Immunology 1981 **44** 187

The adjuvant activity of fatty acid esters. The role of acyl chain length and degree of saturation

R. BOMFORD *Department of Experimental Immunobiology, the Wellcome Research Laboratories, Beckenham, Kent*

Accepted for publication 9 April 1981

Summary. Water-in-oil emulsions of metabolizable fatty acid esters, with the non-toxic surfactant Pluronic L122 as emulsifying agent, potentiated the humoral response to bovine serum albumin and staphylococcal toxoid in the mouse. Adjuvant activity was increased by changing the chemical nature of the esters as follows: (i) using a series of ethyl esters, adjuvant activity appeared when the acyl chain length of the fatty acid component was 16 or greater; (ii) isobutyl and isopropyl esters of palmitic acid (C16:0) were superior to ethyl; (iii) the ethyl esters of oleic (C18:1) and linoleic (C18:2) acids were better than stearic (C18:0). Since emulsions prepared with longer chain saturated esters are very viscous or solid at room temperature, and unsaturated esters are chemically reactive, emulsions were prepared with differing proportions of ethyl caprate (C10:0) and butyl stearate. At a ratio of 9:1 the emulsions possessed the low viscosity of ethyl caprate, but gained the adjuvant activity of butyl stearate. ^{125}I-labelled BSA was retained in the footpad to a significantly greater extent than with a caprate emulsion, but reasons are given for believing that slow release of antigen is not the only mechanism of adjuvant activity. The ester emulsions caused more acute but less chronic local inflammation (footpad swelling) than Freund's incomplete adjuvant.

Correspondence: Dr R. Bomford, Department of Experimental Immunobiology, The Wellcome Research Laboratories, Beckenham, Kent.

0019-2805/81/0900-0187$02.00

INTRODUCTION

The incorporation of antigens into the aqueous phase of a water-in-mineral oil emulsion as a means of immunopotentiation was introduced by Freund (reviewed in Freund, 1956). Subsequently various alternatives to the mineral oil have been tested, usually with the objective of reducing the inflammatory response. These include straight chain (Shaw, Alvord & Kies, 1964) or branched (Wilner, Evers, Troutman, Trader & McLean, 1963) hydrocarbons, plant oils (Hilleman, 1966; Holt, 1967; Audibert & Chedid, 1975; Reynolds, Harrington, Crabbs, Peters & Di Luzio, 1980), squalene and fatty acid esters (Holt, 1967; Whitehouse, Orr, Beck & Pearson, 1974).

The present paper is concerned with the adjuvant properties of stable water-in-oil (w/o) emulsions of fatty acid esters prepared with the non-ionic surfactant Pluronic L122, a block co-polymer of polyoxyethylene and polyoxypropylene, as emulsifying agent. It was hoped that this system would provide good immunopotentiation without chronic inflammation, since the fatty acid esters are metabolizable (Blickens & Di Luzio, 1965) and the pluronics are non-toxic (Hymes & Beck, 1968; Grover, Herton, Newman & Patton, 1969). The influence of the chemical structure of the ester, particularly the length and degree of

saturation of the acyl chain, on immunopotentiation was also investigated.

MATERIALS AND METHODS

Mice

CBA T6T6 male mice, aged about 12 weeks, and conventionally bred in this department, were used.

Antigens

Bovine serum albumin (BSA), fraction V, was purchased from Armour Pharmaceuticals Ltd, Eastbourne, Sussex.

Staphylococcal toxoid, purified from culture supernatants by glass bead chromatography (Cassidy & Harshman, 1976) was kindly provided by Dr P. Ward and Dr C. Adlam, Bacteriology Department, Wellcome Research Laboratories.

Adjuvants

These were purchased from the following sources: ethyl caprylate, caprate and laurate (Aldrich Chemical Co.); ethyl palmitate and stearate (Eastman Kodak); isobutyl palmitate (Kessler Chemicals Division, Armour Industrial Chemicals Ltd); isopropyl palmitate (Boake Roberts Co., London); ethyl oleate and linoleate (Sigma); Pluronic L122 (BSAF Wyandotte Corp. U.S.A.); Freund's incomplete adjuvant, FIA, (Difco).

