Semipermeable Microcapsules

Abstract. *Simple methods have been developed for encapsulating aqueous solutions of protein within polymer membranes. Stable microcapsules 1 to 100 μ in diameter, with semipermeable membranes, can be made by depositing polymer around emulsified aqueous droplets, either by interfacial coacervation or by interfacial polycondensation. Aqueous suspensions of enzyme-loaded microcapsules act well on small-molecular substrates both in vitro and in vivo.*

Enzymes in nature are commonly encapsulated within small aqueous compartments—cells or subcellular organelles—whose limiting membranes prevent their escape but are permeable to the smaller molecules of their substrates and products. I have found that artificial microcapsules of comparable dimensions and properties can be made simply and in large numbers, and that enzymes and other proteins loaded into these particles retain useful biological activity in vitro and in vivo. Described in this report are the methods that I have found most satisfactory for making the microcapsules, and some of the results obtained with them.

Many techniques have proved satisfactory but all entail three main steps. (i) The aqueous protein solution is emulsified in an organic liquid: for example, in the case of interfacial coacervation (collodion membrane), buffered erythrocyte hemolysate is mechanically emulsified in 10 times its volume of ether; in the case of interfacial polycondensation (nylon membrane), an erythrocyte hemolysate solution containing 50 percent by volume of 1,6-hexanediamine solution (0.4*M* in 0.45*M* $NaHCO_3$-Na_2CO_3 buffer; *p*H of buffer, 9.8) is mechanically emulsified in 5 times its volume of a chloroform-cyclohexane (1 : 4) mixture with 15 ml percent of Span 85 detergent (*1*) as emulsifying agent. (ii) On addition of a suitable material to the continuous phase of the stirred emulsion, a permanent membrane is formed at the interface of each microdroplet either by interfacial coacervation [as when an equal volume of organic liquid containing an alcohol-free ether solution of collodion (USP grade) is added] or by interfacial polycondensation (*2*) (as when an equal volume of cyclohexane-chloroform organic liquid containing 0.018*M* sebacoyl chloride is added, with continuous stirring for 3 minutes at 0°C). (iii) The microcapsules so formed are transferred to an aqueous medium, for example, by centrifugation and resuspension in an appropriate series of fluids. In the coacervation procedure, these fluids may be *n*-butyl benzoate containing Span 85, followed by Tween 20 (*1*) detergent solution (50 percent by volume in water) and then by water; in the polycondensation procedure, *n*-butyl benzoate is omitted. Typical procedures will be described in full elsewhere; the details are important, especially for preparing satisfactory microcapsules of mean diameter less than 15 μ.

Typical microcapsules made by interfacial polycondensation are shown in Fig. 1, *A* and *B*; those made by interfacial coacervation are similar in appearance. The size of the microcapsules is determined in the first step, mainly by the speed of the mechanical emulsifier and the concentration of emulsifying agents. The thickness of the membrane is determined mainly by the concentration of the material(s) used to form them. The coacervation technique can be used to form membranes from polymers other than collodion that are soluble in organic solvents, for example, polystyrene. The polycondensation technique can be used with diamines other than hexanediamine, or with polyamines. When the aqueous phase contains protein, the nylon membrane is always commingled with cross-linked protein—indeed, stable membrane can be formed by reacting sebacoyl chloride with protein alone. A particularly satisfactory membrane is formed with sebacoyl chloride from a 50 percent (by volume) mixture of 1,6-hexanediamine and 4,4′-diamino-2,2′ diphenyldisulfonic acid. The resulting copolymer membrane carries a strong negative charge; microcapsules made with it do not clump and are readily suspended in aqueous media without the aid of emulsifying agents. The presence in the aqueous phase of protein in fairly high concentration facilitates the formation of spherical microcapsules, apparently because osmotic pressure of the protein helps to maintain turgor. Besides protein and other macromolecular solutes, aqueous suspensions of particulate matter can be enclosed without particular difficulty. Thus larger microcapsules may be made to contain smaller ones, or, if a bland organic medium such as a low-viscosity silicone is used, even erythrocytes or other kinds of cells.

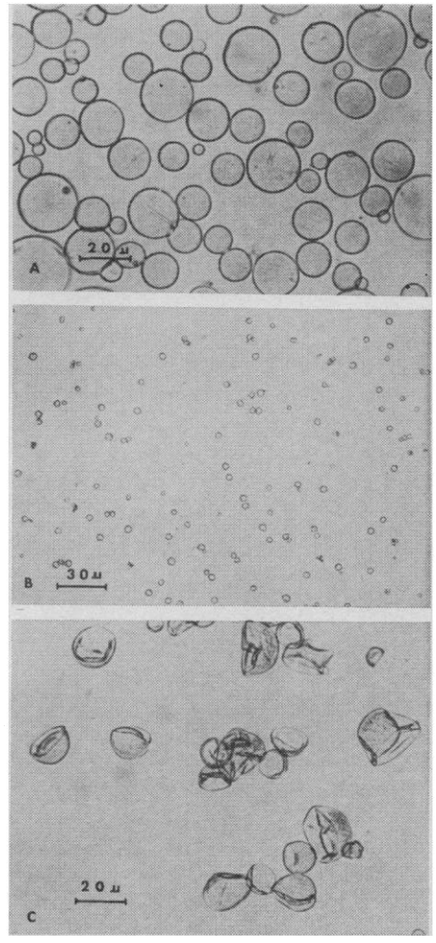

Fig. 1. Microcapsules containing erythrocyte hemolysate in nylon membranes: *A*, in water, and *C*, shortly after immersion in 0.5*M* K_2HPO_4, mean diameter 15 μ; *B*, in water, mean diameter 5 μ.

