In summary, all the results indicate that the optimal effect of L-asparaginase is obtained when the enzyme is injected intramuscularly in immobilized form in small doses.

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Acrylic Microspheres *In Vivo* VII: Morphological Studies on Mice and Cultured Macrophages

PETER EDMAN *, INGVAR SJÖHOLM *x, and ULF BRUNK

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* Present address: Department of Drugs, National Board of Health and Welfare, Division of Pharmacy, S-751 25 Uppsala, Sweden.

Abstract □ Intravenously injected microparticles of polyacrylamide were cleared from the circulatory system in mice predominantly in liver, spleen, and bone marrow in mice by macrophages belonging to the reticuloendothelial system. In these cells, particles were found in dilated secondary lysosomes. The lysosomotropic character of the particles was further demonstrated using cultured peritoneal mouse macrophages. Histological changes of the liver, spleen, and bone marrow detectable by light and electron microscopy could only be seen after administration of massive doses of microparticles corresponding to 160 mg/kg body weight. In such cases, a medium-coarse vacuolization of liver parenchymal cells could be seen 1–2 days after particle administration. After 3–5 days, degeneration and necrotic cellular alteration occurred in the liver, spleen, and bone marrow. One week after particle administration, regeneration started under formation of granulomas which replaced the necrotic areas. The tissues later became normalized (after 2–3 weeks), but small granulomas remained for several weeks. The damage was parallelled by changes in the liver and spleen weights. Electron microscopy of the liver revealed that the initiated vacuolization of the parenchymal cells was due to mitochondrial swelling with rupture of the mitochondrial cristae.

Keyphrases □ Acrylic microspheres—*in vivo*, morphological studies on mice and cultured macrophages, polyacrylamide, liver, spleen, marrow □ Macrophages, cultured—acrylic microspheres, *in vivo*, morphological studies on mice, polyacrylamide, liver, spleen, marrow □ Polyacrylamide—acrylic microspheres, *in vivo*, morphological studies on mice and cultured macrophages, liver, spleen, marrow

Microparticles of highly cross-linked polyacrylamide, or derivatives, have recently been introduced as a slowly degradable carrier system for immobilized enzymes *in vivo* (1). Such particles have a pronounced porous structure allowing substrates to freely penetrate the particles and interact with the immobilized enzymes. They are small enough (mean diameter 0.1–0.4 μm) to be injected intravenously without causing respiratory complications. After intravenous or intraperitoneal injection, the microparticles are taken up by cells of the reticuloendothelial system, essentially in the liver and spleen, where they are probably localized in the lysosomal vacuome, although no direct evidence has yet been presented. Microparticles with immobilized L-asparaginase have been used to depress the level of circulating L-asparagine with resulting growth inhibition of the ascites tumor 6C3HED in mice (2). In addition, particles containing dextranase have successfully been used to treat an artificial storage disease in mice, which is produced by polyacryldextran (3).

In all studies performed to date, no signs of acute toxicity have been detected. Thus, the growth rate and survival time have been unaffected, and no tissue incompatibility has been noticed, besides the normal encapsulation of implants or intramuscularly injected microparticles. Very large doses of microparticles (100–200 mg/kg of body weight), however, have produced a transient hepatosplenomegaly in mice, lasting 4–8 weeks, resulting from the localization of the spheres in the liver and spleen. The purpose of the present work was to study microscopically the possible morphological alterations of several organs following the administration of such provocative doses of

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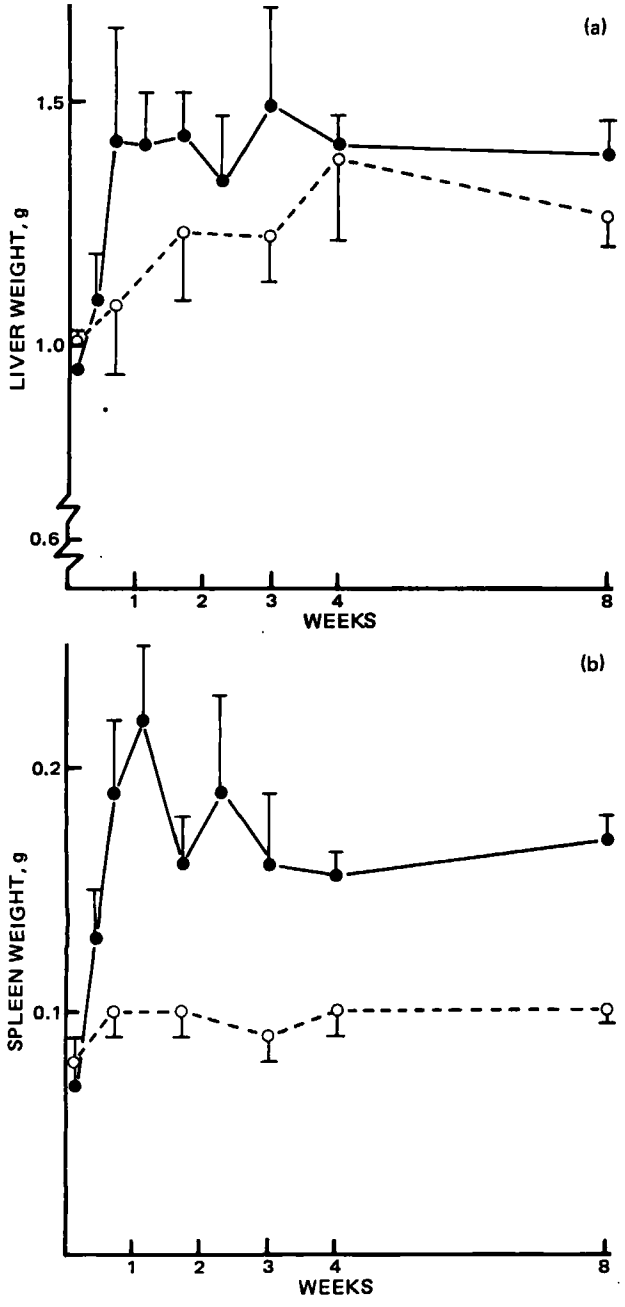


Figure 1—*(a) Weight of the liver after injection of 4 mg of polyacrylamide microparticles iv (●) or physiological saline (○) in mice. Each point shows the mean ±SD from the five animals. (b) Weight of the spleen after injection of 4 mg of polyacrylamide microparticles iv (●) or physiological saline (○) in mice. Each point represents mean ±SD from five animals.*

microparticles to mice. Concomitantly, the lysosomotropic character of the particles has been established using cultured mouse peritoneal macrophages.

