(14) G. S. Tarnowski, F. A. Schmid, H. G. Satterwhite, E. M. Solney, and R. K. Barclay, *Biochem. Pharmacol.*, **17**, 989 (1968).
(15) R. T. Matthews and C. Y. Chion, *Biochem. Pharmacol.*, **28**, 405 (1979).
(16) G. J. Goldenberg and B. K. Sinha, *Cancer Res.*, **33**, 1253 (1973).
(17) M. Ogawa, D. E. Bergsagel, and E. A. McCulloch, *Cancer Res.*, **33**, 3172 (1973).
(18) P. G. W. Plagemann and D. P. Richey, *Biochim. Biophys. Acta*, **344**, 263 (1974).
(19) G. Owens and I. Hatiboglu, *Ann. Surg.*, **154**, 895 (1961).

ACKNOWLEDGMENTS

A preliminary report of this work has been presented [S. A. Naujokaitis, *Proc. Am. Assoc. Cancer Res.*, **20**, 1012 (1979)].

Simple Rapid Method for the Preparation of Enteric-Coated Microspheres

I. MAHARAJ *, J. G. NAIRN ‡, and J. B. CAMPBELL *x

Received August 31, 1982, from the **Department of Microbiology, Faculty of Medicine*, and ‡*Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada. M5S 1A1.* Accepted for publication November 15, 1982.

Abstract □ A method is presented for encapsulating high molecular weight biological materials such as viral antigen, concanavalin A, and other proteins with cellulose acetate phthalate. The method is simple, inexpensive, and rapid; the process takes ~15 min. Capsules generated by this method are produced as microspheres 1–3 mm in diameter. They are stable for at least 6 h in simulated gastric conditions, but disintegrate rapidly under simulated intestinal conditions. Encapsulation had no effect on the activity of the biological materials. The method has potentially wide application for encapsulation of drugs and other substances.

Keyphrases □ Microspheres—enteric-coated, method for rapid preparation, encapsulation, cellulose acetate phthalate □ Encapsulation—method for the rapid preparation of enteric-coated microspheres, cellulose acetate phthalate □ Cellulose acetate phthalate—method for the preparation of enteric-coated microspheres, encapsulation □ Delivery systems—enteric-coated microspheres, cellulose acetate phthalate, method for rapid preparation

Cellulose acetate phthalate (I) has been used extensively as an enteric coating. Due to the presence of ionizable phthalate groups, the polymer is insoluble in acid media ≤pH 5, but is soluble when the pH is ≥6 (1). Since it is also remarkably inert *in vivo* (2), it is used to coat material for the release of drugs and other substances in the intestine. In recent years, I-coating technologies have been applied to the encapsulation of many biologically active materials, ranging from low molecular weight drugs [*e.g.*, sodium salicylate and phenacetin (3, 4)] to microorganisms [*e.g.*, viruses and bacteria (5–7)].

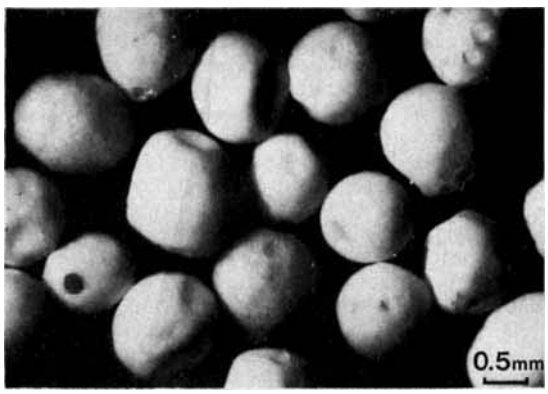

Figure 1—*Sucrose microspheres prepared as detailed in the text (formation time: 5 min).*

This report describes the development of an enteric coating for an oral vaccine used to protect wildlife against rabies. Studies on the vaccine itself will be reported elsewhere. The present report describes the principles of a method for encapsulation of the vaccine in the form of quasi-spherical particles ~1–3 mm in diameter (microspheres). The method is simple, rapid, and can be used to encapsulate a wide variety of materials. Therefore it has potential applications other than vaccine encapsulation.

