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Characterization of Polyacryl Starch Microparticles as Carriers for Proteins and Drugs

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Abstract □ Biodegradable microparticles of cross-linked starch (maltodextrin) have been designed as carriers of proteins and low molecular weight drugs *in vivo*. Methods are presented for the synthesis of acryloyl starch and its polymerization to microparticles. Macromolecules were immobilized in the microparticles in high yields, *i.e.*, up to 40% of the dry weight consisted of the immobilized protein. The optimal conditions of immobilization were investigated by varying the concentration of starch (D), the concentration of acryloyl groups (T), and the amount of additional cross-linking agent (C). Exclusion of the cross-linking agent gave maximal immobilization of the macromolecules. Enzyme kinetics, release profiles, surface localization, and heat stability of the immobilized macromolecules are also presented. Microparticles based on starch with small amounts of acryloyl groups were completely degraded after incubation with amyloglucosidase. The degradation of microparticles in serum and in the target organelle, the lysosome, was investigated *in vitro*. The polyacrylic starch microspheres (mean diameter, 0.5 μm) constitute an attractive alternative to other drug and enzyme carriers.

Keyphrases □ Drug carrier systems—biodegradable, starch microparticles, lysosomal degradation □ Microspheres—drug carrier system, lysosomal degradation, polyacryl starch

Enzymes have hitherto had relatively limited applications *in vivo* and have essentially been used only for the treatment of blood coagulation disorders. Several problems are associated with the use of enzymes *in vivo*, *e.g.*, the targets of the enzymes are often strictly compartmentalized to specific tissues and cell compartments and administered enzymes cannot readily pass the membranes of these compartments. Furthermore, free enzymes available from human sources are rapidly degraded by proteolytic enzymes. Foreign enzymes, when injected, will induce the formation of antibodies and other immunologic reactions. Systems that will protect the enzymes from degradation and direct them to their specific targets are not yet available. Similarly, suitable carrier systems for low molecular weight drugs are still unavailable for targeting *in vivo*, although several different carriers have been extensively tested (1–4).

Spherical microparticles (<1 μm) made of polyacrylamide or polyacrylic dextran can be used for the immobilization of proteins (5, 6). When the particles are injected intravenously in rats and mice, they are rapidly cleared from the circulatory system by macrophages in the reticuloendothelial system (RES). After uptake, the particles are localized in the lysosomal vacuome (7, 8). This "passive targeting" of microspheres to the RES has been used successfully to treat an artificially induced storage disease in mice (9). However, any system used as an *in vivo* drug carrier must fulfill certain requirements: it must be biocompatible and not induce toxic reactions; it must be biodegradable to avoid accumulation in the tissues; it should be small to avoid respiratory distress after intravenous injection; it must be compartmentalized in such a way that the therapeutic index of the drug or enzyme carried is improved.

This paper describes the preparation and characteristics of biodegradable starch microparticles. The biodegradability of the spheres is investigated *in vitro* with hydrolytic enzymes and an isolated lysosome-enriched rat liver fraction.

EXPERIMENTAL SECTION

Materials—Human serum albumin[1], immunoglobulin G[1], carbonic anhydrase[2], α-amylase[3], amyloglucosidase[2], *N*-acetyl-β-glucoseamidase[2], and lysosyme[4] were used without further purification. *N,N'*-Methylenebisacrylamide[5], 4-methylumbelliferyl-*N*-acetyl-β-D-glucoseamidine[2], *p*-nitrophenyl acetate[2], and the other chemicals used were analytical grade. Hydroxyethyl starch[6] (mol. wt., 400,000; degree of derivatization, 0.7), maltodextrin[7] (mol. wt. 5000), [^{14}C]paraformaldehyde[8], and [^{14}C]starch (prepared from *Nico-*

[1] KABI AB, Stockholm.
[2] Sigma Chemical Co.
[3] Boehringer Mannheim.
[4] Merck Co.
[5] Eastman Kodak Co.
[6] Hydroxyethyl starch was obtained as a gift from Dr. Tony de Belder, Pharmacia, Uppsala, Sweden.
[7] Maltodextrin was obtained as a gift from Dr. Lars Svensson, Stadex AB, Malmö, Sweden.
[8] Amersham, England.

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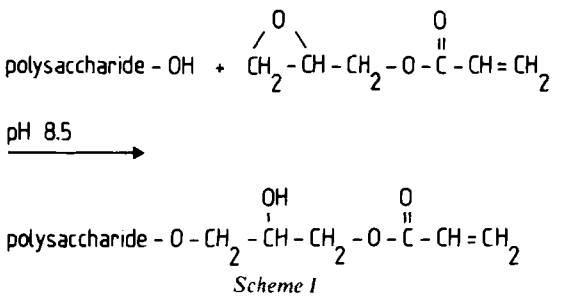

Scheme I

tiana tabacum[9]; mol. wt., 7000–8000) were used without further purification.

Acryloylation of Polysaccharides—Chemical modification of the polysaccharides (Scheme I) with acrylic acid glycidyl ester followed the procedure outlined by Edman *et al.* (7). Maltodextrin or hydroxyethyl starch (10 g) was dissolved in 0.2 M phosphate buffer (pH 8.5) to give a solution of appropriate concentration. Acrylic acid glycidyl ester[10] (10 mL) was added, and the two-phase system was magnetically stirred for up to 15 d. Samples (10 mL) were withdrawn on days 1, 3, 5, 7, 10, and 15 and centrifuged for 15 min at 3000×*g*. Unreacted acrylic acid glycidyl ester was discarded, and the aqueous solution with the derivatized polysaccharide was extracted with 10 parts of toluene to remove remaining unreacted glycidyl ester.

Determination of the Degree of Derivatization of Polysaccharides—The degree of derivatization (acrylic groups/glucose residue) was analyzed by ^{1}H-FT-NMR according to the method of Lepistö *et al.* (10). The measurements were made at 100 MHz on a spectrometer[11] using the water-elimination Fourier transform method. After phase correction of the region containing the acrylic and anomeric proton resonances (δ 4.8–6.7 ppm) the region was integrated. The signals of the anomeric proton (δ 4.8–5.8 ppm) were used as internal standard.