Allowance being made for their thinness, the permeability of microcapsular membranes made by these methods resembles that of extended membranes made from the same materials by conventional methods. Outward leakage of protein is usually undetectable even with agitation or after prolonged storage. Microcapsules are not ruptured by osmotic changes unless their membranes are made very thin, but they crenate almost instantaneously when placed in a hypertonic solution of a crystalloid (Fig. 1*C*). This crenation is spontaneously reversible with-

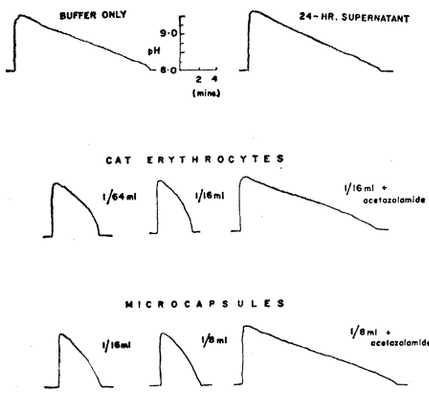

Fig. 2. Carbonic anhydrase activity, measured as the rate of fall of p*H* while CO_2 bubbles through 12 ml of alkaline buffer: method as in (*3*), but with 0.15*M* NaCl added to the medium. Fall of *p*H is accelerated by the presence of erythrocytes or of nylon microcapsules containing erythrocyte hemolysate diluted 1 : 1 (nylon microcapsules made from the same volume of hemolysate solution); no acceleration occurs in the presence of acetazolamide or when the microcapsules are replaced by supernatant from a 50 percent suspension of microcapsules stored for 24 hours.

in seconds or minutes if the solute species is one that penetrates the membranes: for a given osmolarity, the duration of crenation rises steeply with the molecular weight of the solute in the case of nonelectrolytes, and with the radius of the largest hydrated ion in the case of electrolytes. With small water-soluble species such as urea, crenation is not detected at concentrations below 3*M*, but large hydrated ions such as $SO_4^=$ and $HPO_4^=$ and large nonelectrolyte molecules such as sucrose cause long-lasting crenation.

One would expect the activity of an encapsulated enzyme, tested on a substrate present in the external phase, to be less than that of the enzyme in free solution, since the diffusion of its substrate or product, or both, would usually be the rate-limiting process. In favorable cases the activity in vivo is approached. Figure 2 shows that carbonic anhydrase from a red cell hemolysate, enclosed in nylon microcapsules of 10-μ mean diameter, did not leak out but catalyzed the hydration of CO_2 nearly as efficiently as before the cells were hemolyzed, and was sensitive to the inhibitor acetazolamide. Trypsin (on dipeptide but not on protein substrates) and urease also acted fairly efficiently after incorporation into microcapsules.

The stability of enclosed enzymes is variable: the carbonic anhydrase of an encapsulated hemolysate retained its activity after weeks of storage, while an encapsulated solution of crude urease lost most of its activity in a day if a preparation of soya bean trypsin inhibitor was not included. Some enzymes and proteins probably would be partly or wholly denatured when encapsulated by these procedures; this is so with hemoglobin which, however, retains more than 50 percent of its oxygen-carrying capacity if a favorable environment is specially provided for preparation and storage of the microcapsules.

I have occasionally tested in vivo the toxicity and the enzymatic activity of injected microcapsules. The toxicity of subcutaneously or intraperitoneally administered microcapsules is low and significant enzymatic activity is retained. Injection of encapsulated urease can markedly increase the concentration of ammonia in the blood. Intravenously injected microcapsules are not toxic provided they are washed free of Tween 20 and are administered in small doses; those prepared by the methods described herein usually produce some circulatory disturbance if the dosage exceeds 1 to 2 ml per kilogram of body weight, and like other foreign particles they do not survive for long in the blood stream. Some progress has been made toward a procedure for making microcapsules with a longer lifetime in the circulation: small negatively-charged capsules seem to survive longest.

It seems very likely that a number of applications, both in vitro and in vivo, will be found for these small objects. An obvious but not an immediate possibility is the therapeutic replacement of an enzyme lost in a genetic accident.

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References and Notes

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Gene-Enzyme Relations in Histidine Biosynthesis in Yeast

Abstract. *Each of the histidine mutants of* Saccharomyces cerevisiae *has been correlated with a particular step in histidine biosynthesis. The relations of the genes controlling histidine biosynthesis in yeast appear to be identical with those found in* Neurospora *and clearly distinct from those found in* Salmonella.

Although the original theory, now more than 20 years old, which suggests that genes control enzymes came largely from observations on biosynthesis in *Neurospora* (*1*), the identification of genes with their enzyme products has proceeded slowly. Until recently the assignment of a gene for every enzyme in a biosynthetic pathway had been achieved only in bacterial systems where the genetic material, unlike that of higher organisms, is contained in a single chromosome. However, application of the detailed methods of Ames and his collaborators (*2–4*) on the histidine pathway recently permitted the correlation of each of the histidine mutants of a eucaryotic organism, *Neurospora crassa*, with a particular step in histidine biosynthesis (*5*). The pathway of histidine biosynthesis is the same in *Neurospora* and *Salmonella*, but the relations of the genes controlling the enzymes in the pathway are different. To determine whether the gene-enzyme relations in the histidine pathway of other fungi also differ from *Salmonella* and whether they resemble *Neurospora* the present work was undertaken with the histidine mutants of *Saccharomyces cerevisiae*.

The mutational blocks were determined by enzyme assays and by characterization of accumulation products. Since some of the substrates before imidazole-glycerol phosphate (IGP) are not available, the yeast mutants were assayed by incubating them with adenosine triphosphate (ATP), 5-phos-