Preparation of emulsions

Esters. The choice of Pluronic L122 as emulsifying agent was based on unpublished prior experiments by Dr R.W. Baker and colleagues at Bend Research Inc., Bend, Oregon, U.S.A., who determined that this surfactant provides stable w/o emulsions with esters of fatty acids of low carbon chain length. To 2 ml of a mixture of ester and Pluronic (10%, v/v) in a bijou was added a saline solution of antigen (0·5 ml) and the mixture homogenized at top speed for 1 min on an MSE blender: a second 0·5 ml aliquot of antigen solution was added, and the blending repeated.

FIA. Equal volumes of antigen solution and FIA were blended for 1 min.

The nature of the emulsions was tested by shaking a few drops in cold water. If the emulsion remained as discrete droplets it was considered to be water-in-oil; a uniform milky suspension would have indicated an oil-in-water (o/w) or a water-in-oil-in-water (w/o/w) emulsion.

Measurement of viscosity of emulsions

This was performed at 25° on a Brookfield Viscometer (Brookfield Engineering, Stoughton, Mass.) by Mr P.A. Everett of Analytical and Environmental Monitoring Services, Wellcome Research Laboratories.

Injection of mice

Emulsion (0·2 ml) was injected subcutaneously (s.c.) in the left flank.

Assay of antibodies

Bovine serum albumin. The method of Farr (1958) as modified by Bell & Shand (1973) was used. Log_{10} ABC/35 values were calculated according to the method of Brownstone, Mitchison & Pitt-Rivers (1966).

Staphylococcal toxoid. The inhibition of the haemolysis of rabbit erythrocytes by staphylococcal toxin was measured. Serial dilutions of sera in isotonic borate buffer, pH 7·0, plus 1% w/v BSA, were made in microtitre plates, and 25 μl of toxin, containing approximately two haemolytic units, was added. After incubation at room temperature for 30 min the plates received one drop of fresh washed 2% erythrocytes, were incubated at 37° for 90 min with shaking at 30 and 60 min and then left at 4° overnight.

Measurement of local reaction to injection of emulsions

Emulsion (0·05 ml) was injected into a hind footpad, the size of which was afterwards measured with a dial gauge caliper (Schnelltaster, H.C. Kroplin GmbH, Hessen, West Germany).

Statistics

The arithmetic means of log_{10} ABC values were compared by the Student's t test.

RESULTS

The effect of different chain length of the fatty acid or alcohol component of the fatty acid ester

The immune response to BSA was chosen as the first test for adjuvant activity, since this antigen induces little antibody formation in the mouse without adjuvants, and responds very well to Freund's incomplete adjuvant (Mitchison, 1964; Bomford, 1980).

In order to determine the effect of fatty acid chain length on adjuvanticity, mice were injected with 500 μg of BSA in saline, or in w/o emulsions of the ethyl esters

of caprylic (C8:O), capric (C10:O), lauric (C12:O), palmitic (C16:O) and stearic (C18:O) acids. The effect of changing the nature of the alcohol component of the esters was investigated by including the isobutyl and isopropyl esters of palmitic acid.

The antibody response of those groups in which the $\log_{10}$ ABC attained a positive value is shown in Fig. 1. BSA in saline, or emulsified in ethyl caprylate, caprate or laurate, failed to achieve this level. Emulsions made with ethyl stearate gave a higher response than ethyl

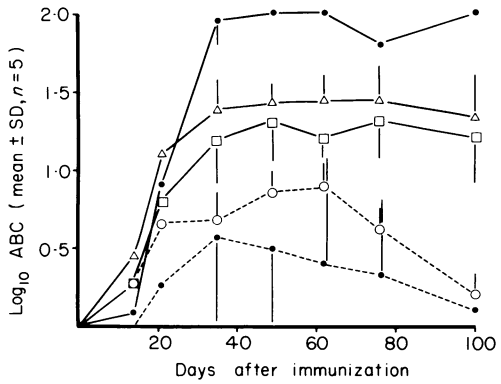

Figure 1. The adjuvant activity of w/o emulsions of fatty acid esters. The effect of varying the acyl chain length of the fatty acid component of the ester, or of using ethyl, isobutyl or isopropyl esters. Free BSA (500 μg) of ethyl caprylate, caprate or laurate emulsions did not achieve a positive $\log_{10}$ ABC value. Ethyl palmitate (●– –●), ethyl stearate (○– –○), isobutyl palmitate (□——□), isopropyl palmitate (△—△), FIA (●——●).

palmitate, although the difference was not significant. However, isopropyl and isobutyl palmitate were significantly better adjuvants than the ethyl ester, although they did not reach the level of FIA.

