observed upon the addition of the latter drug to the coenzymes must be attributed to an interaction between the two species.

Whether this interaction provides a basis for the pharmacological activity of the drug is, of course, mere speculation at this stage. Obviously, further and more detailed studies are needed, utilizing other phenothiazines and employing other techniques. The main thrust of the investigation at this point is that electrochemical methods can and do provide a direct and feasible approach to the study of the possible interactions between the drugs and the coenzymes; further studies should lead to the acquisition of some definite knowledge in this area.

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Polymerized Micelles and Their Use as Adjuvants in Immunology

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Abstract □ A polymerization process is described for the preparation of hydrophilic micelles containing solubilized drug molecules in a colloidal aqueous system of dissolved monomers. A hydrocarbon medium constitutes the outer phase. After secondary solubilization with the aid of selected surfactants, polymerization of micelles under different conditions takes place. The formulation of drugs including labile proteins in the ultrafine polymer components ("nanoparts") and their isolation are possible without noticeable destruction of the encapsulated molecules. Entrapped tagged material (human ^{125}I-immunoglobulin G) shows a stable fixation in such nanoparts during long-term *in vitro* liberation trials. Nanoparts are visible with the aid of an electron microscope. They are mostly spherical in shape and smaller than 80 nm in diameter. They form real colloidal aqueous solutions. Nanoparts are suitable to embed antigenic material (tetanus toxoid and human immunoglobulin G) for parenteral use. These preparations show intact biological activity and high antibody production in animals.

Keyphrases □ Polymerization—hydrophilic micelles containing drug molecules (nanoparts), use as immunological adjuvants □ Micelles, hydrophilic—containing drug molecules, polymerization, use as immunological adjuvants □ Nanoparts—hydrophilic micelles containing drug molecules, polymerization, use as immunological adjuvants □ Adjuvants, immunological—hydrophilic micelles containing drug molecules, polymerization □ Immunological adjuvants—hydrophilic micelles containing drug molecules, polymerization

"Nanoparts" are solidified micelles containing drugs; they are spherical particles of nontoxic polymeric material with entrapped bioactive materials. The extremely small diameter of nanoparts, smaller than 80 nm, is measured by electron scanning microscopy. The lower limit for the particle diameter ranges between 20 and 35 nm, as shown by ultrafiltration with special diaphragms of an average pore size between 20 and 50 nm.

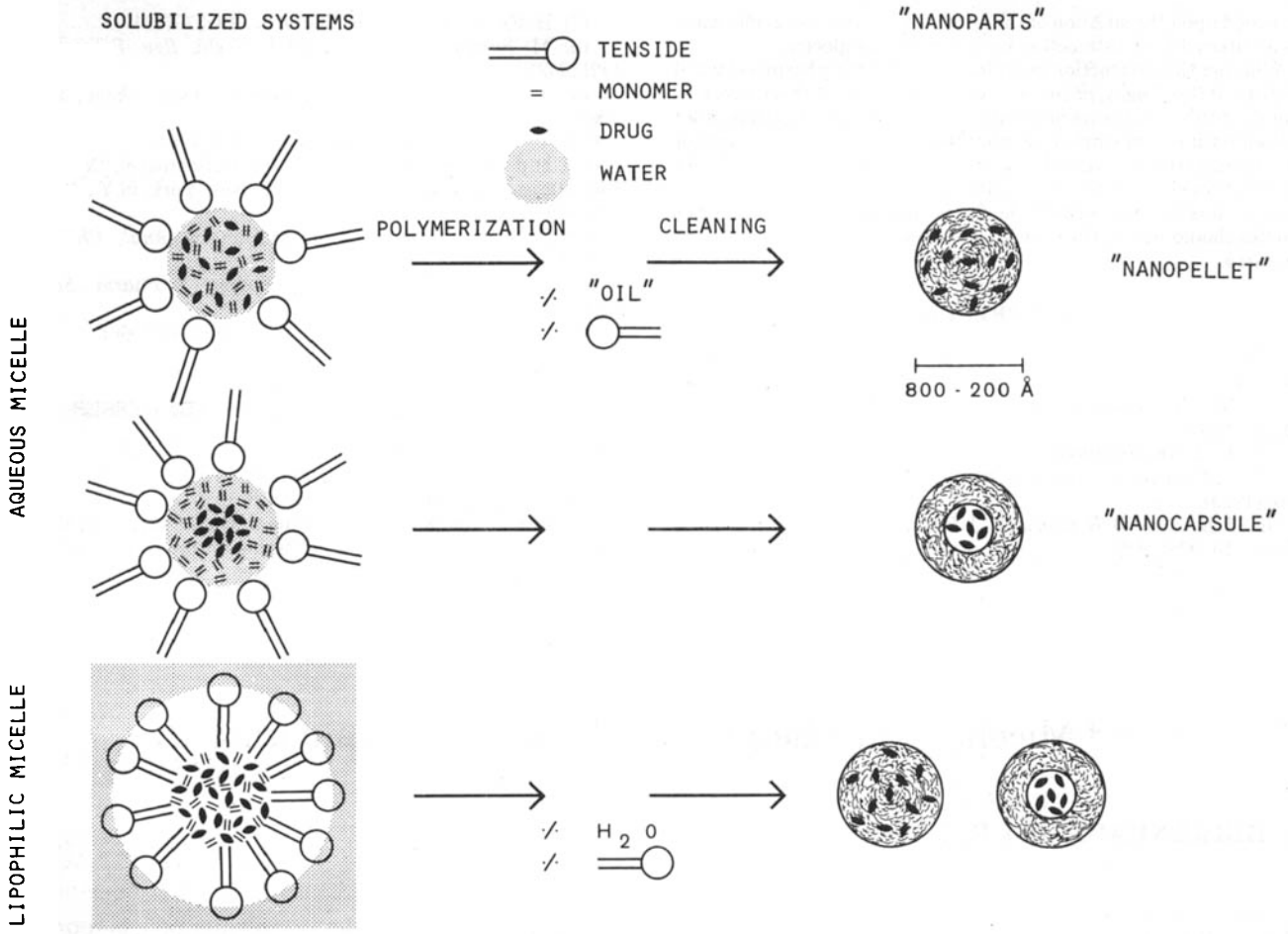
These ultrafine capsules or polymer pellets represent a narrow distribution in particle size. The electron scanning photographs show an aggregation of homogeneous, spherical particles of nearly the same shape and size, being small enough to give colloidal aqueous solutions.

