# Metabolic Fate of Mineral Oil Adjuvants Using $^{14}$C-Labeled Tracers I: Mineral Oil

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**Abstract** □ This investigation was undertaken to determine the metabolic fate of injected mineral oil using *n*-hexadecane as the tracer. Female white rats and female squirrel monkeys were injected either subcutaneously or intramuscularly with an emulsion containing 1-$^{14}$C-*n*-hexadecane made with mineral oil and mannide monooleate. The amount of radioactivity remaining at the site of injection and the distribution of radioactivity in the major organs were determined at varying time periods up to 10 months after injection. One week after injection, 85–98% of the 1-$^{14}$C-*n*-hexadecane-labeled mineral oil remained at the site of injection. After 3 months, 50–60% of the mineral oil remained; and after 10 months, approximately 25–30% of the labeled mineral oil was still at the site of injection. The mineral oil tracer, which migrated from the site of injection, was readily incorporated into lipids. Aside from the site of injection, there did not appear to be any accumulation of the 1-$^{14}$C-*n*-hexadecane-labeled mineral oil in the major organs.

**Keyphrases** □ Mineral oil—metabolic fate □ 1-$^{14}$C-*n*-Hexadecane–mineral oil–mannide monooleate emulsion—distribution □ Radioactivity accumulation—labeled mineral oil injection, monkeys, rats □ Scintillometry—analysis □ TLC—analysis

Mineral oil emulsions are used in many bacterial and viral preparations in present experimental immunization programs. However, little is known concerning the metabolic fate of the mineral oil. Many observations, both present and in the past, show that mineral oil emulsions are not always well tolerated (1). The safety of mineral oil adjuvants has been questioned on the basis of data that indicate mineral oil alone or Freund's (2) complete or incomplete mineral oil adjuvant, given with or without added antigens, may cause or be associated with induction of plasma cell tumors, autoimmune reactions, or excessive formation of focal granulomata (3–6). Some of the questions that have arisen from these observations are whether these adverse effects are a function of an incapability of animals to mobilize and metabolize mineral oil, adverse cellular effects due to emulsifying agents, or the result of bacterial and viral biochemical action on the adjuvant preparation itself or at the site of injection.

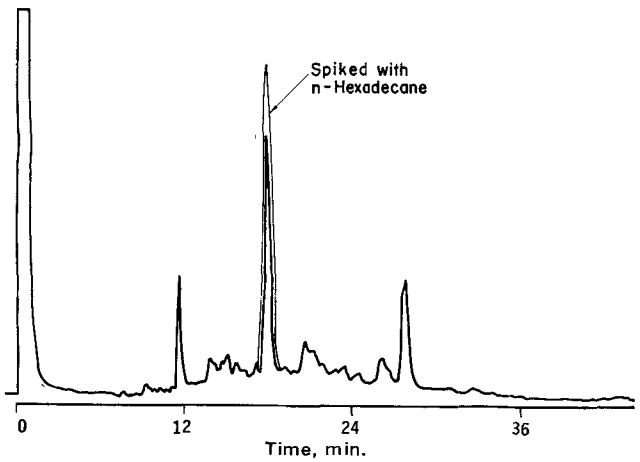


**Figure 1**—*Gas-liquid chromatograph of mineral oil. Material, mineral oil; column 45.72 m. (150 ft.) capillary coated with Apiezon L; and column temperature, 200°.*