The effect of changing the degree of saturation of the fatty acid chain

The immune response to 500 μg of BSA emulsified in FIA or the ethyl esters of stearic (C18:O), oleic (C18:1) or linoleic (C18:2) acids is shown in Fig. 2. Both the unsaturated esters potentiated the response more than ethyl stearate, and this was particularly relevant early in the response (days 21 and 28), when they raised the antibody level more rapidly than even FIA: however later on, after day 85, their performance deteriorated relative to FIA.

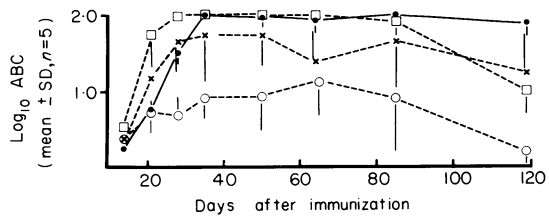

Figure 2. The adjuvant activity of esters of unsaturated fatty acids. BSA (500 μg) was emulsified with ethyl stearate (○——○), oleate (x– –x) or linoleate (□– –□) or FIA (●——●).

The adjuvant activity of ethyl linoleate emulsion for staphylococcal toxoid

It was important to check that the adjuvant activity of the fatty acid esters is not restricted to BSA.

Figure 3 shows the day 25 primary response to 5·4, 0·54 or 0·054 i.u. of toxoid in saline or emulsified with FIA or with ethyl linoleate, and the secondary response 14 days after a subcutaneous boost with 5 i.u. of toxoid in saline on day 60. The ethyl linoleate emulsion promoted the primary response to all doses of antigen and, with the lower doses of antigen, improved the secondary response. The level of potentiation was usually greater than that with FIA.

The relationship between emulsion viscosity and adjuvant activity

The emulsions prepared from the ethyl esters of

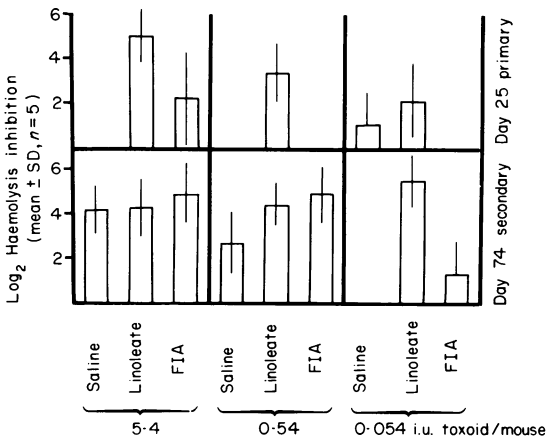

Figure 3. The adjuvant activity of ethyl linoleate and FIA for staphylococcal toxoid. Mice were injected s.c. with toxoid 5·4, 0·54 or 0·054 (i.u.) in saline, or emulsified in linoleate or FIA, and boosted s.c. with toxoid (5 i.u. in saline) on day 60.

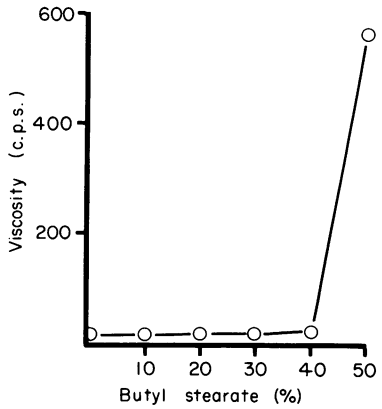

Figure 4. Emulsions prepared with mixtures of ethyl caprate and butyl stearate. The relationship between viscosity and content of butyl stearate.

saturated fatty acids became more viscous as the carbon chain lengths of the fatty acids increased, progressing from liquid (ethyl caprylate and caprate) to semi-solid (ethyl palmitate) and finally solid at room temperature (ethyl stearate). However it is unlikely that this physical change is responsible for the improved adjuvant activity, since the adjuvant-active emulsions of the unsaturated oleate and linoleate esters were of similar viscosity to those prepared from caprylate or caprate.

The role of viscosity was investigated quantitatively by comparing the consistency and adjuvant activity of emulsions made from ethyl caprate mixed with increasing proportions of butyl stearate. The latter was chosen as the active ester because it has a lower melting point than ethyl stearate, and is more stable and cheaper than the unsaturated esters. It was hoped that a mixture could be found which would possess the low viscosity and stability of the caprylate and caprate emulsions, together with the adjuvanticity of the longer-chain esters.

