Characterization of Mannide Monooleate

H. J. O'NEILL*▲, T. N. YAMAUCHI*, P. COHEN†, and M. C. HARDEGREE†

Abstract ☐ Two lots of a specially treated mannide monooleate emulsifier were fractionated by column chromatography and characterized by TLC and GC; individual components were identified by mass spectrometry. Silica gel fractionation indicated the presence of three major classes of components: diesters, monoesters, and polymeric carbohydrate material. The diester contents of Lots 114B and 115B were 36.3 and 51.8 wt. % while the monoester content was 52.2 and 31.4 wt. %, respectively. The carbohydrate polymer content amounted to 11.2 and 15.8 wt. %, respectively. The mannide monooleate (as the chemical entity) content within Lots 114B and 115B was 33.6 and 20 wt. %, respectively, while the mannide dioleate content varied from 21.9 wt. % (114B) to 29.2 wt. % (115B). Six cyclic fatty acids were observed in these two lots as well as in two toxic lots identified as 8B and 3589. The UV absorptions at 267, 278, 298, and 315 nm. were approximately fivefold greater for Lot 8B than Lot 114B and approximately 20-fold greater for Lot 3589. Nonconjugated *trans*-olefins were present in Lots 114B and 115B to the extent of 5.9 and 6.0 wt. %, respectively, while the conjugated *trans-trans*-diene content amounted to about 1.6 wt. % for both specially treated lots. In both lots, 14 components were found to comprise the monoester class, while 13 components comprised the diester class. The carbohydrate material was not characterized.

Keyphrases ☐ Mannide monooleate commercial emulsifier—fractionation, characterization ☐ Toxicity—commercial mannide monooleate formulation ☐ Adjuvant formulations—characterization of mannide monooleate emulsifier ☐ Emulsifying agents—characterization of mannide monooleate commercial formulation

Mannide monooleate[1] is employed both in Freund's incomplete adjuvant (1) and Adjuvant 65 (2–4) as an emulsifying agent in the preparation of various water-in-oil vaccines. Although this product has currently found widespread experimental use in adjuvant formulations, the safety of this material for humans has been of concern due to various tissue reactions associated with its use (5–9).

In a review of the chemical and biological properties of mannide monooleate, Berlin (10) and Berlin and Wyman (11) associated its toxicity with certain production batches and indicated that improved production methods by the manufacturer substantially reduced the toxic components and their undesirable effects. Also, to ensure further the safety of this product, particularly for human use, several short-term toxicity screening procedures were advanced (12–14). Berlin (13) described both an intraperitoneal test in mice for assessing weight gain and chemical peritonitis and an intracutaneous test in guinea pigs for evaluating local tissue inflammations. More recently, Barile and Hardegree (14) reported a culture assay, using L-929 mouse fibroblast cells, which agreed quite well with the Berlin mouse test and indicated a possible correlation between the level of free fatty acids in the emulsifier and its toxicity. A similar correlation was described earlier by Hardegree and Pittman (7) for two types of water-in-oil emulsified tetanus toxoids. Hardegree and Kirschstein (8) suggested that unidentified components associated with the free fatty acid fraction, but not necessarily the fatty acids themselves, may be responsible for abscesses.

Until recently, little information was available regarding the detailed chemical composition of emulsifying agents such as mannide monooleate. However, studies conducted by Sahasrabudhe and Chadha (15) for the mono fatty acid esters of sorbitan demonstrated the heterogeneous nature of such materials. An early review by Goldsmith (16), relating to the conditions employed for the preparation of such products, indicated the methods of preparation of such esters to vary widely on both the mole ratios of the initial reactants and on the conditions (catalyst and temperature) used.

In view of these data, a detailed analysis of mannide monooleate was carried out to characterize the composition of this material.

MATERIALS[2] AND METHODS

Mannide monooleate, Lots 114B and 115B, were obtained from a commercial source[3]. Two aged lots of the emulsifying agent, approximately 10 years old (8B and 3589), were also used[4]. These lots were considered toxic as demonstrated by the L-929 mouse fibroblast cell culture assay (14).

Silica Gel Fractionation—Initial class separations of the emulsifier components were carried out by column (1.2 × 80 cm.) chromatography over silica gel. Weighed aliquots (1 g.) of each lot were introduced onto the column in benzene solution and eluted with increasing percentages of methanol and finally with water. The 44 fractions were collected in 10-ml. volumes, concentrated under dry nitrogen, and screened by IR, TLC, and GC. Recombination of the individual column fractions was based on the results obtained from these analyses.

