Fractionation of Arlacel A[1] (35493)

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Arlacel A[2] is a synthetic oil-soluble emulsifier for the preparation of water-in-mineral oil emulsions, the type initially described by Freund as immunologic adjuvants (1). Specially-Treated Arlacel A is a partially purified form of Arlacel A that has been processed to remove toxic materials which might be injurious to tissues. The special grade of the emulsifier has been widely employed as a component of water-in-mineral oil emulsions which are used for prevention of respiratory diseases by vaccination and for repository treatment of allergy and only those batches that satisfy stringent biological tests for safety have been used in adjuvant emulsions intended for use in man (2).

Arlacel A is also called mannide monooleate by its manufacturer as an eponym for 1,3:4,6 dianhydromannitol monooleate which is presumably the principal active ingredient of this emulsifier (3). Dianhydromannitol, an anhydrosugar with two hydroxyl groups available for possible esterfication, is apparently formed during the manufacturing process and combines with oleic acid to yield mannide monooleate and mannide dioleate which are thought to be major constituents of Arlacel A. However the composition of Arlacel A is not well known and it is probable that the emulsifier also contains esters of other fatty acids (4, 5). The objectives of the present study were to ascertain the chemical and biologic properties of more highly purified mannide monooleate and mannide dioleate fractions of Arlacel.

A. Methods. 1. Analytical methods. Thin-layer chromatography (TLC) on silica gel[3] matrix was used for qualitative identification of various fractions recovered during the purification process. Chromatograms were developed with a mixture of acetone and chloroform (1:2; v/v), unless otherwise stated, and were stained by exposure to I_2 vapor. Fractions were identified by their characteristic rate of migration on TLC plates.

2. Fractionation methods. a. Distillation. Distillation was done in a closed system at 0.001 mm Hg pressure using an apparatus[4] which spread the materials to be distilled as a thin layer over the inner walls of a heated glass cylinder by means of rotating vanes. The temperature of distillation was controlled by an external heating jacket which was wrapped around the body of the still. Distillate was condensed on the outer walls of a smaller water-cooled glass column which was concentric to the heated cylinder and the condensate was collected in a side-arm flask at the reduced pressure. It was necessary to open the system to ambient pressure to recover either distillate or the undistilled residue.

b. Column chromatography. Appropriate fractions obtained by distillation were subjected to column chromatography as an additional fractionation procedure. Initially, the columns which measured about 20 mm in diameter were made with 40 to 50 g of mag-

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[2] Atlas Chemical Industries, Wilmington, Delaware.

[3] Silica Gel G, Brinkmann Instruments, Inc., Des Plaines, Illinois.

[4] Molecular Still, A. O. Smith and Company, Kankakee, Illinois.

nesium silicate,[5] which was additionally ground by mortar and pestle so as to obtain a tightly packed column which ran at less than 1 ml/min. Various fractions were removed from the columns by successive elution with suitable nonaqueous solvents. Samples of column eluates were collected in 25-ml samples, assayed by TLC; and those containing like fractions were combined and concentrated by evaporating the solvent.

B. Findings. 1. Analysis. Reference samples of mannide dioleate and mannide monooleate were prepared under special laboratory conditions[6] from higher quality mannitol and oleic acid than those customarily employed in the manufacture of Arlacel A. TLC patterns showed that the major constituent of the dioleate reference was a fraction with a R_f value of 0.8 while the major constituent of the monooleate reference was a fraction with a R_f value of 0.5. Each reference sample also contained a distinct secondary fraction: The secondary fraction in the monooleate reference had a R_f value of 0.8 while that for the dioleate reference had a R_f value of 0.5. In addition, the monoleate reference contained a minor fraction with a R_f value of 0.1. In subsequent chromatograms, the R_f-0.5 material is referred to as the monooleate fraction while the R_f-0.8 material is referred to as the dioleate fraction.

The TLC pattern of Specially-Treated Arlacel A showed at least four distinct chromatographic fractions: one with an R_f of 0.4, another with an R_f of 0.5 which presumably was a mannide monooleate fraction, a third fraction with an R_f 0.7 and, lastly, a fraction with an R_f 0.8, presumably containing isomannide dioleate. Several other batches of Arlacel A examined by TLC also contained similar fractions presenting clear evidence that the emulsifying agent was more heterogeneous a material than the mannide monooleate reference supplied by the manufacturer.