Figure 4 shows the relationship between w/o emulsion viscosity and the proportion of butyl stearate. Only a very slight increase in viscosity occurs as the level of butyl stearate is raised to 40%, but at 50% there is a sharp increase. The adjuvant activity of emulsions prepared from ethyl caprate alone, or mixed with 1%, 10% or 50% butyl stearate for 500 μg BSA was tested (Table 1). As before, ethyl caprate was a poor adjuvant. The addition of only 10% butyl stearate, causing a very small increase in emulsion viscosity (Fig. 4), produced as great an improvement in adjuvant activity as the addition of 50% butyl stearate.

The relationship between adjuvant activity, local reaction and retention of antigen at the site of injection

The same degree of local reaction (footpad swelling) was caused by emulsions of ethyl caprate alone or mixed with 10% butyl stearate (Fig. 5). The reaction to the esters was more severe than that to FIA up to 14 days after injection, but afterwards disappeared more quickly.

The rate of loss of ^{125}I from the footpad after injection of ^{125}I-BSA in these emulsions is shown in Fig. 6. With FIA, the proportion of radioactivity retained in the footpad stabilized at about 25% between days 3 and 22 (the last time examined): whereas with the esters it dropped to undetectable levels by day 22. At day 3 significantly more ^{125}I remained in the footpads injected with caprate plus 10% stearate compared with those which had received caprate alone. This result was reproduced in a second experiment.

Table 1. Adjuvant activity of emulsions prepared with ethyl caprate mixed with increasing proportions of butyl stearate

BSA (500 μg) injected with	Day 22 antibody response ($\log_{10}$ ABC, mean $\pm$ SD, $n=5$)
Saline	$-1{\cdot}62 \pm 0{\cdot}56$
FIA	$0{\cdot}49 \pm 0{\cdot}13$
Ethyl caprate	$0{\cdot}03 \pm 0{\cdot}01$
Butyl stearate	$0{\cdot}93 \pm 0{\cdot}04$
Ethyl linoleate	$1{\cdot}79 \pm 0{\cdot}29$
Ethyl caprate: butyl stearate 99:1	$0{\cdot}31 \pm 0{\cdot}12$
Ethyl caprate: butyl stearate 90:10	$1{\cdot}34 \pm 0{\cdot}31$
Ethyl caprate: butyl stearate 50:50	$1{\cdot}04 \pm 0{\cdot}27$

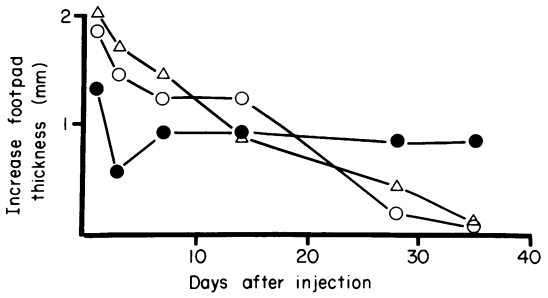

Figure 5. The time course of local reaction (increase in footpad thickness) caused by the injection of 0·05 ml of ethyl caprate (o——o), caprate plus 10% butyl stearate (Δ——Δ) or FIA (●——●) emulsions.

DISCUSSION

This work extends earlier reports of the adjuvant activity of fatty acid esters. Holt (1967) used a w/o emulsion of isopropyl myristate, with Arlacel (mannide monooleate) as the emulsifying agent, to promote the antibody response to diphtheria and tetanus toxoid in guinea-pigs. Whitehouse *et al.* (1974) were able to potentiate the induction of allergic encephalomyelitis in Lewis rats injected with guinea-pig spinal cord by emulsification with methyl oleate.

In the present work the ester emulsions were prepared with a novel emulsifying agent, Pluronic L122, chosen as being non-toxic (Hymes & Beck, 1968; Grover *et al.*, 1969), and providing w/o emulsions of long-term stability. Adjuvant activity was shown to depend on the chemical nature of the ester. Increasing length and unsaturation of the fatty acid carbon chain were both favourable. The positive effect of acyl chain length has been previously noted for hydrocarbons (Shaw *et al.*, 1964) and aliphatic amines (Gall, 1966).