EXPERIMENTAL

Chemicals—Acrylamide[1], *N,N'*-methylenebisacrylamide[1], cationized ferritin[2], *N,N,N',N'*-tetramethylethylenediamine[2], and dextran[3] were of analytical grade. Acrylic acid–glycidyl ester[4] and catalase[4] (E.C. 1.11.1.6) were used without further purification.

[1] Eastman Kodak Co.
[2] Sigma Chemical Co.
[3] Dextran T40 (mol.wt. 40,000), Pharmacia Fine Chemicals, Uppsala, Sweden.
[4] Fluka AG.

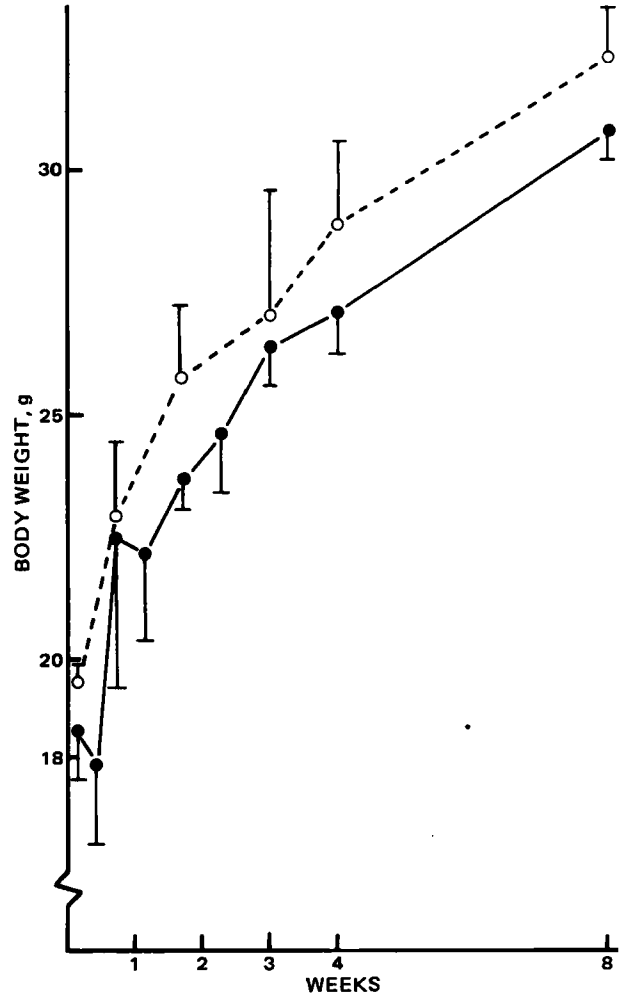


Figure 2—*Body weight of mice given 4 mg of polyacrylamide particles (●) or physiological saline (○) intravenously. Each point represents mean ±SD from five animals.*

Preparation and Administration of Microparticles—Microparticles[5] of polyacrylamide (TC = 8-25)[6] or polyacryldextran (DTC = 11-1-75)[6] were prepared according to a reported method (4, 5). Acrylamide or acryldextran and *N,N'*-methylenebisacrylamide were dissolved in sodium phosphate buffer, pH 7.4. Oxygen was eliminated from this solution with nitrogen gas. The toluene–chloroform mixture (4 + 1) containing a detergent[7] was treated in the same manner. After addition of the catalyst, ammonium peroxydisulfate (dissolved in water), the mixture was homogenized to produce a water-in-oil emulsion. The polymerization of the emulsion was initiated by adding *N,N,N',N'*-tetramethylethylenediamine.

The suspension was stirred for 20 min and the phases were separated by centrifugation. The microparticles located at the bottom of the water phase were washed at least five times, and after the last washing the particles were suspended in physiological saline.

When microparticles with immobilized ferritin or immobilized catalase were prepared, ferritin (52 mg) or catalase (20 mg) was dissolved in 2.5 ml of the acrylic monomer solution. After polymerization, the particles were washed as described above.

The microparticles were resuspended carefully in physiological saline prior to administration. The size of the particles was measured from photographs taken by scanning electron microscopy, which was done as described earlier (4). With the method used, 92.6% of the polyacrylamide particles (TC = 8-25) had a diameter of 0.1–0.4 μm, 2.2% were <0.1 μm, and 5.2% had a diameter of 0.4–0.5 μm. The polyacryldextran particles (DTC = 11-1-75) had essentially a diameter of 0.2–1.2 μm; 72.8% of the particles had a diameter within this range, and 8.9% were <0.2 μm and 5.8% were >2.1 μm.

[5] U.S. Patent 4,061,466.
[6] The nomenclature is explained in Refs. 4 and 5.
[7] Pluronics F-68, Trebac AB Stockholm, Sweden.

