

Mechanism of action of mineral oil adjuvants

I. Conditions influencing the in vitro stability of water-in-oil emulsions

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An experimental model has been developed in which the stability of water-in-oil emulsions in simple environments of known constitution could be studied. A decrease in emulsion stability was effected by decreasing the pH of the emulsion environment with the ionic strength constant, increasing the ionic strength of the environment with the pH constant, or increasing the temperature of the environment with the pH and ionic strength constant. The concentration of protein in the emulsion environment at a given pH was not found to alter the stability of the emulsions. Parallels between in vitro influences upon stability and those produced by an inflammatory response at an emulsion repository site are discussed.

An adjuvant is any substance that, when combined with an antigen, enhances its antigenic properties. Mineral oil adjuvants are water-in-paraffin oil emulsions containing water-soluble or particulate antigens, or both, in the aqueous phase. The effectiveness of mineral oil adjuvants in enhancing and prolonging responses to vaccines is presumably due to protection of the antigen by the oily coating so that it serves as an antigen depot, attraction about the antigen by the paraffin oil of mononuclear cells that may take part in antibody formation, and an accompanying slow, continuous release of antigen that stimulates antibody production in suitable cells.^{1, 2}

There is no information presently available about emulsion-tissue environment interactions and their influence upon the effectiveness of the adjuvant response. Any factors that might conceivably influence the stability of the emulsion at the site of injection necessarily influence the persistence of the depot. These factors can be divided conveniently into those that are intrinsic and those that are extrinsic. Intrinsic factors include the chemical and physical properties of the emulsion and are influenced by³: (1) chemical properties of the oil, (2) chemical properties of the emulsifying agent, (3) proportion of oil to water, (4) per cent of emulsifying agent employed, and (5) droplet size of the discontinuous phase. Extrinsic factors include the interaction of the emulsion with the tissue environment and are influenced by: (1) effect of tissue fluid and its constituents (water, electrolytes, and proteins), (2) effect of tissue cellular

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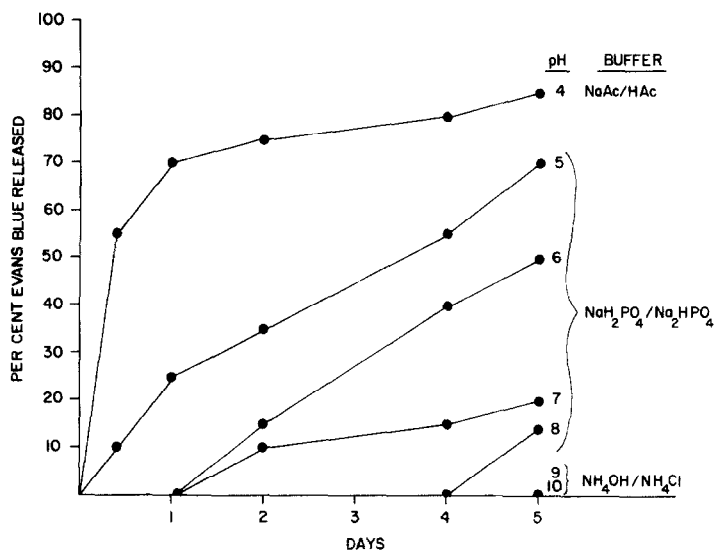


FIG. 1. Effect of environmental buffer pH with constant ionic strength upon emulsion stability.

elements, (3) temperature, and (4) effect of emulsion surface area presented to the tissue fluids.

In order to ascertain the importance of various chemical and physical factors that influence the adjuvant properties of emulsified vaccines, an *in vitro* experimental model was designed that allowed investigation of emulsion stability while varying conditions in the environment such as pH, ionic strength, protein concentration, and temperature.

The model was based on the observation that the color of Evans blue dye incorporated into the aqueous phase of an emulsion appeared, after a period of time, in an aqueous medium into which the emulsion had been placed. Presumably movement of this dye from the emulsion to the surrounding aqueous medium would be representative of any dissolved molecule upon deterioration of the emulsion.

MATERIALS AND METHODS

Experimental procedure

The open end of a 20 c.c. Luer-Lok syringe containing emulsion was centered over the mouth of a round-bottom Beckman spectrophotometric tube (13 ml. light path and 95 mm. height containing 6.0 c.c. of fluid). One drop of emulsion (approximately 0.05 c.c.) was allowed to fall freely into each tube. Those tubes in which the emulsion hit the walls above the water level were replaced. Experiments were carried out at room temperature, unless otherwise specified. The optical density (O.D.) of the aqueous medium environment was measured with a Beckman Model B spectrophotometer at 650 $m\mu$.