2. Fractionation. a. Vacuum distillation. Fractional distillation of Arlacel A was employed as a preliminary step, especially in recovery of mannide monooleate fractions. Because of the mode of operating the still, it was necessary to use a batch-type procedure. A large volume, usually 250 ml, of Arlacel was processed with a jacket temperature of 50 to 60° until the entire sample had passed through the still. Then the apparatus was opened to ambient pressure, the vessel for collecting the distillate was replaced, and the undistilled residue was reprocessed at a slightly higher temperature, usually 20 to 30° higher than the preceding distillation.

Distillates recovered with jacket temperatures from 50 to 100° contained appreciable amounts of a material with an R_f of 0.6 which behaved in the same manner as oleic acid on TLC. Similar material appeared in varying concentrations in distillates obtained over the entire range of temperatures employed and was even obtained from batches of Arlacel A that appeared to be without nonesterified or "free" oleic acid. It is assumed that the R_f-0.6 fraction contained oleic acid which may have been released by thermal decomposition of esters in Arlacel A. Distillates obtained at jacket temperatures between 130 and 170° consisted chiefly of the mannide monooleate fraction but also contained smaller amounts of the mannide dioleate fraction. Finally, the major part of distillates recovered with jacket temperatures above 180° consisted of the mannide dioleate fraction, but even these distillates also contained small amounts of the lower boiling fractions.

Attempts to collect materials using jacket temperatures above 300° resulted in carbonization of the undistilled residue. Redistillation of various distillates at the temperatures at which they were initially produced did not result in further purification.

b. Column chromatography. Final recovery of the fractions desired was obtained by subjecting appropriate distillates from vacuum distillation to several cycles of column chromatography. The choice of solvents used in developing the magnesium silicate columns to which the distillate fractions were applied and the order of application was based on evidence from TLC that the mannide dioleate fraction had a higher R_f value in sever-

[5] Florisil, The Floridin Company, Pittsburgh, Pennsylvania.

[6] Reference samples kindly supplied by the Atlas Chemical Industries, Wilmington, Delaware.

TABLE I. Comparative Mobilities of Isomannide Monooleate and Isomannide Dioleate on Silica Gel.

Solvent	*Rf*[a]	
	Monooleate fraction	Dioleate fraction
1.) $CHCl_3$ alone	0.6	0.9
2.) $CHCl_3$ + acetone (2:1)[b]	0.55	0.8
3.) Hexane–chloroform–acetone (18:1:1)	0.35	0.6
4.) Hexane + methanol (98:2)	0.3	0.55
5.) 5% $CHCl_3$ in hexane [hexane + $CHCl_3$ (19:1)]	0.1	0.1
6.) Skellysolve	0.1	0.1

[a] *Rf* = (distance substance migrated)/(distance solvent front migrated).
[b] Ratio by volume.

al organic solvents than did the mannide monooleate fraction. Moreover both fractions migrated faster in polar solvents than they did in nonpolar solvents (Table I). Accordingly, the columns were developed by successive application of chloroform, acetone and chloroform mixtures, acetone and methanol. The course of the chromatographic procedure was monitored by analysis of the eluates by TLC at appropriate intervals and those eluates exhibiting identical thin-layer chromatograms were combined, and then concentrated by flash evaporation of the organic solvents at partially reduced pressures.

Vacuum distillates recovered at 140 to 150° were used as the source of material for recovery of the mannide monooleate fraction by two cycles of column chromatography. Eluates recovered with 10 and 20% solutions of acetone in chloroform (v/v) were combined and subjected to a second run. The mannide monooleate fraction was eluted from the second column with 10% acetone in chloroform and the chromatogram of this product showed a single spot with an R_f of 0.5. Some dioleate material was recovered in the chloroform eluates of columns which were loaded with 140 to 150° distillates, but this procedure did not result in a high yield. The mannide dioleate fraction was obtained principally by column chromatography of vacuum distillates obtained above 180° and was eluted from the magnesium silicate columns with chloroform. In addition, after the monooleate and dioleate fractions had been removed from the columns, elution with methanol yielded a third, and as yet unidentified, fraction which had a R_f of 0.2.

C. Chemical properties. The mannide monooleate fraction was a pale straw-colored oily liquid which had a refractive index of 1.477 at 23° and sp gr, 1.05 at 23°. The monooleate fraction promoted formation of water-in-oil emulsions when added in a 10% concentration to Drakeol 6VR, a medicinal form of mineral oil. In contrast, the mannide dioleate fraction purified by magnesium silicate was a colorless oily liquid with an index of refraction of 1.473 and sp gr, 0.94. The dioleate fraction was less viscous than the monooleate fraction and, when added to medicinal mineral oil in a 10% suspension, led to production of oil-in-water emulsions which are the opposite type of those desired as adjuvants.

