glycerol-C^{14} into lymph triglycerides of man has been demonstrated(24). It is also known that intragastric administration of glycerol increases the *in vivo* esterification of fatty acids by hamster intestinal mucosa(12).

Summary. Single oral doses of fructose and glycerol administered to normoglyceridemic human subjects after a standard fat meal increase the postprandial hyperglyceridemia while glucose given in a similar way decreases the serum triglyceride response. It is tentatively suggested that the effect of fructose and glycerol is due to a stimulation of intestinal synthesis of triglycerides from endogenous fatty acids. The opposite effect of glucose might be attributed to an accelerated elimination of triglycerides from the blood possibly mediated by insulin.

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A Comparison of *n*-Hexadecane and Mineral Oil Emulsions in Induction of Hypersensitivity in Mice.* (31412)

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The adjuvant action of Freund-type water-in-oil(w/o) emulsions for the immunogenicity of antigens is imperfectly understood. It has been attributed to the various performances of emulsion constituents acting singly as well as to their collective effect in determining the characteristics of the entire emulsion(1,2). Mineral oils are superior to animal and vegetable oils in constituting effective Freund emulsions, probably because they are more poorly catabolized than animal and vegetable oils. Shaw *et al*(1) compared the adjuvant activity of various pure constituents of mineral oils in inducing experimental allergic encephalomyelitis in guinea pigs. They found C_{15-20} alkanes to be better than those of either shorter or longer carbon chains. Thus, they showed that crude oils of variable and complex composition can be replaced by such pure chemicals as *n*-hexadecane, a C_{16} alkane.

We have used *n*-hexadecane with excellent results in our experiments on mouse delayed

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hypersensitivity for several years(3), having selected it originally to reduce experimental variables, and because Hoyt *et al*(4) had shown it to have adjuvant activity. Although on the whole we have found it and light mineral oil comparably effective in inducing hypersensitivity, we have learned recently, as we report here, that under some circumstances *n*-hexadecane is superior.

Materials and methods. CF_1 female mice, used in groups of 10 and fed Rockland mouse food and water, were sensitized and skin-tested with crystalline human serum albumin (HSA)† as described previously(3,5). Briefly, these procedures as applied in the present experiments consist of injecting mice once subcutaneously with a 0.25 mg quantity of HSA in 0.1 ml of w/o emulsion not containing mycobacteria and then skin-testing these animals and appropriate control mice 3, 5, and 7 weeks later with 20 μg of the same protein in 0.02 ml of physiologic phosphate buffer injected intradermally in alternating flanks on successive test dates. Our results are expressed as percent positive for both the edema of Arthus reactions read at 3 hours after testing and the induration of delayed hypersensitivity reactions read at 24 hours after testing(5).

Emulsion constituents employed were mannide monooleate (Arlacel),‡ glycerol monooleate (Myverol),§ n-hexadecane,¶ light mineral oil, U.S.P., and physiologic phosphate buffer of pH 7.4.

Experiments and results. The experiment summarized in Table I compared the sensitizing effectiveness of a single injection of antigen in an Arlacel-oil emulsion, of composition similar to that commonly used by other investigators, with that of antigen in 2 different mixtures of Myverol-hexadecane emulsion. One of these (1:4:10) was only moderately viscous; the other (2:3:10) was of paste-like stiffness. As the results show, the Arlacel-oil emulsion was inferior to both of the hexadecane emulsions in inducing either Arthus or delayed hypersensitivity. There was some delay in the emergence of delayed hypersensitivity in mice receiving the paste-like hexadecane emulsion, but by 7 weeks similar proportions in both hexadecane emulsion groups had developed immediate and delayed hypersensitivities.

TABLE I. Hypersensitivity* in Mice Vaccinated with Arlacel-Oil and Myverol-Hexadecane w/o Emulsions.

Group No.	HSA injected in	3 wk I†	3 wk D	7 wk I	7 wk D
1	No injection	0	0	0	0
2	A:O:B = 2:10: 5	0	0	10	0
3	M:H:B = 1: 4:10	60	30	100	70
4	M:H:B = 2: 3:10‡	70	0	100	60

* Expressed as % reacting, 10 mice per group. In this and the following tables the letters A, M, O, H, and B are used to represent Arlacel, Myverol, oil, hexadecane, and buffer, respectively.