Preparation of Polyacryl Starch Microparticles—Microparticles with or without protein were prepared as described previously (5, 6). Protein and, when applicable, bisacrylamide were dissolved in a solution of acryloylated maltodextrin or in acryloylated hydroxyethyl starch in 0.2 M sodium phosphate buffer, pH 8.5. EDTA (1×10^{-3} M) and ammonium peroxodisulfate were added to the deoxygenated mixture. The water solution (5–10 mL) was homogenized with poloxamer 188[12] in 600 mL of chloroform–toluene (1:4) to produce a water-in-oil emulsion. On addition of *N,N,N′,N′*-tetramethylethylenediamine the water phase droplets were polymerized to microparticles. The microparticles were freed from the organic phase and nonimmobilized protein by repeated washings with buffer.

The microparticle composition is characterized by the D-T-C nomenclature suggested by Hjertén (11), as modified by Edman *et al.* (6). The D-T-C values denote the amounts of the components in the monomer solution used for particle formation: D denotes the concentration of derivatized macromolecules (*i.e.*, acryloylated starch, g/100 mL); T denotes the total concentration of acryloyl groups (g/100 mL); C is the relative amount of the cross-linking agent, bisacrylamide, expressed as the percentage (w/w) of the total amount of acrylic monomers.

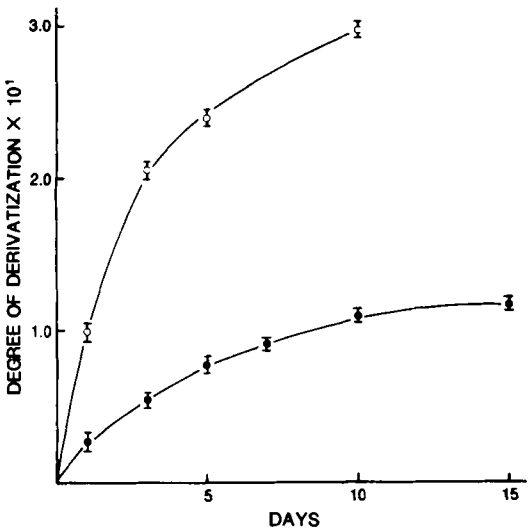

Figure 1—*Derivatization of the polysaccharides. The degree of derivatization (acrylic groups per glucose residue) of maltodextrin (●) and hydroxyethyl starch (○) are expressed as mean values ± SD (n = 5 and 3, respectively).*

[9] New England Nuclear, West Germany.
[10] Fluka AG.
[11] Model JNM-FX 100; JEOL.
[12] Pluronic F68; BASF Wyandotte.

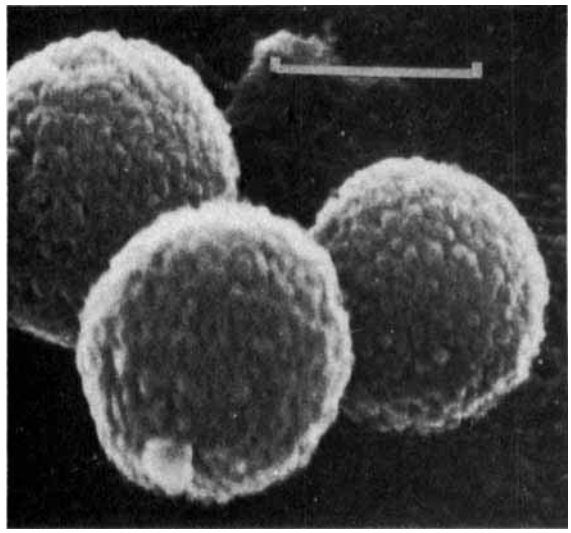

Figure 2—*Scanning electron micrograph (30,000×) of polyacryl starch microparticles (D-T-C 10-0.2-0). The bar indicates 1 μm.*

Radioactive Labeling of Polyacryl Starch Microparticles—Two types of radioactive labels were used. The label was introduced with ^{14}C-labeled *N,N′*-methylenebisacrylamide as the cross-linking agent or with [^{14}C]starch as part of the monomers. In the former case, the labeled derivative was prepared from [^{14}C]paraformaldehyde and acrylamide according to Feuer and Lynch (12) as described by Sjöholm and Edman (7). The radioactive starch was derivatized with acrylic acid glycidyl ester together with the polysaccharides used in the particle preparation.

Protein Determination—The amount of protein incorporated in the microparticles was determined with an automatic amino acid analyzer after hydrolysis in 6 M HCl at 105°C for 20 h. The amount of protein leaking from the microspheres was measured by the method of Lowry *et al.* (13). In each case, the protein studied was used as a standard. In some experiments, ^{125}I-human serum albumin was used to determine the protein content of the microparticles and to follow the leakage of the albumin from the particles.

Determination of Carbonic Anhydrase Activity—The activity of carbonic anhydrase was determined with the chromogenic substrate *p*-nitrophenyl acetate (14). The assay mixture consisted of 4.7 mL of 0.05 M Tris buffer (pH 7.4), 0.25 mL of substrate solution in absolute ethanol at the appropriate concentration, and 0.05 mL of the enzyme (immobilized or in free form) in Tris buffer. The formation of the hydrolysis products was followed spectrophotometrically at 400 nm at room temperature. The velocity of the reaction is the initial increase in absorbance during 1 min. Correction was made for the spontaneous hydrolysis of the substrate in samples that did not contain the enzyme; the particle matrix did not affect the reaction rate (results of control experiments). Furthermore, the particles did not cause light scattering at this wavelength, and no sedimentation was detectable during the time of measurement.

Determination of the Functional Capacity of Immobilized Human Serum Albumin—The functional capacity of immobilized albumin was determined by the method of Kober *et al.* (15). Microparticles containing albumin were incubated with a fixed concentration of [^{14}C]salicylic acid. After centrifugation, the concentration of the free drug was determined by measuring the radioactivity in the supernatant. The amount of salicylic acid bound to albumin was calculated from the percent binding and from a standard curve derived by equilibrium dialysis with different albumin concentrations.

Iodination of Human Serum Albumin—Human serum albumin (10 mg) in 0.5 mL of 0.05 M phosphate buffer (pH 7.3) containing 0.15 M NaCl was labeled with [^{125}I]NaI (1 mCi) in the presence of lactoperoxidase (20 μg) and hydrogen peroxide (0.03% v/v, 20 μL added 3 times at 3-min intervals) according to the method of Phillips and Morrison (16). The reaction was terminated after 10 min by diluting the sample with chilled buffer. The ^{125}I-labeled human serum albumin was isolated from the reaction mixture by gel filtration on a Sephadex G-25 column.

