Incorporation of Macromolecules in Microparticles: Preparation and Characteristics†

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ABSTRACT: Macromolecules can easily be incorporated in microparticles of polyacrylamide by copolymerization with acrylamide in a water-in-oil emulsion. The microparticles, preferably around 1-3 μm in diameter, will have a macroporous structure formed by the polymeric network. The amount of incorporation of the macromolecules will depend on the structure of the network, which, in turn, will depend on the total amount of monomer (T) and the relative amount of cross-linking agent (C) in the monomeric solution. Two mechanisms are responsible for the incorporation; all macromolecules are, independently of the size, fixed in the threads of polyacrylamide and large ones are entrapped within the network formed by the threads. The amount *entrapped* will depend on the size of the macromolecule and the mean pore radius of the gel. In microparticles with a total concentration of monomers of 8% and a cross-linking of 25% (T-C = 8-25) the biological properties of incorporated macromolecules are retained, due to the macroporous structure, as found in binding studies with albumin. The density of the particles will also depend on C and T and, to some extent, on the protein concentration. Due to the fixation in the polyacrylamide threads, some of the incorporated macromolecules will be exposed on the surface, allowing them to react with, for instance, cells, which cannot penetrate the particles. The optimal conditions for the incorporation of macromolecules in the microparticles are investigated.

In recent years, several groups of biologically active macromolecules, immobilized in different kinds of solid matrices, have been extensively used for different purposes. In most cases, the macromolecules have been covalently bound to the support by normal coupling reactions. Generally, a high yield of bound macromolecules can be obtained also with small molecules and the coupling procedure also ensures that a significant part of the immobilized molecules is available on the surface of the solid support. However, the gel entrapment technique, initially introduced by Bernfeld and Wan (1963), offers several advantages. It is simple, no chemical modifications of the entrapped molecules are needed, the biological activity is retained, and the entrapped molecules are, within certain limits, accessible when polyacrylamide gels are used for the entrapment through large macropores formed in the gel (Fawcett and Morris, 1966). The entrapped macromolecules will, moreover, be well protected in the gel against bacterial degradation and even proteolytic enzymes (Mori et al., 1974).

Macromolecules immobilized in polyacrylamide gel polymerized from monomeric acrylamide have found applications as immunosorbents (Bernfeld and Wan, 1963, Carrel et al., 1969), in chemical analysis (Hicks and Updike, 1966), or in columns for different purposes (Mosbach and Mosbach, 1966, Wada and Kishizaka, 1968). For this latter purpose, the introduction of the bead polymerization technique, in order to produce spherical particles with narrow size distribution (Mosbach and Mosbach, 1966, Nilsson et al., 1972, Johansson and Mosbach, 1974), meant a significant improvement. Spherical particles have found technical applications in enzyme column reactors, in which a substrate is modified when passing through the column. However, one drawback of the method is that the diameter of the beads has to be at least 100-250 μm to guarantee that the flow rate through the column is sufficiently high. This means that the enzyme activity will be decreased due to a rate-limiting diffusion process within the particles (Mori et al., 1974), if the gel polymer network is too tight.

In a preliminary note (Ekman and Sjöholm, 1975), we have recently reported that small particles can conveniently be prepared by the bead polymerization technique. An interesting feature of the polyacrylamide particles, which can be most efficiently and spectacularly demonstrated, is the partial localization of the bound macromolecules on the particle surface. This finding, which has not been observed earlier, opens up wide perspectives for several new applications for microparticles in, e.g., cell studies. In such microparticles with a mean diameter less than 10 μm, the activity of the immobilized macromolecules can be efficiently utilized. The present paper will describe, in detail, the preparation and properties of the microparticles and discuss the possible mechanisms for the immobilization of macromolecules in the particles.

