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Studies on Microcapsules. V.[1] Preparation of Polyamide Microcapsules containing Aqueous Protein Solution

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A study was made of the preparation of polyamide microcapsules containing aqueous bovine serum albumin solution. The size and size distribution were found to be independent of the concentration of bovine serum albumin. The formation of double microcapsules was frequently observed. Part of the amino residues of the encapsulated bovine serum albumin molecules was supposed to be chemically incorporated in the microcapsule membranes through the reaction with acid chloride.

The microencapsulation of aqueous solutions of proteins and enzymes has drawn attention because the microcapsules thus obtained may be very useful in the medical and pharmaceutical fields. Chang and his coworkers, for instance, prepared the nylon microcapsules containing aqueous solutions of enzymes,[3,4] and developed interesting applications of the microcapsules in physiology.[5-9] From the physicochemical point of view, however, there still remain fundamental problems to be solved in the preparation and properties of protein or enzyme containing microcapsules.

This paper and later one[10] deal with physicochemical studies of the prepartion and properties of polyamide microcapsules containing bovine serum albumin solutions.

Experimental

Materials——Crystalline bovine serum albumin (BSA) was obtained from Nutritional Biochemicals Corp., U.S.A., and sebacoyl chloride was a product of Eastman Organic Chemicals, U.S.A. Span 85 and Tween 20 were supplied by Nihon Surfactant Ind., Inc., Tokyo. All other chemicals used in this study were of reagent grade.

Preparation of Microcapsules——The preparation of polyamide microcapsules was done by a method similar to that in a previous paper.[11] Thus, the procedure consisted of the following three steps. 1) To 1.5 ml of aqueous BSA solution in a 200 ml beaker surrounded by ice was added an equal volume of 0.4M 1,6-hexamethylenediamine or piperazine solution in 0.45M $NaHCO_3$–Na_2CO_3 buffer of pH 9.8. To this solution was added 15 ml of a mixed solvent (chloroform–cyclohexane, 1:3 (v/v), containing 10% (v/v) Span 85 as an emulsifing agent). The mixed solution was then mechanically emulsified with a Chemistirrer

1) Part IV: M. Koishi, N. Fukuhara, and T. Kondo, *Can. J. Chem.*, **47**, 3447 (1969).
2) Location: *a) 41-8, 3-chome, Takada, Toshima-ku, Tokyo; b) 12 Funagawara-machi, Ichigaya, Shinjuku-ku, Tokyo.*
3) T.M.S. Chang, *Science*, **23**, 524 (1964).
4) T.M.S. Chang, F.C. MacIntosh, and S.G. Mason, *Can. J. Physiol. Pharmacol.*, **44**, 115 (1966).
5) T.M.S. Chang, *Trans. Am. Soc. Artificial. Internal. Organs*, **12**, 13 (1966).
6) T.M.S. Chang, L.J. Johnson, and O.J. Ransome, *Can. J. Physiol. Pharmacol.*, **45**, 705 (1967).
7) T.M.S. Chang and N.J. Poznansky, *J. Biomed. Mater. Res.*, **2**, 187 (1968).
8) T.M.S. Chang, A. Pont, L.J. Johnson, and N. Malave, *Trans. Am. Soc. Artificial. Internal. Organs*, **14**, 163 (1968).
9) T.M.S. Chang, and M.J. Poznansky, *Nature*, **218**, 243 (1968).
10) M. Shiba, Y. Kawano, M. Koishi, and T. Kondo, *Bull. Chem. Soc. Japan*, to be submited.
11) M. Koishi, N. Fukuhara, and T. Kondo, *Chem. Pharm. Bull.* (Tokyo), **17**, 804 (1969).

(Tokyo Rika Kikai Co., Tokyo, Model B-100) at 512 rpm for 5 minutes to yield a water-in-oil emulsion. 2) Without stopping the stirring, 15 ml of sebacoyl chloride or *p*-phthaloyl dichloride solution in the mixed solvent was added to the emulsion, and the stirring was continued for another 3 minutes. 3) To this dispersion was added 30 ml of the mixed solvent, and the dispersion was centrifuged for 30 seconds at 350 g to separate the newly formed microcapsules. The microcapsules were then completely dispersed in 10 ml of aqueous 50% (v/v) Tween 20 solution, to which was added 50 ml of distilled water. The dispersion was centrifuged again for 1 minute at 350 g, and the separated microcapsules were resuspended in 25 ml of 0.9% NaCl solution. The resulting microcapsule dispersion, containing a small amount of Tween 20, could conveniently be stored in a refrigerator at 2—4°C.