According to polarity, dielectric constant, and steric effect of the reactants, a more or less shell-like polymer construction ("nanocapsule") or a compact polymer particle ("nanopellet") can be proposed (Scheme I). The minuteness of the nanoparts and the homogeneity of the final product also enable the formulation of parenteral drug delivery systems.

EXPERIMENTAL

Synthesis—Water-soluble polymerizable monomers and the enclosure material, *e.g.*, the biologically or pharmacologically active agent, are dissolved in water to form a true, or at least a colloidal, solution. This solution is distributed with the aid of surfactants by stirring in a hydrophobic phase (*n*-hexane) in which the precursors and the drugs are practically insoluble. Minute aqueous micelles, containing the polymerizable monomers, drugs, and possibly other auxiliary agents, are thus solubilized in a relatively large volume of the lipophilic outer phase and form extremely small reaction regions for the ensuing polymerization. The polymerization is induced by known methods (1–7).

The mechanism of micelle polymerization is definitely different from emulsion polymerization. In most cases, mainly in micellar reaction regions, water-insoluble monomers polymerize in water during emulsion polymerization. Here, the radical-containing polymerizing regions may swell to many times their original size. This swelling is due to diffusion of monomers from a stock of monomer droplets in the emulsion into growing latex polymer particles (8, 9). In this process, polymerization is strictly restricted to the micellar regions, since



Scheme I—Schematic presentation of nanoparts

the hydrophobic outer phase contains no polymerizable material. A diffusion of monomers into this phase is largely prevented because of the low partition coefficient.

To encapsulate water-insoluble materials, the system can be modified so that a lipophilic phase with the dissolved weak polar drug can be solubilized in a hydrophilic outer medium, usually water. In this case, the precursors must have a high partition coefficient and the diffusion of monomers into the hydrophilic outer phase has to be prevented (Scheme I). The polymer micelle formed can usually be cleaned and isolated by precipitation or by ultrafiltration, dialysis, or centrifugation.

The procedure for preparing nanoparts, however, requires the following three steps: (*a*) solubilization of enclosure material (drug) and polymerizable molecules to form micelles, (*b*) polymerization of the monomers, and (*c*) purification and isolation of the solidified micelle.

Solubilization—All materials used were analytical grade.

Procedure A—A mixture of 12.0 g of bis(2-ethylhexyl) sodium sulfosuccinate[1] (I) and 6.0 g of polyoxyethylene 4 lauryl ether[2] (II), with an average of four ethylene oxide units in the chain, was dissolved in 80 ml of *n*-hexane by stirring at room temperature until the solution was clear. By adding 5.0 ml of distilled water dropwise into the solution and by continuous stirring, the crystalline monomers, 0.25 g of *N,N'*-methylenebisacrylamide[3] (III) and 2.00 g of acrylamide[3] (IV), were dissolved.