GC—GC analyses were performed on a gas chromatograph[5] employing a dual hydrogen flame-ionization detector. Analyses conducted on the individual and recombined column chromatography fractions were carried out by treating approximately 2–5-mg. aliquots with 1–2.0 ml. of the silylation reagent and allowing the reaction to proceed for 15 min. at 50°. Analyses were then carried out on approximately 2 µl. of the trimethylsilyl ether derivatives. The neutral, Fraction A, components were separated on a 0.9-m. × 0.45-cm. (3-ft. × 0.187-in.) column of 3 wt. % OV-1 at 325°. Fraction B, from the column chromatography, was separated on a 2.4-m. ×

[1] In this article, mannide monooleate is the generic term used to refer to the commercial product, Arlacel A (Atlas Chemical Industries, Wilmington, Del.). When mannide monooleate is used to refer to the specific chemical content, it is so indicated.

[2] Fatty acid reference standards for GC and silica gel (Adsorbosil ADN-1, 25 wt. % $AgNO_3$) were purchased from Applied Science Laboratories. Silylation reagent (Tri-Sil) was purchased from Pierce Chemical Co. Silica gel for column chromatography, mesh 200–235, was purchased from the Grace Division Co., and silica gel G for TLC was obtained from Brinkmann Instruments, Inc. GC column packings consisted of 12 wt. % diethylene glycol succinate on Anakrom ABS, 50–60 mesh (Analabs, Inc.), and 3 wt. % OV-1 on 80–100-mesh Chromosorb G-HP (Supelco, Inc.).

[3] Hilltop Laboratories, a distributor for the Atlas Chemical Industries.

[4] Supplied by the Division of Biologics Standards, National Institutes of Health.

[5] Loenco model 70.

Table I—Silica Gel Fractionation Data for Mannide Monooleate, Lots 114B and 115B

Combined Fraction Number	Column Fractions[a]	Solvent Mixture	114B Sample, Wt., mg.	114B Wt. %	114B Component Classification	115B Sample Wt., mg.	115B Wt. %	115B Component Classification
1c	1–6	100% benzene	210.2	21.2		416.0	44.2	
2c	7–10	0.5% methanol–benzene	62.1	6.3	A (36.3%) Diesters	33.5	3.5	A (51.8%) Diesters
3c	11–15	1.0% methanol–benzene	55.9	5.6		38.0	4.0	
4c	16–19	2.0% methanol–benzene	31.9	3.2		9.7	0.1	
5c	20–25	5.0% methanol–benzene	169.5	17.1		138.7	14.6	
6c	26–30	10.0% methanol–benzene	164.4	16.6	B (52.2%) Monoesters	158.7	16.7	B (31.4%) Monoesters
7c	31–35	50% methanol–benzene	183.0	18.5		4.7	0.1	
8c	36–39	100% methanol	31.9	3.2	C (11.2%) Carbo-	11.5	1.2	C (15.8%) Carbo-
9c	40–44	100% water	79.2	8.0	hydrates	138.3	14.6	hydrates
			988.1	99.7%	Recovery	949.1	99.0%	Recovery

[a] Fractions collected in 10-ml. quantities and combined as shown.

0.45-cm. (8-ft. × 0.187-in.) column of 3 wt. % OV-1 at 250°. Fraction C was also converted to its trimethylsilyl ether derivatives and screened on both columns.

The fatty acid distribution of the various lots of mannide monooleate was carried out as their methyl esters after interesterification with methanolic hydrochloric acid. The concentrated fatty acid methyl esters were diluted to 20 wt. % in hexane, and 1 μl. of the sample was used for analysis. The analyses were carried out by temperature programming from 140 to 315° (5°/min.) on a 3.7-m. × 0.45-cm. (12-ft. × 0.187-in.) column containing 3 wt. % OV-1. The GC distribution of the various unsaturated fatty acids in each carbon range was determined by isothermal (185°) analysis on a 2.4-m. × 0.45-cm. (8-ft. × 0.187-in.) column containing 12 wt. % diethylene glycol succinate.

Mass Spectroscopy[6]—Identification of the individual fatty acids was made by combination GC–mass spectrometry employing the OV-1 column. The GC peaks were scanned up to mass 500 at a scan speed of 7 sec. Individual column fractions from the neutral range class (A) were analyzed by a direct sample inlet system operated at 395° at 10^{-6} mm. Hg, while the components comprising Class B were identified as their trimethylsilyl ether derivatives.