Table I—Characteristics of Microparticles with Immobilized ^{125}I-Human Serum Albumin [a]

Microparticle Composition, D-T-C	Total Yield of Microparticle, %	Total Protein Yield, %	Total Protein Content, mg/mg dry weight	Functional Protein Content, %	Leakage in 6 Weeks, %
		Maltodextrin Particles			
5-0.2-0	7.9	2.2	0.218	41	34
7-0.2-0	5.4	3.4	0.386	—[b]	34
10-0.2-0	28.1	7.3	0.206	44	62
10-0.3-0	58.1	13.8	0.192	75	52
10-0.4-0	46.2	27.5	0.373	58	38
10-0.4-5	44.6	9.8	0.180	40[c]	43
10-0.4-25	19.4	3.8	0.164	47[c]	56
10-1.2-60	55.6	22.4	0.287	74	40
		Hydroxyethyl Starch Particles			
10-0.4-0	31.4	3.8	0.108	79	32
10-0.8-0	70.3	12.7	0.153	58	43
10-1.0-0	93.5	24.7	0.209	42	33
10-1.2-0	87.3	39.4	0.311	63	36
10-1.5-50	69.3	17.0	0.197	95	38

[a] The study was carried out with 100 mg of ^{125}I-labeled human serum albumin/mL of aqueous buffer. The protein content of the particles was determined in a gamma counter. The amount of functionally active protein in the particles was determined by estimating the capacity of the albumin to bind [^{14}C]salicylic acid (15). [b] Not determined. [c] Determined after 50 d.

Determination of Radioactivity, Particle Size, and Density—β-Emitting isotopes were determined in a scintillation counter[13]; the counting efficiency was calculated with an external standard. Iodine-125 was measured in a gamma scintillation counter[14].

The size of the particles was determined from photographs taken with a scanning electron microscope (7). Using this technique, 85% of the microparticles had a diameter of 0.2–1.0 μm; 15% were 1.0–1.6 μm in diameter.

The densities of the microparticles were determined by density gradient centrifugation in colloidal silica coated with polyvinylpyrrolidone[15] (17) utilizing a gradient ranging from 1.02 to 1.10 g/cm^3, obtained with a dual-chamber gradient maker. Density marker beads[16] were used to monitor the gradient according to the manufacturer's specification. The gradient volume was 10 mL. Microparticles (0.5–2.5 mg) in 300 μL of physiological saline were applied to the top of the gradients, which were centrifuged at 400×g for 25 min using a swing-out rotor.

Isolation of a Lysosomal Fraction from Rat Liver Homogenate—The isolation of the lysosomal fraction followed the technique outlined by Beaufay (18). Livers from male Sprague–Dawley rats, which were fasted for 12 h, were removed rapidly and immersed in a beaker containing ice-cold 0.25 M sucrose (pH 5.5). After weighing, the tissue was cut into pieces and homogenized with 3 volumes of the medium in a Potter–Elvehjelm homogenizer. The homogenate was centrifuged at 4°C and 750×g for 10 min. The sediment was redispersed three times and centrifuged. The resulting supernatants were pooled and centrifuged at 12,000×g for 10 min. The supernatant was discarded and the pellet was dissolved in 0.25 M sucrose and centrifuged for 10 min at 6500×g. This step was repeated three times and the supernatants were combined and centrifuged for 15 min at 12,000×g. The resulting sediment was dispersed in water (pH 4.5) and made up to a final volume equal to that of the original tissue. The suspension was frozen and thawed twice before use. The isolated lysosome-enriched fraction was usually preserved with penicillin G. The partition of lysosomes in the different fractions during the preparation was followed by assay of the lysosomal enzyme, N-acetyl-β-glucoseamidase. The enzyme was determined by using 4-methylumbelliferyl-N-acetyl-β-D-glucoseamidine as substrate in 0.15 M acetate buffer, pH 4.2 (19).

Enzymatic Solubilization of Microparticles—α-Amylase (10 U/mL in 0.1 M phosphate buffer, pH 5.5) or amyloglucosidase (0.1–10 U/mL in 0.1 M citrate buffer, pH 4.5) was added to 0.5 mg of radioactive microparticles in the same buffer to give a final volume of 2.0 mL. In other experiments, 1.0 mL of pooled human serum or 1.0 mL of the lysosomal fraction was added to 1.0 mL of radioactive microparticles (0.5 mg dry weight). After incubation at 37°C for 0.5–6 h the particles were centrifuged (3000×g) and the radioactivity in 1.0 mL of the supernatant was measured before or after filtration through a 0.45-μm membrane filter. In some of the experiments, the radioactive degradation products were separated on a Sephadex G-150 column. The degradation products (5 mL) were applied on a 2.6 × 100-cm column. The separation was performed with 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl as the elution medium.

Size Determination of Microparticle Aggregates—Microparticles (100 μL, 50 μg) were suspended in 150 mL of particle-free buffer[17]. The suspensions were used in a particle counter[18], which simultaneously counts particles in 14 size classes. The instrument was fitted with a 50-μm orifice tube. During all measurements the speed and stirrer position were constant. The mean diameters of each size class of suspended microparticles were calculated[19] from the particle distribution.

In some experiments, microparticles containing human serum albumin were incubated with a specific antiserum (*i.e.*, 50 μL of rabbit anti-human serum containing 3.6 mg of immunoglobulin G/mL) in an end-over-end mixer for 45 min prior to the estimation of the mean diameter. In control experiments, the microparticles containing human serum albumin were incubated with 0.15 M NaCl, or empty microparticles were incubated with the corresponding antiserum.

RESULTS

Derivatization of Polysaccharides—The introduction of acryloyl groups into hydroxyethyl starch and maltodextrin with acrylic acid glycidyl ester was followed by NMR for 10 and 15 d, respectively. Hydroxyethyl starch reacted more rapidly and more extensively than maltodextrin (Fig. 1); every 3rd glucose residue was substituted after 10 d compared with every 10th glucose unit for maltodextrin. The higher reaction rate with hydroxyethyl starch can most probably be ascribed to the presence of the hydroxyethyl groups. With maltodextrin, a plateau was reached after ~10 d.