In the use of this model 4 assumptions were made: (1) the amount of color present in the aqueous medium surrounding the emulsion was a measure of the amount of emulsion that had broken; (2) the distribution of the color in the environmental solutions was homogeneous: preliminary experiments showed that the color was homogeneously distributed when it appeared in the environment; (3) propensity for emulsion breakdown is the same in each part of a well-made water-in-oil emulsion: preliminary experiments showed that drops of emulsion randomly selected from individual emulsion batches showed no significant variation with respect to breakdown; (4) the emulsion remained unchanged in the syringe during the

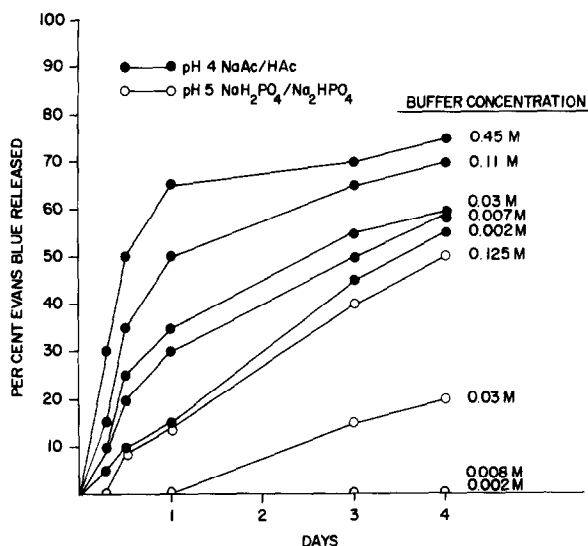


FIG. 2. Influence upon emulsion stability of change in environmental ionic strength by varying the concentration of the buffer system.

conduct of the experiment: the first experiment was repeated at the conclusion of the experiments with identical results, thus confirming this assumption.

Emulsions

Equal parts of an aqueous phase containing 0.1 per cent Evans blue dye (T-1824) (Warner-Chilcott Lab., Morris Plains, N. J.) in 5 per cent sodium citrate solution (0.14M), pH of this phase being 8.4, were added to an oily phase consisting of 10 per cent Arlacel A in Drakeol 6VR (Atlas Chemical Industries, Wilmington, Del.). Emulsification was accomplished by transferring the materials 50 times between two 20 c.c. Luer-Lok syringes connected by a double-hub 18-gauge needle.⁴ The drop test was employed to determine the quality of emulsion obtained.⁵ The emulsions were stored at 10° C. and, before being used in an experiment, 10 further transfers between syringes were made. The same emulsion was used throughout the experiment.

Buffers

The following buffer solutions were prepared with a constant ionic strength ($\mu = 0.1$): HAc/NaAc - (pH 4); Na₂HPO₄/NaH₂PO₄ - (pH 5, 6, 7, 8); NH₄OH/NH₄Cl - (pH 9, 10).⁶

The color of Evans blue was shown to be stable over the pH range 4 through 9 with the three different buffer systems used. At pH 10 the O.D. was less.

RESULTS

Effect of environmental buffer pH with constant ionic strength upon emulsion stability

One drop of emulsion (approximately 0.05 c.c.) was placed in each of a triplicate series of tubes containing 6.0 c.c. of buffer over a pH range from 4 to 10. NaAc/HAc - (pH 4), NaH₂PO₄/Na₂HPO₄ - (pH 5, 6, 7, 8), and NH₄OH/NH₄Cl - (pH 9, 10). The ionic strength of each of these buffers (μ) = 0.1. The optical density was read at intervals for 5 days. The pH values were re-measured in each tube at the termination of the experiment, and in this and all subsequent experiments no change in pH had occurred. Fig. 1 indicates that the stability of the emulsions decreased as the pH decreased.