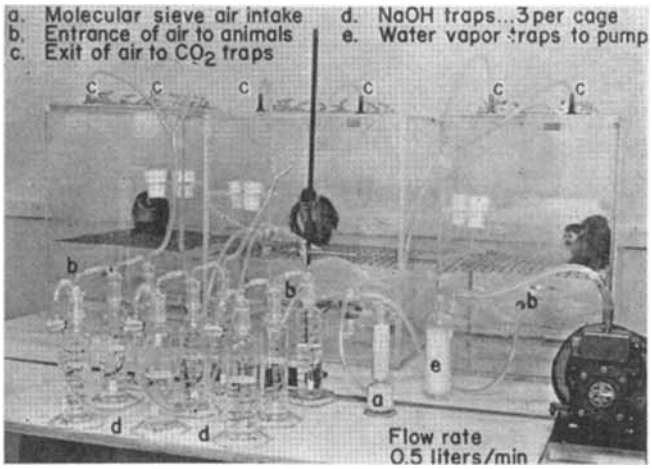


**Figure 2**—*Plexiglas metabolism cages designed for monkeys.*

Several investigators have attempted to answer some of these questions. Peck *et al.* (7) and Woodhour *et al.* (8) have substituted a vegetable oil for mineral oil in their adjuvant preparation. These investigators reported that the clinical, gross, and histomorphologic reactions were much less than were those obtained in comparable test with Freund's (2) incomplete mineral oil adjuvant. These data, however, could be interpreted to reflect that the ability to metabolize the vegetable adjuvant oils was a factor in lowering toxicity. However, since the extent of metabolism was not measured, no definite conclusion could be drawn. On the other hand, several investigators have shown that straight-chain hydrocarbons (C-16–C-18) are metabolized to fatty acids (9–11).

Investigations by Berlin (12) involving a nonionic emulsifier[1] indicated that the toxic reactions of mineral oil emulsions may be, in part or in whole, related to the level of ester hydrolysis and oxidative changes of the emulsifier. More recently, Hardegree and Pittman (13) have shown that the emulsified tetanus vaccines, which caused sterile abscesses in humans, contained free fatty acids and that a variety of antigens were capable of releasing free fatty acids from a water emulsion of mannide monooleate. At any rate, it would appear that emulsions containing vegetable oil instead of mineral oil would yield as high or higher free fatty acid levels with added antigens. Peck *et al.* (7), however, found less toxicity (guinea pig dermal irritation and mouse peritoneal irritation) with peanut oil than with mineral oil in influenza virus vaccine preparations.

[1] Arlacel A, Atlas Chemical Industries, Inc., Wilmington, DE 19899

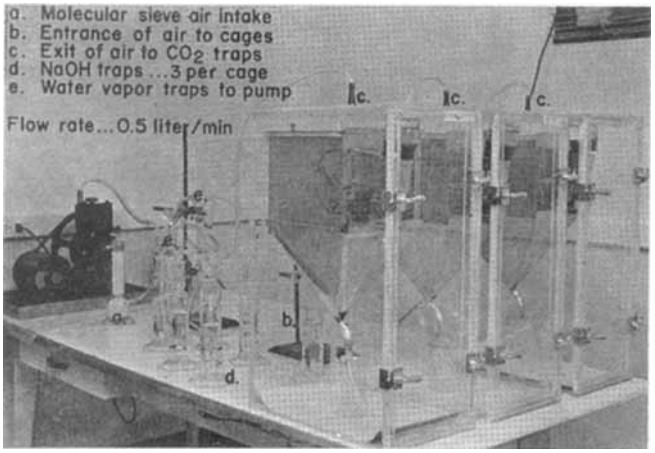


**Figure 3**—*Plexiglas metabolism cages designed for rats.*

It is the purpose of this study to determine the metabolic fate of the mineral oil in an injected adjuvant emulsion using a major mineral oil component, *n*-hexadecane, as the tracer.

## EXPERIMENTAL

**Adjuvant Components**—A gas-liquid chromatographic analysis of mineral oil[2] is shown in Fig. 1.

TLC analysis of the surfactant, mannide monooleate,[3] shows this material to contain at least 15 components. Identification of the various components has recently been accomplished by O'Neill and Yamauchi (14).

The emulsion used in this study was prepared by using 1 volume surfactant, 9 volumes mineral oil, and 9 volumes water. The resulting emulsions, prepared either by sonic vibration or the double-hubbed needle, double-syringe method, passed the suggested tests for stability (1). However, to eliminate any difficulties with emulsion stability in this investigation, all emulsions were administered within 30 min. after preparation.

**$^{14}$C-Labeled Tracers**—A representative $^{14}$C-labeled paraffin (*n*-hexadecane) was selected to serve as the $^{14}$C-tracer on the basis of its similarity to one of the major components of the mineral oil (Fig. 1), as well as its ability to serve as an effective adjuvant (15). Tritium-labeled materials were considered but are less desirable, since there always exists the possibility of tritium-exchange reactions which invariably complicate interpretation of experimental data (9).