† I = "immediate" (Arthus) and D = "delayed" hypersensitivities.

‡ This emulsion was a paste, whereas that used in group 3 was a viscous liquid.

Findings from this experiment did not indicate whether the inferiority of the Arlacel-oil emulsion was due to its ingredients or to its proportional composition. This question was examined in another experiment with results presented in Table II. From these it is evident that the 2:10:5 mixture of Arlacel, oil, and buffer used in the first experiment not only is inferior to emulsions employing hexadecane, but also that using it in a double-injection schedule with injections given 1 week apart does not enable it to sensitize mice as well as a single injection of the 1:4:10 mixture of Myverol, hexadecane and buffer

TABLE II. Hypersensitivity Induced in Mice by Vaccination with Emulsions of Various Composition.

Group No.	HSA injected in	3 wk I	3 wk D	7 wk I	7 wk D
1	No injection	0	0	0	0
2	A:O:B = 2:10:5	20	0	66	12
3	A:O:B = 2:10:5 (×2)*	70	10	78	10
4	A:H:B = 2:10:5	40	20	90	50
5	A:O:B = 1:4:10	10	0	66	0
6	M:O:B = 1:4:10	50	0	30	10
7	M:H:B = 1:4:10	88	50	88	74

* Two vaccinations given 1 wk apart instead of only a single one.

† A product of Pentex, Incorporated, Kankakee, Ill.

‡ "Specially Treated Arlacel A", Atlas Powder Co., Wilmington, Del.

§ Myverol Type 18-71-E, a gift of Distillation Products Industries, Rochester, N. Y.

¶ Practical grade, purchased from Distillation Products Industries, Rochester, N. Y.

TABLE III. Anamnestic Hypersensitization of Mice with Various Emulsion Mixtures.

Group No.	HSA injected in	Primary 7 wk I	Primary 7 wk D	Secondary* 1 wk I	Secondary* 1 wk D	Secondary* 3 wk I	Secondary* 3 wk D
1	No injection	0	0	20	0	10	0
2	M:H:B = 1:4:10	78	44	63	63	100	100
3	M:O:B = 1:4:10	20	0	100	30	100	100
4	A:H:B = 1:4:10	80	20	80	50	100	78
5	A:O:B = 1:4:10	30	0	100	70	90	30

* Given 8 wk after primary injection. For each group the emulsions used for first and second injections were the same.

(compare Groups 3 and 7). But replacing oil with hexadecane in this type of emulsion, while maintaining the proportions of emulsifier, "oil", and buffer the same (Group 4), increased its adjuvant activity; changing its ingredient proportions from oil-rich (2:10:5) to oil-poor (1:4:10) had no effect (Group 5). Hence, within the range of variations being studied, the nature of the oil is more important than either the nature of the emulsifier or the proportions in which the various constituents are mixed. This finding is corroborated by the relative sensitizing efficacies of emulsion used in Groups 5 and 6 as compared with that in Group 7. In all 3 the proportions of ingredients were the same; Arlacel was used as surfactant in 5 and Myverol in 6, and in both the oil was mineral oil. In neither was hypersensitization as good as in Group 7 mice receiving hexadecane in place of mineral oil; indeed, neither emulsion induced delayed hypersensitivity in a significant number of mice, whereas the emulsion constituted with hexadecane sensitized 74% of the mice as determined by the 7-week skin-testing.

Confirmatory experiments were performed, and in some of these the effect of reinjecting the antigen several weeks later in the same emulsion was examined to determine whether the distinctions revealed above between mineral oil and hexadecane emulsions might also extend to eliciting anamnestic responses. Results from one of these experiments are presented in Table III. Once again, the superiority of hexadecane-containing adjuvant for inducing primary immediate and delayed hypersensitivity is evident (7-week skin-testing). But also evident is that this distinction is lost for eliciting an anamnestic response. The slightly poorer response in mice of Group 5 as indicated by the 3-week post-boosting testing probably was an experimental anomaly, since it has not been seen in later experiments.

