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Characterization of Adjuvant Mineral Oils

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Abstract □ Analysis of two lots of mineral oil indicated the overall composition to be quite similar with two major hydrocarbon ranges: a low range from C_{14} to C_{20} with a maximum at C_{16} and a high range from C_{20} to C_{30} with a maximum at C_{25}. The low range components represented 40.8 and 49.3 wt.% of Lots Q-9 and Q-10, respectively. Urea fractionation of the paraffin–naphthene fraction from silica gel chromatography yielded 28.2 and 28.8 wt.% for Lots Q-9 and Q-10, respectively. The nonadductable material consisted of highly branched and cyclic structures containing up to four-ring systems. The total butyl phthalate content in Lots Q-9 and Q-10 was 13.2 and 7.0 p.p.m., respectively, with the ratio of monobutyl phthalate to dibutyl phthalate being 9:1. No evidence was found to indicate the presence of polynuclear aromatic hydrocarbons.

Keyphrases □ Mineral oils, adjuvant—separation, UV and mass spectrometry characterization □ Phthalate esters—identification in adjuvant mineral oils □ Adjuvant mineral oils—separation, UV and mass spectrometry characterization □ GC—separation of mineral oil components □ UV spectrophotometry—characterization of mineral oils □ Mass spectroscopy—characterization of mineral oils

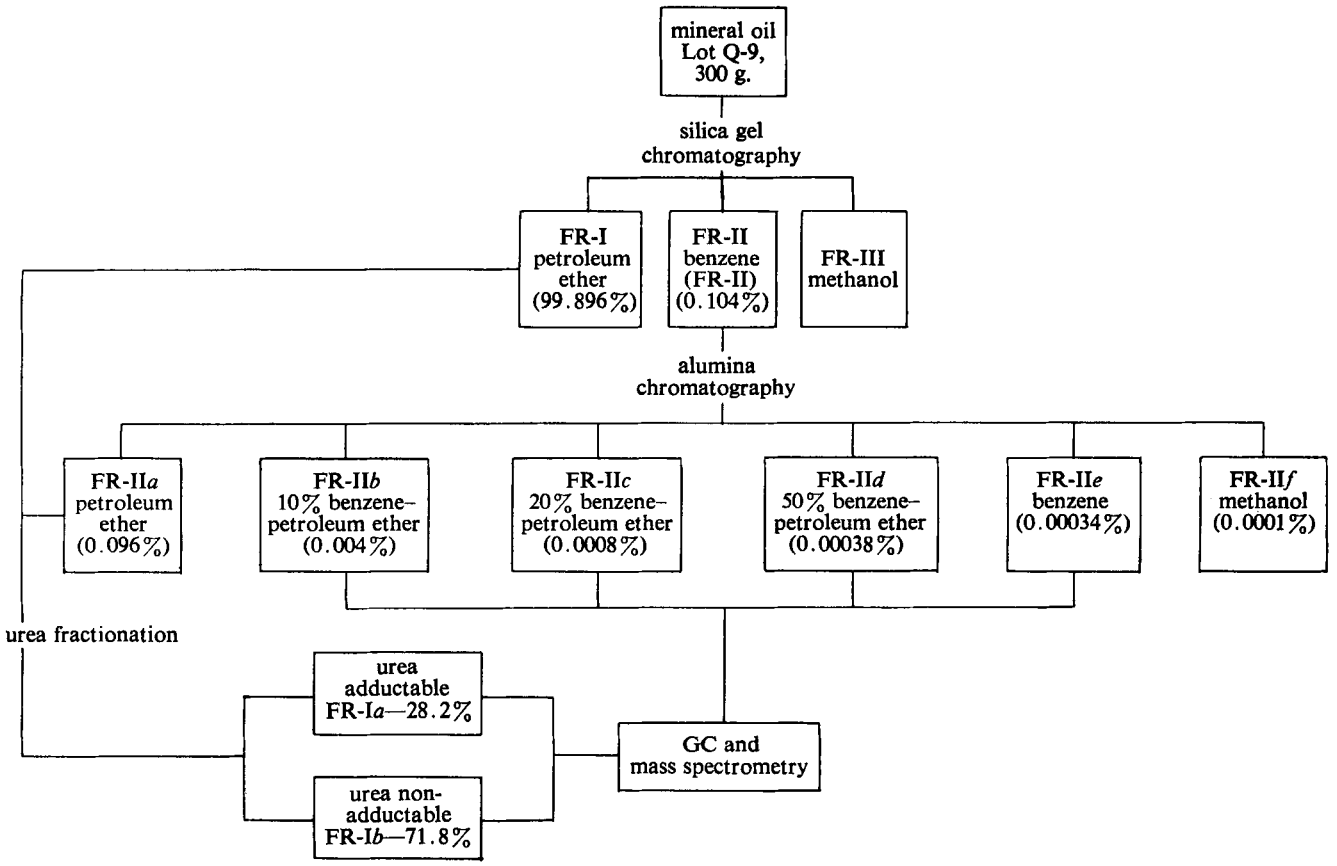
Mineral oil[1] for adjuvant use is a light oil consisting of a blend of oleum-treated charge stocks (1) and, along with the emulsifier mannide monooleate[2], constitutes Freund's incomplete adjuvant preparation (2). This adjuvant has found widespread experimental use over the years for enhancing antibody responses to viral and bacterial antigens and for hyposensitization of certain allergic individuals (3). Recently, the safety of this adjuvant for use in humans has been questioned due to the long-term persistency (4, 5) of the mineral oil in the tissue at the injection site and because of various pathological changes which have been observed in experimental animals and man (3, 6–8). In view of these findings as well as those of others relating to either the carcinogenic or cocarcinogenic properties of mineral oils in general (9–11), an analytical study was initiated.