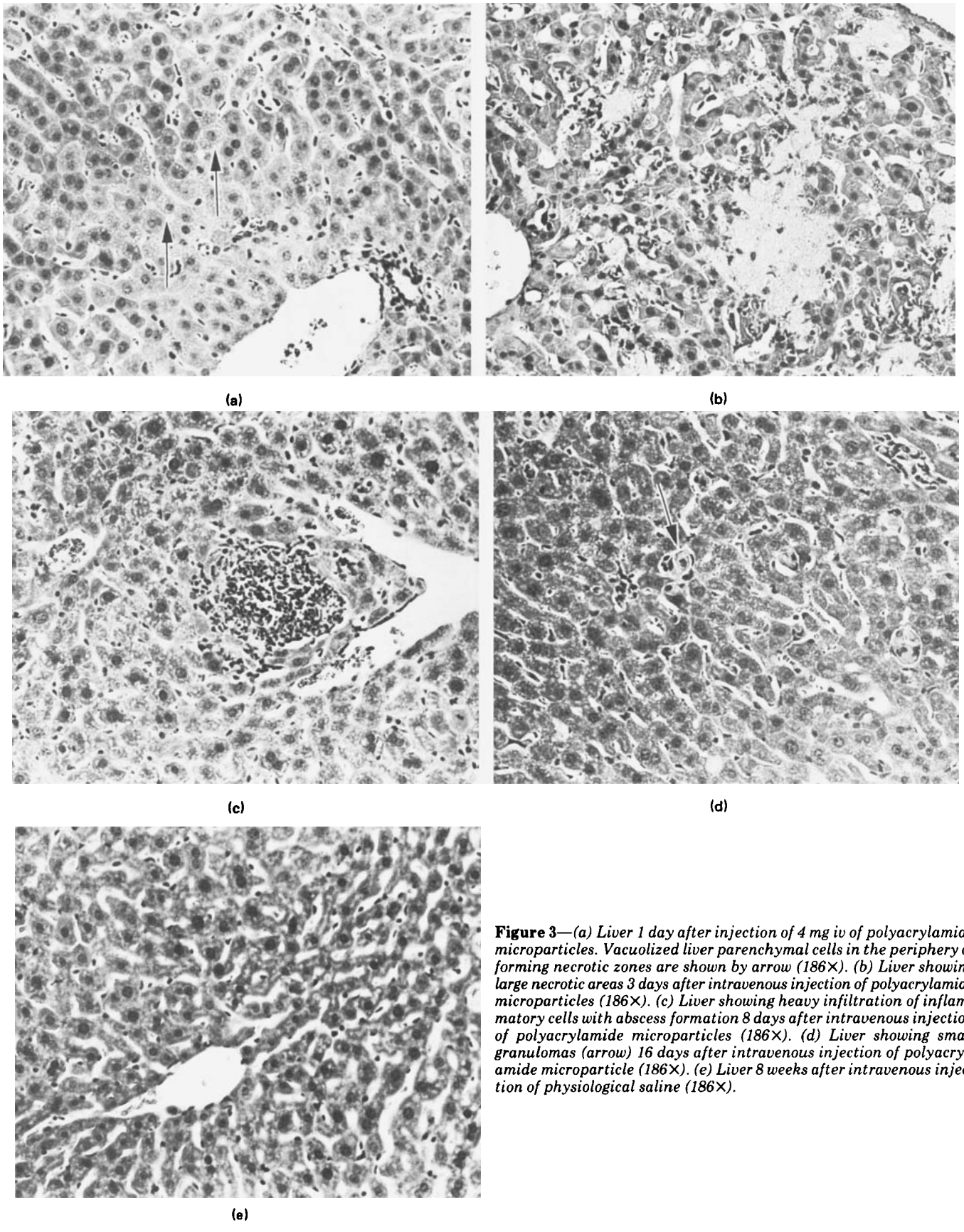


Figure 3—*(a) Liver 1 day after injection of 4 mg iv of polyacrylamide microparticles. Vacuolized liver parenchymal cells in the periphery of forming necrotic zones are shown by arrow (186×). (b) Liver showing large necrotic areas 3 days after intravenous injection of polyacrylamide microparticles (186×). (c) Liver showing heavy infiltration of inflammatory cells with abscess formation 8 days after intravenous injection of polyacrylamide microparticles (186×). (d) Liver showing small granulomas (arrow) 16 days after intravenous injection of polyacrylamide microparticle (186×). (e) Liver 8 weeks after intravenous injection of physiological saline (186×).*

Male mice[8] weighing 20–25 g were used throughout. Microparticles of polyacrylamide or polyacryldextran were suspended in physiological saline (0.1 or 0.2 ml) and injected intravenously. The doses corresponded to 1–4 mg of lyophilized microparticles.

[8] NMRI-mice, Anticimex, Stockholm, Sweden.

Preparation of Tissue Samples for Light and Transmission Electron Microscopy—At various time intervals (1–56 days) after exposure to the microparticles, five animals from each group were sacrificed by cervical dislocation, and the liver, spleen, heart, lungs, kidneys, brain, and bone marrow from the spine were collected. At each time interval, three mice from an untreated group were sacrificed and their organs used