EXPERIMENTAL

Materials—Core materials (*i.e.*, high molecular weight materials) that were encapsulated included rabies antigen (ERA-H strain of virus grown in BHK-21 cells and inactivated with β-propiolactone[1]), concanavalin A[1], and bovine serum albumin[1]. Radiolabeling of these materials with iodine-125 was carried out essentially as described by Thorell and Larson (8). Before use, the labeled preparations were passed through columns of Sephadex G-25[2] and extensively dialyzed against phosphate-buffered

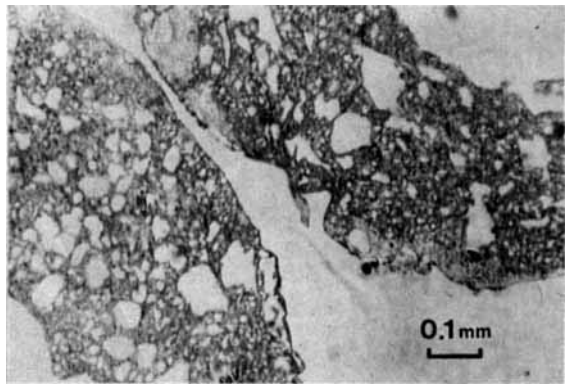

Figure 2—*Paraffin section (hematoxylin–eosin stain) of part of two sucrose microspheres showing the I matrix and the randomly distributed pockets that contained microparticles of the sucrose/core material. Hollow interiors of the microspheres are at the top right and bottom left of the photomicrograph.*

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Pharmacia, Uppsala, Sweden.

0022-3549/84/0100-0039$01.00/0

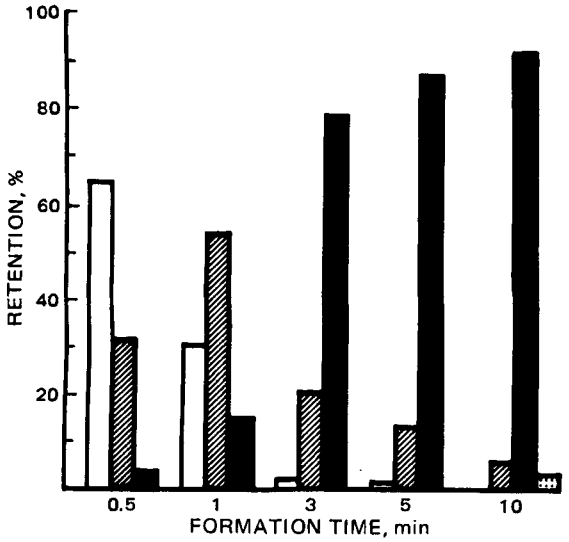

Figure 3—*Influence of formation time on the size of the microspheres. Formation was terminated by the addition of chloroform. Formed microspheres were collected, dried in air, and graded by passage through a series of U.S. standard stainless steel sieves mounted on a portable sieve shaker*[9]*, before being weighed. Key: (□) No. 50 sieve; (▨) No. 20 sieve; (■) No. 16 sieve; (⊞) No. 12 sieve.*

saline to remove any free iodine. In the final preparations, >99% of the radioactivity was associated with trichloroacetic acid-precipitable (*i.e.*, high molecular weight) material.

Two lots of cellulose acetate phthalate[3] (A and B) were employed as the encapsulation material. Lot A had been stored for several years and had a pungent smell of acetic acid. Lot B was newly purchased and was practically odorless. Although both preparations were acceptable, they required slightly different conditions for optimal formation of microspheres.