TLC—TLC of the original products and individual column chromatography fractions were carried out on silica gel G employing chloroform–methanol–ethyl acetate (97:2:1) and benzene–methanol (98:2) as the solvent phases. Visualization was achieved by chromate–sulfuric acid spraying and charring. Both solvent systems performed quite well and were consistent with the column studies since three major classes of components were observed: (A) a fast migrating nonpolar class, (B) an intermediate polar class, and (C) a nonmigrating polar class.

The fatty acid methyl ester fractions were subjected to TLC separations on 25 wt. % $AgNO_3$–silica gel G, employing chloroform–methanol (98:2) as the solvent. This separation confirmed the presence of *trans*-olefins as indicated in the IR spectrum of the original materials. Their quantitative determination was made by preparing a standard calibration curve with methyl elaidate and screening at 10.3 μ (9.0–11.0 μ) in the IR using a 0.1-mm. cell path. Aliquots of the fatty acid methyl ester samples of Lots 114B and 115B were then diluted in carbon disulfide (0.50 g./10 ml.) and scanned in a similar fashion.

UV Absorption Studies—The UV absorption spectra of the fatty acid methyl ester fractions from each lot (114B, 115B, 8B, and 3589) were obtained on a recording spectrophotometer[7] in the range of 200–400 nm. in isooctane at a scan rate of 10 Å/sec.

RESULTS

Silica gel chromatography of mannide monooleate revealed the composition of the emulsifier to consist of three major classes of components: Fraction A, a high molecular weight, nonpolar fraction made up primarily of mannide diesters; Fraction B, a moderately polar fraction representing the monoester class; and Fraction C, a highly polar fraction consisting predominantly of a solid carbohydrate residue. A summary of these results is presented in Table I. Similar distribution data were obtained by column chromatography with larger samples (20 g.) by the proportionate increase in the relative parameters of column dimensions and weight ratios of gel to sample. The tentative identification of each class was based on IR and TLC screening. IR showed the early fractions (Fractions 1–19), representing Class A, to be free of hydroxy groupings and to exhibit spectra very similar to neutral lipids. These components also migrated at the solvent front during

Table II—Summary of Distribution Data for Mannide Monooleate Lots 114B and 115B

Component Class	Identification	Wt. % of Arlacel A 114B	Wt. % of Arlacel A 115B
A[a]			
1A	Mannide octadecenyl, tetra-	0.07	0.23
2A	decenoate	0.48	1.1
3A	Mannide octadecenyl, penta- decenoate	0.15	0.22
4A	Mannide octadecenyl, hexa-	1.2	N.D.
5A	decenoate	1.8	6.4
6A	Unknown		
7A	Mannide octadecenyl, hepta-	0.96	0.83
8A	decenoate	5.6	9.95
9A	Unknown	1.3	1.3
10A	Mannide dioctadecenoate	3.1	0.93
11A		18.8	28.3
12A	Mannitan dioctadecenoate	0.36	1.5
13A		2.0	0.11
B[b]			
1B	Mannide monotetradecenoate	0.21	0.09
2B		1.0	0.57
3B	Mannide monopentadecenoate	0.26	0.13
4B	Mannide monohexadecenoate	0.64	0.50
5B		3.5	2.4
6B	Unknown	1.7	0.53
7B	Mannide monoheptadecenoate	0.74	0.34
8B		0.79	0.78
9B	Mannide monooctadecenoate	5.4	3.3
10B		28.2	16.7
11B	Mannitan monooctadecenoate	1.9	1.2
12B		0.36	0.22
13B		3.1	1.8
14B		4.4	2.84
Carbohydrate polymer		11.2	15.8
	Recovery[c]	99.70	99.00

[a] Structures within this class based on mass spectrometric identification of component 11A and on retention data. [b] Structures confirmed by mass spectrometry. [c] Recovery based on silica gel chromatography data.

[6] Analyses were carried out on a Hitachi-Perkin-Elmer model RMU-6D mass spectrometer using the Perkin-Elmer model 881 gas chromatograph for separation.

[7] Cary model 14.