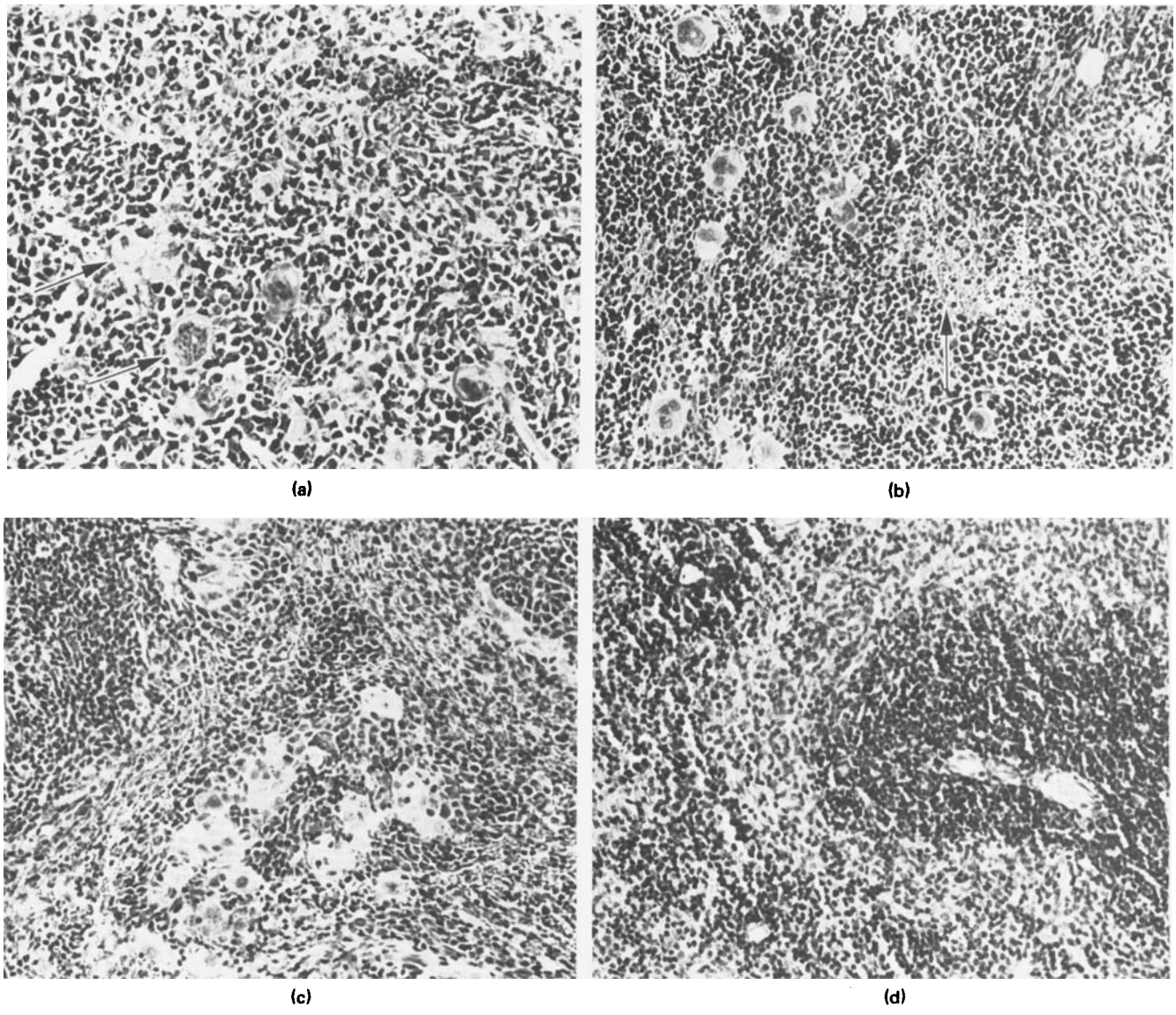


Figure 4—*(a) Spleen 3 days after intravenous injection of polyacrylamide microparticles. The architecture is much altered, with no demarcation between the red and white pulp and heavy infiltration of inflammatory cells. Small abscesses are shown by arrow (186×). (b) Spleen with forming granulomas (arrow) 8 days after intravenous injection of polyacrylamide microparticles (186×). (c) Spleen 21 days after intravenous injection of polyacrylamide microparticles; reconstitution of red and white pulps but remaining granulomas (186×). (d) Spleen 8 weeks after intravenous injection of physiological saline (186×).*

as the control group. The liver, spleen, heart, lungs, brain, and kidneys were weighed, and thin (2 mm) tissue specimens were cut from the organs immediately, and immersed in chilled 5% formaldehyde in 0.15 *M* phosphate buffer, pH 7.2. The specimens were subsequently embedded in paraffin, cut at 2–3 μm, stained with hematoxylin and eosin, and studied under the light microscope.

For transmission electron microscopy, small samples (<1 mm thick) were removed from the liver immediately after the animals were sacrificed. The specimens were immersed in ice-cold 2% glutaraldehyde in 0.1 *M* sodium cacodylate buffer with 0.1 *M* sucrose (pH 7.2; total osmotic pressure 510 mOsm, effective osmotic pressure 300 mOsm) (6, 7). After glutaraldehyde fixation for 24 hr, the specimens were rinsed in 0.15 *M* sodium cacodylate buffer, pH 7.2, and cut into small pieces which were postfixed in 1% OsO_4 in 0.15 *M* sodium cacodylate buffer for 90 min at room temperature. The specimens were dehydrated in an ethanol series, stained *en masse* with 2% uranyl acetate in 50% ethanol for 12 hr, and embedded in expoxy[9]. Thin sections were cut with diamond knives, stained with lead (8), and examined at 60 kV with an electron microscope[10].

Transmission Electron Microscopy of Negatively Stained Microparticles—A suspension of ferritin-labeled microparticles was placed as a tiny drop on formvar coated 300-mesh copper electron microscopy grids and most of the fluid was immediately removed with a piece of filter paper. A drop of a 0.2% water solution of uranyl acetate was applied for 30 sec and removed with filter paper. After drying, the grids were examined at 80 kV with the electron microscope[10], using a 50-μm objective aperture.

Cultures of Mice Peritoneal Macrophages—Mouse macrophages were collected by washing the unstimulated peritoneum of adult male animals[8] with 3 ml of warm (37°) phosphate-buffered saline. The cells were further suspended in 10 ml of cold (0°) phosphate buffer and mildly spun down at 180×*g* for 10 min. The pellet was resuspended in F-10 medium (9) with 20% newborn calf serum and 15 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer, supplemented with 10 μg/ml of streptomycin and 100 U/ml of penicillin. The cells were seeded into 50-mm plastic petri dishes at 5×10^5 cells/dish and cultured at 37° in 5% carbon dioxide in air at 85% humidity. The dishes were carefully rinsed with new, complete medium 24 hr after the initial seeding, to eliminate red blood cells, lymphocytes, and macrophages that had not attached to the solid support. During the ensuing cultivation period the medium was changed every 48 hr.

Cultures were exposed to 0.1 mg/ml of microspheres containing ferritin as a marker 24 hr after seeding (immediately after being rinsed). The cells were exposed to these particles for 48 and 72 hr and then fixed in 2% glutaraldehyde in 0.1 *M* sodium cacodylate buffer with 0.1 *M* sucrose (pH

[9] Epon 812.
[10] JEOL 100-C microscope.