EXPERIMENTAL

Materials—Two commercial lots of mineral oil (Lots Q-9 and Q-10) were obtained from the manufacturer and consisted of three basic charge stocks representing 61, 31, and 8% of the total oil[3]. Column chromatography was conducted employing silica gel[4] (mesh 60–200) and alumina[5] (chromatographic grade F20). All solvents were reagent grade and were distilled before use.

Column Chromatography—Mineral oil, Lot Q-9, was chromatographed over silica gel as the first step in the overall fractionation procedure (Scheme I). For this separation, 300 g. of mineral oil was dissolved in 500 ml. petroleum ether (30–60°) and introduced over 1140 g. of silica gel. The column was eluted sequentially with petroleum ether, benzene, and absolute methanol. Alumina chromatography of the derived benzene fraction was carried out by

[1] Drakeol 6VR mineral oil manufactured by the Pennsylvania Refining Co., Butler, Pa.
[2] Arlacel A, Atlas Chemical Industries, Wilmington, Del.
[3] Personal communication, Mr. Charles Steenbergen, Pennsylvania Refining Co., Butler, Pa.
[4] Davison Chemical, Baltimore, Md.
[5] Alcoa Chemicals, Bauxite, Ark.



Scheme I—Fractionation scheme for mineral oil Lot Q-9

charging 270 mg. of sample over 25 g. of adsorbent. The sample was eluted with successive volumes of: (*a*) petroleum ether, (*b*) 10% benzene in petroleum ether, (*c*) 20% benzene in petroleum ether, (*d*) 50% benzene in petroleum ether, (*e*) 100% benzene, and, finally, (*f*) methanol.

Urea Fractionation of Paraffin–Naphthene Fraction—The combined paraffin–naphthene fraction (FR-I and FR-II*a*) from the column chromatography separation of Lot Q-9 was subjected to fractionation by urea complexation. This separation was necessary to class fractionate the paraffinic and naphthenic components prior to GC–mass spectrometry characterization.

The general procedure used for the urea fractionation (12) involved the addition of a weighed aliquot of the hydrocarbon material (FR-I and FR-II*a*) to a mixture of acetone and urea, and the contents were stirred vigorously for 1 hr. and filtered. The solid residue was then slurried with a saturated solution of urea in acetone and filtered, and the solid filter cake was transferred to a flask containing hot distilled water to decompose the urea–hydrocarbon clathrate. The cooled water–urea–hydrocarbon mixture was extracted three times with hexane. The first extraction yielded virtually all of the normal hydrocarbon material. A second extraction yielded a trace of oil, and a third extraction failed to yield any additional product. The naphthenic material was recovered from the acetone filtrates by diluting with cold distilled water and extracting the oil with *n*-hexane. The hexane extract was dried over anhydrous sodium sulfate and concentrated.