Immobilization of Proteins in Microparticles of Different Composition—Table I summarizes results from the incorporation of ^{125}I-labeled human

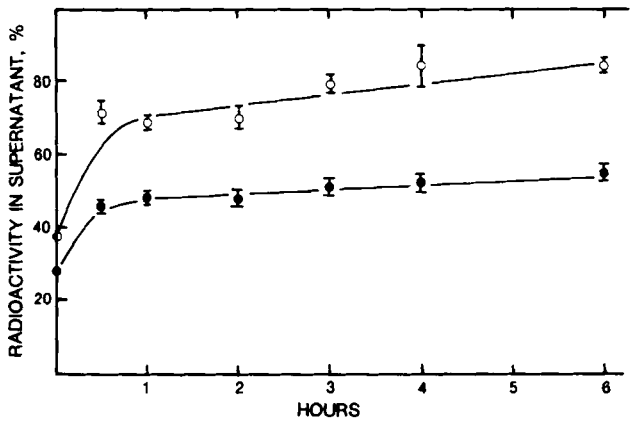

Figure 3—In vitro *digestion of ^{14}C-labeled microparticles in a lysosome-enriched fraction. Maltodextrin particles with D-T-C of 10-0.2-0 (○) and 10-0.4-0 (●) were preincubated in pooled human serum for 1 h prior to incubation with the lysosomal fraction. Mean values* ± SD (n = 3–6).

[13] TriCarb 2405 Spectrometer; Packard.
[14] Auto-Gamma 5110; Packard.
[15] Percoll; Pharmacia AB, Uppsala, Sweden.
[16] Pharmacia AB, Uppsala, Sweden.
[17] Isoton II; Coulter Electronics, England.
[18] Model TAII; Coulter.
[19] Model 9825 computer, Hewlett-Packard.

Table II—Degradation of Maltodextrin Microparticles by Amylogucosidase *In Vitro*

Microparticle Composition, D-T-C	Label	Soluble Radioactivity After 3 h[a]
10-0.2-0	[^{14}C]Starch	103 ± 3
10-0.2-0	[^{125}I-Human serum albumin	100 ± 7
10-0.4-0	[^{14}C]Starch	59 ± 2
10-0.4-25	[^{14}C]Starch	69 ± 2
10-0.4-25	[^{14}C]Bisacrylamide	64 ± 4

[a] Mean in percent of total ± *SD*; $n = 3$.

serum albumin in polyacryl starch microparticles. Three series of experiments were run, varying total concentration of starch (D), total concentration of acrylic groups (T), and degree of cross-linking (C). A decrease of acryloylated starch (D) in the particles decreases the total yield of immobilized protein as well as the yield of particles.

Increase of the concentration of acrylic groups (T) (acryloylated maltodextrin or hydroxyethyl starch) in the aqueous phase resulted in an increase in the amount of albumin immobilized in the particles. The maximal amount of albumin which could be incorporated corresponded to 37% of the dry weight (particles based on maltodextrin and a D-T-C value of 10-0.4-0). The relative amount of cross-linking agent (C) also had an influence on the total protein yield; particle yield and protein content decreased when the cross-linking agent bisacrylamide was introduced into maltodextrin microparticles. The functional capacity of immobilized albumin to bind salicylic acid varied between ~40 and 95%. No systematic trend could be detected; high C values possibly improve the functional capacity.

Microparticle Density, Surface Structure, and Protein Leakage—The density of polysaccharide microparticles was 1.04–1.07 g/mL, which is somewhat lower than that obtained with polyacrylamide particles (5). The density essentially followed the content of acryloyl groups, when both maltodextrin and hydroxyethyl starch were studied. Thus, when T was increased from 0.2 to 0.4, the density of maltodextrin particles increased from 1.04 to 1.06 g/mL. The density was the same when the particles contained up to 30% human serum albumin.

Microparticles based on maltodextrin or hydroxyethyl starch have a surface structure quite different from that of highly cross-linked polyacrylamide particles. The particles have a relatively smooth surface structure with smaller pores (Fig. 2) than polyacrylamide particles with a T-C value of 8-25 (5).

The leakage of ^{125}I-human serum albumin from the microparticles (Table I) was investigated to study the influence of the microparticle composition on the protein fixation within the polymer. Variations in leakage were observed with T and C changes. The maltodextrin microparticles with few acryloyl groups leaked more than particles with higher T values. The hydroxyethyl starch microparticles were generally not as leaky as the maltodextrin spheres; no systematic change was found when T was varied.

When the cross-linking agent *N,N'*-methylenebisacrylamide was added to the maltodextrin microparticles, the leakage of ^{125}I-human serum albumin was accelerated. This is consistent with previous findings that microparticle pore radius increases with the addition of a cross-linking agent (5).

Storage of the microparticles at −20°C was also studied. Maltodextrin microparticles with compositions of 10-0.2-0, 10-0.4-0, and 10-0.4-25 and a batch of hydroxyethyl starch microparticles (10-0.8-0) lost no particle-bound protein when stored at −20°C for 8 weeks.

Table III—Degradation of Maltodextrin Microparticles in Serum and in a Lysosome-Enriched Fraction[a]

Microparticle Composition, D-T-C	Solubilized Radioactivity, %[b] Incubation in Serum, 1 h	Incubation in Lysosomal Fraction, 1 h	Incubation in Lysosomal Fraction, 6 h
	Empty Microparticles		
10-0.2-0	21.8 ± 1.8	44.4 ± 3.2	51.1 ± 3.2
10-0.4-0	12.6 ± 0.8	30.8 ± 1.4	36.4 ± 3.2
10-0.4-20	10.8 ± 2.3	29.2 ± 3.3	53.5 ± 7.8
	Protein-Containing Microparticles[c]		
10-0.2-0	38.0 ± 0.9	68.3 ± 1.4	83.5 ± 2.4
10-0.4-0	28.5 ± 2.3	47.9 ± 3.2	54.3 ± 3.4
10-0.4-20	40.6 ± 1.5	66.5 ± 2.1	73.8 ± 2.4

[a] The [^{14}C]starch-labeled particles were preincubated for 1 h in serum before further incubation in the lysosomal fraction. Mean values ± *SD* for incubation in lysosomes ($n = 3$–6) and incubation in serum ($n = 18$). [b] Cumulative values. [c] Human serum albumin, 100 mg/mL of the aqueous phase.