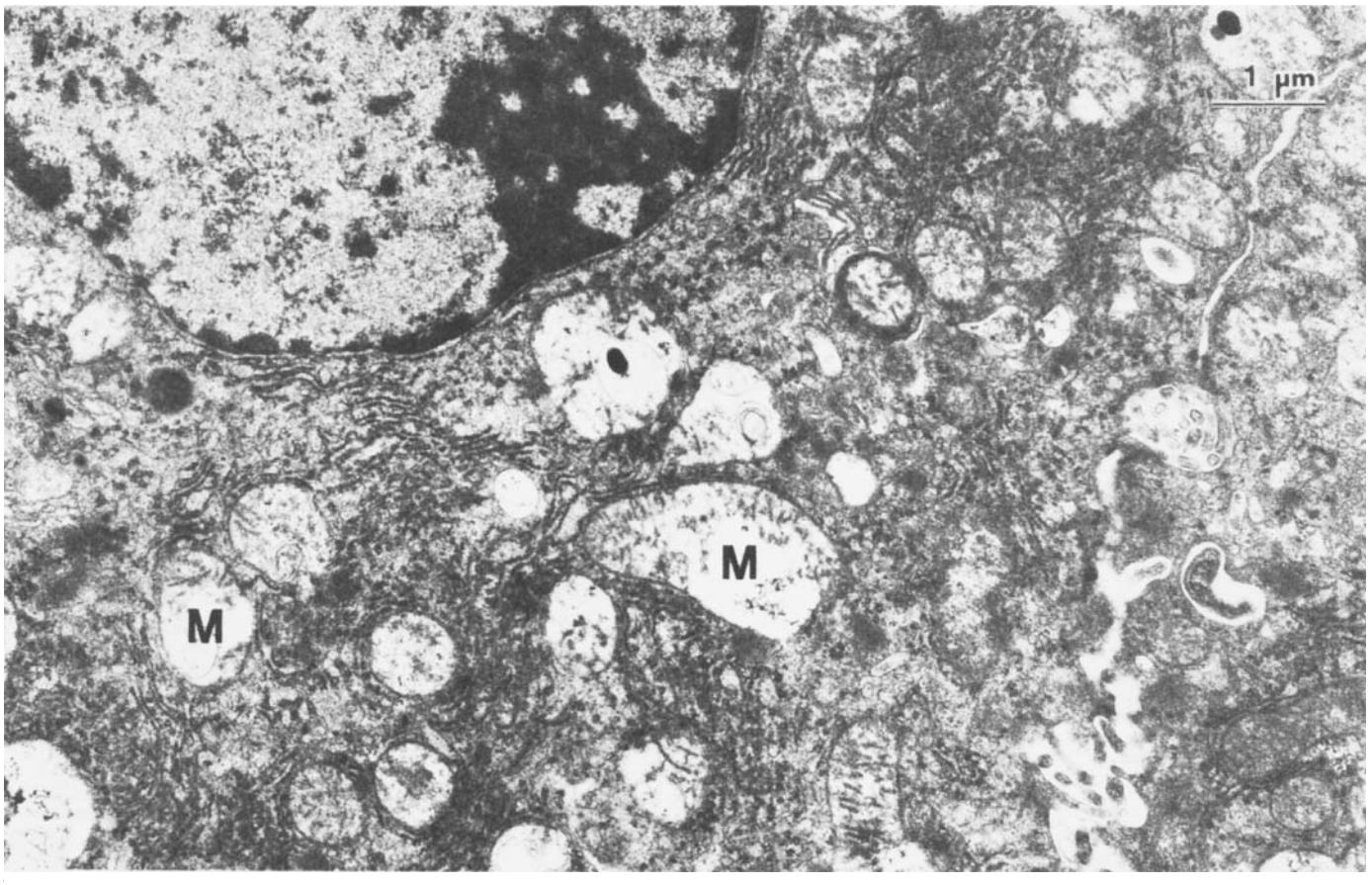


Figure 5—*Liver parenchymal cells 2 days after injection of 4 mg iv polyacrylamide microparticles containing catalase. Several swollen mitochondria (M) with ruptured cristae can be seen.*

7.2, total osmotic pressure 510 mOsm, effective osmotic pressure 300 mOsm (6, 7) for 60 min at 0°, rapidly rinsed in 0.15 *M* sodium cacodylate buffer and postfixed in 1% OsO_4 in 0.15 *M* sodium cacodylate buffer for 90 min at room temperature. The cells were then dehydrated *in situ* in an ethanol series, counter-stained *en masse* for 12 hr in 2% uranyl acetate in 50% ethanol, and embedded in small capsules in epoxy as described earlier (10). Untreated control cells were harvested at the same time intervals. Thin sections were cut with diamond knives, stained with lead citrate (8), and examined at 60 kV with the electron microscope[10].

RESULTS

Gross Distribution of Microparticles After Intravenous Injection—It was shown in an earlier study (11) that the distribution of large doses of microparticles to the reticuloendothelial system in mice after intravenous injections results in a transient hepatosplenomegaly. To correlate the megaly with the microscopic findings, the weight changes were studied in some detail.

Figures 1a and 1b show the variations in weight of the liver and the spleen, respectively, with time after 4-mg iv injections of microparticles. The liver weight reached its maximum after 5–8 days and was essentially normalized after 4–8 weeks. The effects of the microparticles on the growth of the spleen were relatively greater than on the liver (Fig. 1b). One week after the injection, the weight was ~220% of that of the controls. The megaly was successively retracted, but the organ weight was not normalized within the 8-week study period. In the earlier work (11), the weight of the spleen was normalized after 16–24 weeks. With smaller doses of polyacrylamide (1 mg) or of polyacryldextran (2 mg) enlargement of the liver and spleen was not as great. This weight increase was strictly dose dependent.

The variation in body weight after intravenous injection of 4-mg polyacrylamide microparticles is shown in Fig. 2. As is apparent from the figure, there were no significant differences between the control and test groups. Although the overall weight of the test group was lower at the time of injection, the increase in weight with time was the same. Consequently, the administration of large doses of microparticles to the mice did not affect growth.

Light Microscopy of Mouse Tissue—*Following* the administration of 1 mg of particles, no changes were detected by light microscopy in any of the studied organs. No demonstrable changes were noted in the brain, heart, kidneys, or the lungs, while the liver, spleen, and the bone marrow showed pronounced alterations after injection of 4 mg of particles.

As early as 1 day after particle administration, the parenchymal cells of the liver showed a medium coarse vacuolization, which had a patchy distribution (Fig. 3a). After a few more days, necrotic areas of various sizes had developed corresponding to the areas of initial vacuolization, as well as unicellular necrosis (Fig. 3b). There was a diffuse, sparse infiltration of inflammatory cells (Figs. 3c and d). Later, regeneration started with mitotic activity within the parenchymal liver cell population. After 7–10 days, granulomas composed of macrophages, epithelioid cells, lymphocytes, and polymorphonuclear cells formed and replaced the necrotic cells and areas (Fig. 3d). These granulomas remained for several weeks. The number of Kupffer cells was found to have increased at later time intervals. The normal ultrastructural appearance of the liver 8 weeks after intravenous injection of physiological saline is shown in (Fig. 3e).

In the bone marrow there was an early (after 1 day) and massive infiltration of mature, irregularly distributed polymorphonuclear cells and macrophages with the formation of granulomas. After 7–10 days, the bone marrow had regained an apparently normal appearance.

The spleen showed early and pronounced changes. After 1–3 days, the limits between the white and the red pulps were blurred, the sinusoids were dilated and lined with prominent endothelial cells, and the number of megakaryocytes was increased (Fig. 4a). Later (4–10 days) there was a heavy infiltration of various inflammatory cells under formation of small abscesses and granulomas (Fig. 4b). The tissue became normalized later, but small granulomas, composed of histiocytes, remained for many weeks (Fig. 4c). The normal appearance of the spleen is shown in (Fig. 4d).

Transmission Electron Microscopy of Liver Tissue—The vacuoles of the liver cells, which were so strikingly observed 1 or 2 days after the administration of large amounts of particles, were found to be due to mitochondrial swelling with rupture of the mitochondrial cristae (Fig. 5). There was no obvious alteration of the lysosomal vacuome of the parenchymal cells. The Kupffer cells showed many dilated lysosomes filled with an amorphous material.