In addition, the monooleate fraction was more easily hydrolyzed than the dioleate fraction. When initially recovered from the magnesium silicate column, the monooleate fraction contained little free unbound acid as shown by an acid number[7] of 1.0, but after 3-years storage at 4 to 10°, the acid number increased to 16. In contrast, no free acid was found in the dioleate fraction either when freshly isolated or after 3-years storage. The monooleate fraction was also readily hydrolyzed when refluxed with sodium hydroxide. An ether extract of acidified hydrolysate yielded an oily material which accounted for 64% of the weight of the starting fraction and also had an acid number of 208. Pancreatic

[7] Number of milligrams of potassium hydroxide to neutralize an alcoholic solution equivalent to 1 g of lipid (6).

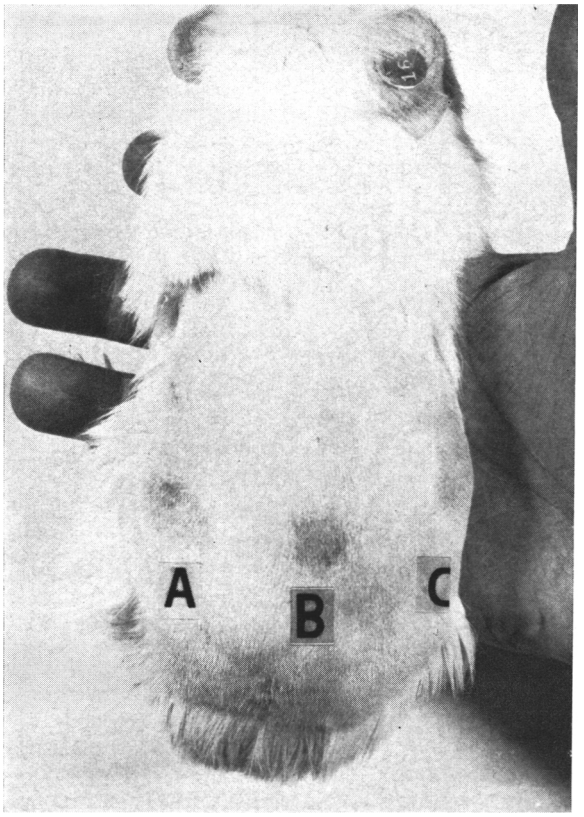

FIG. 1. Skin reactions to intradermal injections of: (A) component R_f approx. 0.2; (B) component R_f approx 0.5 (mannide monooleate fraction); and (C) component R_f approx 0.8 (mannide dioleate fraction).

lipase had greater lipolytic activity with the monooleate fraction than with the dioleate fraction, as shown by incubating each fraction at 37° overnight with the equivalent of 2 g of crude lipase[8]/g of lipid and measuring the amount of potassium hydroxide to neutralize the products of this hydrolysis. The equivalent of 150 mg of potassium hydroxide/g of monooleate fraction contrasted sharply with the value of 29 mg of potassium hydroxide/g of dioleate fraction.

D. Biologic properties. The mannide monooleate and mannide dioleate fractions were tested for intradermal toxicity in two guinea pigs using procedures based on previously described tests for biologic safety (2). Figure 1 is a photograph of the back of one guinea pig 24 hr after intradermal injection of 0.1-ml amounts of freshly obtained monooleate and dioleate fractions. The same volume of the unidentified fraction with a R_f of 0.2 was also tested in these guinea pigs. The monooleate and R_f-0.2 fractions caused severe inflammatory reactions and local tissue necrosis comparable to that seen in guinea pigs injected intradermally with toxic lots of Arlacel A or with oleic acid (2). In contrast, mannide dioleate had no greater effect than that observed in other tests following injection of acceptable batches of Specially-Treated Arlacel A. Figure 1, illustrates the greater toxicity of the R_f 0.2 and monooleate fractions. As expected, lesions caused by the toxic materials healed leaving noticeable scars.

In addition, a simple biological assay was employed to compare the tissue toxicity of the monooleate fraction with that of oleic

[8] Pancreatic Lipase, Sigma Chemicals, St. Louis, Missouri.

TABLE II. Biologic Assay of Isomannide Monooleate.

Solute/solvent ratio[a] (v/v)	Intradermal reaction; solute: Monooleate fraction a	Monooleate fraction b	Oleic acid a	Oleic acid b
Undiluted	18(N)[b]	25(N)	18(N)	20(N)
3:1	20	ND	18(N)	20(N)
1:1	20	20	20(N)	20(N)
1:3	10	15	18(N)	20(N)
Peanut oil alone	8	8	—	—

[a] Ratio of volume of isomannide monooleate fraction or oleic acid to volume of peanut oil: 0.1 ml intracutaneous.