Formation of Microspheres—The core material (maximum 50 mg) was suspended in 200 mL of 5% sucrose (w/v), shell-frozen, and then freeze-dried. The resulting powder was then triturated in a 1:4 ratio with finely divided sucrose containing up to 5% cornstarch and pressed through a No. 50 U.S. stainless steel sieve. This powder was then suspended in 200 mL of white paraffin oil[4] contained in a 400-mL beaker. The mixture

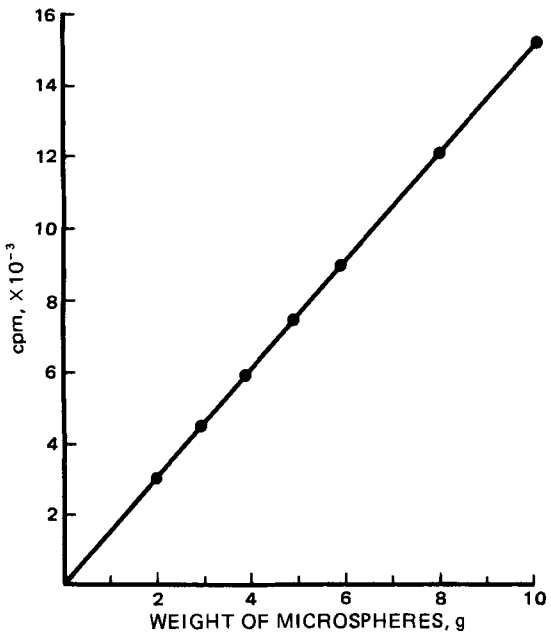

Figure 4—*Relationship between weight and core material content of the microspheres. Core material was [*^{125}*I]viral antigen, measured by radioactivity.*

[3] Lots #193(A) and #A9E(B); Eastman Kodak, Rochester, N.Y.
[4] Anachemia Chemicals Ltd., Mississauga, Ont.

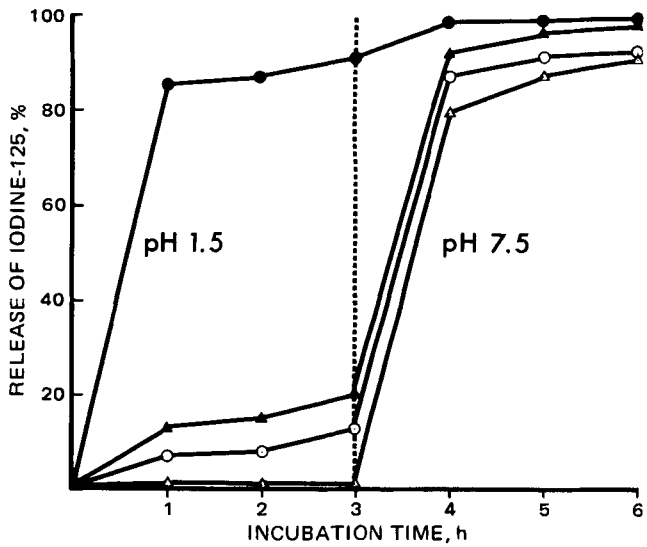

Figure 5—*Influence of pH of the incubation medium on the release of encapsulated viral radiolabel at 37°C. Key: (●) uncoated; (▲) I coated; (○) I–diethyl phthalate coated; (△) I–diethyl phthalate–wax coated.*

was dispersed by stirring at ~260 rpm with a 44-mm polyethylene three-blade paddle fitted to a high-torque stirrer[5]. To the suspension was added 20 mL of 10% (w/v) I in acetone–95% ethanol (9:1). Stirring was continued for 5 min to allow the microspheres to form, and then 75 mL of chloroform was added. The suspending medium was then decanted, and the microspheres were briefly resuspended in 75 mL of chloroform and air-dried at ambient temperature.

With one lot of I (B), some clumping of the microspheres was observed when they were removed from the chloroform. This was circumvented by stirring the suspension for 10–15 min after addition of the first volume of chloroform, prior to decanting.

In some preparations, the plasticizer diethyl phthalate[6] was included since it has been shown to increase the pliability and reduce the amount of moisture absorbed by I-coated capsules (9). The plasticizer (3% w/v) was dissolved in the I solution before addition to the encapsulation medium.