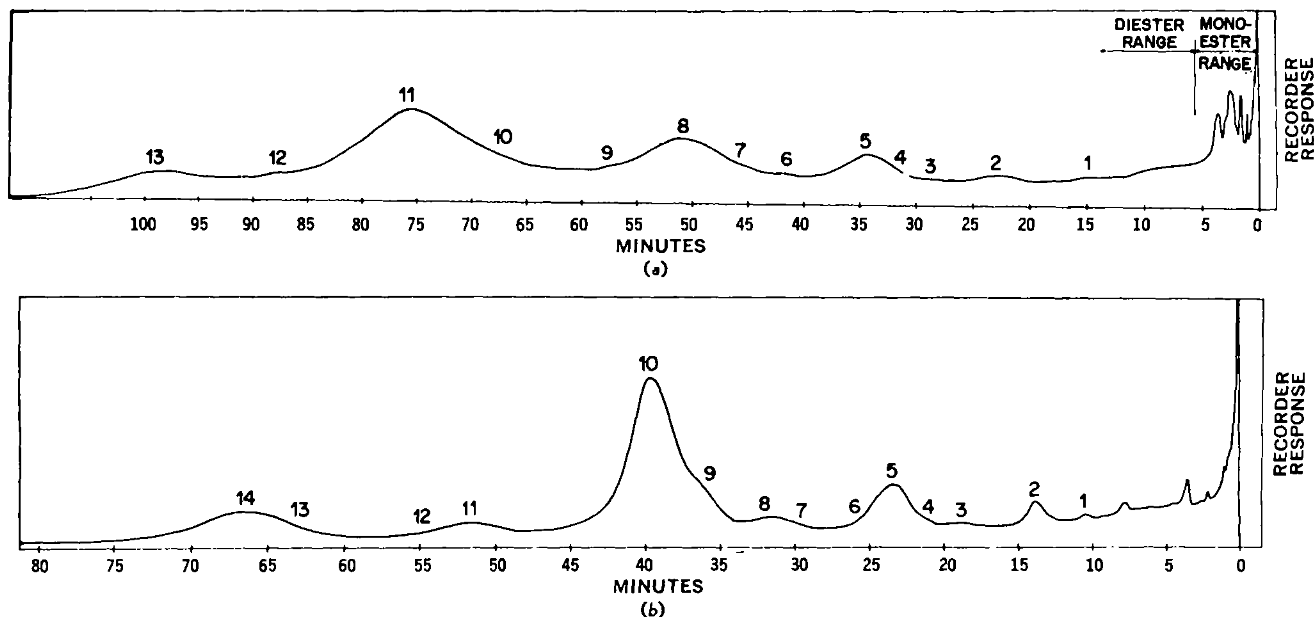

Figure 1—(a) *Distribution of Class A components comprising the neutral diester fraction of mannide monooleate. Separation was carried out on a 0.9-m. × 0.45-cm. (3-ft. × 0.187-in.) column containing 3 wt. % OV-1 at 325°.* (b) *Distribution of Class B components comprising the monoester raction as their trimethylsilyl ether derivatives. Separation was carried out on a 2.4-m. × 0.45-cm. (8-ft. × 0.187-in.) column of 3 wt. % OV-1 at 250°. All peak identifications are consistent with Table II.*

the TLC separation. The IR spectra of the intermediate fractions (Fractions 25–35) were characterized by the appearance of an O—H stretching mode at 3630 cm.$^{-1}$ and by components with R_f values ranging from 0.1 to 0.60 by TLC using benzene–methanol. The remaining fractions (Fractions 36–44) exhibited typical carbohydrate spectra with the addition of a very weak carbonyl band. Dilution studies with these fractions revealed no shifting of the O—H stretching band and, consequently, very little intermolecular hydrogen bonding. Under the condition described, these fractions did not migrate by TLC.

Direct GC analyses of the combined A fractions from each lot indicated a distribution of 13 components, whereas the trimethylsilyl ether derivatives of Fraction B revealed a pattern of 14 components. Fraction C consisted of a white semisolid mass which could not be eluted by GC as the trimethylsilyl ether derivative. Mass spectrometry of the individual GC peaks of Class B identified them as the mono fatty acid esters of dianhydromannitol. Individual peak identification for Class A components could not be obtained due to the bleed rate of the column substrate at the temperature required for its separation. The component distribution data, obtained by GC for Classes A and B among the two lots of emulsifier, are presented in Table II.

The tentative structural assignments given to the Fraction A components were based on several facts:

1. The GC profile, with the exception of components 12A and 13A, yielded an identical elution pattern with or without its prior conversion to the trimethylsilyl ether derivatives.
2. The IR spectrum was consistent with the structure of a neutral or peracylated carbohydrate, presumably of the dianhydromannitol (mannide) structure.
3. The GC screening of column Fraction 12 of Lot 114B indicated a purity of 93 wt. % for GC peak 11A. This fraction, on mass spectrometric analysis using a direct sample inlet port and a temperature of 395° at 10^{-5} mm. Hg, yielded a parent ion peak (M^+) at *m/e* 674. The latter (peak 11A) molecular weight is consistent with the structure of mannide dioleate. The mannide dioleate content represented 51.8 and 54.8 wt. % of the neutral fraction (A) for Lots 114B and 115B, respectively. This component thus represented 18.8 and 28.3 wt. % of the total sample in the order given. If one includes peak 10A, the total mannide dioleate content would represent 21.9 and 29.3 wt. % for Lots 114B and 115B, respectively. Presumably, peak 10A represents a different mannide configuration. The remaining peaks of Class A are shown in the upper part of Fig. 1 and were assigned tentative structures as based on the correlation of their relative retention data with the identified components of Class B.

