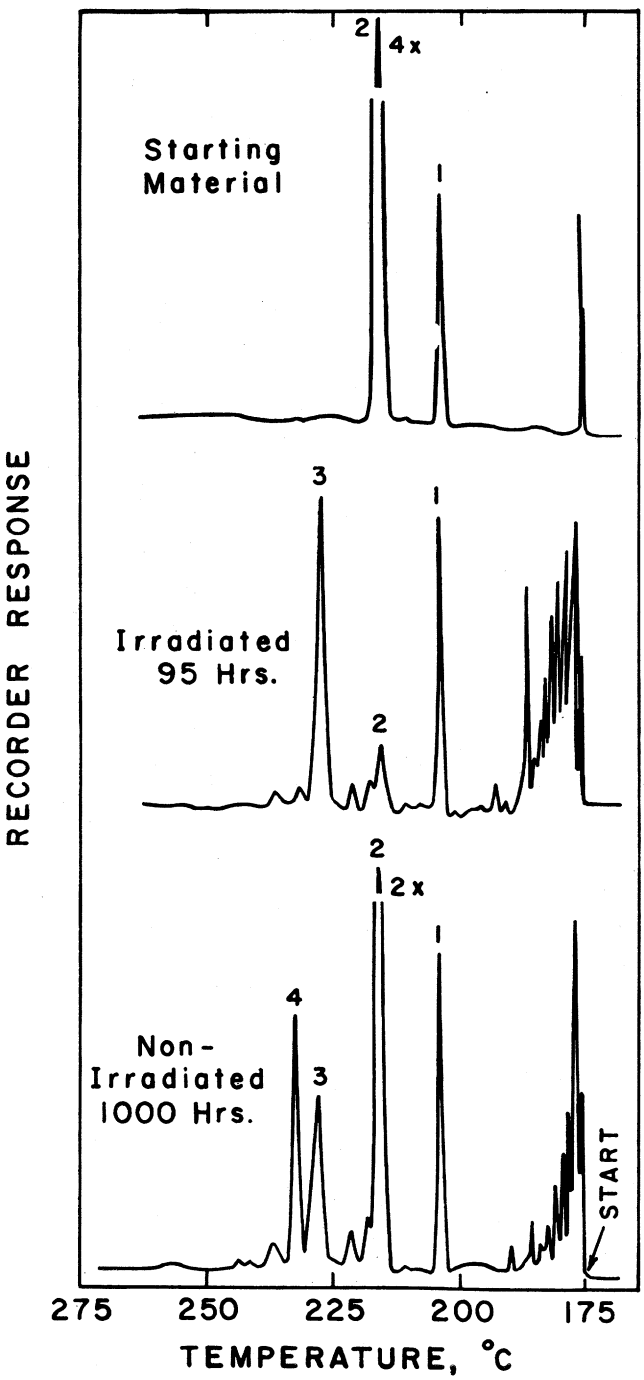

FIG. 1. Programmed GLC on SE30 of recovered oil from emulsion containing histidine plus iron: peaks—(1) methyl palmitate, (2) methyl oleate, (3) and (4) see text.

Results and Discussion

GLC data were obtained by comparing the decreased peak area of methyl oleate with the peak area of the internal standard, methyl palmitate. Although the internal standard was present during the reaction, it was essentially unreacted. This was verified by the addition of C_{15} methyl ester to the reaction mixture as a second internal standard after the oxidation had been carried out.

Monounsaturated hydroperoxides may be converted to dienes during GLC (14). However, under the operating conditions of our GLC, hydroperoxides were not converted to dienes. This was determined by the injection of peroxide concentrates (70% peroxide) from autoxidized methyl oleate, an important step, because the C_{18} methyl esters would all have about the same retention time on the SE-30 column which was used for most of the analyses. Any dienes formed from hydroperoxides would overlap the oleate peak. Some peroxidized samples were analyzed on the polar EGS column which separates monoenes from dienes. No dienes were found. Consequently hydroperoxides were shown not to interfere in determining oleate peak area.

Fig. 1, in addition to showing the relative retention of the reactants and products, shows the extent to which methyl oleate was oxidized. After irradiation with ultraviolet light for 95 hr, less than 5% methyl oleate remains whereas in the nonirradiated experiment the oil phase still contains about 30% unreacted oleate even after 1,000 hrs.

Unirradiated Emulsion. Other papers in this series (3,4) have shown that the histidine-catalyzed autoxidation of methyl linoleate emulsion was a specialized reaction which takes place only under certain limited conditions. The interaction of histidine and iron was emphasized as it occurs at the oil-water interface. Explanations were offered to explain these effects.

Table I shows the effect of histidine and iron on the rate of methyl oleate oxidation. Results confirm previous findings, obtained by oxygen-uptake studies, that histidine and histidine plus iron are prooxidants at the concentration under study. Also the same order of prooxidant effect was found in both studies, i.e., histidine plus iron > histidine > iron > control.