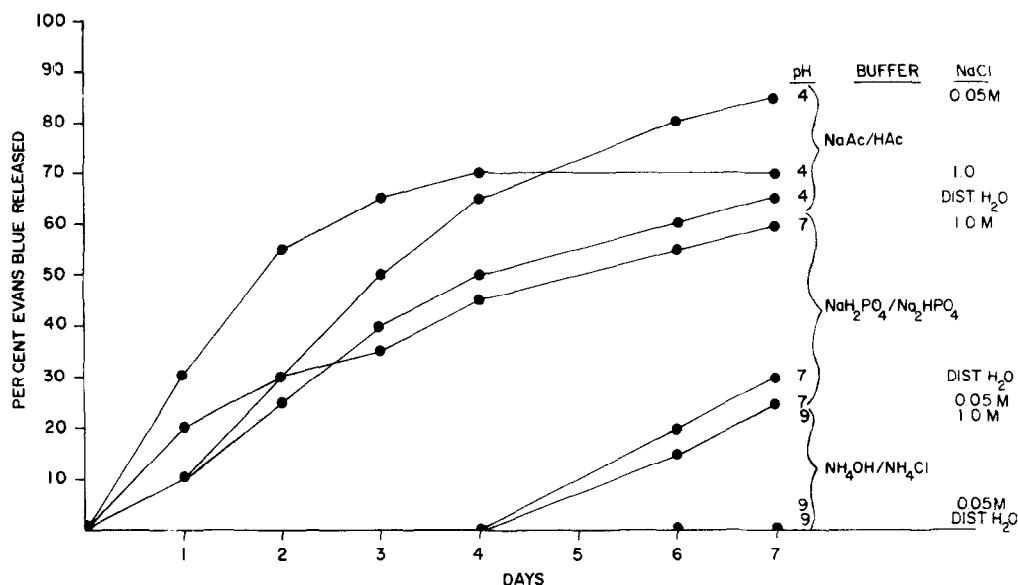


FIG. 3. Effect upon emulsion stability of change in environmental ionic strength by varying concentrations of NaCl in weakly buffered systems.

Influence of change in environmental ionic strength by varying the concentration of the buffer system

One drop of emulsion was placed into each of a duplicate series of tubes containing NaAc/HAc buffer (pH 4) at 0.45, 0.11, 0.03, 0.007, and 0.002M and Na₂HPO₄/NaH₂PO₄ (pH 5) at 0.125, 0.030, 0.008, 0.002M. The tubes were read at intervals for 4 days.

Fig. 2 indicates that within a given buffer system, an increase in ionic strength of the buffer caused a decrease in emulsion stability.

Effect of change in environmental ionic strength by varying concentrations of NaCl in weakly buffered systems

Solutions containing 1M, 0.05M NaCl, and distilled water were prepared in a 0.001M NaAc/HAc buffer (pH 4); 0.001M Na₂HPO₄/NaH₂PO₄ (pH 7); 0.001M NH₄OH/NH₄Cl (pH 9). Preliminary experiments showed that at each ionic strength of the buffer employed, addition of NaCl up to 6M shifted the pH no more than 0.6, but NaCl concentrations greater than 3M precipitated the dye. One drop of emulsion was placed in each of a duplicate series of tubes containing 6.0 c.c. of buffer at a given pH value and NaCl concentration. The tubes were read at intervals for 7 days.

Fig. 3 indicates that the behavior of the emulsions fell into two groupings. As the pH of the buffer decreased, the emulsions became less stable and, within each pH grouping, increasing concentration of NaCl decreased emulsion stability.

Influence of temperature at varying pH

One drop of emulsion was placed into each of a duplicate series of tubes containing Na₂HPO₄/NaH₂PO₄ buffer (pH 5.8, 6.2, 6.6, 7.0, and 7.4). One

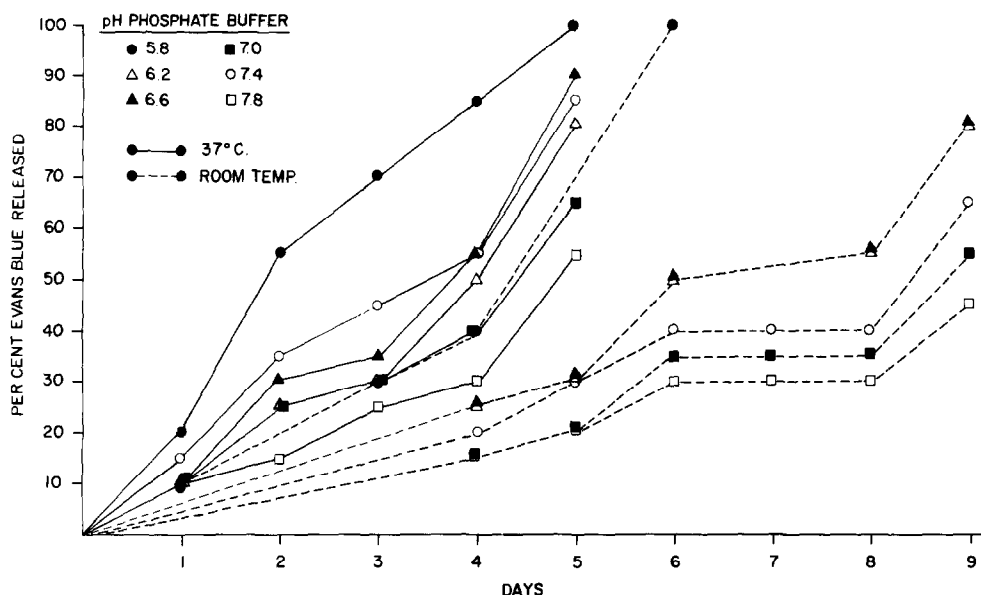


FIG. 4. Influence of temperature upon emulsion stability at varying pH.

series of tubes was incubated at room temperature, the other at 37° C. The tubes were read daily for 9 days.