Materials and Methods

Materials. Human serum albumin was prepared essentially according to McMenamy et al. (1971) and treated with activated charcoal at pH 3.0 as described by Chen (1967). A highly purified preparation of albumin was also obtained as a gift from AB KABI, Stockholm. Normal human immunoglobulin G (IgG[1]), from AB KABI, Stockholm, and bovine β-lactoglobulin (as a mixture of the A and B forms), from Sigma Chemical Co., were used without further purification. [^{14}C]Salicylic acid and [^{14}C]warfarin were purchased from The Radiochemical Centre, Amersham, England. The corresponding unlabeled drugs were obtained as gifts from the

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[1] Abbreviations used are: Bis, *N,N'*-methylenebisacrylamide; Temed, *N,N,N',N'*-tetramethylethylenediamine; IgG, normal human immunoglobulin G; PPO, 2,5-diphenyloxazole; POPOP, 1, 4-bis[2-(5-phenyloxazolyl)]benzene.

different manufacturers. Pluronic F 68 (polyoxyethylene-derived polyoxypropylene), manufactured by Wyandotte Chemicals Corp., Wyandotte, Michigan, was obtained as a gift through Trebec AB, Stockholm, Sweden. Acrylamide and *N,N'*-methylenebisacrylamide (Bis) were purchased from Eastman Kodak Co. *N,N,N',N'*-tetramethylethylenediamine (Temed) was purchased from Sigma Chemical Co. Other chemicals were of analytical grade.

Preparation of Microparticles of Polyacrylamide. Acrylamide (e.g., 1.5 g) and Bis (e.g., 0.5 g)[2] were dissolved in 25 ml of 0.005 M sodium phosphate buffer, pH 7.4. When a macromolecule was to be immobilized, an appropriate amount was dissolved in the same solution. It was cooled to about 0 °C with ice and freed from oxygen with nitrogen gas. At the same time, a mixture (300 ml) of toluene and chloroform (4:1, density ~0.98 g/ml) was prepared with the detergent Pluronics F68 (0.25 g), cooled, and flushed with nitrogen. A catalyst system of Temed (100 μl) and 100 μl of ammonium peroxosulphate (0.5 g/ml of water) was then added to the water phase, which, in turn, was immediately added to the organic mixture and suspended in it with a homogenizer. Two types of homogenizer have successfully been used, either a Silverson (Waterside, Chesham, Bucks, U.K.) or, for 5–10 ml volumes of monomer solution, an Ultraturrax TP 18-10.

A water-in-oil emulsion is formed easily and the polymerization of the acrylamide monomers generally starts immediately. The speed of the homogenizer is then decreased and the temperature, which is slightly increased during the polymerization, is raised to about 30 °C in a water bath. The suspension is stirred for about 10 min, after which time the polymerization generally is complete.

After the polymerization, a phosphate buffer (50 ml) is added and the mixture is stirred slowly with a magnetic stirrer for a further 5 min. The phases are then allowed to separate. The particles are found at the bottom of the water phase, over which the organic phase is layered. The separation can be accelerated by mild centrifugation. The organic phase can be regained and purified by fractionated distillation.

Macromolecules not bound in the microparticles can be isolated from the water phase and the first washing of the microparticles, which are washed at least five times with the phosphate buffer. After the initial centrifugation, a higher *g* number can be used to isolate the particles without any risk that the particles stick together in the centrifuge tube. During the washing procedure, a crude separation of the microparticles according to size can be achieved by differential centrifugation.

Protein Determination. The amount of protein bound in the particles was determined after hydrolysis in 6 M hydrochloric acid at 105 °C for 20 h and subsequent amino acid analysis with an automatic amino acid analyzer, when exact information was needed. For practical purposes, the method of Eskamani et al. (1974) was generally sufficient. The protein content was then estimated from the tryptophan amount determined after hydrolysis of the particles in formic acid, hydrochloric acid, and ninhydrin at 110 °C for 20 min. Appropriate controls with blank samples (particles without protein) and standards (the incorporated protein) were run.

Radioactivity measurements were made in a Beckman Scintillation Counter, LS 100-C. The samples (100 μl) were dissolved in 2 ml of ethanol and 10 ml of a toluene solution (PPO, 5 g; dimethyl POPOP, 0.3 g in 1000 ml of toluene). The efficiency was calculated from standard samples and an external standard.

The binding of ligands to albumin was studied with microparticles or by equilibrium dialysis. Lyophilized microparticles (25 mg) were suspended in 1.0 ml of buffer (0.05 M phosphate, 0.1 M KCl, pH 7.4) in 2-ml plastic tubes. Incubation with ligand was carried out by slowly rotating the tubes for 30 min at room temperature. The suspension was then centrifuged. The amount of free ligand was determined in 100 μl of the supernatant. Equilibrium dialysis with albumin and ligands was done in special cells with 1-ml samples, as described earlier (Sjöholm et al., 1976) under the same conditions as used with the microparticles containing albumin. The data are plotted according to the Scatchard equation (Scatchard, 1949)

$$\frac{r}{D} = (nK_a) - (rK_a) \qquad (1)$$

where r = mol of bound ligand/mol of albumin, D = concentration of unbound ligand, n = number of binding sites, and K_a = the apparent association constant.