GC of Fractionation Products—GC analyses[6] were performed on all the Q-9 fractions derived from the various fractionation procedures shown in Scheme I. For this analysis, a temperature-programmed (2°/min.) run was made from 125 to 325° on a 3.7-m. × 0.5-cm. (12-ft. × 0.19-in.) OV-1 (3 wt. %) column using dual hydrogen-flame detection. Samples were prepared for analysis by diluting 10 mg. of each fraction to 1.0 ml. with *n*-hexane and injecting 1 μl. of the solution. Fractions yielding less than 10 mg. were diluted proportionately with a 100-μl. syringe.

[6] Loenco model 70.

Mass Spectroscopy[7]—GC–mass spectrometry studies were conducted on the OV-I column by scanning each peak up to mass 500 at a scan speed of 7 sec.

UV Absorption Data[8]—The UV absorption spectra obtained for the various alumina chromatography fractions (Lot Q-9) were carried out by preparing known concentrations of each fraction in isooctane and scanning the region between 200 and 400 nm. at 10 Å/sec.

RESULTS AND DISCUSSION

In the preliminary silica gel chromatography fractionation of Lot Q-9, the petroleum ether eluate (FR-I, 99.9%) contained the paraffin–naphthene components, while the benzene fraction (FR-II, 0.10 wt.%) concentrated any aromatic or olefinic material present. Absolute methanol was used as a polar solvent phase to displace the benzene from the column and, on subsequent concentration, was free of any solute material. IR analysis of the benzene fraction indicated the presence of residual hydrocarbon material and thus necessitated the further separation of this fraction over alumina. The recoveries obtained for the alumina separation (Table I) indicated about 92.7 wt.% of neutral paraffin–naphthene material, characteristic of Fraction I, was carried over into Fraction II. This hydrocarbon material, identified as FR-II*a*, was recombined with FR-I of the silica gel fractionation. The remaining fractions from the alumina chromatography (II*b*, II*c*, II*d*, II*e*, and II*f*) amounted to 0.00562 wt.% (56.2 p.p.m.) of the original mineral oil (Lot Q-9). A modified procedure, replacing silica gel with silicic acid, yielded a total aromatic content for Lot Q-10 of 0.0025 wt.%. The weight ratio of the two benzene fractions (Q-9/Q-10 = 2.30) was in general agreement with UV absorption data obtained on the two original samples. In this case the ratio

[7] GC–mass spectrometry analyses were carried out on a Perkin-Elmer model 881 gas chromatograph coupled to a Hitachi-Perkin-Elmer RMU-6D mass spectrometer.

[8] Cary model 14.

Table I—Alumina Chromatography of Benzene Eluate from Silica Gel Chromatography of Mineral Oil (Lot Q-9, Charge = 270 mg.)

Fraction	Solvent Phase	Yield[a] g.	Wt.% FR-II	Wt.% (Q-9)
II*a*	Petroleum ether (30–60°)	0.2505	92.7	0.0964
II*b*	10% benzene in petroleum ether	0.0107	3.97	0.0041
II*c*	20% benzene in petroleum ether	0.0021	0.78	0.0008
II*d*	50% benzene in petroleum ether	0.0010	0.37	0.00038
II*e*	100% benzene	0.0009	0.33	0.00034
II*f*	100% methanol	—	—	—
		0.2652	98.15	0.10202

[a] Recovery 98.15%.

of the two specific absorption coefficients (Q-9/Q-10) at 272 nm. was 1.89. The difference between these two ratios can be attributed to residual levels of paraffinic material in these two fractions, while the UV values would be influenced by the nature of the material exhibiting the 272-nm. absorption.

By subjecting the paraffin–naphthene (petroleum ether eluate) fractions to urea complexation, a separation was made of the straight-chain hydrocarbons from the multibranched and cyclic structures. The yields of the urea adductable (straight-chain) fractions from Lots Q-9 and Q-10 amounted to 28.2 and 28.8 wt.%, respectively, while the nonadductable (multibranched, cyclic) fractions of Q-9 and Q-10 amounted to 71.8 and 71.2 wt.%, respectively. The urea adductable and nonadductable fractions isolated from Lot Q-9 were then submitted to GC analysis along with aliquots of the two original fluids.