The GC elution profile obtained for Class B components for Lot 115B, analyzed isothermally as their trimethylsilyl ether derivatives, is shown in the lower portion of Fig. 1. In the B series, the individual components were transferred to the mass spectrometer as they eluted from the gas chromatograph. As can be observed in Table II, the components of the B class encompass both the dianhydromannitol (mannide) and monoanhydromannitol (mannitan) structures. Based on these results, the total mannide monooleate (as the chemical entity) contents (peaks 9B and 10B) for Lots 114B and 115B are 33.6 and 20.0 wt. %, respectively. However, only 28.2 and 16.7 wt. % are characteristic of the isomannide configuration (peak 10B), which is presumed to represent the major contribution of each monoester structure. The peaks immediately preceding the major ones (1, 4, 7, 9, 11, and 13) for each fatty acid ester probably represent the esters of the 1,5-2,6-dianhydromannitol structure.

The GC distribution of the total fatty acid content for Lots 114B, 115B, 8B, and 3589 was determined on both a polar (diethylene glycol succinate) column and a nonpolar (OV-1) column (Table III). The use of polar and nonpolar columns was required for differentiating between the individual olefinic fatty acids. A typical gas chromatogram for the fatty acid methyl esters of Lot 115B, as obtained on the OV-1 column, is shown in Fig. 2. The elution sequence observed on the OV-1 column revealed a series of acids (peaks 18–23) eluting immediately after methyl octadecanoate (C_{18})

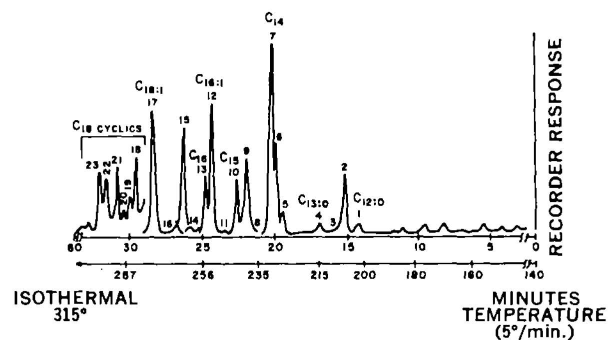

Figure 2—*Separation of the fatty acid methyl ester fraction from mannide monooleate, Lot 115B. Separation was carried out on a 3.7-m. × 0.45-cm. (12-ft. × 0.187-in.) column containing 3 wt. % OV-1. Column temperature was programmed from 140 to 315° (5°/min.).*

Table III—Summary of Fatty Acid Distribution among Mannide Monooleate Lots 114B, 115B, 3589, and 8B[a]

Fatty Acid Identification[b,c]	Relative Wt. %[e]			
	114B	115B	3589	8B
12:0	0.05	0.03	0.04	0.04
12:1	—[d]	—[d]	0.26	0.03
br 13:0	—[d]	—[d]	—[d]	0.02
13:0	0.01	0.02	0.04	0.07
br 14:0	0.06	0.04	0.10	0.08
14:0	2.2	2.2	1.6	1.4
14:1	1.4	2.0	0.84	0.70
14:2	0.01	0.02	0.13	0.01
br 15:0	0.30	0.20	0.48	0.41
15:0	0.25	0.20	0.23	0.20
br 16:0	0.07	0.04	0.08	0.06
16:0	4.4	4.6	3.6	3.5
16:1	7.9	8.5	8.2	9.0
16:2	1.0	1.3	0.42	0.74
br 17:0	0.10	0.20	0.05	0.2
17:0	0.06	0.07	0.07	0.05
17:1	1.6	1.8	0.95	1.6
18:0	0.56	0.55	0.64	0.50
18:1	75.2	73.7	76.5	76.0
18:2	1.5	1.5	1.8	1.4
18:2 (t − t)	1.0	0.90	1.4	1.2
18:3	0.64	0.62	0.50	0.80
cy 18:1	0.3	0.22	0.43	0.35
cy 18:1 + 19:1	0.1 }	0.14 }	0.14 }	0.15 }
cy 18:1	0.03 } 1.69[f]	0.02 } 1.51[f]	0.04 } 2.07[f]	0.05 } 1.99[f]
cy 18:0	0.23 }	0.22 }	0.43 }	0.44 }
cy 18:2	0.51 }	0.60 }	0.58 }	0.50 }
cy 18:0	0.52	0.31	0.45	0.50