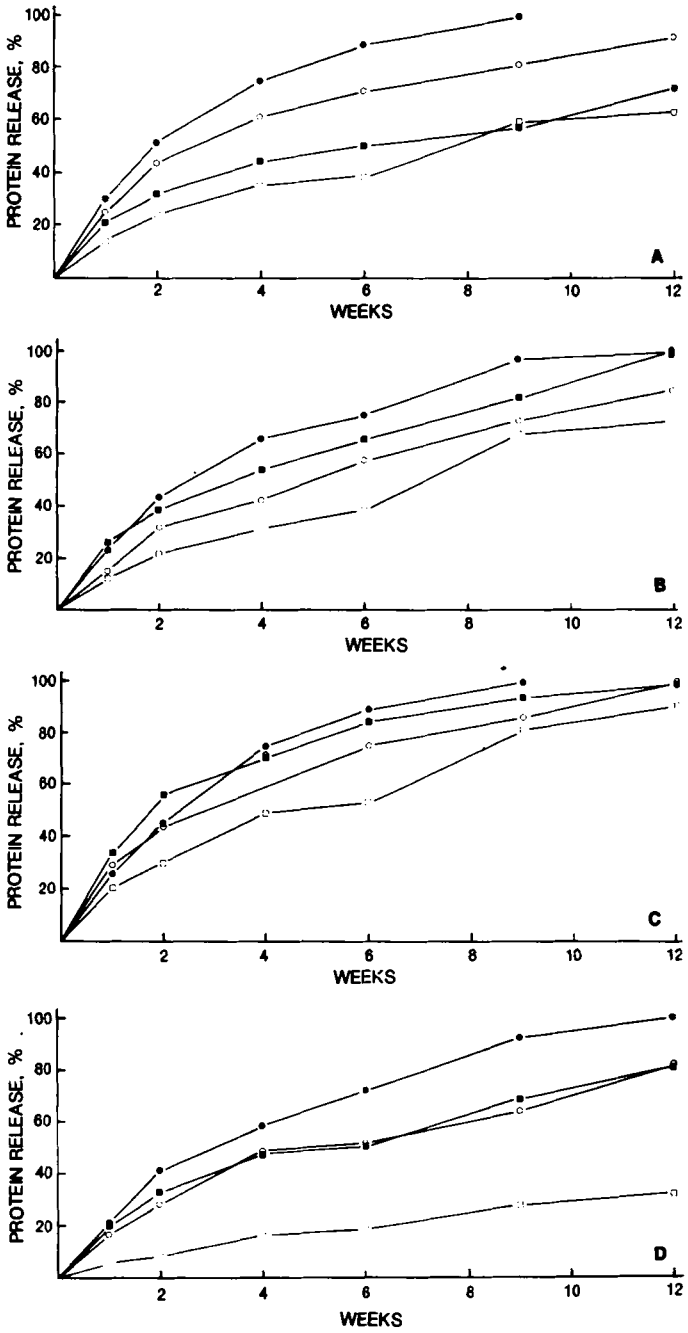

Figure 4—*Release of immobilized proteins during storage at room temperature from microparticles of maltodextrin with D-T-C of 10-0.2-0 (A), 10-0.4-0 (B), and 10-0.4-25 (C) and hydroxyethyl starch with D-T-C of 10-0.8-0 (D). The immobilized proteins were lysozyme (●), human serum albumin (○), carbonic anhydrase (■), and immunoglobulin G (□).*

Enzymatic Degradation of Polyacryl Starch Microparticles—The particles in Table I (0.5 mg dry weight) were incubated with α-amylase, and the release of ^{125}I-human serum albumin was compared with the controls. Maltodextrin microspheres with the D-T-C compositions of 10-0.2-0, 10-0.3-0, and 10-0.4-0 released 27, 24, and 26% of the entrapped radioactivity in 1 h, respectively. Spheres containing bisacrylamide had a lower release, *e.g.*, 15% (D-T-C 10-1.2-60). No significant increase in radioactivity was found in the supernatants when hydroxyethyl starch microspheres were incubated with α-amylase.

The degradation of the starch matrix by amyloglucosidase was investigated for maltodextrin microparticles of different compositions (D-T-C 10-0.2-0, 10-0.4-0, and 10-0.4-25) containing acryloylated ^{14}C-labeled starch and albumin (Table II). The particles were incubated with amyloglucosidase (10 IU/mL) for 3 h at 37°C. Particles with few acrylic groups (D-T-C, 10-0.2-0) were totally solubilized, and all radioactivity was regained in the supernatant

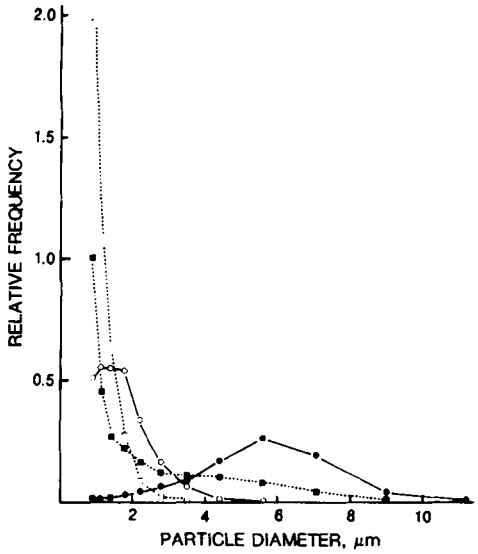

Figure 5—*Size distribution of maltodextrin microparticles (D-T-C 10-0.4-25), including the frequency by weight before (○) and after (●) incubation with specific antiserum and the frequency by number before (□) and after (■) antiserum addition. The relative frequency was obtained by division of the frequency values by the class width.*

after 3 h. When the degradation mixture was gel-filtered on a Sephadex G-150 column, 30% of the radioactivity was obtained in the void volume and 70% in the low molecular weight region. Identical results were obtained when particles with the same composition containing ^{125}I-human serum albumin were incubated with amyloglucosidase. The ^{125}I-albumin-containing microparticles were completely dissolved in 3 h. Spheres containing 0.4% (w/w) acrylic groups (D-T-C 10-0.4-0) or a cross-linking agent (D-T-C 10-0.4-25) were more stable, and 59 and 69%, respectively, of the radioactivity was detected in the supernatant after incubation for 3 h.