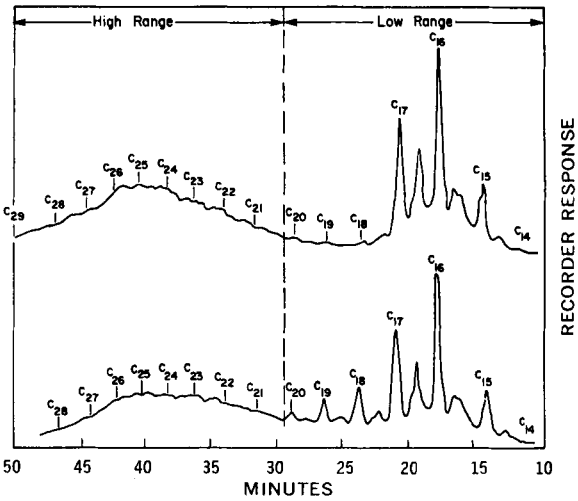


Figure 1—*GC separation of original mineral oil samples, Lots Q-9 (top) and Q-10 (bottom).*

The GC elution profiles of the original mineral oils, as expected, revealed an extremely complex distribution of components (Fig. 1). In general, both lots (Q-9 and Q-10) appeared very similar and exhibited two principal hydrocarbon ranges: a low hydrocarbon range of components from C_{14} to C_{20} and a high hydrocarbon portion ranging from C_{21} to C_{30}. The average component distribution throughout the light range exhibited a maximum at hexadecane (C_{16}). The average hydrocarbon chain length of the high hydrocarbon range was C_{25} (pentacosane), but component resolution in this range was represented by a poorly defined continuum. This type of curve is frequently encountered when the plate effi-

Table II—GC Data as Based on Urea Adductable and Nonadductable Fractions of Lot Q-9 and on Original Mineral Oil Fluids (Lots Q-9 and Q-10)

Peak Number	Equivalent Carbon Length	Weight Percent of Original Fluid[a–d] Urea Adduct (FR-I*a*)	+ Nonurea Adduct (FR-I*b*)	= Total Q-9	Weight Percent of Original Q-9	Q-10
			Low Range			
1	14.00	0.03	—	0.03	0.12	0.41
2	0.41	—	0.28	0.28	0.26	0.18
3	0.66	0.07	0.86	0.93	0.81	0.77
4	15.00	2.10	2.0	4.1	4.0	4.3
5	0.47	0.27	6.7	7.30	3.4	3.0
6	0.64	0.33 } 0.60			3.6 } 7.0	4.1 } 7.1
7	16.00	5.53	5.8	11.33	11.2	12.4
8	0.46	0.60	7.6	8.80	5.7	6.2
9	0.64	0.60 } 1.20			2.4 } 8.1	1.7 } 7.9
10	17.00	3.38	2.9	6.28	7.2	8.2
11	0.48	0.11	0.72	0.83	0.82	1.4
12	0.64	0.08	N.D.	0.08	N.D.	0.37
13	18.00	0.21	0.14	0.35	0.24	3.0
14	0.47	N.D.	N.D.	N.D.	N.D.	0.91
15	19.00	0.08	0.09	0.17	0.24	1.4
16	0.62	0.07	—	0.07	0.26	0.31
17	20.00	0.18	0.18	0.36	0.51	0.65
			High Range			
18	20.1 to 21.00	0.58	2.9	3.48	2.3	1.8
19	21.1 to 22.00	1.08	4.0	5.08	4.0	4.7
20	22.1 to 23.00	1.56	5.6	7.16	6.9	7.2
21	23.1 to 24.00	1.95	6.3	8.25	7.0	7.2
22	24.1 to 25.00	2.24	7.4	9.64	9.6	8.9
23	25.1 to 26.00	2.34	6.9	9.24	9.1	8.2
24	26.1 to 27.00	1.94	4.9	6.84	8.0	6.9
25	27.1 to 28.00	1.18	3.4	4.58	6.0	3.8
26	28.1 to 29.00	0.65	2.2	2.83	3.2	1.6
27	29.1 to 30.00	1.07	0.79	1.85	2.4	0.4
28	30.1 to 31.00	N.D.	0.14	0.14	0.74	N.D.