Partition of Diamines between Aqueous and Organic Phases——In order to investigate the effect of BSA on the formation of polyamide microcapsules, the determination was made of the diamine partitions between the aqueous and the organic phases under the same conditions as those used in the preparation of microcapsules. In each case an emulsion was formed, which was centrifuged to completely separate into two liquid layers.

The transferred amount of diamine was determined by titrating the separated organic liquid layer with 0.01N alcoholic hydrochloric acid using BCG-BTB mixed indicator. The alcoholic hydrochloric acid was standaridized for 0.01N alcoholic sodium hydroxide before use. The initial concentration of diamine in the aqueous phase was then determined by the titration with aqueous hydrochloric acid. Finally, the partition coefficients between the aqueous and the organic phases were calculated by the following equation

K = (initial concentration of diamine in the aqueous phase—final concentration of diamine in the organic phase)/final concentration of diamine in the organic phase.

Determination of Size and Size Distribution——Several samples from each batch of the microcapsules were photographed under an optical microscope. Calculations were made of the length mean diameter, size distribution, standard deviation, mean surface diameter, and mean volume diameter by using an IBM 1620 computer as described in the previous paper.[11] The size distribution of microcapsules dispersed in 0.9% NaCl solution were also measured by a Celloscope (AB Lars Ljungberg & Co., Sweden), which works upon the Coulter principle.

Electrophoretic Mobilities of Microcapsule Membranes——The stocked microcapsule dispersion was dialysed in a cellulose tubing (Visking) against distilled water at 40° until a constant conductance of the dispersion was attained. And then, the microcapsules were destroyed by subjecting to a high centrifugal field, and the collected membranes were washed twice with acetate buffer solution on the centrifuge. The washed membranes were finally dispersed in the above buffer solution.

Electrophoretic measurements on the membranes were conducted in a quartz flat microelectrophoretic cell supplied by Mitamura Riken Kogyo & Co., Tokyo. For each measurement 40 specimens were timed in each direction to eliminate the polarization effect of the electrodes. Mobilities were taken at room temperature and corrected to 25°.

Results and Discussion

Formation of Microcapsules

The all polyamide microcapsules made from the combinations of 1,6-hexamethylenediamine–sebacoyl chloride (HS), 1,6-hexamethylenediamine–*p*-phthaloyl dichloride (HP), piperazine–sebacoyl chloride (PS), and piperazine–*p*-phthaloyl dichloride (PP) were invariably

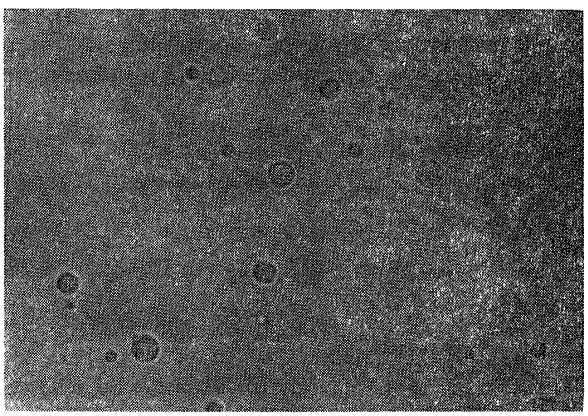

Photo. 1. Effect of the Concentration of BSA on the Size of HP Microcapsules
concentration of BSA 0.5% (w/v)

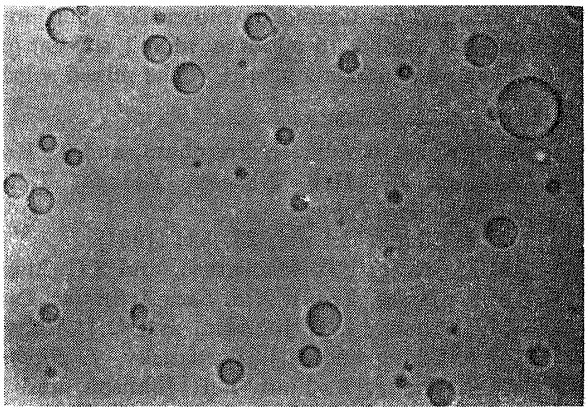

Photo. 2. Effect of the Concentration of BSA on the Size of HP Microcapsules
concentration of BSA 2.5%(w/v)

spherical in shape, but varied in mechanical resistance to centrifugation according to the increasing order PS<HS<HP<PP. In the case of PS, most of the microcapsules were ruptured by centrifugation. The HS microcapsules were not resistant to repeated centrifugation. Therefore, the following experiments were carried out with the combinations, HP and PP, which gave fairly strong membranes.