The data in Table I at the 240-hr mark lend further support to the suggestion that histidine and iron act synergistically because the initial rate of oxidation with histidine plus iron is slightly more than the sum of the individual rates of histidine and iron alone.

Table I also shows the amount of peroxide and secondary products formed with increased oxidation. Although the amount of peroxide is greatest in the emulsion which is catalyzed by iron alone after 1,000 hr, the histidine and histidine-plus-iron emulsions are more highly oxidized, as evidenced by the decrease in methyl oleate determined by GLC. Thus, as is well known, reliance on peroxide determinations only

TABLE I

Analysis of Recovered Oil from Autoxidized Emulsion

		Catalyst			
		None[a]	Histidine[b]	FeIII	Histidine + FeIII
Nonirradiated					
% Oxidized	240 hr	2.6	23.0	9.9	38.0
	1000 hr	9.7	48.2	41.5	60.5
% Peroxide	240 hr	1.1	14.3	10.3	13.3
	1000 hr	6.5	29.5	31.3	27.4
% GLC Peak 3	240 hr	Trace	1.9	< 1	4.5
	1000 hr	Trace	6.7	1.9	8.6
% GLC Peak 4	240 hr	Trace	2.2	< 1	5.0
	1000 hr	Trace	6.1	2.5	6.5
I_2 Number	240 hr	77.4 (75.6)[c]	72.9 (70.7)	77.4 (76.9)	67.4 (59.3)
	1000 hr	76.5 (74.0)	68.0 (64.7)	70.2 (68.8)	57.3 (52.8)
UV Irradiated					
% Oxidized	50 hr	57.9	53.0	61.7	57.8
	95 hr	85.1	85.3	85.0	85.5
% Peroxide	50 hr	14.9	16.2	12.6	20.7
	95 hr	0.5	14.8	9.5	19.8
% GLC Peak 3	50 hr	8.3	11.2	9.2	11.2
	95 hr	12.1	18.9 [19.1][d]	15.5 [14.5]	16.5 [19.2]
I_2 Number	50 hr	54.5 (39.5)	57.0 (44.8)	48.5 (34.4)	47.5 (44.3)
	95 hr	45.0 (4.4)	29.0 (15.9)	28.0 (11.9)	25.5 (19.9)

[a] Control (no catalyst) contains 0.6400 g of oil (89.9% Me oleate, 10.1% Me palmitate) plus 0.0144 g of sodium dodecylsulfate in 25 ml of water.
[b] Histidine (0.0388 g) and/or FeIII (4 ppm) were added to the control.
[c] Calculated I_2 number is in parentheses.
[d] Durbetaki determination of epoxide content is in brackets.

as a measure of the extent of oxidation can be misleading.

Iodine number and pH decreased with increased autoxidation. The initial and final pH's of the emulsions were: control (6.2–6.2), histidine (7.2–6.1), FeIII (3.9–3.5), and histidine plus FeIII (7.1–5.0). Iodine numbers were determined on reduced oils recovered from peroxide determinations. The calculated iodine numbers are equal to the sum of the contributions from unreacted oleate plus reduced peroxide plus the compound represented by GLC Peak 4, which will be shown to be unsaturated. Table I shows that these three contributions make up most of the iodine number determined experimentally.

In the emulsion catalyzed by iron alone, the calculated iodine number and the experimentally determined value are almost the same. This is undoubtedly owing to the fact that there are fewer break-down products in this case and that almost all the methyl oleate which was oxidized formed peroxide.

Fig. 2 shows the effect of histidine concentration on the rate of oleate oxidation and peroxide formation. At 10^{-4} M there is little effect. The rate increases with the increasing concentration and reaches a maximum at 10^{-2} M. Then the rate decreases at 10^{-1} M. Similar results were obtained with methyl linoleate emulsions (3).

Lewis and Wills (15) found that dilute solutions of hemoglobin (4×10^{-6} M) caused a rapid oxidation of linoleic acid emulsions but that a 4×10^{-5} M solution inhibited oxidation. They concluded that hematin proteins in high concentration inhibit peroxide formation, but lower concentrations, within a certain critical range, catalyze oxidation and peroxide formation of unsaturated fatty acids. Our experience with histidine shows a similar relationship between catalyst concentration and rate of oxidation, but a complete explanation of histidine's role must await further investigation.

Irradiated Emulsions. Table I shows the stimulating effect of ultraviolet light on methyl oleate oxidation. The initial rate of oxidation is about 100× faster than the nonirradiated control, and the effect of adding histidine and iron is almost negligible although there seems to be some stabilization of peroxide in the presence of histidine and iron.