[b] Recordings in two guinea pigs (a) and (b) denotes the diameter (ml) of the area of inflammation surrounding the site of injection at 3 days; N, the occurrence of local necrosis; ND, not done.

acid which might be a major irritant in undesirable batches of Arlacel A. Oily mixtures made with differing proportions of the monooleate fraction and peanut oil or mixtures made with similar proportions of oleic acid and peanut oil were injected in 0.1-ml amounts intradermally in the skin on the back of two guinea pigs and the local reactions were observed. Sites injected with undiluted monooleate fraction or with oleic acid developed the expected local inflammation and necrosis. The findings at 3 days (Table II), which were representative of the relative toxicity of the various materials injected, indicate that the toxicity of the monooleate fraction was rapidly diluted out by peanut oil, whereas that of oleic acid was not appreciably changed even when diluted fourfold with peanut oil. The interpretation given to these findings was that the monooleate fraction was less toxic than oleic acid by at least fourfold factor.

Discussion. These findings demonstrated that three fractions, at least, were obtained from Specially-Treated Arlacel A by fractional distillation and column chromatography. The principal products, the mannide monooleate and dioleate fractions, had disparate chemical and biologic properties. The monooleate fraction was more viscous, more dense, more readily hydrolyzed, and more toxic than the dioleate fraction. The finding suggests a possible direct relationship between the relative instability of the monooleate fraction and its toxicity in the skin of the guinea pig.

The product obtained by alkaline hydrolysis of the monooleate fraction is consistent with the assumption that this fraction consists largely of mannide monooleate. First, the acid number of the lipid recovered from the monooleate fraction approximated that for oleic acid (7). Second, the proportion by weight (64%) of the ether-soluble material recovered from the monooleate fraction approximated the proportion of oleic acid (mol wt, 282) in mannide monooleate (mol wt, 426).

A possible explanation for the tissue toxicity is that some or all of the components of the monooleate fraction are as readily hydrolyzed by tissue lipases *in vivo* as by pancreatic lipases *in vitro*. Presumably, the enzymic hydrolysis releases free fatty acids which then have direct toxic effects similar to those described by Freinkel (8). Thus it appears reasonable to expect that tissue toxicity of Arlacel A would be reduced if means could be found to prevent release of fatty acids. Evidently, those lots of Arlacel A which pass biologic tests for safety in animals do not exhibit significant toxicity in man despite their content of mannide monooleate. One explanation for the apparent lack of toxicity might be that mannide dioleate and other constituents of Arlacel A reduce the concentration of mannide monooleate in Arlacel A below the toxic level by simple dilution. In addition, these other components might also limit any enzymic hydrolysis as well as the inherent tendency of mannide monooleate to hydrolyze spontaneously.

Hardegree and Pittman (9) reported a correlation between the presence of free fatty acids in tetanus toxoid vaccines and other vaccines with the induction of abscess formation. They found that certain antigens like cholera and tetanus vaccines promoted the release of free fatty acids *in vitro* and surmised that the effect may have been due to a bacterial lipase. The susceptibility of purified mannide monooleate fraction observed in

the present studies and the ease with which it was hydrolyzed by a number of methods is consistent with the observation by Hardegree and Pittman and also suggests that it is the mannide monooleate in those vaccines which is acted upon by esterases and lipases *in vivo*.

These present studies also support an earlier concept for the production of stabilized emulsified systems. Water-in-oil emulsions may be stabilized if, in addition to an emulsifying agent which promotes that type of emulsion, a small amount of an emulsifying agent which promotes the opposite kind of emulsion is included (10). The mannide monooleate fraction served as a water-in-oil emulsifier, although it is toxic, whereas the dioleate fraction which minimized the toxicity served to produce oil-in-water emulsions, albeit an unstable one. Thus the mixture of the monooleate and dioleate fractions in Arlacel A works toward an optimal emulsifier.

Summary. Mannide monooleate and mannide dioleate fractions were obtained by vacuum distillation and column chromatography of Arlacel A. Different densities, stabilities, and different toxicities were found. The monooleate fraction was readily hydrolyzed by pancreatic lipase with consequent release of free oleic acid and it exhibited also severe tissue toxicity when injected intradermally into guinea pigs. In contrast, the dioleate fraction was not so susceptible to hydrolysis and showed minimal effects when injected intradermally. The findings support the hypothesis that the toxic effect of certain batches of Arlacel A is due to release of free oleic acid *in vivo*.

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