The soluble portion was filtered through membrane filters[4] to produce a sterile solution. Under aseptic conditions, 5.0 g of tetanus toxoid solution containing 3100 limit flocculation (Lf) units/ml[5] were added dropwise, with stirring, until a clear solution resulted.

Procedure B—A mixture of 12.0 g of bis(2-ethylhexyl) sodium sulfosuccinate and 6.0 g of polyoxyethylene 4 lauryl ether was dissolved in 80.0 ml of *n*-hexane at room temperature. Then 35 ml of distilled water was slowly added while the solution was stirred. Then the crystalline monomers, 0.50 g of III and 4.00 g of IV, were dissolved in this solution. The solution was sterile filtered[4], and 0.30 g of freeze-dried urease[6] was added to form a micellar solution with a weak Tyndall effect.

Procedure C—A mixture of 45.0 g of I and 25.0 g of II was dissolved in 215 ml of *n*-hexane at room temperature. Then 2.5 ml of ethanol, 2.5 ml of methanol, 40.0 ml of distilled water, 1.00 g of III, and 8.00 g of IV were added and dissolved successively.

The solubilized mixture was sterile filtered, and *n*-hexane was added to form 340 ml of solution. While stirring under aseptic conditions, 10.0 ml of a ^{125}I-labeled human immunoglobulin G (IgG) solution[5] (aggregate free in tromethamine buffer with 0.100 g of sodium chloride corresponding to ~1.4% human IgG) was added dropwise at room temperature.

Human IgG was alcohol fractioned, with approximately 10–15% of aggregated material, and was purified by molecular sieving with dextran gel[7]. Labeling with iodine-125 was performed by the method of Helmkamp *et al.* (10).

Polymerization with γ-Radiation (1)—Solutions prepared according to Procedures A and C were placed under nitrogen, sealed tightly, and exposed continuously to the radiation of a cobalt-60 source at about 20–30°. A dosage of 0.3 Mrad ensured complete polymerization. The end-point of polymerization, *i.e.*, the disappearance of the monomers, could be determined with the aid of an acidimetric color titration method to determine unsaturated compounds by reaction with morpholine (11).

[1] Fluka, Ltd., Buchs, Switzerland.
[2] Brij 30, Atlas Powder Co.
[3] Ciba-Geigy Ltd., Basle, Switzerland.
[4] Millipore HA 45 nm and GS 22 nm, Millipore, Bedford, Mass.
[5] Serum- & Impfinstitut, Bern, Switzerland.
[6] Merck Ltd., Darmstadt, Germany.
[7] Sephadex G 200.

Polymerization with Visible Light (3–7)—A solution prepared according to Procedure B was placed in a cylindrical, temperature-stable, double-walled reaction vessel of Pyrex glass with an internal diameter of 6 cm[8]. Then 0.2 mg of riboflavin 5′-sodium phosphate[1] and 0.2 mg of potassium peroxodisulfate[6], as an initiator, were added and dissolved. While stirring at 35 ± 1°, the solution was continuously perfused with a weak stream of nitrogen gas. The opalescent solution was then radiated from outside the column at a distance of 15 cm with an electric bulb of 300 w for 7 hr until the monomers disappeared completely.

Polymerization with UV Light (2)—One solution was prepared according to Procedure B and placed in a cylindrical reaction vessel as already described. It then was continuously stirred at 35 ± 1° and perfused by a weak stream of nitrogen gas while being radiated from inside by a UV dipping lamp (quartz burner of 70 w) for 45 min until the monomers completely disappeared.

Isolation and Purification According to Procedure C—After complete polymerization, the *n*-hexane hydrophobic phase was removed by azeotropic distillation at room temperature under vacuum. From the remaining aqueous residue containing the nanoparts and the surfactants, the surfactants were removed completely by ultrafiltration and repeated washing with distilled water under nitrogen pressure (~2–4 atm). Ultrafiltration[9] assays of the polymerized nanoparts were performed in special cells of 65, 200, or 400 ml and with a special diaphragm.

Finally an opalescent aqueous solution of the polymer product was obtained. Freeze drying of this solution gave a white free-flowing powder, which was redispersable in aqueous solution.