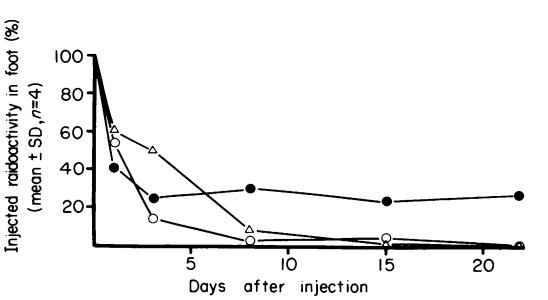

Figure 6. The retention of radioactivity in the footpad after injection of ^{125}I-BSA in ethyl caprate (o——o), caprate plus 10% butyl stearate (Δ——Δ) or FIA (●——●).

The mechanism of action of the ester emulsions was studied by exploiting the fact that addition of 10% of butyl stearate to ethyl caprate caused a considerable increase in adjuvant activity. It did not raise the inflammation induced by the emulsions, ruling this out as the sole determinant of immunopotentiation. Although the viscosity of the two emulsions was almost identical, the active caprate-stearate combination did retain the antigen at the site of injection to a greater extent at 3 days after injection. This is compatible with the view that slow release of antigen contributes to adjuvant activity; however it is unlikely that this type of action is sufficient by itself, since injecting the non-immunogenic dose of 500 μg of fluid BSA as multiple smaller doses (50 μg ten times every other day) failed to improve the immune response (Bomford, unpublished results). Herbert (1966), who performed an experiment of similar design with ovalbumin, obtained a strong response equivalent to that generated by Freund's adjuvant, and concluded that slow release of antigen could fully explain adjuvant activity. It is likely that this applies only to such relatively strongly immunogenic antigens as ovalbumin, which do not possess the tolerogenic properties of BSA (Mitchison, 1964).

The hypothesis that the esters exert an immunostimulating effect on cells of the host immune system is supported by reports of their action on macrophages. Injected i.v. they depress the mononuclear phagocytic system (Stuart, Biozzi, Stiffel, Halpern & Mouton, 1960; Di Luzio & Wooles, 1964), and can either depress or promote the antibody response to a subsequent i.v. injection of SRBC (Stuart & Davidson, 1964; Barrie & Cooper, 1964). This modulation of the SRBC response could be caused by the same mechanism as that demonstrated for dextran sulphate which, by blockade of the mononuclear phagocytic system, caused a greater proportion of i.v.-injected SRBC to reach the spleen (Bradfield, Souhami & Addison, 1974). This type of mechanism is inapplicable to an s.c. injection of antigen in ester emulsions, where it is more feasible to think in terms of an effect on antigen-presenting cells or lymphocytes in the draining lymph node. It is possible that fatty acids released from the esters after hydrolysis could mediate adjuvant activity. Oleic acid possesses immunopotentiating properties (Dresser, 1961); although linoleic acid suppresses cell-mediated responses such as mitogen and antigen-induced lymphocyte prolifer-

ation (Mertin & Hughes, 1975), and the cytotoxic response and allograft survival (Mertin, 1976).

In conclusion, an emulsion prepared from a mixture of an inactive ester of a short carbon chain saturated fatty acid with a small proportion of an active ester of a long chain saturated acid, provides an easily-injectable and stable (both in terms of emulsion integrity and chemical composition) emulsion which, whilst not possessing the full potency of FIA, does not cause chronic inflammation.

ACKNOWLEDGMENTS

The author thanks Mrs S. Wishart for skilful technical assistance, and Dr O. Basarab and Mr J. Tilleray for critical reading of the manuscript.

REFERENCES

AUDIBERT F. & CHEDID L. (1975) Enhancement of the immune response by a water in metabolizable oil adjuvant. *Comptes Rend. Hebdom. Acad. Sci.* **280(D),** 1629.

BARRIE J.U. & COOPER G.N. (1964) Enhancement of the primary antibody response to particulate antigens by simple lipids. *J. Immunol.* **92,** 529.

BELL E.B. & SHAND F.L. (1973) Cellular events in protein tolerant inbred rats: the fate of thoracic duct lymphocytes and memory cells during tolerance induction to human serum albumin. *Europ. J. Immunol.* **3,** 259.

BLICKENS D.A. & DI LUZIO N.R. (1965) Metabolism of methyl palmitate, a phagocytic and immunologic depressant, and its influence on tissue lipids. *J. Reticuloendoth. Soc.* **2,** 60.