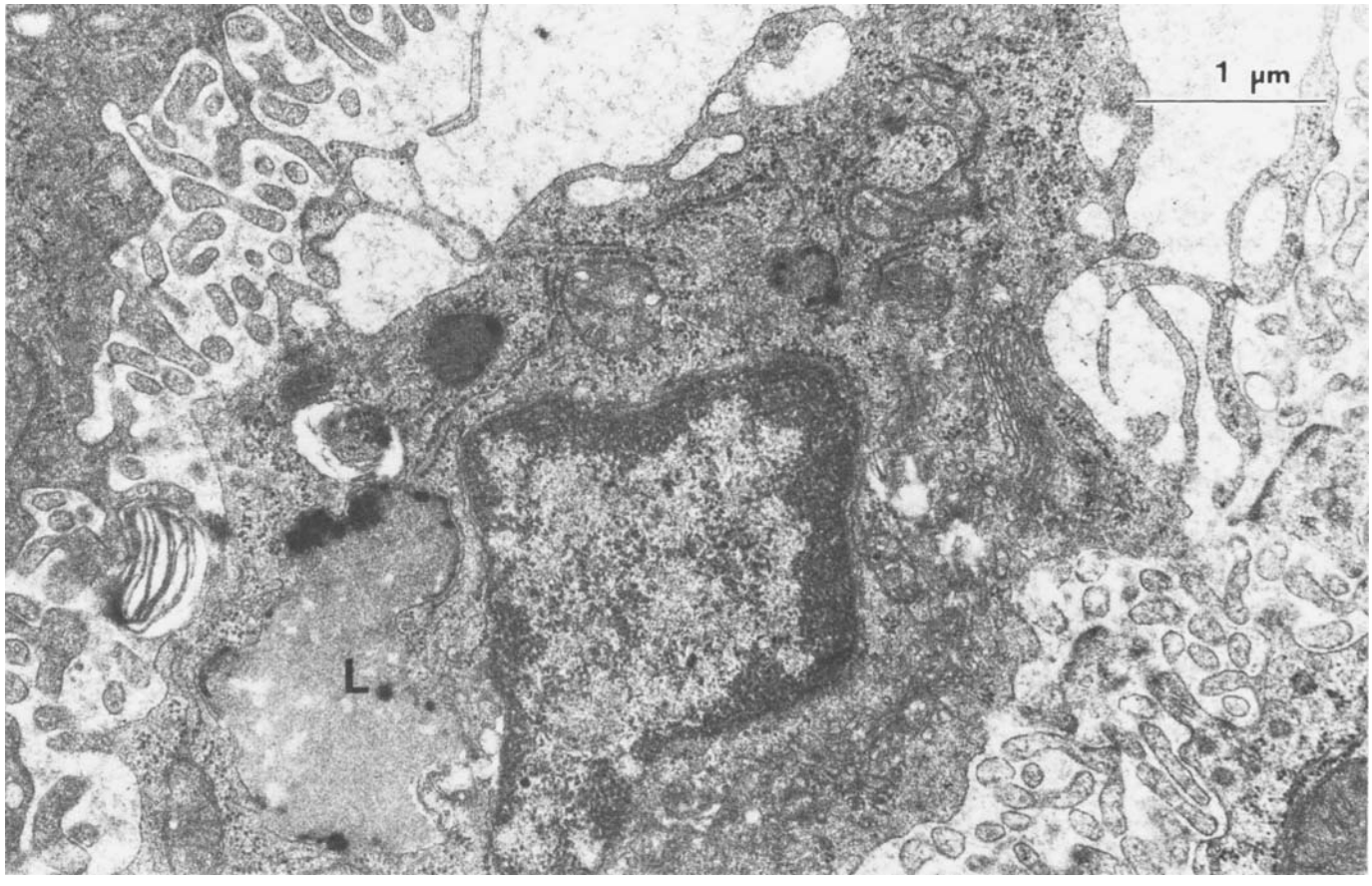


Figure 6—*Kupffer cell 2 months after injection of 4 mg iv polyacrylamide microparticles loaded with catalase. Note large vacuoles (L, secondary lysosomes) filled with an amorphous material.*

At later time intervals, the tissue degeneration prevented detailed studies on cellular alterations until the acute inflammatory response had declined. In the specimens studied ~2 months after the administration of the catalase-loaded particles, these lysosomes were still frequently observed (Fig. 6). It was, however, not possible to discern individual particles either in liver parenchymal or in Kupffer cells. Obviously, the particles have an electron density about equal to that of the embedding resin and do not bind osmium, uranyl, or lead.

Transmission Electron Microscopy of Negatively Stained Microparticles—Transmission electron microscopy of the ferritin-loaded microparticles indirectly confirmed (12) that the polyacrylamide particles were spherical and had a diameter between 0.1 and 0.4 μm. Ferritin gave the particles a dotted appearance.

Transmission Electron Microscopy of Cultured Macrophages—Macrophages exposed to 0.1 mg of microparticles with ferritin per milliliter of medium for 72 hr showed a high increased rate of autophagocytosis and an increased number of secondary lysosomes containing an amorphous material with tiny electron dense particles corresponding to the ferritin of the labeled microspheres. In most lysosomes the electron dense particles were concentrated toward the periphery of the organelles (Figs. 7a and b).

DISCUSSION

As mentioned earlier, no signs of acute toxicity were detected when microparticles of acrylic polymers were used as carriers for enzymes in various experiments on mice and rats (1). When the distribution of such small particles was studied in mice (11) only a transient hepatosplenomegaly was noticed. The present work has, moreover, demonstrated that a large number of spheres must be administered in order to produce any pathological morphological alterations in the liver, spleen, and bone marrow, which constitute the tissues where the microparticles are localized *in vivo* (11). The effects described in the present study are generally obtained after treatment with doses corresponding to 160 mg of dried material per kg of body weight. Such a very large dose is necessary to produce the adverse reactions, which are important to know, since the microparticles are an attractive alternative as enzyme carriers for future human applications.