[a] Weight percent of component calculated from GC of FR-I*a* and FR-I*b* and corrected for weight percent of each fraction in original mineral oil, Lot Q-9; 28.2% (FR-I*a*) and 71.8% (FR-I*b*). [b] See Table III for qualitative identification of urea and nonurea adductable components. [c] N.D. = peak not detected. [d] Conditions of analysis: column 3.7 m. × 0.5 cm. (12 ft. × 0.19 in.), 3 wt.% OV-1, temperature progression 125–325° (2°/min.), dual hydrogen-flame detection.

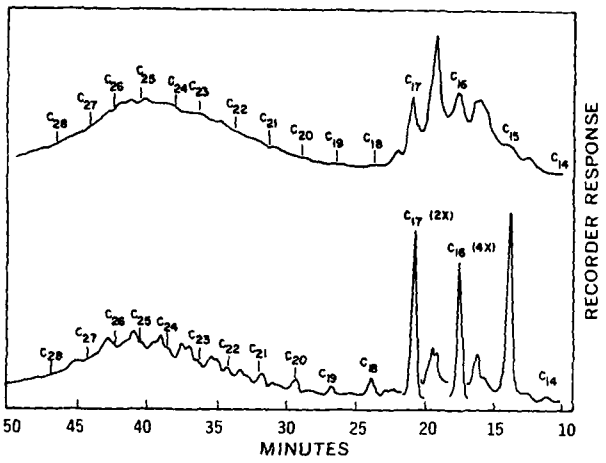


Figure 2—*GC separation of urea adductable FR-Ia (bottom) and urea nonadductable FR-Ib (top) from mineral oil Lot Q-9.*

ciency of a column is not sufficient to resolve individual components effectively or, conversely, when the nature of the material is so complex that several components overlap within a narrow time interval. This form of elution gives the appearance of a "smearing" effect which makes accurate quantitation virtually impossible.

To circumvent this problem, the distribution of components within this high hydrocarbon range was classified and quantified according to equivalent carbon length. This approach made possible the estimation of these components to determine their relative level of contribution to the total sample. The location of normal hydrocarbons eluting within this range was made by seeding both the original and urea fractionated samples with known levels of standard hydrocarbon blends. This approach not only made possible the assignment of specific regions in the gas chromatogram but also served as a method for advancing the equivalent carbon values of the various unknown peaks. The distribution of components, as obtained on the original fluids, is presented in Table II.

In view of the complexity of the original mineral oil, GC analyses were repeated on the urea adductable (FR-I*a*) and nonadductable (FR-I*b*) fractions from Lot Q-9 (Fig. 2). The distribution of components obtained from this fractionation procedure is also shown in Table II for comparison purposes. The latter values are presented both with respect to the individual component distribution in each of the two fractions (FR-I*a* and I*b*) as well as their relative percentage in the original fluid. No attempt was made to conduct a similar tabulation for Lot Q-10, since both the GC and urea fractionation data for Lots Q-9 and Q-10 were virtually identical.

In performing the GC–mass spectrometry studies on the original fluids, it became apparent that the resolution offered by the GC separation was not sufficient for qualitatively identifying all components in these fractions. The two main reasons attributing to this fact are: (*a*) the high concentration of branched and naphthenic material (71.8%, Q-9; 71.2%, Q-10) in these fractions, and (*b*) the excessive overlap of these components both among themselves and with the normal hydrocarbons.

The advantage in conducting a preliminary urea separation is well demonstrated in a comparison of the results of the GC analyses for the original fluids (Fig. 1) and the urea adductable and nonadductable fractions (Fig. 2). For example, a complete homologous series of normal straight-chain hydrocarbons was evident in Fraction I*a*, with hexadecane constituting the major component. Fraction I*b* components yielded fragmentation patterns indicative of highly branched and multicyclic naphthenic structures containing up to four rings within the low range. The results of these analyses are summarized in Table III. The general classes of components observed herein were very similar to those characterized by Fiero (13) for white mineral oils. In his mass spectrometry studies, naphthenic structures exhibiting up to six rings characterized the heavier oils (SSU-355), while the lighter (SSU-75) naphthenic oils contained up to five rings.