The microparticles were also affected by fresh human serum (Table III). Incubation of 0.5 mg of ^{14}C-labeled particles in serum for 1 h at 37°C gave a release of 11-41% of the radioactivity, depending on the particle composition and immobilized protein content. "Empty" spheres and spheres with a high amount of acrylic groups (high T value) were relatively stable in serum, *e.g.*, particles with a D-T-C of 10-0.2-0 containing no protein were 22% digested compared with 38% when albumin was present. Microparticles (D-T-C, 10-0.4-0) containing albumin were solubilized 28% after a 1-h incubation, compared with 38% when spheres with D-T-C 10-0.2-0 were used. However, the rate of degradation was increased when the particles contained protein and a cross-linking agent. The increased rate probably depends on a better penetration of the particles by the enzymes, as both the proteins and increased C may increase the pore radius of the particles. No further degradation was seen when the incubation time was increased to 6 h.

In an attempt to simulate the biological conditions of particles after intravenous injection, the spheres were preincubated in fresh serum for 1 h prior to incubation with isolated rat liver lysosomes for 1 and 6 h (Table III, Fig. 3). Particles with a D-T-C of 10-0.2-0 were highly affected, and ~84% of the radioactivity in the spheres was regained in the supernatant after incubation for 6 h. Particles with larger T, (D-T-C, 10-0.4-0) were more stable; 54% of the spheres were metabolized after 6 h. Corresponding "empty" microspheres were more inert under these conditions. Empty spheres with a D-T-C of 10-0.2-0 were degraded 51% as compared with 84% when the particles contained albumin (Table III).

When the microparticles were incubated directly in the lysosomal fraction, *i.e.*, without preincubation in serum, the amount of solubilized radioactivity after a 6-h incubation was the same as with preincubation. Thus, the metabolism of polyacryl starch microparticles is related to the protein content and the composition of the particle.

Immobilized Proteins in Polyacryl Starch Microparticles—Further studies were performed with three different batches of maltodextrin microparticles and one batch made of hydroxyethyl starch. The maltodextrin microparticles had compositions 10-0.2-0 (I), 10-0.4-0 (II), and 10-0.4-25 (III). The hydroxyethyl starch microspheres (IV) had a composition of 10-0.8-0.

Release Profiles—Microparticles were prepared with proteins of various sizes: lysozyme [mol. wt. 14,400 (20)], carbonic anhydrase [mol. wt. 31,000 (21)], human serum albumin [mol. wt. 66,500 (22)], and human immunoglobulin G [mol. wt. 150,000 (23)]. The spheres were suspended in buffered physiological saline, pH 7.4 with sodium azide (0.1%) added as a preservative.

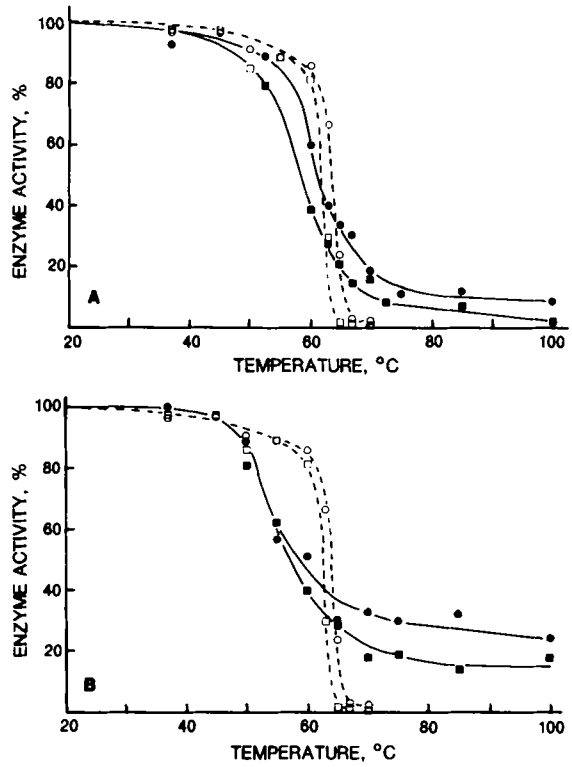

Figure 6—*Thermostability of carbonic anhydrase immobilized in maltodextrin microparticles with D-T-C of 10-0.2-0 (A) and 10-0.4-0 (B) compared with that of the free enzyme. The remaining activity was measured after heating the enzyme for 5 (○, ●) and 30 (□, ■) min. The open symbols represent free enzyme; the solid symbols represent particle-bound enzyme.*

The samples were left at room temperature (22°C) for 1, 2, 4, 6, 9, and 12 weeks. The protein released from the spheres after centrifugation (Fig. 4) was determined by the method of Lowry *et al.* (13).

The most pronounced release, irrespective of particle composition, was obtained when lysozyme was used (Fig. 4A): 100% of the lysozyme was released in 9-12 weeks, depending on the gel composition. Also, release rates generally decrease with increasing molecular weight independent of particle composition. Microparticles containing bisacrylamide as a cross-linking agent (III) (Fig. 4C) released the proteins most rapidly; ~90-100% of the incorporated protein was lost after 12 weeks, irrespective of molecular weight.

Surface Localization—The localization of proteins on the surface of the microparticles can be studied conveniently by adding specific antiserum and following particle aggregation with a Coulter Counter equipped with a computer program. The Coulter Counter cannot detect particles <0.8 μm with the 50-μm orifice tube and does not give an exact measure of the true mean diameter of the microparticles. The results, therefore, cannot be compared with those obtained with the electron microscope, but the method does give a relative measure of the changed size distribution (by number and volume) when the antiserum is added to the microparticles. The mean diameter, based on volume, increased from 2.04 ± 0.82 μm to 5.66 ± 1.65 μm when III microparticles with immobilized human serum albumin were incubated with rabbit anti-albumin serum (Fig. 5). The mean diameter, by number, was also calculated, and a similar increase was noted (Fig. 5). Increases in diameters were also detected for microparticles of other compositions. Empty microparticles did not aggregate after addition of the antiserum.

To further investigate the nature of the surface-localized albumin, the study was repeated after 8 weeks with the same batch of particles. At that time ~50% of the protein had leaked from the spheres (Fig. 4). No significant changes in the aggregation patterns of the washed microparticles could be detected with the Coulter Counter. Obviously, a significant fraction of the immobilized albumin is stably bound on the surface and available for interaction with the antibodies.