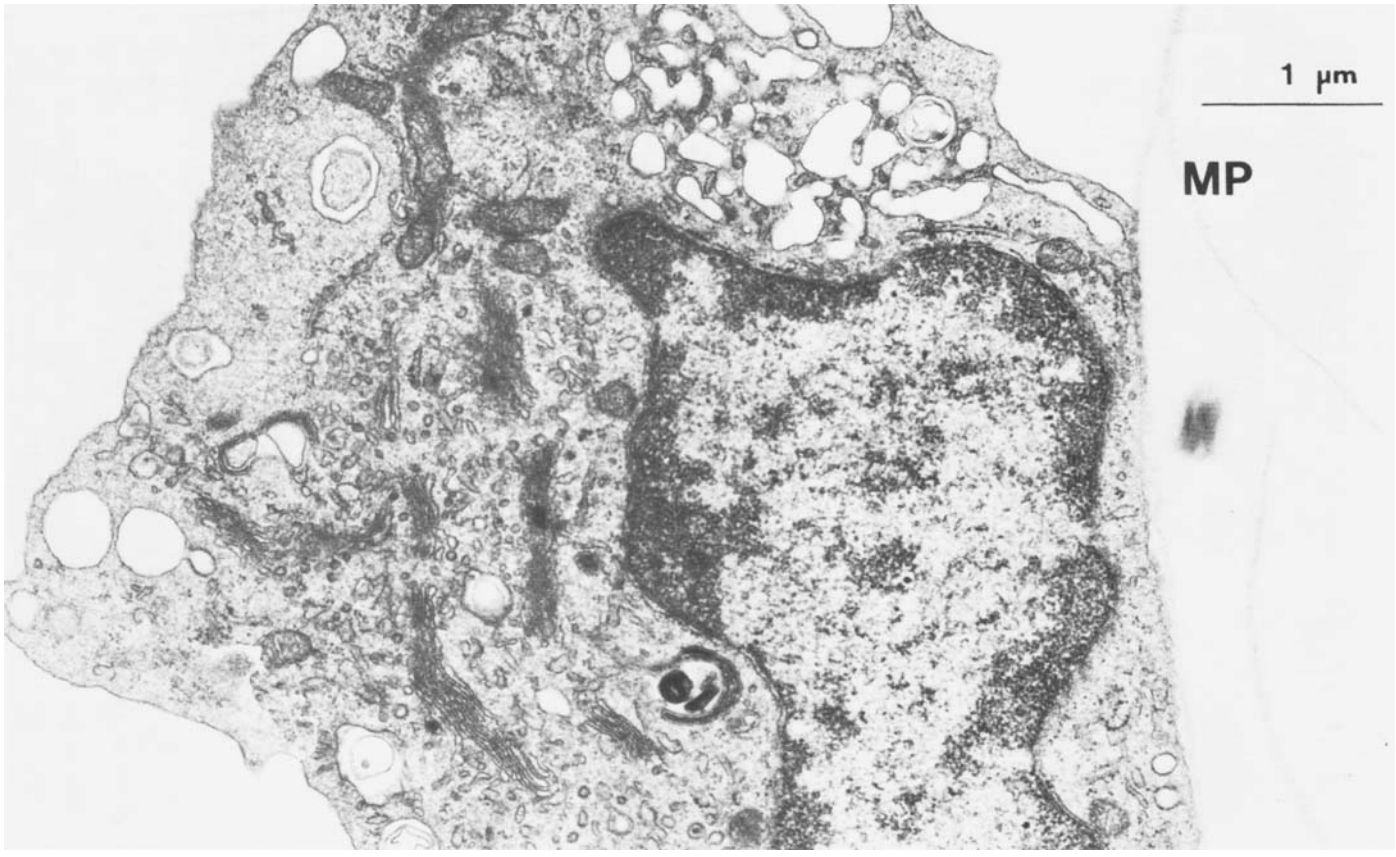
It is important to stress that when dose levels of 40 mg/kg were used (corresponding to the administration of 1 mg of particles to the mice), only insignificant, hardly detectable morphological reactions could be seen in the liver, spleen, and bone marrow.

The first general reaction in the liver tissue, detected by light microscopy and transmission electron microscopy, on exposure to the high doses of microparticles, was the swelling of the mitochondria of the parenchymal cells, which were eventually ruptured as part of cellular degeneration. Necrosis of the tissues followed, and inflammatory cells migrated into the area, with a megaly as a consequence. The weight changes of the liver and spleen followed very closely the morphological changes seen with the light and electron microscopes and reached maxima at the same time as the maximum of the tissue damage. Thus, the megaly was probably essentially due to the increased number of cells in the liver and spleen, and only to a minor extent a result of cellular edema.

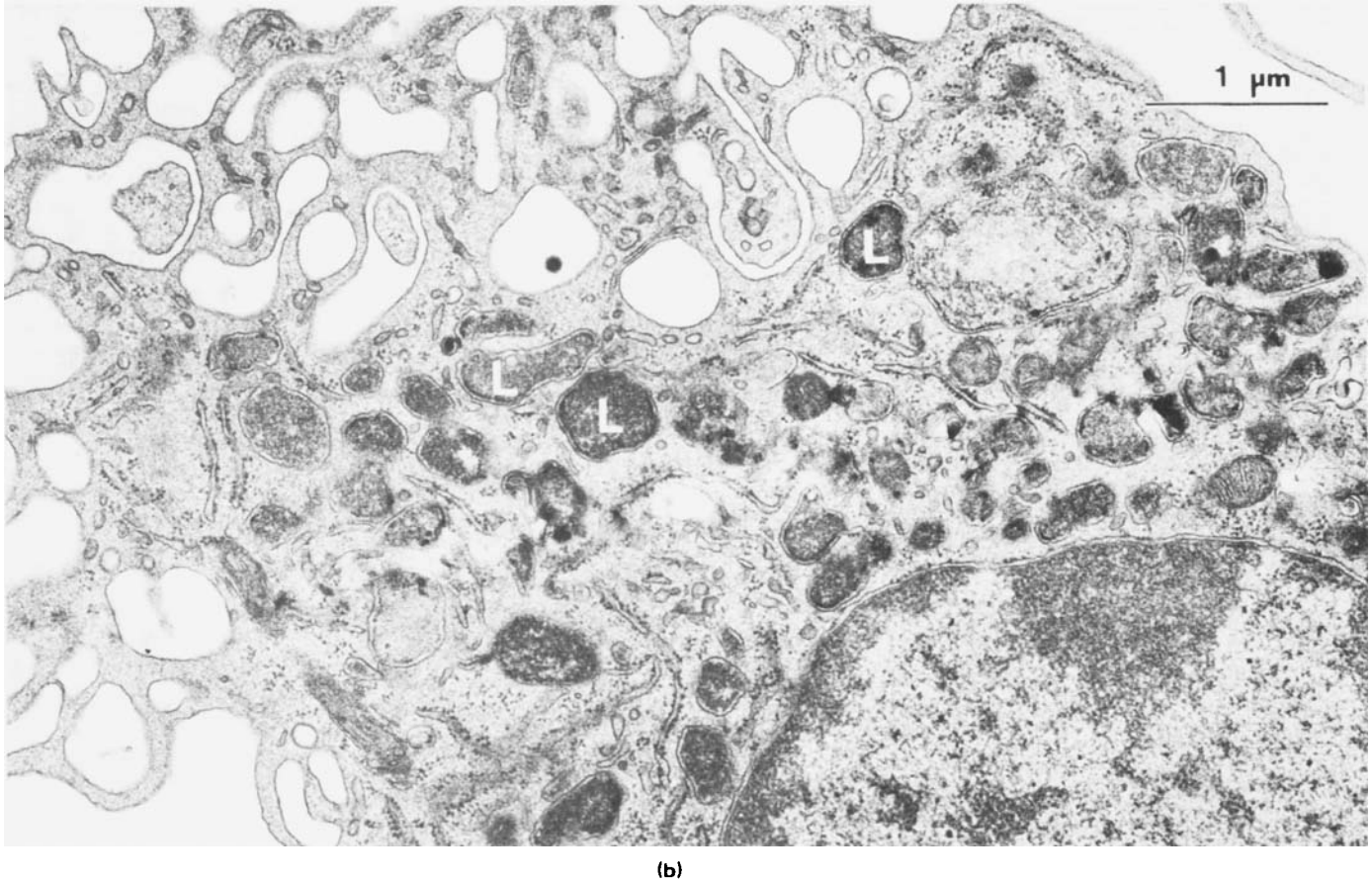
The reason for the initial hepatocellular degeneration is not obvious, and the interpretation of our results is hampered by the fact that the microparticles could not be seen directly with the electron microscope. The spherical form of the ferritin-loaded microparticles was confirmed by negative staining, and ferritin could be used as a marker in the *in vitro* experiments with cultured macrophages to trace the microparticles.