[a] Column conditions: polar: 12 wt. % diethylene glycol succinate (Cs) on Anakrom ABS, 60–70 mesh; column 2.4 m. × 0.45 cm. (8 ft. × 0.187 in.); column temperature, 185°; detector temperature, 240°; injector temperature, 220°; detector, dual hydrogen flame. Nonpolar: 3 wt. % OV-1 on Chromosorb G-HP, 80–100 mesh; column 3.7 m. × 0.45 cm. (12 ft. × 0.187 in.); column temperature, programmed 140–315° (5°/min.); detector temperature, 240°; injector temperature, 220°. [b] Eight additional components detected in trace quantities in all samples at longer retention times (br = branched; cy = cyclic). [c] Identification based on log–log plot of relative retention times observed on polar and nonpolar columns. [d] Presence not detected. [e] Relative weight percentages based on distribution of fatty acid methyl esters. [f] Identification of cyclic structures based on GC–mass spectrometry.

and exhibited parent ion masses of one and two methylene units less than what would be expected for their position in the elution sequence.

The UV absorption spectra obtained for these same fatty acid methyl ester fractions indicated the presence of both linear and cyclic conjugated olefins. However, the bands associated with the conjugated dienes (232 nm.) and trienes (267 nm.) were masked by additional bands at 225, 239, 258, 278, 298, and 315 nm. Although accurate quantitative data could not be advanced for any specific structure due to the severe overlap, the specific absorption coefficients (K) calculated for the 225-, 232-, and 239-nm. bands were similar for all four lots (114B, 115B, 8B, and 3589). In comparing the ratios of the specific absorptivity coefficients of Lots 8B and 3589 with that of Lot 114B, a marked increase was observed at all of the longer wavelengths. For example, Lot 8B exhibited increases of 7.8 (267 nm.), 4.9 (278 nm.), 5.1 (298 nm.) and 3.7 (315 nm.), while Lot 3589 exhibited increases of 20.3, 13.9, 23.3, and 30.6 for the bands in the order given. Undoubtedly, the toxic Lot 3589 exhibited a much higher degree of conjugation and cyclization than any of the other lots, including the other toxic lot, 8B. Accurate quantitation of these bands was impossible due to their overlap and the large variance in the molar extinction values of the various contributing structures.

The total level of nonconjugated *trans*-olefins present in Lots 114B and 115B was calculated to be 5.9 and 6.0 wt. %, respectively, based on the 10.3-μ IR band. For the estimation of the level of *trans-trans* conjugated diene, the UV absorption band at 232 nm. was used. By employing a literature value of 111.4 for the specific absorption coefficient of this band (18), the level of the conjugated *trans-trans*-diene structure was calculated to be approximately 1.6% for both emulsifier lots, 114B and 115B. These values are somewhat larger than those (1.0 and 0.9%, respectively) obtained by GC on the polar column. The differences observed could be represented by the contribution of other structures to the 232-nm. absorption.

Water determinations were carried out by the Karl Fischer titration method on all four lots to observe any change that might be related to the opaqueness, age, or toxicity associated with the two aged lots. Lots 114B and 115B exhibited a water content of 0.125 and 0.109 wt. % water, respectively, while the values for 8B and 3589 were 0.25 and 0.55 wt. %, respectively. The opacity associated with the two aged samples did not appear to correlate with the water content but rather with the insoluble carbohydrate material present in the sample, which was isolated by centrifugation and identified by IR.

DISCUSSION

The analytical data advanced in this study have revealed several interesting features with respect to the use of emulsifiers as adjuvants. Perhaps the most noteworthy feature is the relation of heterogeneity to efficacy. For example, an emulsifying agent, by virtue of its mode of action, should contain both hydrophilic and lipophilic groupings to reduce the surface tension at the oil–water interface. In the case of mannide monooleate, only the monoester class (B) fulfilled this requirement and this fraction represented only about 30–50 wt. % of the total product. Based on these values, it would appear that the use of a purified form of the monoester class would substantially reduce the total amount of emulsifying agent required for use in these adjuvants. However, Bollinger (17) suggested that the retention of mineral oil at the site of injection, employing Freund's incomplete adjuvant, was probably due to a faster removal of certain surfactant components from the site than that of the mineral oil itself. The esterified components of the surfactant persisted longer at the site than the nonesterified. The author indicated that perhaps increased quantities of pure surfactant might be beneficial to the mobilization of the mineral oil from the tissue.