Heat Stability—To investigate the effect of immobilization in polyacryl starch microparticles on the stability of proteins, carbonic anhydrase was incorporated into the microparticles. The spheres were exposed to elevated temperatures for 5 or 30 min. After treatment, the spheres were immediately cooled in an ice bath for 5 min. The samples were then incubated at room temperature for 30 min, and the remaining activity was determined. It is apparent that the microspheres protect the enzyme against heat denaturation

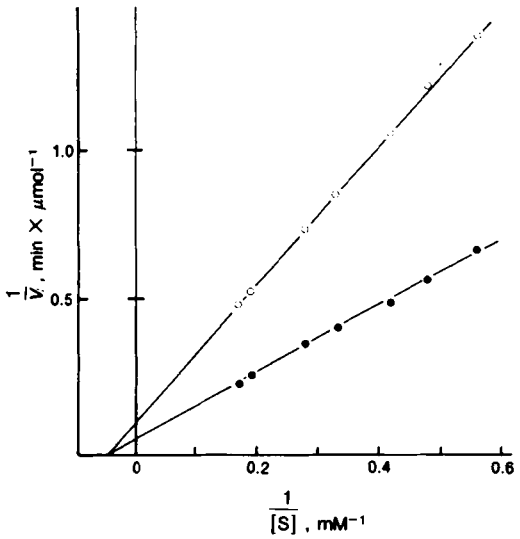

Figure 7—*Lineweaver-Burk plots for the enzymatic activity of carbonic anhydrase in maltodextrin microparticles (D-T-C 10-0.2-0) (○) and free (●) form.*

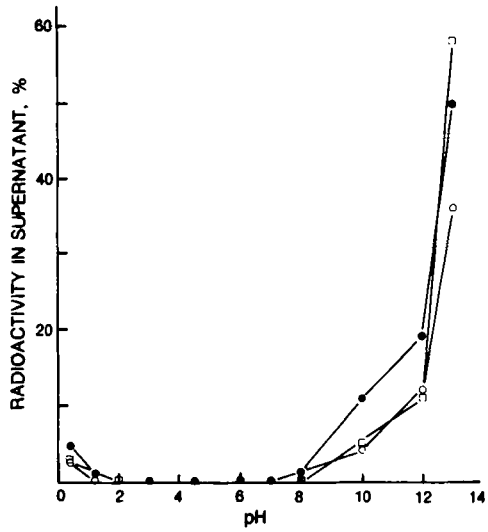

Figure 8—*Stability of ^{14}C-labeled maltodextrin microparticles at different pH. The particles were incubated at 37°C for 12 h. Key to D-T-C values: (●) 10-0.2-0; (○) 10-0.4-0; (□) 10-0.4-25.*

(Fig. 6). Depending on the composition of the spheres, the enzymatic activity after 30 min at 100°C varied between <5 and 20%. Microparticles containing 0.4% acrylic groups (w/w), *i.e.*, II (Fig. 6B), gave better protection against heat than spheres with T = 0.2 (I) (Fig. 6A). The use of a cross-linking agent (III) or hydroxyethyl starch (IV) did not improve the thermal stability of carbonic anhydrase.

Enzymatic Properties of Immobilized Carbonic Anhydrase—To assess the effect of entrapment on the function of the immobilized enzymes, the Michaelis-Menten constant (K_m) of carbonic anhydrase was determined. Regression analysis showed that the Lineweaver-Burk plots for immobilized and soluble enzyme were linear over the entire range of concentrations studied. The calculated K_m for native carbonic anhydrase was 18 mM; for immobilized enzyme, it was 18-24 mM. The increase in K_m by immobilization (irrespective of microsphere composition) is probably due to a limited diffusion rate of substrate and/or reaction products, as observed in other immobilized enzyme systems (24-26).

The maximal reaction rate (v_{max}) of the immobilized enzyme, in I particles, was compared with that of the native enzyme (Fig. 7). The v_{max} of the immobilized enzyme, as calculated from the ordinate intercept, was 58% of that of the free enzyme. Based on amino acid analysis, the amount of enzyme in the two experiments was the same. The enzymatic activity, therefore, is more inhibited in the polyacryl starch particles than in macroporous polyacrylamide microparticles (27).

pH Stability of Polyacryl Starch Microparticles—The pH stability of the polymer matrix was investigated with ^{14}C-labeled starch. The hydroxyethyl starch particles (IV) could not be studied because labeled hydroxyethyl starch is not available and incorporation of ^{14}C-acryloylated starch in these particles would result in inadequate labeling of the particle matrix. The maltodextrin microspheres were incubated at pH 0.4-13 for 12 h at 37°C. Aliquots were withdrawn from the supernatants after centrifugation, and the radioactivity was measured (Fig. 8). The microparticles were stable at pH 2-8. At pH 12, ≤19% of the particles were solubilized; at pH 12.8, ≤58% of the radioactivity was lost from the microparticles. Acidic pH (≥1.2) did not significantly affect the microparticles; at pH 0.5, 5% (at most) was solubilized in 12 h.

DISCUSSION

In our earlier work, small particles were prepared for protein immobilization by polymerization of either acrylic groups in acrylamide and *N,N'*-methylenebisacrylamide to form polyacrylamide microparticles (5) or acrylic groups of acryloyldextran and *N,N'*-methylenebisacrylamide to form polyacryldextran particles (6). These two types of microparticles are only slowly metabolized *in vivo*, with $t_{1/2}$ values of 10-20 weeks (7, 28). The half-life is directly related to the amount of hydrocarbon chains formed during the polymerization (and to some extent to the presence of dextran, which is slowly degraded in the lysosomes). These particles will probably find only limited applications *in vivo*.

In the present paper, a low molecular weight fraction of starch (maltodextrin; mol. wt. 5000) and a preparation of hydroxyethyl starch (mol. wt. 400,000), which are water soluble, were used. The starch compounds were modified with acrylic acid glycidyl ester and polymerized in a water-in-oil emulsion to form small particles; the particle size is essentially determined by the dispersion degree of the emulsion. The cross-links in the microparticles, the hydrocarbon chains, are formed by the acryloyl groups, but their contribution to the matrix structure can be much lower (0.3-0.4% in the polymerization mixture) than in polyacrylamide and polyacryldextran particles. This means that the main part (>95%) of the matrix is composed of maltodextrin or hydroxyethyl starch and will be easily degraded by lysosomal enzymes (maltodextrin particles). The degradation of the maltodextrin microparticles has been followed with ^{14}C-labeled starch from *Nicotiana tabacum* (about the same molecular weight as maltodextrin) included in the microparticles and treated as described for maltodextrin. Unfortunately, no labeled hydroxyethyl starch could be obtained, and consequently, no reliable studies to follow the degradation of hydroxyethyl starch particles have been possible. However, information is available on the metabolism of underivatized hydroxyethyl starch, which is extensively used as a plasma expander in humans. The half-life of hydroxyethyl starch (mol. wt. 450,000; degree of derivatization 0.70) in the spleen and liver of the rat is ~64 and 132 d, respectively (29). It may therefore be concluded that hydroxyethyl starch microparticles are more slowly metabolized than maltodextrin microparticles. Such microparticles are, therefore, the carrier of choice when a prolonged protection of the immobilized material is more essential than a rapid degradation of the particle matrix.