Experiments in which the initial concentration of methyl oleate was increased to 0.194 M as compared with the usual 0.08 M oleate in the uncatalyzed reaction showed that the rate of oxidation was 1.6×10^{-5} moles oxidized per hour as compared with 1.9×10^{-5} moles at the lower concentration. A plot of methyl oleate concentration vs. time gave initially a straight line. Hence the reaction is apparently zero order, and the limiting factor is probably the intensity of ultraviolet light. Experiments with various ultraviolet light intensities were not investigated.

Iodine number and pH again decreased with increased oxidation. The initial and final pH's of the emulsions were: control (6.4–2.7), histidine (7.2–3.2), FeIII (3.7–2.7), and histidine plus FeIII (6.6–3.4). The calculated iodine numbers, based on unreacted oleate and reduced peroxide, were lower than the experimental values and indicated large amounts of unsaturation in the break-down products.

Maximum conversion of methyl oleate to epoxy stearate was obtained with ultraviolet light. A comparison of a range of epoxide values determined by the Durbetaki method (13) with GLC Peak 3 area gave approximately the same amount. Table I shows three of these comparisons.

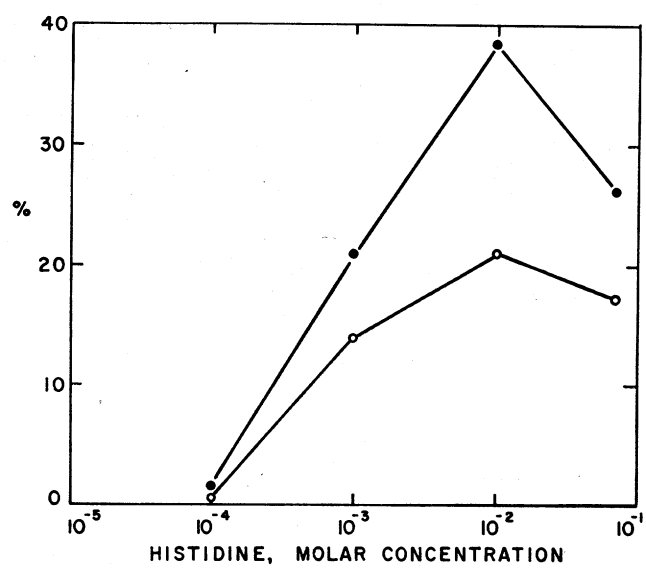

FIG. 2. Effect of histidine concentration on the 500-hr nonirradiated oxidation of methyl oleate emulsion: ● percentage of methyl oleate oxidized, ○ percentage of peroxide formed.

Isolation and Identification. Hydroperoxides, epoxides, α,β-unsaturated carbonyls, hydroxides, aldehydes, and other compounds have been found in autoxidation mixtures of fatty acids. Two recent publications (16,17) gave a comprehensive list of these products. Most products isolated from autoxidation mixtures were by the tedious methods of crystallization, solvent extraction, and column chromatography. Privett (6) however pointed out the usefulness of TLC techniques in studying autoxidation mixtures. Some of these techniques have been adopted and modified for use in the present study.

The identification of every compound formed in the autoxidation mixture was beyond the scope of this work. In early experiments two major peaks, 3 and 4 (Fig. 1), appeared upon the GLC. Hence the identification of these was the prime objective because they potentially represented the largest yields of individual compounds.

Reference compounds of hydroxy, keto, and epoxy stearate methyl esters had similar GLC retention times with Peak 3 (Fig. 1). Determinations of epoxide content of the oxidized oil by the Durbetaki method approximated values calculated from GLC data when it was assumed that Peak 3 corresponded to methyl epoxy stearate. Both the oxidized oil and a reference sample of methyl epoxy stearate were treated with HCl in dioxane. Reference and sample chlorohydrins so prepared gave identical retention on GLC. Further confirmation of the identity of epoxy stearate was obtained by TLC.

Fig. 3 shows that both the irradiated and the unirradiated oxidized oils have a substance with the same R_f value as *trans* 9,10-epoxystearate and 9-ketostearate. These two classes of compounds were distinguished by spraying a similar plate with 2,4-dinitrophenylhydrazine, thereby showing that the suspected epoxide was not a carbonyl. The *cis* epoxy stearate could not be used for identification because it was found to have a slightly lower R_f value than the *trans*. This observation had already been reported (18). A preparative TLC plate was spotted with 20 mg of unirradiated oil and divided into five zones. Zone 2 (R_f 0.55–0.65, Fig. 3) was scraped off and eluted with diethyl ether. GLC of the recovered material showed it to be 85% pure, based on area percentage, with a retention time identical with Peak 3 (Fig. 1). An infrared curve made on this recovered material was found to match a curve made on epoxidized methyl elaidate especially in the region of 900–875 cm^{-1}, thus confirming the compound as *trans* methyl epoxy stearate.