In all of the above experiments incomplete adjuvant was employed, that is, no mycobacteria were used. Part of the experiment reported in Table I was performed to determine whether adding mycobacteria to the oil-containing adjuvant emulsions would cancel their relative ineffectiveness, but as results in Table IV indicate this apparently does not occur.

Discussion. These observations not only confirm previous reports(1,3,7) of the utility of hexadecane as a simple, chemically defined, and effective substitute for mineral oil in Freund adjuvants, they indicate also that in some experimental situations hexadecane constitutes a more effective adjuvant than mineral oil. The difference is rather small and possibly quantitative, because it can be overridden by utilizing a 2-injection sensitization protocol in which the second injection is given after anamnestic responsiveness has had time to develop, but it is nonetheless significant: in single-vaccination experiments it can

TABLE IV. Hypersensitivity in Mice Vaccinated with Arlacel-Oil and Myverol-Hexadecane w/o Emulsions Containing Mycobacteria.*

Group No.	HSA injected in	3 wk I	3 wk D	7 wk I	7 wk D
1	No injection	0	0	0	0
2	A:O:B = 2:10:5+T.B.	0	0	50	10
3	M:H:B = 1:4:10+T.B.	0	0	80	80
4	M:H:B = 2:3:10+T.B.	50	38	100	88

* *Mycobacterium tuberculosis*, avirulent strain H37Ra, used at 0.25 mg dry wt per injection.

determine whether or not hypersensitivity will develop.

The present results provide no explanation for the superiority of hexadecane over mineral oil, but one might speculate from the data of Shaw *et al*(1) that most of the ingredients of mineral oil have little or no adjuvant activity (*e.g.*, saturated hydrocarbons with chains of either less than 15 carbons or more than 24), and that these inert ingredients interfere passively with the adjuvant effect of active constituents of the oil, whereas a pure and effective hydrocarbon like hexadecane has no such contaminants to dilute or impair its adjuvancy.

Glycerol monooleate (Myverol) has not been used very often for preparing Freund emulsions to be used in animal experiments (5-7); the emulsifier most commonly used has been mannide monooleate. Our original use of glycerol monooleate was prompted by its property of forming a stiff gel instantaneously when mixed with water; thus, we could study antigen depot effects in induction of immune responses with or without the added presence of oil. Since that time we have adopted this chemical for routine use as an emulsifier replacing mannide monooleate, because our experience has been that it forms emulsions more readily and stably under a wider variety of conditions, and that has been confirmed partially by Prigal(8). But as the present experiments indicate, and as we have reported previously(5), we have not found glycerol monooleate-constituted emulsions either better or worse as adjuvants than those made with mannide monooleate.

Many different proportions of emulsifier, oil, and aqueous phase can be used to prepare w/o emulsions, and we have tried several. Although our experience with these does not match that of Berlin(9), our findings have been essentially the same as his as suggested, for example, by results from Table I, Group 4. So long as the emulsion is one of water-in-oil, emulsion viscosity seems to be a minor factor in its ultimate adjuvant effectiveness.

The distinct disadvantage, shown here, of using mineral oil rather than hexadecane in making emulsions for inducing delayed hypersensitivity in mice may account for some of the failures as reported in the literature to elicit such hypersensitivity in this species, since w/o emulsions so far used by other workers (*e.g.*, 10) have been made with mineral oil.

Summary. The pure alkane *n*-hexadecane not only is a useful substitute for crude mineral oils as a constituent of Freund water-in-oil adjuvant mixtures used to sensitize mice to protein antigen, but also it provides a stronger adjuvant effect. This superiority appears to be quantitative, for it becomes negligible when a two-injection immunization schedule is used with allowance of adequate time between injections for anamnestic responsiveness to develop. Our results suggest that immunization with water-in-oil emulsions can be improved if the mineral oils usually used in these emulsions are replaced by pure saturated hydrocarbons of appropriate carbon chain length, such as *n*-hexadecane. Replacing the commonly used emulsifier, mannide monooleate (Arlacel) with glycerol monooleate (Myverol) increased the ease of emulsification but had no influence on the immunologic effectiveness of the emulsions studied.

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