The occurrence of cyclic fatty acid moieties in the two lots of mannide monooleate is not too surprising in view of the relatively high levels of *trans*-isomers existing among the unsaturated fatty acids. Numerous reports of similar cyclic fatty acid structures were published relative to both their formation and nutritional losses during excessive heating (19–26). In most of these studies, methyl linoleate was used as the model compound and was exposed to temperatures of about 200° for 200 hr. The presence of various inorganic catalysts or alkali greatly reduces the time necessary for isomerization and cyclization.

The detection and identification of the cyclic structures in the fatty acid methyl ester fraction became evident when it was noticed that a series of components that did not correspond to their molecular weight assignments, based on a semilog plot of their relative retention volumes, were eluted by GC. Mass spectrometry of the individual peaks indicated each component to be C_{18}-acids, but their fragmentation patterns were not typical of straight-chain structures. The tentative assignments given to these components were later found to be consistent with the retention data previously advanced for C_{18}-saturated cyclic acids and C_{18}-unsaturated cyclic fatty acids (23, 28).

Pharmacological studies (19, 26, 27) with purified fractions of cyclic fatty acids indicated a high toxicity when administered *via* the dietary route. In this respect, Michael *et al.* (19) conducted acute toxicity tests with a low boiling, nonurea adductable fraction from thermally abused methyl linoleate fed to weanling rats at a level of 25 g./kg. Toxicity was established, and death resulted in one or more animals within 3 days. Survivors were sacrificed after 10 days and usually exhibited losses in body and thymus weight and suffered severe diarrhea throughout the entire test period. It is difficult to correlate relative levels of these cyclic fatty acids administered in the diet with dosages introduced as the vaccine, since little information is available on the absorption rates of these structures.

In view of the finding that cyclohexyl-type acids were present in the acidic fractions of this product, efforts were made to establish whether aromatic acids were present. Although additional components were detected at very low levels and at longer retention times in the GC screening on the OV-1 column, their concentrations were too low to advance structural assignments to them by mass spectroscopy. However, significant increases (~20-fold) in the UV absorption were observed at all of the longer wavelengths (258, 267, 298, and 315 nm.) for the fatty acid methyl ester fractions of the two aged lots (8B and 3589) in comparison to the nontoxic lots (114B and 115B). In this regard, Michael (20) reported absorption maxima at 263, 267, and 270 nm. for aromatic acids derived from cyclized methyl linoleate. Absorption maxima were also reported at 256, 263, 267, and 272 nm. for aromatic acids arising from heated ethyl linoleate (22). Because of the severe overlapping of the spectrum in all fatty acid methyl ester fractions in this region, it was not possible to advance any definitive conclusions regarding the presence of aromatic acids in these samples. It is perhaps coincidental that the relative UV absorption values observed for the four sample lots are in the same order as their free fatty acid content. Free fatty acid values of 11, 14, 282, and 500 μmoles/ml. were reported for Lots 114B, 115B, 8B, and 3589, respectively (14). Although there is little reason to suspect that disubstituted aromatic acids would have any more tendency to exist in the free form than the long-chain fatty acids, the fact that the UV-absorbing structures do represent a 20-fold increase in concentration among the total fatty acid population suggests a similar increase to be distributed in the free fatty acid fraction. The presence of nonpolar and polar dimer acids, also reported to be present in these same types of thermally abused products (19), could not be confirmed in any of the samples screened.

At present, it is not known whether cyclic fatty acids as such are responsible for destructive tissue changes associated with the use of certain batches of mannide monooleate. Certainly, the toxicity of this product is not dependent on their presence. For example, Berlin and Wyman (11) isolated the monooleate and dioleate fractions of this emulsifier by distillation and column chromatography and compared the toxicity of the monooleate class to oleic acid in various dilutions of peanut oil. Intradermal injections on the backs of guinea pigs indicated the monooleate fraction to be toxic at concentrated levels, but dilution with peanut oil decreased its effect. In contrast, oleic acid toxicity was not appreciably reduced on dilution but yielded similar tissue changes as the monooleate. The authors also found the monooleate fraction to be more readily hydrolyzed by pancreatic lipase than the dioleate fraction and suggested that the susceptibility of the monooleate class to enzymatic hydrolysis may have a direct relationship to its *in vivo* stability and, accordingly, its toxicity as compared to the dioleate. An explanation offered for the variability in toxicity with respect to various batches is that the dioleate level may dilute the monooleate level below its toxic level or that other components observed to be present in the material may reduce the hydrolytic susceptibility of the monooleate. Although it is difficult to offer specific levels of dilutions that might distinguish a toxic from a nontoxic batch, both lots involved in this study were of the "specially treated" product and they varied in their ratio of monoester to diester by a factor of 2. In this case, it may be possible that the levels of diester in both lots were in sufficient quantity to minimize the monooleate toxicity if, indeed, this is its function. However, in view of the large increase in the UV absorption properties at the longer wavelengths of the two toxic lots (8B and 3589) as compared to the specially treated lots, it may suggest an additional explanation as to the variability among batches.