The 1-$^{14}$C-*n*-hexadecane[4] used in this study had a specific activity of 2.35 mc./mmole. The radiochemical purity was determined with the aid of TLC and a liquid-scintillation spectrometer.[5] Hexane was used to develop and resolve the 1-$^{14}$C-*n*-hexadecane on thin-layer plates coated with 125 $\mu$ of silica gel.[6] Removal of a 3% impurity was accomplished by using a 5 × 0.5-cm. silicic acid column. The impurity remained on the column after elution of the 1-$^{14}$C-*n*-hexadecane with glass-distilled hexane. Subsequent analysis indicated that there was less than 0.001% radioactive impurity remaining in the 1-$^{14}$C-*n*-hexadecane tracer.

Emulsions containing the radioactive tracer were prepared by prior mixing of the 1-$^{14}$C-*n*-hexadecane tracer into the unlabeled mineral oil.

**Metabolism Studies**—Two types of experimental animals were used in this investigation: albino female rats[7] weighing approximately 250 g. and female squirrel monkeys[8] weighing 500–700 g. A total of 36 rats and 36 monkeys was used.

The dose was administered into the right rear thigh, either subcutaneously or intramuscularly. Prior to injection, several 1-$\mu$l. samples were removed from the $^{14}$C-labeled emulsion, and a check was made on the distribution uniformity of the tracer within the emulsion. The size of dose administered was 0.1 ml. (approximately 5 $\mu$c.) of the emulsion for rats and 0.3 ml. (approximately 15 $\mu$c.) for monkeys. The dose was administered with a 1-ml. disposable syringe equipped with a 23-gauge needle. A similar but unlabeled dose of the emulsion was injected into the left thigh. The control legs were examined by a veterinary pathologist during the study and immediately upon death.

After injection of the radioactive emulsions, the animals on 1-, 2-, and 7-day experimental periods were immediately housed in Plexiglas metabolism cages (Figs. 2 and 3). These cages were constructed in such a manner as to allow reasonable freedom of movement and easy access to water and food. The cages were opened for 15–20 min. every 24 hr. for cleaning and to replenish food and water.

In the long-term studies, 1, 2, 3, and 10 months, animals were housed in metal metabolism cages which allowed monitoring of urine and feces but not $CO_2$.

After the 1-, 2-, or 7-day experimental periods or the 1-, 2-, 3-, or 10-month experimental periods, the animals were killed by exsanguination following anesthetization with sodium pentobarbital. All animals were immediately examined by gross necropsy with particular emphasis placed on the site of injection of an unlabeled emulsion in the left, or control, leg.

To ensure complete removal of the site of injection for radioactivity determinations, entire thighs were excised. The thighs were then placed in a 600-ml. stainless steel beaker, frozen with liquid nitrogen, and shattered with a steel 5.08-cm. (2-in.) engine valve and a hammer. This technique produced a very fine powder out of the muscle, bone, and skin. The crushed legs were then placed into pint jars; 100 ml. of a 2:1 chloroform–methanol solution was added, and the mixture was homogenized with a high-speed blender.[9] After separation into two phases (methanol–water and chloroform), the two phases and the residue were analyzed for radioactivity.

A portion of the major organs of both monkeys and rats was removed for the determination of the radioactivity. Major organs examined were liver, depot fat, spleen, kidney, small intestine, ovary, lung, and, in some cases, the heart, blood, and brain. The inguinal lymph nodes of monkeys were also removed and assayed for radioactivity.

Leg extracts as well as some organ extractions were fractionated by TLC, and attempts were made to ascertain the nature of radioactivity present by the migration on thin-layer plates relative to known standard materials.

**Radioactivity Determinations**—Respired $^{14}CO_2$ was trapped in NaOH as $Na_2CO_3$ and then precipitated as $BaCO_3$. The $BaCO_3$ was