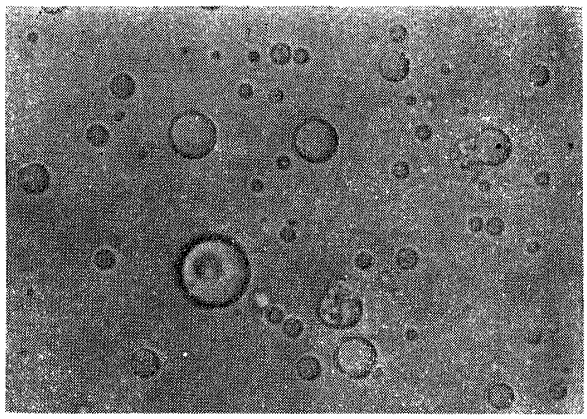

Photo. 3. Effect of the Concentration of BSA on the Size of HP Microcapsules
concentration of BSA 5.0% (w/v)

The strength of HP membranes was clearly affected by the concentration of BSA. Thus, the microcapsules formed in the organic phase in the absence of BSA were mostly destroyed by centrifugation, leaving only very small ones, whereas many large microcapsules could survive even after centrifugation when they were prepared in the presence of BSA. Typical examples showing this tendency are shown in Photo. 1 to 3.

Size Distribution and Mean Microcapsule Size

Fig. 1 and Fig. 2 show the size distribution of microcapsules transferred from the organic to the aqueous phase after centrifugation. In Fig. 3 is given the size distribution of PP microcapsules as obtained by the Celloscope method.

In these cases, the size distribution curves were practically independent of the concentration of BSA, and had almost the same shape.

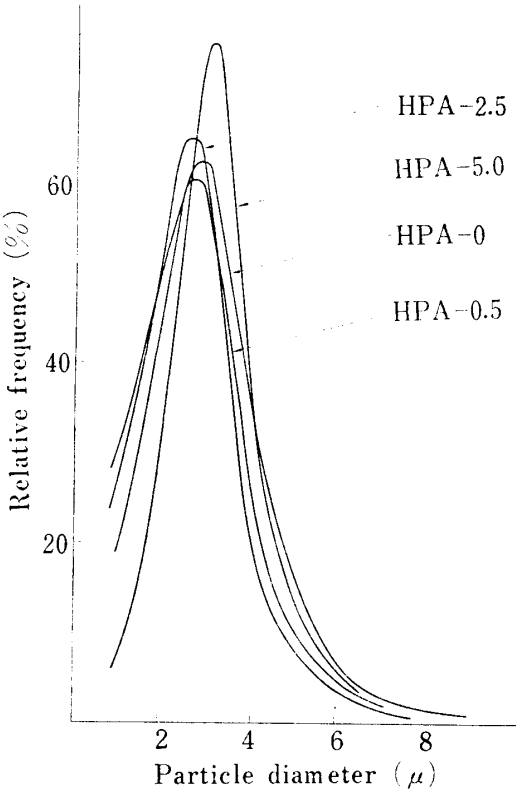

Fig. 1. Effect of the Concentration of BSA on the Size Distribution of Centrifuged HP Microcapsules as obtained by the Photomicroscopic Method (Photomicroscopic Method)

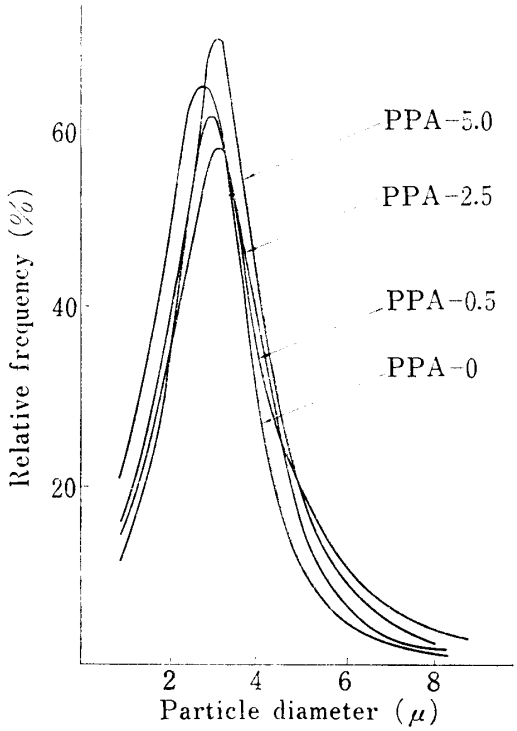

Fig. 2. Effect of the Concentration of BSA on the Size Distribution of Centrifuged PP Microcapsules (Photomicroscopic Method)