The degradation of the starch particles has been followed *in vitro* in a lysosome-enriched fraction from rat liver prepared by differential centrifugation, in serum, and with isolated enzymes. Maltodextrin particles were relatively rapidly degraded by the lysosomal enzymes; >80% of the radioactivity (originating from the starch matrix) was found in the supernatant after 6 h from particles with a D-T-C of 10-0.2-0, and ~50% from particles with a D-T-C of 10-0.4-0. In serum, with α-amylase, or with amyloglucosidase, the solubilization was faster with the less-modified maltodextrin. When the cross-linking degree was increased by adding *N,N'*-methylenebisacrylamide (yielding particles with a D-T-C of 10-0.4-25) the rate of degradation consistently increased. An increased rate of degradation was also seen when the particles contained immobilized protein. In both of these situations the enzymes could better penetrate the particles and hydrolyze the starch, since both proteins and increased C increase the pore radius (5).

In addition to ^{14}C-labeled starch, the degradation of the different particles was studied with immobilized ^{125}I-human serum albumin or ^{14}C-labeled *N,N'*-methylenebisacrylamide, which gave consistent results. As expected, hydroxyethyl starch particles were resistant to α-amylase and amyloglucosidase treatment. On the other hand, maltodextrin particles (D-T-C 10-0.2-0) were totally solubilized by amyloglucosidase in 3 h, and gel filtration revealed that ~70% of the radioactively labeled starch was regained in the low molecular weight fraction. We thus conclude that the derivatization of starch with acrylic acid glycidyl ester has not profoundly changed the biodegradability; enzymes normally found in animal tissues can dissolve maltodextrin microparticles *in vitro*. Whether the same extensive metabolism of these microparticles can happen *in vivo* and the degradation products can be completely eliminated from the macrophages of the reticuloendothelial system

remains to be seen. Preliminary results[20] indicate that maltodextrin microparticles are metabolized and eliminated *in vivo*, which means that degradation products can also pass the lysosomal membrane.

It is obvious that T (specified essentially by the derivatization degree of the starch) and C (the fraction of added *N,N'*-methylenebisacrylamide) are important factors in the microparticle matrix structure and, therefore, also in biodegradation. The structure will determine the amount of proteins immobilized, their leakage, and the stability of the immobilized proteins towards heat denaturation. The macrostructure of maltodextrin particles, as seen in the electron microscope, is obviously smoother than that of polyacrylamide (T-C 8-25) which exhibits a macroporous structure (5). In the polyacrylamide microparticles, thick bundles of polymer threads are formed, in which the proteins are fixed. The proteins are partly exposed on the surface and can interact with cells (30). Even if biodegradable starch microparticles are not cross-linked to the same extent, proteins obviously can be stably bound on the surface in such a way that they interact with antibodies and form large aggregates.

The pores of polyacryl starch particles are thus smaller than those of highly cross-linked polyacrylamide particles. This means that the initial yield of immobilized proteins will be significantly larger because a larger fraction will be entrapped inside the formed network; the larger the Stokes radius of the protein, the more will be immobilized. Under standard conditions (100 mg of protein/mL of aqueous phase), 35-40% of the dry weight is protein, as shown with human serum albumin. The functional capacity, however, is generally not >40-60%, and ~50% of the protein is released from the particles in 6 weeks. These figures indicate that the fraction of protein trapped inside the particles, and not physically fixed in the polymeric network, is much larger than in the polyacrylamide particles. Moreover, the diffusion of low molecular weight ligands or substrates becomes restricted as the functional binding capacity of human serum albumin is decreased, and the enzymatic activity (K_m, v_{max}) of carbonic anhydrase is impaired. The polymer concentration (T) in the microparticles also influences the thermal stability, which is not greatly increased when T = 0.2%. It is important to note that the polymer concentration in the particles is high enough to guarantee that molecules are localized on the surface and that antibodies can form aggregates with the microparticles. This means that polyacryl starch microparticles could be used for specific targeting within the central compartment *in vivo*. In such a system, specific antibodies may be the instrument to target the microparticles to pathological cells in the circulation (*e.g.*, leukemia cells) or to eliminate circulating macromolecules (*e.g.*, antibodies or immune complexes).

Any drug carrier system designed for injection must be degradable. The metabolism of the polyacryl starch microparticles can be varied within wide limits, and the bulk of the matrix (the maltodextrin fraction) can be completely broken down by endogenous enzymes. The metabolism of the remaining part (the hydrocarbon chains from the acryloyl groups) must be further studied, but in the present system the fraction is small. However, the hydrocarbon chains are the decisive factor in the rate of degradation *in vivo*, which must be chosen according to need in the specific situation. As has been clearly demonstrated, polyacryl starch particles (D-T-C 10-0.4-0 or 10-0.2-0) are degraded in normal serum, and 30-40% of the starch is solubilized in 1 h. The half-life of microparticles in the circulatory system after intravenous injection is also ~1 h (7, 24). It is thus obvious that the derivatization of the starch must be high enough to give a reasonable stability in the circulation to ensure transport of the immobilized material to the target. Our experiments have shown that the leakage of immobilized proteins largely follows the degradation of the microparticles. The composition of the carrier system is thus a compromise: it must persist long enough in the circulatory system and yet must be metabolized rather quickly within the target organ. This principle is valid when the carrier is used for the immobilization of low molecular weight substances (*e.g.*, drugs) or proteins (*e.g.*, enzymes). Moreover, in some situations it is essential to control the release of active material from the carrier within the target cells, *e.g.*, to correlate the rate of release and the rate of denaturation of an enzyme. In such situations, it is important that the metabolism of the carrier can be anticipated and controlled. This is true for the polyacryl starch microparticles.

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