**Table I**—Percentage of $^{14}$C-Tracer Remaining in Monkeys and Rats at the Site of Injection after the Administration of Mineral Oil–Mannide Monooleate Emulsion Containing 1-$^{14}$C-Hexadecane as a Tracer

| Route and Time after Injection[a] | Average Percentage Remaining Monkeys, %[b] | | Rats, %[b] | |
|---|---|---|---|---|
| IM, 1 day | 3[c] | 99.0 | 3[c] | 91.2 |
| Sub Q, 1 day | 3 | 96.8 | 3 | 100.0 |
| IM, 2 days | 3 | 98.2 | 3 | 86.6 |
| Sub Q, 2 days | 2 | 91.5 | 3 | 85.2 |
| IM, 7 days | 3 | 98.8 | 3 | 85.2 |
| Sub Q, 7 days | 3 | 96.1 | 3 | 89.5 |
| IM, 1 month | 2 | 63.9 | 2 | 75.4 |
| Sub Q, 1 month | 2 | 66.9 | 2 | 67.3 |
| IM, 2 months | 3 | 77.2 | 2 | 77.9 |
| Sub Q, 2 months | 2 | 81.7 | 2 | 40.0 |
| IM, 3 months | 2 | 65.9 | 2 | 57.9 |
| Sub Q, 3 months | 2 | 61.8 | 2 | 49.4 |
| IM, 7 months | 1 | 22.7 | — | — |
| IM, 10 months | 4 | 29.1 | 6 | 27.1 |

[a] IM, intramuscular, and Sub Q, subcutaneous injections. [b] Average percentage based on the total amount recovered and the total amount administered. [c] Number of animals.

[2] Drakeol 6-VR, Pennsylvania Refining Co., Butler, Pa.
[3] Hilltop Laboratories, Inc., Cincinnati, Ohio.
[4] Mallinckrodt Nuclear, St. Louis, MO 63160
[5] Packard Instrument Co, Inc.
[6] Silica gel F254 (Brinkmann), silica gel G (Analabs).
[7] Simonsen Laboratories, Calif.
[8] Charles Chase Corp., Fla.
[9] Omni-Mixer, Ser Vall.

**Table II**—Thin-Layer Chromatographic Separation and Liquid-Scintillation Counting of the Radioactive Material Remaining at the Site of Injection after 3 and 10 Months

| TLC[a] Fraction | TLC Area | 3 Months | | | | 10 Months, |
|---|---|---|---|---|---|---|
| | | IM[b] Rats | IM Monkeys | Sub Q Rats | Sub Q Monkeys | IM Monkeys |
| | | Percentage Values[c] | | | | |
| 1 | Phospholipid | 1.6 | 0.2 | 2.6 | 0.1 | 0.4 |
| 2 | Free sterol | 0.4 | 0.0 | 0.4 | 0.0 | 0.0 |
| 3 | Free fatty acid | 0.6 | 1.0 | 1.5 | 0.2 | 0.0 |
| 4 | Triglyceride | 3.8 | 0.6 | 3.3 | 0.3 | 0.0 |
| 5 | Sterol ester | 0.8 | 0.3 | 1.0 | 0.2 | 4.0 |
| 6 | Hydrocarbon | 92.7 | 97.6 | 91.0 | 98.8 | 95.7 |

[a] Pooled leg extracts were applied to silica gel-coated TLC plates and developed first with hexane (to resolve hydrocarbons) and then with 90:10:1 hexane–diethyl ether–acetic acid to resolve lipids. [b] IM, intramuscular injection and Sub Q, subcutaneous injection, of the right thigh. [c] Average percentage of duplicate determinations of the radioactivity recovered from thin-layer chromatographic plates.

**Table III**—Average Level of Radioactivity in the Major Organs of Monkeys after the Administration of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-$^{14}$C-*n*-Hexadecane as a Tracer

| Time after Injection | Number of Animals | Average Counts/min./100 mg. Wet Tissue | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Liver | Fat | Spleen | Kidney | Small Intestine | Ovary | Lung | Lymph |
| 1 day | 6 | 142 | 54 | 18 | 11 | 26 | 16 | 21 | 13 |
| 2 days | 6 | 695 | 472 | 40 | 67 | 88 | 31 | 49 | 27 |
| 7 days | 6 | 1232 | 1701 | 161 | 183 | 130 | 112 | 204 | 46 |
| 1 month | 4 | 316 | 2606 | 222 | 397 | 135 | 137 | 324 | 80 |
| 2 months | 5 | 49 | 1949 | 59 | 77 | 72 | 71 | 41 | 181 |
| 3 months | 4 | 144 | 1596 | 98 | 147 | 68 | 52 | 123 | 20 |
| 10 months | 4 | 9 | 462 | 15 | 15 | 13 | 16 | 12 | 28 |