Fig. 4 indicates that temperature increase caused a considerable decrease in emulsion stability. The average half-life for all pH values at room temperature was 6.6 days as compared with a half-life of 3.7 days for all pH values at 37° C.

Effect of human serum

One drop of emulsion was placed in each of a triplicate series of tubes containing human serum diluted to concentrations of 50, 25, 12.5, 6.0, 3.0, 1.5 per cent, and distilled water. Preliminary experiments showed that Evans blue remained at the top of the tubes containing serum instead of diffusing down. Before being read, therefore, the tubes were centrifuged for 10 minutes at 15,000 r.p.m. to mix the dye uniformly. A different triplicate set of tubes were read daily for 4 days. The pH of each tube was read before the addition of emulsion and at the termination of the experiment.

Fig. 5 indicates that varying the concentration of human serum did not alter the stability of the emulsions. The pH at the beginning of the experiment was identical to that at its termination.

DISCUSSION

In the environmental conditions usually found in normal tissues (pH 7.4, 37° C.) the emulsions used for vaccines might be expected to be reasonably stable. However, these adjuvants have been shown to stimulate an inflammatory response in vivo at repository sites.⁷ Melkumova⁸ has demonstrated in the rabbit, using microelectrodes, that tissue fluids become considerably more acidic during the development of a subcutaneous inflammatory response induced by xylene.

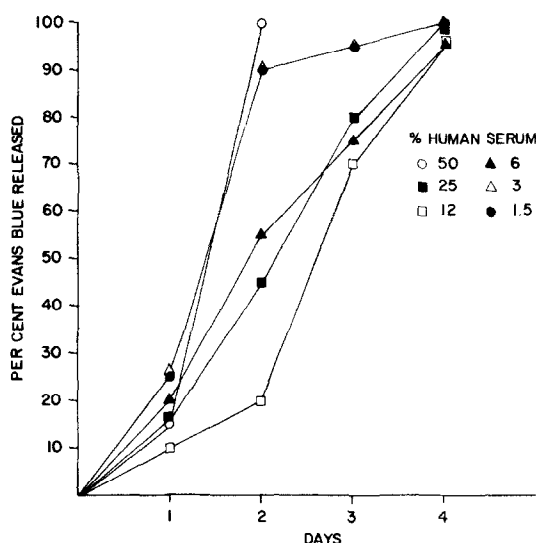


FIG. 5. Effect upon emulsion stability of varying concentrations of human serum.

Our data indicate that these pH changes could cause a decrease in emulsion stability. Szporny and associates⁹ have shown that the acute inflammatory edema induced by xylene in the guinea pig's skin is a bulk filtrate of plasma. The increase in ionic strength resulting from the translocation of Na^+ , K^+ , Cl^- ions from the serum compartment to the inflammatory edema fluid would be expected to contribute to a decrease in emulsion stability. Transudation of serum proteins into acute inflammatory edema fluid has been shown by several authors¹⁰; however, our experimental data do not show any significant effect of protein concentration using human serum. With the passage of time there may be an increase in ionic strength in inflammatory tissue fluids due to the breakdown by cellular enzymes of accumulated serum proteins and other substances present. The in vitro data indicate that such changes could have a deleterious effect upon emulsion stability.

Elevated temperature could contribute to a decrease in emulsion stability, as the in vitro data suggests; however, there is no experimental data to show that this occurs in an inflamed repository depot. That the changes in tissue environment induced by the inflammatory response may be additive in their effect upon emulsion stability is suggested by the experiment in which an increase in ionic strength obtained by adding NaCl to weakly buffered systems resulted in an accentuation of the instability noted at low pH.

In vivo studies employing radiolabeled proteins dissolved in the aqueous phase indicate much more rapid release of dissolved materials at "normal" tissue conditions than we have observed in our in vitro model.¹¹ This finding tends to support the notion that the local environment rapidly becomes unfavorable to the continued stability of the emulsion. As emulsions are injected intramuscularly, local motion could also fragment the emulsion depot, resulting in a greater surface area for breakdown.⁷ If indeed, in vivo depots with a

prolonged half-life are desirable, emulsions that would be physiologically stable in a locally inflamed site should be sought. The in vitro model allows the accurate study of stability under the changing conditions known to occur in tissues.

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