REFERENCES

(1) J. Freund, *Advan. Tuberc. Res.*, **7**, 130(1956).

(2) M. R. Hilleman, *Prog. Med. Virol.*, **28**, 131(1966).

(3) A. F. Woodhour, W. J. McAleer, A. Friedman, R. E. Weibel, J. Stokes, Jr., and M. R. Hilleman, *Proc. Soc. Exp. Biol. Med.*, **131**, 501(1969).

(4) C. C. Porter and D. C. Titus, *ibid.*, **124**, 500(1967).

(5) M. Potter, *J. Exp. Med.*, **115**, 339(1962).

(6) J. E. Salk, *J. Amer. Med. Ass.*, **151**, 1169(1953).

(7) M. C. Hardegree and M. Pittman, *Proc. Soc. Exp. Biol. Med.*, **123**, 179(1966).

(8) M. C. Hardegree and R. L. Kirschstein, *Ann. Allergy*, **26**, 259(1968).

(9) J. O. MacFarlane, D. N. Roberts, C. A. Bailey, A. Monley, P. Cohen, and M. C. Hardegree, *Fed. Proc.*, **29**, 503(1970); Abstr. 1454.

(10) B. S. Berlin, *Ann. Allergy*, **21**, 82(1963).

(11) B. S. Berlin and R. Wyman, *Proc. Soc. Exp. Biol. Med.*, **136**, 1363(1970).

(12) H. M. Peck, A. F. Woodhour, D. P. Metzgar, S. E. McKinney, and M. R. Hilleman, *ibid.*, **116**, 523(1964).

(13) B. S. Berlin, *Ann. Allergy*, **20**, 472(1962).

(14) M. F. Barile and M. C. Hardegree, *Proc. Soc. Exp. Biol. Med.*, **133**, 222(1970).

(15) M. R. Sahasrabudhe and R. K. Chadha, *J. Amer. Oil Chem. Soc.*, **46**, 8(1969).

(16) H. A. Goldsmith, *Chem. Rev.*, **33**, 257(1943).

(17) J. N. Bollinger, *J. Pharm. Sci.*, **59**, 1088(1970).

(18) B. A. Brice and M. C. Swain, *J. Opt. Soc. Amer.*, **35**, 532 (1945).

(19) W. R. Michael, J. C. Alexander, and N. R. Artman, *Lipids*, **1**, 353(1966).

(20) W. R. Michael, *ibid.*, **1**, 359(1966).

(21) *Ibid.*, **1**, 365(1966).

(22) R. B. Hutchison and J. C. Alexander, *J. Org. Chem.*, **28**, 2522(1963).

(23) J. P. Friedrich, *J. Amer. Oil Chem. Soc.*, **44**, 244(1967).

(24) J. P. Friedrich, H. M. Tetter, J. C. Cowan, and G. E. McManis, *ibid.*, **38**, 392(1961).

(25) A. G. McInnes, F. P. Cooper, and J. A. MacDonald, *Can. J. Chem.*, **39**, 1906(1961).

(26) E. W. Crampton, R. H. Common, F. A. Farmer, A. F. Wells, and D. Crawford, *J. Nutr.*, **49**, 333(1953).

(27) E. W. Crampton, R. H. Common, E. T. Pritchard, and F. A. Farmer, *ibid.*, **60**, 13(1956).

(28) R. A. Eisenhauer, R. E. Beal, L. T. Black, and J. P. Friedrich, *J. Amer. Oil Chem. Soc.*, **43**, 515(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 9, 1971, from the **IIT Research Institute Chicago, IL 60616*, and the †*Division of Biologics Standards National Institutes of Health, U. S. Public Health Service, Bethesda, MD 20014*

Accepted for publication February 3, 1972.

Supported by the Division of Biologics Standards, National Institutes of Health, under Contract PH-43-67-1101.

The authors acknowledge the valuable assistance of Dr. R. G. Scholz, IIT Research Institute, and Dr. R. Bell, School of Pharmacy, University of Illinois, for mass spectral data.

▲ To whom inquiries should be directed.