To further reduce the permeability of the microspheres, a wax coating was applied. Carnauba wax (1 g) was dissolved in 200 mL of white paraffin oil at 70°C and cooled to <45°C. The formed microspheres were then suspended in this for 15 min, with constant stirring. The wax solution was then decanted, and the microspheres were collected on filter paper to absorb the excess wax solution.

Release of Core Material—Stability of the microspheres was studied under conditions simulating those of the stomach and intestine. Breakdown or dissolution of the particles was monitored by measuring the release of iodine-125 and sucrose into the supernatant medium.

In most experiments, 2.5-g aliquots of the microsphere preparations were suspended in 20 mL of simulated gastric juice USP, without pepsin (*i.e.*, 0.08 M HCl containing 0.2% NaCl, pH 1.2). The suspension was incubated for 3 h at 37°C, with constant shaking at 160 rpm, on a clinical rotator[7]. At the end of each 1-h period, 1.0 mL of supernatant was removed, clarified by low-speed centrifugation (~7000×*g* for 10 min), and assayed for the presence of released encapsulated materials. Immediately following the 3-h incubation period, the simulated gastric medium was aspirated off, and the remaining microspheres were rinsed briefly with 5 mL of warm distilled water which was then discarded. The particles were incubated for a further 3 h at 37°C with 20 mL of simulated intestinal juice USP, without pancreatin (*i.e.*, 0.05 M KH_2PO_4 adjusted to pH 7.5 with 0.04 M NaOH). The hourly sampling process was repeated as before. The pH of the suspension was monitored by the addition of a few drops of 0.001% phenol red and adjusted when necessary with small volumes of alkali (usually 75 μL of 10 M NaOH after 1 and 2 h).

Assays—Levels of iodine-125 in the supernatant were measured by direct counting in a gamma-radiation counter[8]. Sucrose was measured by the anthrone reaction (10). There was no significant interference in this colorimetric reaction by dissolved I at the dilutions used.

[5] Type RZR1, "Caframo", Wiarton, Ont.
[6] BDH Chemicals, Toronto, Ont.
[7] Fisher Scientific Co., Fair Lawn, N.J.
[8] Gammacord; Ames Co., Elkhart, Ind.

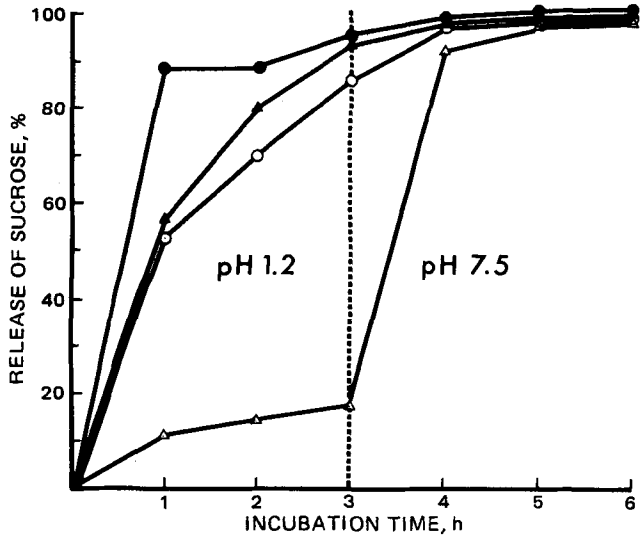

Figure 6—*Influence of pH of the incubation medium on the release of encapsulated sucrose at 37°C. Symbols are the same as in Fig. 5.*