**Table IV**—Average Level of Radioactivity in the Major Organs of Rats after the Administration of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-$^{14}$C-*n*-Hexadecane as a Tracer

| Time after Injection | Number of Animals | Average Counts/min./100 mg. Wet Tissue | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Liver | Fat | Spleen | Kidney | Small Intestine | Ovary | Lung |
| 1 day | 6 | 34 | 16 | 10 | 14 | 8 | 5 | 14 |
| 2 days | 6 | 145 | 92 | 56 | 46 | 74 | 46 | 47 |
| 7 days | 6 | 132 | 365 | 60 | 69 | 53 | 73 | 57 |
| 1 month | 4 | 87 | 555 | 54 | 114 | 119 | 45 | 24 |
| 2 months | 4 | 30 | 717 | 25 | 36 | 23 | 109 | 14 |
| 3 months | 4 | 82 | 2263 | 40 | 103 | 28 | 82 | 36 |
| 10 months | 6 | 10 | 647 | 5 | 9 | 12 | 26 | 8 |

removed from the aqueous phase by filtration and dried in an oven at 120° for a minimum of 6 hr. After $BaCO_3$ dried, it was pulverized to a fine powder. Approximately 50-mg. samples were removed and suspended in a scintillation-gel (16) for radioactivity determination. The liquid-scintillation counting efficiency under the study conditions was 86%. Total radioactivity removed *via* respiratory $^{14}CO_2$ was then calculated from the total $CO_2$ collected over 24-hr. experimental periods.

Urine and feces collected over 24-hr. experimental periods for the 1st week after administration were also assayed for radioactivity. The amount of radioactivity in the feces was determined by a perchloric acid–hydrogen peroxide digestion of 100-mg. samples (17). Radioactive urine was determined by using Bray's method (18). One milliliter of urine was counted, and the total activity eliminated *via* the urine was calculated from total urine volumes.

Radioactivity in the major organs, including blood and feces, was determined by the method of Mahin and Lofberg (17). This method involves perchloric acid digestion of 100-mg. wet tissue samples (0.2 ml. blood). The color is removed from the digested samples with hydrogen peroxide. These samples were then taken up in 20 ml. toluene and ethylene glycol–monoethyl ether (Cellosolve), 2:1, containing the scintillator PPO (6 g./l. counting solution) and counted. Counting efficiency in this system was about 53%.

The fractions obtained from serial scraping of thin-layer chromatographic plates were assayed for radioactivity in the gel-scintillation system as described for $Ba^{14}CO_3$ determinations. Serial scrapings were obtained by the aid of mineral oil and lipid standards[10] and $I_2$ vapor visualization.

[10] Applied Science Laboratories, State College, PA 16801

## RESULTS

The amount of 1-$^{14}$C-*n*-hexadecane remaining at the site of injection from 1 to 10 months after administration is summarized in Table I. In general, the mineral oil was slowly removed from the site of injection. In monkeys, 96–98% was still present after 1 week, while in rats the amount remaining at the site of injection ranged from 85 to 90% after 1 week. After 1 month, approximately 65–75% remained, and by 3 months both groups of animals had retained 50–60% of the total radioactivity at the site of injection. Ten months after injection, approximately 25–30% was still at the site of injection.

Several mathematical equations (linear and semilogarithmic derived equations) were prepared to predict the length of time required to remove all of the oil from the site of injection. However, since there were too few animals at each time point as well as a considerable amount of scatter of the data, it was found that a confident mathematical prediction could not be made.

Virtually all (95–99%) of the activity remaining at the site of injection in monkeys after 3 and 10 months was present in the hydrocarbon fraction (Table II). On the other hand, 3–4% of the radioactivity found in the legs of rats had been incorporated into triglycerides with 91–93% still associated with the hydrocarbon fraction. A reason for this difference was that the rats had a larger amount of depot fat (triglycerides) in the thigh area than did the monkeys. In both animal groups, a certain amount of radioactivity (1–3%) also was associated with the phospholipids of the leg muscles. McCarthy (11) demonstrated that C-16 and C-18 hydrocarbons were converted *in vivo* into fatty acids of the same chain lengths, suggesting that the incorporation of radioactivity into the various leg lipid fractions was the result of 1-$^{14}$C-*n*-hexadecane