The UV screening of Fractions II*a* and II*b* from Lot Q-9 exhibited a sharp doublet at 272 and 280 nm. Subsequent GC–mass spectrometry analyses conducted on these same two fractions revealed the presence of three major components along with lower levels of other components. The first two major components in the elution sequence were identified by mass spectrometry as monobutyl phthalate and dibutyl phthalate. The ratio of monobutyl phthalate to dibutyl phthalate was calculated from area measurements to be 9:1. The third component was too low in concentration to yield a definitive mass spectrum.

The succeeding fractions from the alumina chromatography (II*c*, II*d*, and II*e*) revealed a complex distribution of components as FR-II*b* but very little UV absorption. Based on an average specific extinction coefficient for the two phthalate ester components (K = 4.26), Fractions II*a* and II*b* contained a total phthalate ester content of 9.4 and 3.8 p.p.m., respectively. Based on the measured ratio of 9 to 1 for monobutyl phthalate to dibutyl phthalate, the total level of 13.2 p.p.m. would be distributed as 11.9 p.p.m. monobutyl phthalate and 1.3 p.p.m. dibutyl phthalate. Since the UV absorption value at 272 nm. for Lot Q-10 was approximately one-half that of Lot Q-9, the relative level of the above two components in Lot Q-10 would be approximately 7.0 p.p.m. A similar screening of component 1, representing 61% of the formulation of Lot Q-9, yielded a total phthalate ester content of 29.0 p.p.m. This value would thus amount to 17.7 p.p.m., based on the weight percentage that this component represents in the original oil. It would, therefore, appear evident that component 1 represents the source of these two phthalate derivatives in the original fluid.

Table III—GC–Mass Spectrometry Analysis of Urea Adductable (FR-I*a*) and Urea Nonadductable (FR-I*b*) Fractions from Mineral Oil (Lot Q-9)

Components in Fraction[a]			
Ia	Ib	Equivalent Carbon Length	Structural Assignments Based on Mass Spectral Data
1		14.40	*n*-Tetradecane
	2	14.41	Mixture of methyl-branched saturated hydrocarbons plus monocyclo and dicyclo structures
	3	14.66	Complex mixture of cyclic hydrocarbons with up to four condensed rings in C_{15}–C_{16} range with some methyl branching; at least four components comprising peak
4		15.00	Predominantly *n*-pentadecane; appears to be trace levels of branched C_{16} and cyclic C_{15} components
5		15.47	Consists primarily of a cyclohexylnonane (C_{15}); position of ring uncertain; lesser quantities of dicyclo structure and dimethyl-substituted branched chain hydrocarbon (C_4-substituted)
	5	15.47	Mixture of cycloalkanes with up to four rings in C_{16}–C_{17} range; some methyl branching; at least four components comprising peak
6		15.64	4-Methylpentadecane; trace of mono- and dicyclohexyl structure
7		16.00	*n*-Hexadecane
8		16.46	Predominantly 5-cyclohexyldecane; small level of dimethyl-substituted C_{17}
	8	16.46	Major component a multi (four or greater) methyl-branched C_{18} hydrocarbon; low levels of cycloalkanes with 1-, 2-, and 3-ring systems; condensed rings in C_{18} range
9		16.64	4-Methylhexadecane
10		17.00	*n*-Heptadecane
	10	17.00	Multibranched (probably 3) methyl C_{18}; low levels of cycloalkanes of 1- and 2-ring systems with additional branching in C_{17} range
11		17.48	—[b]
12		17.64	—[b]
13		18.00	*n*-Octadecane

[a] Peak numbers consistent with Table II. [b] Concentration too low to yield distinctive mass spectral patterns.

Samples representing components 2 and 3 in the original Lot Q-9 were not available for analysis.

Fraction II*b*, in addition to exhibiting the 272- and 280-nm. bands, also revealed distinct shoulders at 263 and 293 nm. On this basis, it seems most probable that other aromatic species might be contributing to the total absorption in this region, although no other components could be unequivocally identified by mass spectrometry. In this respect, no direct evidence was found to suggest the presence of any polynuclear aromatic hydrocarbon components in any of the fractions analyzed.