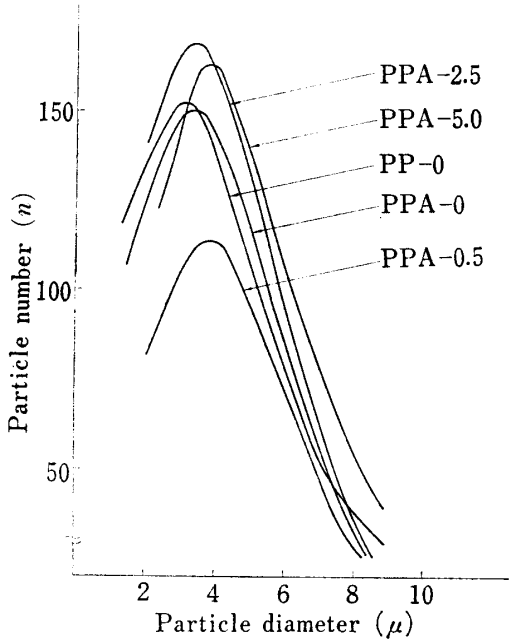

Fig. 3. Effect of the Concentration of BSA on the Size Distribution of Centrifuged PP Microcapsules (Celloscope Method)

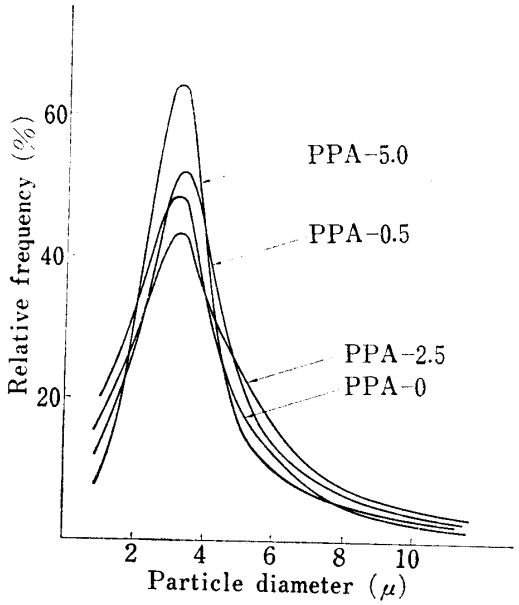

Fig. 4. Effect of the Concentration of BSA on the Size Distribution of Non-centrifuged PP Microcapsules (Photomicroscopic Method)

Fig. 4 illustrates the size distribution of PP microcapsules dispersed in the aqueous phase without using the centrifuge after preparation.

As in Fig. 4, the distribution curves were independent of the concentration of BSA.

In Table I are summarized the results on the calculation of the length mean diameter, standard deviation, mean surface diameter, and mean volume diameter in the above mentioned cases.

The modal diameters of distribution curve as obtained by the photomicroscopic method nearly comformed to the calculated length mean diameters, while those as obtained by the Celloscope method roughly coincided with the calculated mean volume diameters.

Table I. Effect of the Concentration of BSA on the Mean Diameters and Standard Deviation of Microcapsules

Microcapsules[a]	Centrifugation	Mean diameter[b]			Standard deviation
		$d_1(\mu)$	$d_2(\mu)$	$d_3(\mu)$	
HP-0	+	2.9	3.2	3.4	1.30
HPA-0	−	4.0	4.8	5.5	2.53
HPA-0.5	+	2.5	2.8	3.1	1.22
HPA-2.5	+	2.5	2.7	3.0	1.14
HPA-5.0	+	3.0	3.3	3.6	1.29
PP-0	+	2.8	3.2	3.7	1.54
PPA-0	+	2.8	3.1	3.3	1.20
PPA-0.5	+	3.0	3.3	3.7	1.43
PPA-2.5	+	3.2	3.6	4.1	1.67
PPA-5.0	+	2.6	3.0	3.4	1.36
PP-0	−	3.7	4.6	5.6	2.76
PPA-0	−	3.4	4.4	5.7	2.76
PPA-0.5	−	4.1	5.0	6.1	2.90
PPA-2.5	−	3.7	4.4	5.2	2.43
PPA-5.0	−	4.2	5.2	6.3	3.03

a) HP- and PP-, HPA- and PPA-, and (-0) mean the microcapsules containing Na_2CO_3 sol., buffer sol., and buffered BSA sol., respectively.