Table V—Distribution of Radioactivity from 1-$^{14}$C-*n*-Hexadecane Tracer in Major Lipid Classes of Monkey Tissue 2 Days after Administration

| Lipid Classes | Organs | | | | | |
|---|---|---|---|---|---|---|
| | Liver, %[a] | Fat, % | Kidney, % | Spleen, % | Ovaries, % | Leg, % |
| Phospholipids | 19.6 | 0 | 10.6 | 0 | 0 | 0 |
| Free fatty acids | 45.6 | 0 | 7.4 | 18.6 | 10.0 | 0 |
| Triglycerides | 4.4 | 57.7 | 7.4 | 0 | 0 | 0 |
| Hydrocarbons | 30.5 | 42.3 | 74.6 | 81.4 | 90.0 | 100.0 |
| Radioactivity, average c.p.m./100 mg. tissue | 525.0 | 831.0 | 58.0 | 30.0 | 39.0 | |

[a] These percents were obtained from serial scraping of TLC and based on the total radioactivity applied to the plates.

Table VI—Distribution of Radioactivity within the Various Lipid Classes of Organs of Monkeys 1 and 3 Months after Administration of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-$^{14}$C-*n*-Hexadecane as a Tracer

| TLC Fraction[b] | Tissues[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Liver, % | Fat, % | Spleen, % | Kidney, % | Intestine, % | Ovary, % | Lung, % | Brain, % | Lymph, % |
| **1 Month** | | | | | | | | | |
| Phospholipids | 6.0 | 0.5 | 14.3 | 15.1 | 1.1 | 0.0 | 15.0 | 13.8 | 0.0 |
| Free sterols | 24.1 | 10.9 | 42.9 | 11.5 | 7.9 | 16.4 | 30.7 | 31.3 | 0.0 |
| Free fatty acids | 7.5 | 50.0 | 30.0 | 20.9 | 0.0 | 32.9 | 17.3 | 15.6 | 0.0 |
| Triglycerides | 32.3 | 23.5 | 5.7 | 5.0 | 9.6 | 27.4 | 5.5 | 20.6 | 18.2 |
| Sterol esters | 26.3 | 11.5 | 0.0 | 30.9 | 72.5 | 0.0 | 12.6 | 13.8 | 72.8 |
| Hydrocarbon | 3.8 | 3.7 | 7.2 | 13.7 | 9.0 | 23.3 | 18.9 | 5.0 | 9.1 |
| **3 Months** | | | | | | | | | |
| Phospholipids | 59.3 | 0.9 | 76.6 | 70.5 | 73.0 | 68.6 | 88.6 | 93.2 | |
| Free sterols and free fatty acids | 22.0 | 0.0 | 16.2 | 12.9 | 15.2 | 17.2 | 2.5 | 0.0 | |
| Triglycerides | 13.6 | 96.4 | 4.6 | 11.8 | 6.1 | 0.0 | 3.3 | 0.0 | |
| Sterol esters | 3.4 | 0.0 | 1.2 | 2.7 | 1.5 | 10.9 | 4.9 | 0.0 | |
| Hydrocarbons | 1.7 | 2.7 | 1.2 | 2.2 | 4.6 | 3.1 | 1.0 | 6.7 | |

[a] Pooled tissue extracts. [b] Serial scraping from TLC. Silica gel plates developed with hexane initially, then with hexane–diethyl ether–acetic acid (90:10:1) system.

Table VII—Elimination of Respiratory $^{14}CO_2$ for 1 Week after the Injection of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-$^{14}$C-*n*-Hexadecane

| Animal | Route of Injection | Days after Injection | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Total |
| | | **Percentage Values[a]** | | | | | | | |
| Monkeys | IM | 0.01(9)[b] | 0.16(6) | 0.31(3) | 0.33(3) | 0.25(3) | 0.19(3) | 0.28(3) | 1.53(3) |
| | Sub Q | 0.01(6) | 0.16(5) | 0.24(2) | 0.28(2) | 0.37(2) | 0.33(2) | 0.25(3) | 1.66(3) |
| Rats | IM | 0.05(9) | 0.18(6) | 0.07(3) | 0.10(3) | 0.08(3) | 0.12(3) | 0.10(3) | 0.55(3) |
| | Sub Q | 0.12(9) | 0.11(6) | 0.13(3) | 0.32(3) | 0.22(3) | 0.14(3) | 0.17(3) | 1.20(3) |

[a] Each percentage value represents an average percent of the total radioactive dose eliminated as $^{14}CO_2$ over 24-hr. periods. [b] Numbers in parentheses represent the number of individual determinations.

mobilization to the liver, where it was converted to fatty acids and returned in the form of various lipid classes to leg muscle tissue.