BOMFORD R. (1980) The comparative selectivity of adjuvants for humoral and cell-mediated immunity 1. Effect on the antibody response to bovine serum albumin and sheep red blood cells of Freund's incomplete and complete adjuvants, alhydrogel, *Corynebacterium parvum*, *Bordetella pertussis*, muramyl dipeptide and saponin. *Clin. exp. Immunol.* **39,** 426.

BRADFIELD J.W.B., SOUHAMI R.L. & ADDISON I.E. (1974) The mechanism of the adjuvant effect of dextran sulphate. *Immunology*, **26,** 383.

BROWNSTONE A., MITCHISON N.A. & PITT-RIVERS R. (1966) Biological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. *Immunology*, **10,** 481.

CASSIDY P. & HARSHMAN S. (1976) Purification of staphylococcal and toxin by adsorption chromatography on glass. *Infect. Immunol.* **13,** 982.

DI LUZIO N.R. & WOOLES W.R. (1964) Depression of phagocytic activity and immune response by methyl palmitate. *Am. J. Physiol.* **206,** 939.

DRESSER D.W. (1961) Effectiveness of lipid and lipidophilic substances as adjuvants. *Nature (Lond.)*, **191,** 1169.

FARR R.S. (1958) A quantitative chemical measure of the primary interaction between 1* BSA and antibody. *J. Infect. Dis.* **103,** 239.

FREUND J. (1956) The mode of action of immunological adjuvants. *Adv. Tuberc. Res.* **7,** 130.

GALL D. (1966) The adjuvant activity of aliphatic nitrogenous bases. *Immunology*, **11,** 369.

GROVER F., HERTON M.W., NEWMAN M.N. & PATTON B.C. (1969) Effect of nontoxic surface active agent on blood viscosity and platelet adhesiveness. *Circulation*, **39–40** (Suppl.), 249.

HERBERT W.J. (1966) Antigenicity of soluble protein in the presence of high levels of antibody: a possible mode of action of the antigen adjuvants. *Nature (Lond.)*, **210,** 747.

HILLEMAN M.R. (1966) Critical appraisal of emulsified oil adjuvants applied to viral vaccines. *Prog. med. Virol.* **8,** 131.

HOLT L.B. (1967) Oily adjuvants. In: Symposium Series Immunobiol. Standard., vol. 6, p. 131. Karger, Basel and New York.

HYMES A.C. & BECK K. (1968) A comparison of pluronic F68, low molecular weight dextran and saline as priming agents in the heart lung apparatus. *J. Thorac. Cardiov. Surg.* **56,** 23.

MERTIN J. (1976) Effect of polyunsaturated fatty acids on skin allograft survival and primary and secondary cytotoxic response in mice. *Transplantation*, **21,** 1.

MERTIN J. & HUGHES, D. (1975) Specific inhibitory action of polyunsaturated fatty acids on lymphocyte transformation induced by PHA and PPD. *Int. Archs. Allergy appl. Immun.* **48,** 203.

MITCHISON N.A. (1964) Induction of immunological paralysis in two zones of dosage. *Proc. Roy. Soc. Ser. B.* **161,** 275.

REYNOLDS J.A., HARRINGTON D.G., CRABBS C.L., PETERS C.J. & DI LUZIO N.R. (1980) Adjuvant activity of a novel metabolizable lipid emulsion with inactivated viral vaccines. *Infect. Immunol.* **28,** 937.

SHAW C.M., ALVORD E.C. & KIES M.W. (1964) Straight chain hydrocarbons as substitutes for the oil in Freund's adjuvants in the production of experimental 'allergic' encephalomyelitis in the guinea pig. *J. Immunol.* **92,** 24.

STUART A.E., BIOZZI G., STIFFEL C., HALPERN B.N. & MOUTON D. (1960) The stimulation and depression of reticuloendothelial phagocytic function by simple lipids. *Brit. J. exp. Pathol.* **41,** 599.

STUART A.E. & DAVIDSON A.E. (1964) Effect of simple lipids on antibody formation after injection of foreign red cells. *J. Path. Bact.* **87,** 305.

WHITEHOUSE M.W., ORR K.J., BECK F.W.J. & PEARSON C.M. (1974) Freund's adjuvants: relationship of arthritogenicity and adjuvanticity in rats to vehicle composition. *Immunology*, **27,** 311.

WILNER B.I., EVERS M.A., TROUTMAN H.D., TRADER F.W. & MCLEAN I.W. (1963) Vaccine potentiation by emulsification with pure hydrocarbon compounds. *J. Immunol.* **91,** 210.