b) d_1, length mean diameter; d_2, mean surface diameter; d_3, mean volume diameter.

The centrifugation appeared to scarcely affect the modal diameter, although it exerted influence on the calculated mean diameter, suggesting the breakdown of large microcapsules in the centrifugal field. This is shown in Fig. 5.

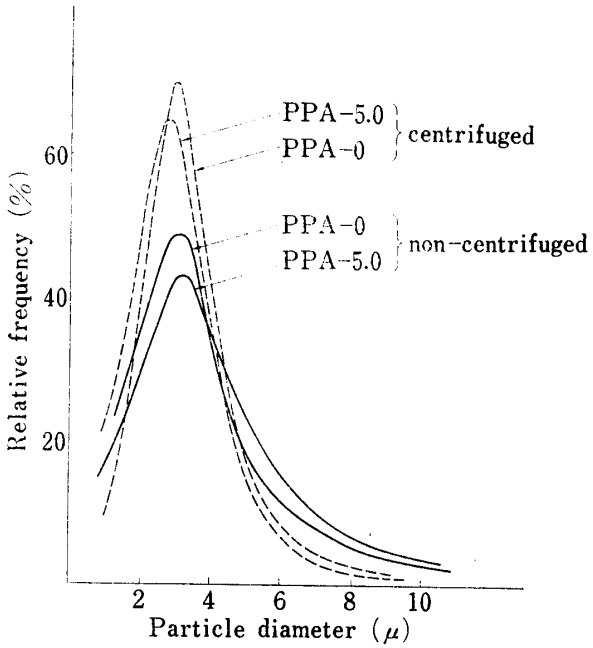

Fig. 5. Effect of the Centrifugation on the Size Distribution of PP Microcapsules

Partition of Diamines between Aqueous and Organic Phases

Table II gives the partition coefficients of 1,6-hexamethylenediamine between the organic phase and Na_2CO_3 solution, buffer solution ($NaHCO_3$–Na_2CO_3, pH 9.8), and the buffer plus BSA solution.

In the absence of Span 85 in the organic phase, the transfer of the diamine from the aqueous to the organic phase showed an upward tendency with an increase in the stirring speed as described in the previous paper.[11] The presence of Span 85 in the organic phase did not affect appreciably the variation of parti-

Table II. Effect of the Constitution of Encapsulated Aqueous Phase on the Partition Coefficient of 1,6-Hexamethylenediamine

Aqueous phase	Concentration of BSA % (w/v)	Partition coefficient $K(C_{aq}/C_{org})^{a)}$
0.45M Na_2CO_3	0	1.03
0.45M buffer	0	6.39
0.45M buffer	0.1	9.35
0.45M buffer	0.5	8.68
0.45M buffer	1.0	8.87

a) The volume ratio of aqueous to organic phase was 1: 5.

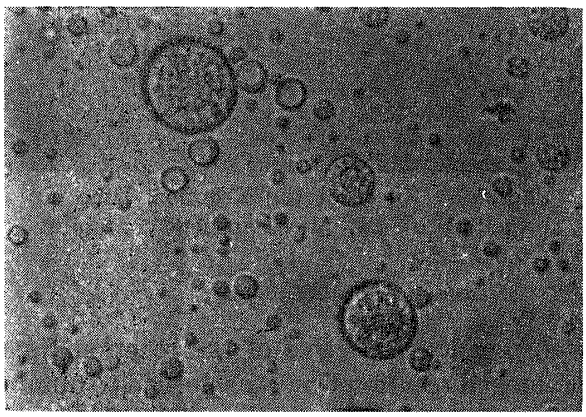

Photo. 4. Effect of the Reaction Time on the Size of PP Microcapsules containing 0.5% BSA