Tables III and IV show the average level of radioactivity found in the major organs of monkeys and rats, respectively, after the administration of a mineral oil mannide monooleate emulsion containing 1-$^{14}$C-*n*-hexadecane tracer. After the first 24 hr., all organs tested contained radioactivity. The liver had the highest activity of any organ 24 hr. after injection. Two days after injection of the emulsion containing 1-$^{14}$C-*n*-hexadecane, the level of radioactivity in the liver increased four- to fivefold, as did the level in the depot fat. The radioactivity in all other organs increased to varying degrees. Radioactivity in the depot fat of both animals continued to increase until it became about fourfold over that amount present after 2 days. After 1 month, the amount of radioactivity in the liver of both monkeys and rats decreased; after 2 months, it had returned to those levels recorded for the first 24-hr. period after injection. The specific activity of the depot fat remained high throughout the 3-month period. After 10 months, the radioactivity in the depot fat had decreased to about one-fourth the level measured at 3 months. However, the level of radioactivity in the depot fat was still 50- to 100-fold greater than any other organ. Other than the liver and depot fat, no organ appeared to accumulate large amounts of radioactivity. Most of the detectable radioactivity in the various organs resided in the fat-soluble fraction, with little or none appearing in the water-soluble and residue fractions.

Table V shows the distribution of radioactivity in chloroform–methanol extracts of various organs taken from monkeys 2 days after the administration of an emulsion containing 1-$^{14}$C-*n*-hexadecane. These data indicate that a substantial portion of the radioactivity in the liver (30%), fat (42%), kidney (74%), spleen (81%), and ovary (90%) was unmetabolized 1-$^{14}$C-*n*-hexadecane tracer. The remaining radioactivity was located in the various lipid classes such as phospholipids, free fatty acids, and triglycerides, indicating that hexadecane was undergoing metabolism.

By 1 month, however, the amount of radioactivity in the various organs associated with 1-$^{14}$C-*n*-hexadecane was greatly reduced, and most of the radioactivity (80–95%) was associated with the major lipid classes. After 3 months the major lipid classes contained as much as 95–98% of the radioactivity (Table VI).

Only a small amount of radioactivity from 1-$^{14}$C-*n*-hexadecane was eliminated the 1st week in the respiratory $CO_2$ by either monkeys or rats (Table VII). The amount eliminated per day varied considerably among animals, but it was usually less than 0.2% and, in many cases, was as little as 0.01%. As a result, the average total amount

eliminated by respiratory $^{14}CO_2$ the 1st week ranged from 0.5 to 2.0%. The major reason for these low levels was that this material was only slowly mobilized from the site of injection, and its conversion to fatty acids and subsequent $CO_2$ was limited by the rate of mobilization. There was little or no difference in the rate of mobilization from subcutaneous and intramuscular injections.

Some radioactivity from 1-$^{14}$C-*n*-hexadecane was eliminated in the urine and feces during the 1st week after administration; however, in both groups of animals, the total amount for the entire week was less than 0.01%.

All animals received an unlabeled dose of the emulsion in the opposite leg. Upon death, each of the control legs was immediately examined by gross necropsy by a veterinary pathologist. At no time was there any indication of any type of infection or a sterile abscess due to the injection.

## SUMMARY

This investigation has shown that the *n*-hexadecane component and most likely other straight-chain hydrocarbon components of a mineral oil adjuvant emulsion are very slowly mobilized from the site of injection in monkeys and rats. Results have been presented which show that as much as 25–30% of the $^{14}$C-*n*-hexadecane tracer remains at the site of injection after 10 months. The *n*-hexadecane tracer, which is mobilized from the site of injection, was readily metabolized to naturally occurring lipids.

Gross necropsy did not reveal any evidence of any pathological states at the sites of injection.

## REFERENCES

(1) Department of Health, Education, and Welfare, Food and Drug Administration, *Ann. Allergy*, **23,** 558(1965).