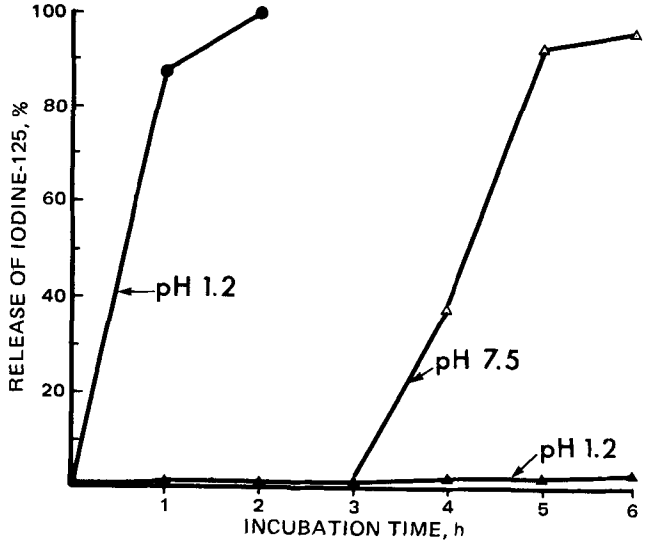

Figure 7—*Influence of pH of the incubation medium on the release of encapsulated concanavalin A at 37°C. Key: (●) uncoated; (▲, △) I–diethyl phthalate–wax coated.*

Before encapsulation, >99% of the iodine-125 activity was associated with trichloroacetic acid-precipitable material. To determine if any degradation or dissociation had occurred during the encapsulation and/or release studies, the hourly samples were adjusted to 10% with respect to trichloroacetic acid and incubated for 30 min at 4°C. Bovine serum albumin (1 mg) was added as a carrier. The resulting precipitates were separated from the supernatant by centrifugation, and both fractions were assayed for radioactivity.

Viral antigen and concanavalin A (a protein with lectin and immunostimulatory activity) were also assayed for biological activity before and after encapsulation. The virus was assayed by its ability to induce circulating antibody formation after intraperitoneal injection into mice, and the concanavalin A was assayed by its ability to agglutinate rabbit erythrocytes.

RESULTS AND DISCUSSION

Figure 1 illustrates the physical appearance of the microspheres. Preparations made with I lot A were dull white in color, whereas those produced with lot B were shiny white. Other characteristics of both preparations were similar. Most experiments presented in this report, however, were carried out with lot A.

Almost all of the microspheres were hollow, and in the large majority of particles the interior was completely sealed. A cross section of the particles revealed that the encapsulated material was dispersed throughout the I matrix in small pockets (Fig. 2). Fully formed quasi-spherical particles were apparent as early as 1 min after addition of the I solution. As seen from the data in Fig. 3, the size of the particles increased with time, reaching a maximum by 5–10 min after initiation. Coacervation of I, sucrose, and core material appeared to be complete since no I and only trace amounts of radioactivity (probably due to free iodine-125) were detected in the paraffin phase after separation of the microspheres.

It appears that formation of the microspheres occurs by stages. Initially, the finely divided suspension of sucrose/core material in the paraffin oil nonsolvent comes in contact with the I solution. Since the paraffin is also a nonsolvent for I, phase separation of the latter occurs with its deposition around the sucrose microparticles. Coated microparticles then aggregate, rapidly forming small hollow spheres. Further deposition of coated material around the aggregate results in the formation of larger spheres until all the suspended material has been utilized.

The data in Fig. 4 show a linear relationship between the weight of the microspheres and the amount of incorporated ^{125}I-labeled antigen. This indicates that the core material is dispersed uniformly among the particles.

Figures 5–7 summarize the disintegration characteristics of the microspheres under simulated gastric and intestinal conditions. From Fig. 5 it can be seen that, with a I–diethyl phthalate–wax coating, <1% of the encapsulated viral radiolabel was released into the gastric medium in 3 h. By comparison, the I–diethyl phthalate and I coatings were not as effective, releasing ~10 and 17%, respectively. Regardless of the coatings applied, however, most of the encapsulated protein was released within the first hour of incubation in the intestinal environment. Only 85–90% of the uncoated viral radiolabel (*i.e.*, lyophilized sucrose–viral antigen alone) was released in the first 3 h. The reason for this is not clear, but it may be due to an artifact in the assay system. There are a number of different proteins in the viral antigen preparation with radiolabel attached. Some of these proteins may have been denatured at the low pH of the simulated gastric juice and may have precipitated, in which case they would not have been measured in the supernatants, after centrifugation, as "released" material. Released under intestinal conditions, the same proteins would have remained soluble.