In these studies it was quite evident that the microparticles had been taken up by the cells and introduced into the lysosomal vacuome, as seen both indirectly by the markedly increased number of lysosomes and their swelling, and directly by the localization of large amounts of electron dense material in the lysosomes (Fig. 7b), which was not seen in the control (Fig. 7a). Consequently, it might be suggested that the degenerative cellular alterations, *e.g.*, the swelling and rupture of the mitochondria, giving rise to the vacuolization of the liver parenchymal cells, are due to an altered function of the lysosomal apparatus. It was thus not possible to directly detect the particles in the hepatocytes in the tissue samples. The indirect evidence, however, suggests an uptake both in the Kupffer and the parenchymal cells. Most probably the small size of the particles allows their passage into the space of Dissé through the existing gaps in the sinusoidal endothelium exposing the microparticles to the liver parenchymal cells which could bring about their endocytosis.

The present work is only semiquantitative, in the sense that the toxic



(a)



(b)

Figure 7—*(a) Normal mouse peritoneal macrophage cultured* in vitro. *MP denotes the microprecipitate to which the cells have attached. (b) Macrophage 72 hr after exposure to ferritin-labeled polyacrylamide microparticles (0.1 mg/ml of medium). Note the large number of lysosomes (L) containing microparticles with ferritin.*

reactions seen in the mice are proportional to the doses given, and only detectable when the mice are exposed to very high doses exceeding 40 mg/kg of body weight. Quantitative studies are not possible *in vivo*, when the relationship between the number of particles taken up per cell cannot be satisfactorily controlled. For such studies, it is necessary to use suitable *in vitro* systems, *e.g.*, cultured peritoneal macrophages, where the relationship between the number of particles and cells can be carefully controlled. Such studies will be presented separately, showing when exposure of cultured macrophages to microspheres will lead to changed intracellular function and cellular degeneration. Subsequent *in vivo* applications with acrylic microspheres must be based on such studies.

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Poly-L-methionine Sulfoxide: A Biologically Inert Analogue of Dimethyl Sulfoxide with Solubilizing Potency

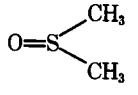
JOSEF PITHA *x, LAJOS SZENTE *, and JUDITH GREENBERG ‡

Received January 18, 1982, from the **National Institutes of Health, National Institute on Aging-GRC, Baltimore, Maryland 21224* and the ‡*National Institute of Dental Research-NIH, Bethesda, Maryland 20205.* Accepted for publication June 8, 1982.

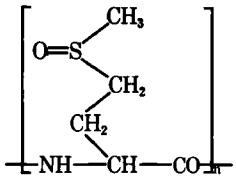
Abstract □ Poly-L-methionine sulfoxide is a water-soluble polymer containing the sulfoxide moiety. The preparation and radiolabeling of this polymer is described and its bioeffects are compared with those of dimethyl sulfoxide. Poly-L-methionine sulfoxide is similar to dimethyl sulfoxide in that it is a potent solubilizer of lipophilic compounds in water. Although the partition coefficient of poly-L-methionine sulfoxide in 1-octanol–water is only 20 times lower than that of dimethyl sulfoxide, it was found not to penetrate into intracellular spaces. In contrast to dimethyl sulfoxide, poly-L-methionine sulfoxide and L-methionine sulfoxide were found to be ineffective in inducing differentiation in murine erythroleukemia cells and inhibiting differentiation of avian neural crest cells, suggesting that compounds effective in these processes must have the ability to penetrate into cells or membrane proteins. Overall lack of bioactivity of poly-L-methionine sulfoxide, combined with low toxicity (2 g/kg, iv, in the mouse with no effect), makes this compound a suitable inert solubilizer and carrier for lipophilic drugs.

Keyphrases □ Dimethyl sulfoxide—poly-L-methionine sulfoxide, biologically inert analogue with solubilizing potency □ Poly-L-methionine sulfoxide—biologically inert analogue of dimethyl sulfoxide with solubilizing potency □ Solubilization potency—poly-L-sulfoxide, biologically inert analogue of dimethyl sulfoxide

A majority of the applications of polymers in pharmacy are based on their mechanical properties and inertness. Also, polymers are able to form complexes with pharmaceuticals; this property has been used relatively rarely, *e.g.*, in decreasing the aggressive properties of iodine by complexation with povidone. Furthermore, polymers possessing complexing power can be used, instead of organic



Dimethyl Sulfoxide



Polymethionine Sulfoxide

solvents, to dissolve nonpolar drugs in aqueous media and to promote sorption of pharmaceuticals. Dimethyl sulfoxide (I), one of the solvents often used for this purpose (1, 2), is relatively nontoxic to cells, yet easily penetrates cellular membranes and, in some cases, triggers very complex biological responses, *e.g.*, induction (3–8) and inhibition (9–12) of cellular differentiation.

It may be advantageous, for both practical applications and for studies of the induction mechanism, to supress some of its bioeffects, *i.e.*, to make a new selective agent by chemical modifications from a pluripotent compound. This may be possible by preparing a macromolecule that contains structural elements of dimethyl sulfoxide. A

Table I—Solubility (μg/ml) of Lipophilic Compounds in Phosphate-Buffered Isotonic Saline in the Presence (5%) or Absence of Poly-L-methionine Sulfoxide

Compound	Saline	Saline and Poly-L-methionine Sulfoxide
β-Ionone, $C_{13}H_{20}O$	7.3	1400
Retinol, $C_{20}H_{30}O$	<4.6	12
β-Carotene, $C_{40}H_{56}$	Nondetectable	6
Lycopene, $C_{40}H_{56}$	Nondetectable	4
Vitamin D_3, $C_{27}H_{44}O$	<0.18	138

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