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

(3) M. Potter and C. L. Robertson, *J. Nat. Cancer Inst.*, **28,** 847(1960).

(4) R. Lieberman, N. Mantel, and W. Humphrey, Jr., *Proc. Soc. Exp. Biol. Med.*, **107,** 163(1961).

(5) S. Levine and E. J. Wenk, *ibid.*, **113,** 898(1963).

(6) J. W. Steiner, B. Langer, and D. L. Schatz, *Arch. Pathol.*, **70,** 424(1960).

(7) H. M. Peck, A. F. Woodhour, T. B. Stein, S. E. McKinney, and M. R. Hilleman, *Proc. Soc. Exp. Biol. Med.*, **116,** 23(1964).

(8) A. F. Woodhour, D. P. Metzgar, T. B. Stein, A. A. Tytell, and M. R. Hilleman, *ibid.*, **116,** 516(1964).

(9) A. G. Ebert, C. R. Schleifer, and S. M. Hess, *J. Pharm. Sci.*, **55,** 923(1966).

(10) M. P. Mitchell and G. Hubscher, *Biochem. J.*, **103,** 23(1967).

(11) R. D. McCarthy, *Biochim. Biophys. Acta*, **84,** 74(1964).

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

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

(14) H. J. O'Neill and T. N. Yamauchi, *J. Amer. Oil Chem. Soc.*, **46,** 99A(1969).

(15) A. J. Crowle and C. C. Hu, *Proc. Soc. Exp. Biol. Med.*, **123,** 94(1966).

(16) J. N. Bollinger, W. A. Mallow, J. W. Register, Jr., and D. E. Johnson, *Anal. Chem.*, **19,** 1508(1967).

(17) D. T. Mahin and R. T. Lofberg, *Anal. Biochem.*, **16,** 500 (1966).

(18) G. A. Bray, *ibid.*, **1,** 279(1960).

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# Metabolic Fate of Mineral Oil Adjuvants Using $^{14}$C-Labeled Tracers II: Mannide Monooleate

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**Abstract** □ This investigation was undertaken to determine the metabolic fate of mannide monooleate when employed in a mineral oil emulsion. Female white rats and female squirrel monkeys were injected subcutaneously or intramuscularly with an emulsion made with mineral oil and surfactant and including either 1-$^{14}$C-oleate or UL-$^{14}$C-mannide-labeled mannide monooleate tracer preparations. It was shown that 30–40% of the surfactant mixture is removed from the site of injection after 24 hr. After 1 week, 40–60% of the surfactant is removed from the site of injection; while after 3 months, 10–30% of the surfactant still remains. The 1-$^{14}$C-oleate-labeled mannide monooleate was largely incorporated into the various lipid classes, while the UL-$^{14}$C-mannide-labeled mannide monooleate preparation was largely eliminated in the urine. There was some indication that the inguinal lymph nodes of monkeys may have contained unusually large amounts of radioactivity.

**Keyphrases** □ 1-$^{14}$C-Oleate-labeled mannide monooleate—metabolic fate □ UL-$^{14}$C-Mannide-labeled mannide monooleate—metabolic fate □ Mineral oil–mannide monooleate emulsion—subcutaneous, intramuscular injection □ Metabolic fate—mannide monooleate in mineral oil emulsion □ TLC—identity

Freund (1) found that "mannide monooleate"[1] was an effective agent in combining antigens and mineral oils in the form of a water and oil emulsion. However, subsequent use has indicated an infrequent occurrence of cysts following injection of vaccines and allergens (2) made with certain lots of this surfactant. Berlin (3) performed studies which showed that the toxic reactions of a mineral oil–"mannide monooleate" emulsion may be, in part or in whole, related to the level of ester hydrolysis and oxidative changes of this material. Recently, Hardegree and Pittman (4) have shown that the tetanus vaccines which caused sterile abscesses in humans contained free fatty acids and that a variety of these antigens were capable of releasing free fatty acids from a water emulsion of "mannide monooleate."

This study was undertaken to obtain information on the mobilization and metabolism of a mannide monooleate surfactant preparation when it is received as an injected mineral oil emulsion.

[1] Arlacel A, Atlas Chemical Industries, Inc., Wilmington, DE 19899. Quotation marks in text of article indicate the impure mixture; absence of quotation marks in text of article indicates pure mannide monooleate.