reaction time 3 min, non-centrifuged

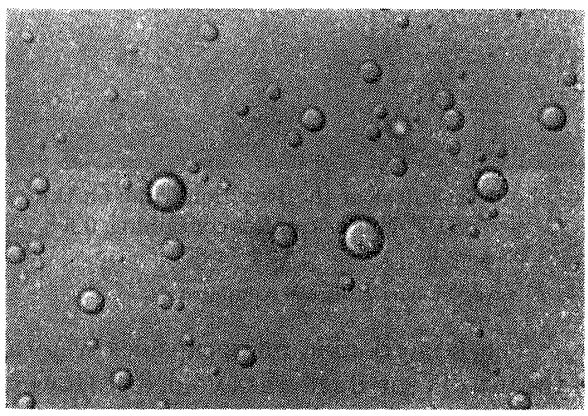

Photo. 5. Effect of the Reaction Time on the Size of PP Microcapsules containing 0.5 % BSA

reaction time 3 min, centrifuged

tion coefficient with the BSA concentration. Consequently, the data are given in Table II for the systems containing no Span 85. The same trend was found in the transfer of piperazine.

The use of buffer solution, instead of Na_2CO_3 solution, deteriorated the transfer of diamine. BSA was also an obstruction to the transfer of diamine from the aqueous to the organic phase.

Formation of Double Microcapsules

In many cases, the microcapsules of large size seemed to contain a number of small microcapsules inside them. An example is shown in Photo. 4. The large microcapsules were easily broken by centrifugation, leaving only small ones as is shown in Photo. 5.

The small microcapsules were undergoing Brownian motion in the large ones when observed under a microscope and never went out. This will be interpreted, therefore, as showing that the small microcapsules are contained in but not attached to the large ones.

The PP microcapsules prepared using various reaction times are shown in photo. 6 to 9.

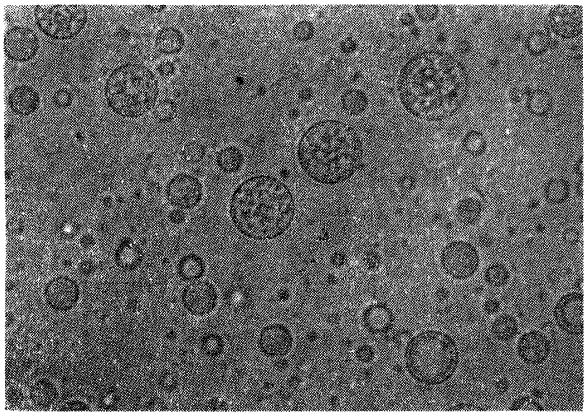

Photo. 6. Effect of the Reaction Time on the Size of PP Microcapsules containing 0.5% BSA

reaction time 1 min, non-centrifuged

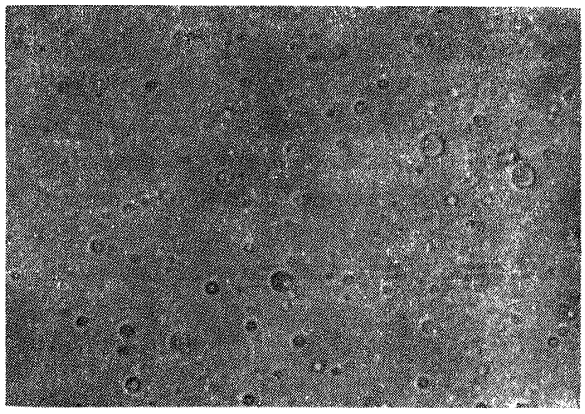

Photo. 7. Effect of the Reaction Time on the Size of PP Microcapsules containing 0.5% BSA

reaction time 1 min, centrifuged

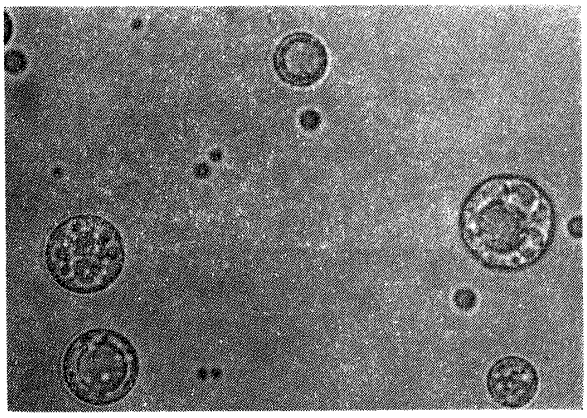

Photo. 8. Effect of the Reaction Time on the Size of PP Microcapsules containing 0.5% BSA