The relative weight percentages advanced for the urea adductable (28.2 wt.%) and nonadductable (71.8 wt.%) fractions of the mineral oil characterize quite well the general composition of this fluid. Thus, the entire urea adductable hydrocarbon content within the low hydrocarbon range of C_{14}–C_{20} of FR-I*a* is representative of only 13.6 wt.% of the total oil. Of this amount, *n*-hexadecane represents almost half or 5.53 wt.%. The remaining light end portion of this fraction consists primarily of straight-chain hydrocarbons, with methyl branching or cyclohexyl ring structures positioned near the terminal end of the chains. This type of structure would be expected, based on the configurational requirements for clathrate trapping with urea, and is exemplified by the identification of 4-methylpentadecane (equivalent carbon length, 15.64) and 4-methylhexadecane (equivalent carbon length, 16.64) in this fraction. The fractional equivalent chain length of 0.65 ± 0.02 reflects predominantly simple methyl branched structures, while values of 0.47 ± 0.02 represent primarily multibranched and condensed ring structures.

The composition of the urea nonadductable fraction (FR-I*b*) is characterized almost exclusively by multibranched and multiring naphthenic structures. The lower carbon range (C_{14}–C_{20}) components of this fraction represent 27.27 wt.% of the total sample; in the higher carbon range (C_{21}–C_{31}), these structures amount to 44.53 wt.% of the total mineral oil. This latter value may be related to the *in vivo* persistence of these oils (4) since highly condensed naphthenic structures would be virtually inert with respect to metabolic processes necessary for their absorption, metabolism, and elimination. This is not true for straight-chain hydrocarbons because such structures have been identified in mammalian lipids (14) and in sebaceous secretions arising from hydrocarbons of dietary origin (15).

Many of the general structures identified in this study are similar to those hydrocarbons that were reported by Horton *et al.* (10) to accelerate the formation of skin tumors in C3H mice. Such hydrocarbons in their study were employed as solvents for benzo[*a*]pyrene, which was introduced at concentration levels of 0.2 wt.%. However, accelerating activity was still found when the concentration of the carcinogen was reduced to 0.04 wt.%. Carcinogen concentration was found to be of lesser importance to tumor-accelerating activity than the nature of the hydrocarbon itself. The minimum normal hydrocarbon chain length for exhibiting accelerating activity appeared to be about *n*-decane (C_{10}).

Although the accelerating activity data reported above are of direct significance with respect to the hydrocarbon content of mineral oil, the presence of four-ring polynuclear aromatic hydrocarbons employed as carcinogens could not be demonstrated in either of the mineral oil samples. Even if such structures were present in the alumina fractions, they would have had to be well below 0.006 wt.%. Since the molar absorptivities of such condensed aromatic structures are greater than 10^4, they would have had to be present at levels less than 60 p.p.b. in order for them to go undetected in the UV scanning of the alumina fractions. The apparent absence of polynuclear aromatic hydrocarbons in this mineral oil is in agreement with data reported by Lijinsky *et al.* (16). In their screening of five mineral oils (USP grade), no polynuclear aromatic hydrocarbons could be detected down to a concentration of 1 p.p.b.

The identification of monobutyl phthalate and dibutyl phthalate in this mineral oil was somewhat unexpected and is of concern in view of the subtle toxicity studies carried out by Guess *et al.* (17). They evaluated butyl octyl phthalate and butyl decyl phthalate at 5 wt.% levels in tissue cultures and chick embryos. The butyl octyl phthalate stimulated growth in both human amnion cells and KB (Eagle–human cancer line) tissue cultures. However, when injected into the allantoic cavities of 9-day-old chick embryos, both toxicity and deformity effects were observed.

More recently, Singh *et al.* (18) reported on the teratogenicity of eight different phthalate esters in the rat by intraperitoneal injection at three concentration levels. Although fertility was not affected, such effects as resorptions, gross abnormalities, skeletal malformations, fetal death, or decreased fetal size were observed at one or more of the concentrations employed. At present, the effects of these compounds in humans are unknown.

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