Isolation and Purification According to Procedures A and C—The polymeric product containing the antigen could be precipitated at −5° with an excess of a mixture containing 60% water and 40% (v/v) methanol. The isolation and purification of the nanoparts were done by centrifugation or ultrafiltration[9] at −5° to remove all surfactants. Freeze drying, preferably with a methanol content of 5% (v/v), removed the solvent and yielded products that formed a white free-flowing powder.

Immunological Assays—The antibody levels of human IgG in guinea pig serum were measured by a passive hemagglutination test (12, 13). For this purpose, sheep erythrocytes, which had been sensitized with the antigen by means of benzidine, were used. Antitoxin titer levels are average values for surviving animals of a group of five to 10 guinea pigs from a serum dilution series with factor 2 (Fig. 1). The antibody levels (antitoxin titers in International Units per milliliter) against tetanus toxoid in guinea pig serum were determined as the LD+ dose of the toxoid (14, 15). The serum of four to 10 animals of one group was analyzed in pools.

***In Vitro* Release Studies**—According to Procedure C, nanoparts containing ^{125}I-labeled human IgG were synthesized by polymerization with γ-radiation. The resulting product was isolated and purified by following ultrafiltration and freeze drying as described for Procedures A and C.

The release of the globulin from the polymer material was studied by a modified beaker method (16). Therefore, 38.44 mg of nanocapsules, corresponding to 1 mg of labeled human IgG, was redissolved in a 300-ml well-closed beaker with 250 ml of aqueous phosphate-buffered medium of pH 7.2 (0.645 g of monobasic potassium phosphate and 2.125 g of dibasic sodium phosphate dihydrate). The solution was magnetically stirred at 60 rpm, and the whole medium was thermostated in a water bath of 37 ± 0.2°.

The liberation of the free globulin from the polymer material was followed by membrane filtration[10]. Therefore, every 24 hr, 0.050 ml of the solution was diluted with 1000 ml of the phosphate buffer and filtered under a nitrogen pressure of 5 atm in a special cell[11] and washed five times with 1000 ml of the buffer. ^{125}I-Activity measurements were performed in a liquid scintillation counter[12], and probes were diluted with Bray solution (4.0 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 60 g of naphthalene, 20 ml of ethylene glycol, 100 ml of methanol, and 815 ml of dioxane).

[8] W. Möller, Glass-Blower Ltd., Zurich, Switzerland.
[9] Diaphragm: Diaflo PK 30, Amicon Ltd., Oosterhout, The Netherlands.
[10] Diaphragm SM 115.30, 35–20-nm pore size, Sartorius GmbH, Göttingen, Germany.
[11] Ultrafiltration cell SM 162 25 with an effective filtration area of 16 cm^2, Sartorius GmbH, Göttingen, Germany.
[12] Packard Tri-Carb liquid scintillation spectrometer 3320, Packard Instrument Corp., Frankfurt, Germany.

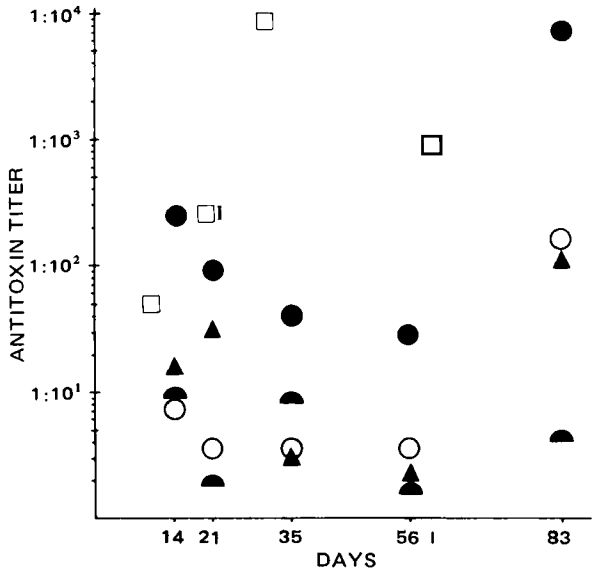


Figure 1—*Immunization of guinea pigs with human IgG preparations (antibody levels as a function of time). Key: ◓, immunization with IgG basic material (aqueous buffered solution, pH 7.2, 0.3 mg of IgG/kg sc); ▲, IgG basic material plus aluminum oxide (4 mg/ml) (0.3 mg/kg sc); ○, IgG in nanoparts (0.3 mg of IgG/kg sc); ●, IgG in nanoparts (1 mg of IgG/kg sc); □, IgG basic material plus complete Freund adjuvant (1:1) (1 mg of IgG/kg im); and I, booster injection after 21 days and booster injections with basic material after 60 days.*

Each day, the activity of 0.050 ml of the test medium was set at 100%. The activity of collected filtrates corresponded to percent free globulin (as could be proven by test filtrations with free globulin in the presence of "empty" nanocapsules in solution).