Although the I and I–diethyl phthalate coatings were effective in retaining the high molecular weight material, they had little effect on the retardation of the sucrose efflux, with almost all sucrose being released into the suspending medium within the 3-h test period. In contrast, when a I–diethyl phthalate-wax coating was applied, <20% was released under the same conditions (Fig. 6).

Figure 7 depicts the release of [^{125}I]concanavalin A from I–diethyl phthalate–wax-coated microspheres. Even after 6 h at low pH, there was <5% release, but there was almost complete breakdown at the intestinal pH of 7.5.

The size of the microspheres to some extent determined their release characteristics. In the experiment summarized in Fig. 8, I-coated microspheres prepared with [^{125}I]bovine albumin as the core material were graded through a series of Tyler sieves mounted on a portable sieve shaker[9]. It can be seen that the smaller the diameter, the more rapid the release. Since the size of the microspheres can be controlled to some extent by the time of addition of the chloroform (Fig. 3), the rate of release of their contents can also be controlled.

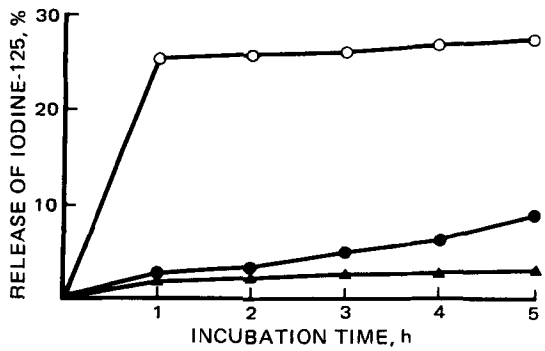

Figure 8—*Influence of size of the microspheres on the release of bovine albumin from simulated gastric juice (without enzymes), pH 1.2, at 37°C. Key: (○) particles retained by a No. 50 sieve; (●) particles retained by a No. 20 sieve; (▲) particles retained by a No. 12 sieve.*

[9] Model RX-24; W. S. Tyler Co. of Canada, Ltd., St. Catherines, Ont.

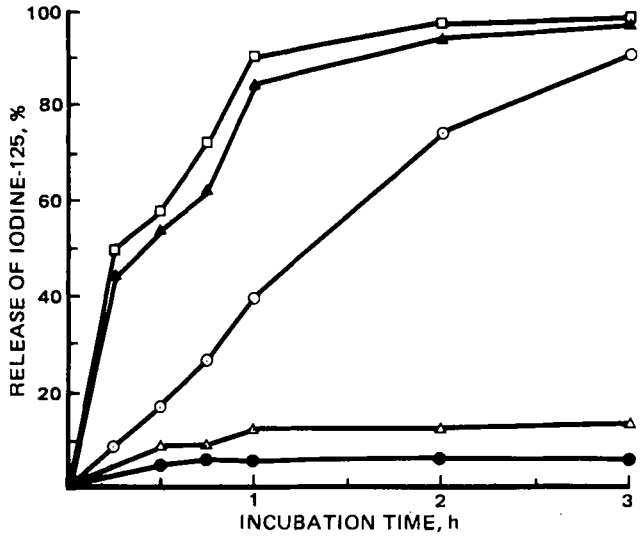

Figure 9—*Effect of pH on the release of viral radiolabel from citrate–phosphate buffer at 37°C. Key: (●) pH 2.5; (△) pH 5.0; (○) pH 6.0; (▲) pH 7.0; (□) pH 8.0.*

The above data indicate that the core materials were effectively released under simulated intestinal conditions. However, the data give no information about the physical state of the labeled material—specifically, whether or not it has been degraded during encapsulation and/or incubation. Studies on trichloroacetic acid-induced precipitation showed that >80% of the viral antigen- and concanavalin A-associated radiolabel was still acid insoluble after 3 h in each of the simulated gastric and intestinal conditions. Additionally, and more importantly, mice injected with the decapsulated viral antigen still produced protective neutralizing antibody titers against rabies virus (indicating that the antigenicity was largely intact), and the decapsulated lectin showed no significant loss of hemagglutinating activity with rabbit erythrocytes.