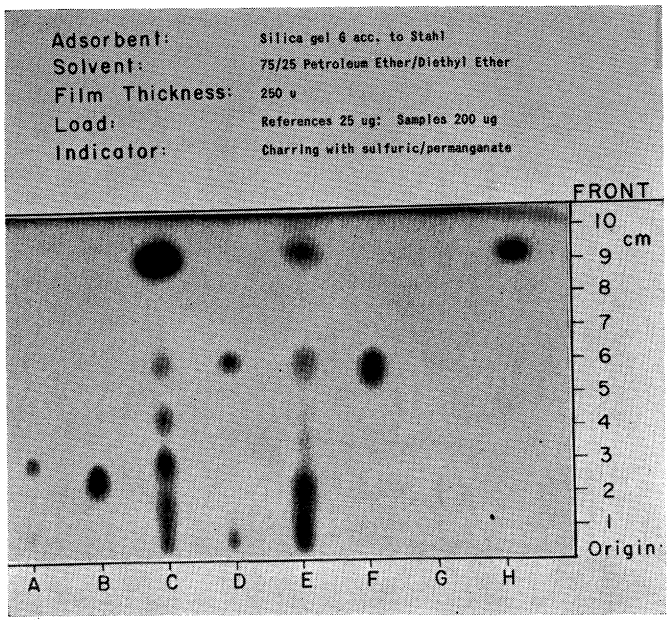

FIG. 3. TLC of recovered oil for irradiated and nonirradiated emulsions. A, methyl azelaldehydate; B, methyl ricinoleate; C, nonirradiated emulsion; D, *trans*-9,10-epoxystearate; E, irradiated emulsion; F, methyl 9-ketostearate; G, pelargonaldehyde; H, methyl oleate-methyl palmitate.

Similar procedures were used to identify the compound represented by Peak 4 (Fig. 1). Fig. 3, Position C, shows a spot following the epoxide. Spraying a similar plate with 2,4-dinitrophenylhydrazine showed the spot to contain a carbonyl, but spraying another similar plate with Schiff's reagent gave negative results. Consequently the spot was presumed to be a ketone. From the preparative plate Zone 3 (R_f 0.4–0.5, Fig. 3) was eluted with diethyl ether. GLC of the recovered material showed it to be 87% pure, based on area percentage. The material had a retention time identical with Peak 4 of Fig. 1. An infrared curve of the material showed strong absorptions at 1675 and 1630 cm^{-1}. These are in the regions given by Bellamy (19) for α,β-unsaturated carbonyl. For further confirmation its ultraviolet absorption spectrum was determined. This spectrum showed a single strong absorption at 224 mμ, which is characteristic of α,β-unsaturated carbonyls (20), and it was concluded that Peak 4 represents mostly a C_{18} α,β-unsaturated carbonyl.

Fig. 1 and 3 show another difference between oxidation in the presence and absence of ultraviolet light. In the presence of ultraviolet light α,β-unsaturated carbonyl is absent as a reaction product. The probable reason for this is that the unsaturated carbonyl absorbs UV light and is decomposed in irradiated emulsions.

Zones 1, 4, and 5 were also scraped from the preparative plate and eluted with diethyl ether. GLC of Zone 1 (R_f 0.85–1.0, Fig. 3) showed only unreacted methyl oleate and the internal standard methyl palmitate. GLC of Zone 4 (R_f 0.25–.35, Fig. 3) showed several peaks with retentions at 221C, 225C, 234C, and 244C in addition to breakdown products which came off the column almost immediately after injection. An infrared curve of Zone 4 showed absorptions in the hydroxyl, unsaturated, and *trans* double-bond regions. Also positive results by using indicator sprays were obtained for carbonyls and peroxides in Zone 4. GLC of Zone 5 (R_f 0.10–0.20, Fig. 3) showed several small peaks with retentions at 221C, 225C, 233C, and 244C. An infrared curve showed absorptions in the hydroxy and *trans* double-bond regions. Positive results were again obtained by using indicator sprays for carbonyls and peroxides.

Acids were detected in the autoxidation mixture by spraying a TLC plate with bromocresol purple. Yellow spots on a blue background were obtained at the origin only.

Fig. 3 shows the disadvantage of using only charring with sulfuric permanganate to visualize TLC spots. Pelargonaldehyde was detected with iodine vapor and 2,4-dinitrophenylhydrazine but was not detected by charring as can be seen in the figure. Although Fig. 3 represents only gross separations, it does show how TLC can be used to isolate and, by the use of indicator sprays, to identify the type of products present in autoxidation mixtures. By comparing these types with reference compounds, identi-

fication of specific compounds can be made. Also various spots can be scraped off the plate and identified by infrared spectroscopy.

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J. S. Ard and C. T. Leander prepared the infrared spectra.

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