RESULTS AND DISCUSSION

Activity and Stability of Biological Material in Nanoparts—Even labile proteins can be embedded in nanoparts. After a "simulated" micelle polymerization, *i.e.*, in the absence of monomers, urease, tetanus toxoid, and human IgG, no noticeable loss of enzymatic reactivity or immunological response was shown. The urea-decomposing activity of urease and the immunoelectrophoretic behavior of the toxoid and the globulin remained unchanged after the "simulated" synthesis (17).

Embedded active substance, *e.g.*, human IgG, forms a deposit in the nanoparts. With labeled human IgG as a model, a maximum of only 25% of the incorporated substance could be released unaltered from nanocapsules over 48 days. It is reasonable to assume that only radioactive fragments of the globulin molecule were recorded, suggesting that the fixed portion of IgG may be greater than 75%.

Adjuvant Effect—In theory, stable antigen fixation and the physical properties of nanoparts offer rich prospects for immunological applications. Favorable characteristics of nanoparts for use as adjuvants include their compatibility with only slight local reactions at the place of application, their stability in solution, their easy method of application in a water-based solution, their protective effect with only slowly expected metabolism or corrosion and with only minimal release of antigenic material, and their possible affinity to lipoid cell membranes. Furthermore, a highly dispersed amount of antigenic material can be incorporated in the nanoparts.

Another advantage is the possible disseminating effect of colloid particles with a tendency to spread over the whole lymphoid system. The antigen appears in the polymerized state as a considerably larger complex. As a result, phagocytosis is strongly stimulated. The antigen–adjuvant complex penetrates into the immunosystem; it is transferred into the lymphatic ganglions and into the spleen, where it is degraded slowly and can be distributed.

Expected good adjuvant effects have been confirmed experimentally. Animal experiments also showed that nanoparts are well tolerated parenterally.

Human IgG—Studies of the adjuvant effect of nanoparts with

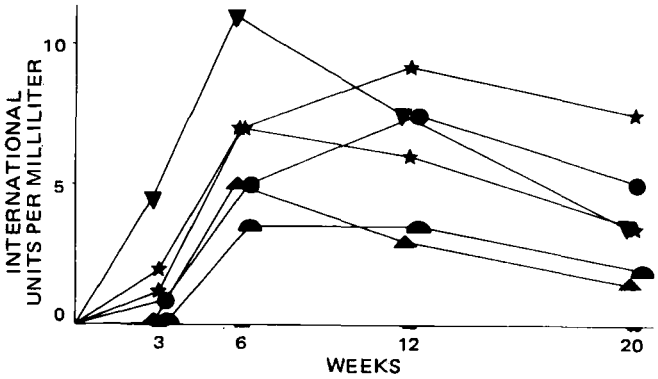


Figure 2—*Immunization of guinea pigs with tetanus toxoid preparations (antitoxin titers as a function of time) using intramuscular vaccination with no booster injection. Key: ▲, tetanus toxoid embedded in nanoparts (5 Lf); ▼, tetanus toxoid embedded in nanoparts (50 Lf); ◓, tetanus toxoid embedded (5 Lf) plus basic material (aqueous buffered solution, 5 Lf); ●, tetanus toxoid embedded (50 Lf) plus basic material (aqueous buffered solution, 50 Lf); ★, tetanus toxoid adsorbed on aluminum phosphate (5 Lf); and ☆, tetanus toxoid adsorbed on aluminum phosphate (50 Lf).*

enclosed human IgG in immunization trials on guinea pigs showed that higher antibody titers are obtained as compared with aluminum oxide as the adjuvant. Furthermore, a level comparable to Freund's adjuvant, the most effective jet toxic adjuvant, is approached (Fig. 1).

Tetanus Toxoid—Nanoparts with tetanus toxoid as antigen produce in guinea pigs slightly less immunization (antibody production) than the standard aluminum phosphate adjuvant (see Fig. 2).

CONCLUSION

The embedding of biologically active materials in ultrafine components (nanoparts) offers considerable technological possibilities and flexibility, as shown with the example of human IgG, tetanus toxoid, and urease. Accordingly, the partition of drugs in such nanoparts seems to be promising as a new parenteral drug delivery system for long-term therapy. Other biological drug models and the mechanism of drug incorporation and release will be studied.

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