Figure 9 illustrates the pH dependency of the release of radiolabel from I-diethyl phthalate–wax-coated microspheres with a viral antigen core material. Conditions were the same as in previous experiments except that the buffer system was 0.1 M citrate–phosphate, ranging from pH 2.5 to 8.0. It is clear that the wax coating does not interfere with the release, which is minimal at pH ≤5. Between pH 5 and 7 there is increasingly rapid release, with no significant further increase above pH 7.

CONCLUSIONS

The encapsulation method described here is simple, inexpensive, and rapid; starting with the core material in the form of a finely divided powder, the whole procedure can take <15 min. Furthermore, no specialized equipment is required. The system has similarities to that described by Kitajima and coworkers (11); in their method, however, the core material is suspended in the I solution and the process required 5 h. In the system described here, it was found that if the stirring process was continued for > ~15 min prior to addition of the chloroform, clumping of the microspheres occurred. Since the microspheres were formed considerably before this time, this was no drawback.

In this report, the encapsulation of complex biologicals has been described, but the method can be applied to the encapsulation of many other substances. For example, microspheres have been prepared with such varied materials as cornstarch, tetracycline, saponin, and barium sulfate individually replacing the sucrose. Sucrose microspheres have been prepared containing the following active ingredients (2–60 mg/g of sucrose): scopolamine butylbromide[10], loperamide[11], trifluoperazine–isopropamide[12], and metoclopramide hydrochloride[13]. Encapsulation of cimetidine[14], however, was unsuccessful. The prime requirements appear to be that the materials to be encapsulated are in a finely divided state and that they are insoluble in both the acetone–ethanol and the paraffin phases. The microspheres can also be color-coded by addition of a dye such as malachite green to the I solution, the requirement here being that the dye is insoluble in the paraffin phase.

REFERENCES

(1) C. J. Malm, J. Emerson, and G. D. Hiatt, *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 520 (1951).

(2) H. C. Hodge, *J. Pharmacol. Exp. Ther.*, **80**, 250 (1944).

(3) H. P. Merkle and P. Speiser, *J. Pharm. Sci.*, **62**, 1444 (1973).

(4) P. L. Madan and S. R. Shanbhag, *J. Pharm. Pharmacol.*, **30**, 65 (1978).

(5) A. Gumma and A. Mirimanoff, *Pharm. Acta Helv.*, **46**, 278 (1971).

(6) D. H. Ferris and A. S. Arambulo, Ger. Offen. 2,343,570 (1974); *Chem. Abstr.*, **81**, 96437u (1974).

(7) R. M. Chanock, R. J. Huebner, and R. B. Couch, U.S. Pat. 3,317,393 (1967); *Chem. Abstr.*, **67**, 31018z (1967).

(8) J. I. Thorell and S. M. Larson, "Radioimmunoassay and Related Techniques. Methodology and Clinical Applications," C. V. Mosby, St. Louis, Mo., 1978.

(9) R. R. Crawford and O. K. Esmerian, *J. Pharm. Sci.*, **60**, 312 (1971).

(10) D. L. Morris, *Science*, **107**, 254 (1948).

(11) M. Kitajima, A. Kondo, S. Asaka, M. Moroshita, and J. Abe, Ger. Offen. 2,105,039 (1971); *Chem. Abstr.*, **75**, 152682k (1971).

ACKNOWLEDGMENTS

This work was supported by Provincial Lottery funds through a contract with the Ontario Ministry of Natural Resources.

[10] Buscopan; Boehringer Ingelheim (Canada) Ltd., Burlington, Ont.
[11] Imodium; Ortho Pharmaceutical (Canada) Ltd., Don Mills, Ont.
[12] Stelabid Forte; Smith, Kline and French (Canada) Ltd., Mississauga, Ont.
[13] Reglan; A. H. Robins Canada Ltd., Montreal, Que.
[14] Tagamet; Smith, Kline and French (Canada) Ltd., Mississauga, Ont.