reaction time 5 min, non-centrifuged

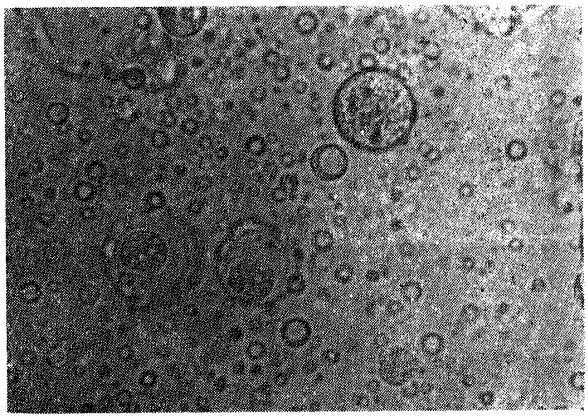

Photo. 9. Effect of the Reaction Time on the Size of PP Microcapsules containing 0.5% BSA

reaction time 5 min, centrifuged

The large double microcapsules were always observed irrespective of the reaction time if the use of centrifuge was avoided. However, a prolonged reaction time was needed to obtain the double microcapsules without breakdown when the centrifugation was used. This will result from the fact that the membrane strength is highly dependent on the reaction time.[12]

12) P.W. Morgan and S.L. Kwolek, *J. Polymer Sci.*, **40**, 299 (1959).

The double microcapsules are, therefore, likely to be formed at an early stage of condensation reaction. This hypothesis seemes to be true because the ratio of surface area to volume is larger for small microcapsules than for large ones, indicating the faster transfer of diamine from the aqueous to the organic phase for the formers than for the latters. The accumulation of diamine at the interface should facilitate the formation of polymer membrane. Accordingly, the small microcapsules having relatively thick membrane will be able to penetrate on collision into the large ones enclosed with very thin membrane during the stirring. As the condensation reaction is still going on, the membrane of large microcapsules will be capable of growing enough to patch up the rents formed by the penetration of small microcapsules.

The formation of double microcapsules is, therefore, closely related to the partition coefficient of diamine. In fact, the double microcapsules were not found to form until the partition coefficient of diamine attained a fairlly large value by the addition of albumin or salt as is seen in Table II.

Electrophoresis of Microcapsule Membranes

The microcapsule membranes moved with a measurable mobility in an electric field in the aqueous acetate buffers. The mobilities of microcapsule memberans are plotted as a function of pH of the medium in Fig. 6.

The observed isoelectric point locates approximately at pH 3.5, whereas the microcapsules containing anyone of the buffers alone did not have any charge detectable by electrophoresis in the same medium. Then, it will be assumed that the surface charge of membranes arises from the charge on the encapsulated BSA. As the isoelectric point of BSA in aqueous phase lies at pH 4.7, part of the amino residues of BSA molecules is likely to have reacted with acid chloride to reduce the number of positively charged sites on the molecules.

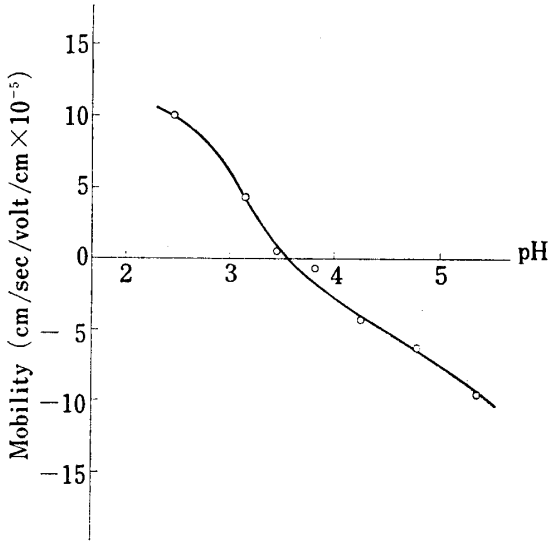

Fig. 6. Mobility of the Microcapsule Membranes as a Function of pH of Acetate Buffer at 25° (Ionic Strength 0.01)

From what have been described so far, it may be concluded that the presence of BSA obstructs the transfer of diamine from the aqueous to the organic phase and makes it easier to form double microcapsules. On the other hand, BSA molecules strengthen the microcapsules by being chemically incorporated as a constituent element in the membranes.

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