Determination of the Density of Microparticles. The density of microparticles was determined by a density gradient centrifugation method described for viruses (Pertoft et al., 1967). Lyophilized microparticles were suspended in colloidal solutions of silica, Ludox^R (E. F. du Pont de Nemours and Co., Wilmington, Delaware), and centrifuged at 111 000*g* in a Beckman L5-65 ultracentrifuge. During the centrifugation for 30 min in an angle-head rotor (50 Ti), a density gradient was generated. The microparticles, which were initially uniformly distributed in the solution, became layered in the gradient at a density level equal to the density of the particles. Linear density gradients could be generated with different density intervals, depending on the initial silica concentration. Concentrations generally used were 7.5, 10, 15, or 20% Ludox SM giving overlapping densities in the region 1.02–1.18. After centrifugation, 0.5-ml fractions were withdrawn from the centrifugation tubes and the density was measured in a density gradient column containing a mixture of kerosene and carbon tetrachloride with known density (Miller and McGasek, 1960). The density was calibrated with sucrose solutions, the density of which was determined by refractometry. The accuracy of the method corresponded to ±0.002 g/ml.

Scanning electron microscopy was done mainly as described by Höglund and Morein (Höglund and Morein, 1973). The microparticles were rinsed with 0.9% NaCl and fixed at 4 °C for about 20 h with glutaraldehyde, which had been previously treated with charcoal and filtered through a glass filter to remove polymerization products in the fixative. Postfixation was performed, after rinsing in 0.9% NaCl, in neutralized 1% osmic acid for 1 h. After rinsing in NaCl, the specimens were freeze-dried. The dehydrated specimens were coated by palladium gold alloy in a JEOL evaporator and analyzed in a JSM-U$_3$ (JEOL) scanning electron microscope at 15 kV accelerating voltage.

Results

Incorporation of Protein in the Microparticles. The extent of incorporation of protein in the microparticles during the polymerization was tested with albumin. Two series of experiments were run with varying total concentration of monomer (T) and with varying degree of cross-linking (C) with a protein concentration of 4 mg/ml. In one series, the total

[2] The gels prepared are characterized according to the nomenclature suggested by Hjertén (Hjertén, 1962). The first numeral (=T) denotes the total amount of monomers (g/100 ml of solvent) and the second numeral (=C) the amount of Bis expressed as the percentage (w/w) of the total amount of monomers. The given example will have T-C = 8-25.

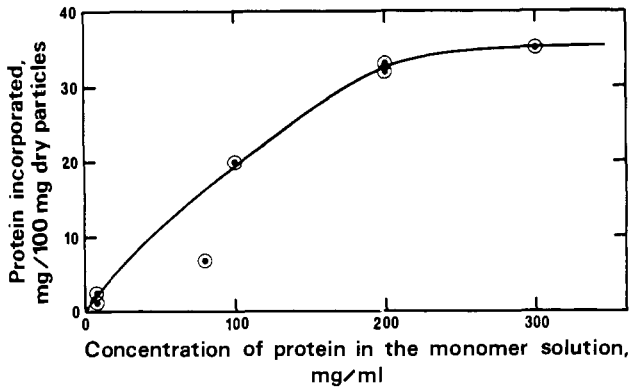

FIGURE 1: The effect of the protein concentration in the water phase on the amount of protein entrapped in polyacrylamide particles (T-C = 8-25%). The protein studied was human serum albumin.

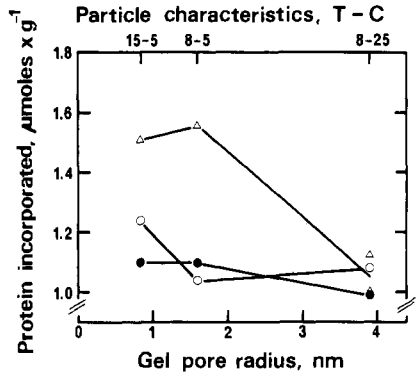

FIGURE 2: The incorporation of bovine β-lactoglobulin (●-●), human serum albumin (○-○), and human immunoglobulin G (△-△) in polyacrylamide gels with different cross-linking (C) and total concentration (T). The protein concentration in the monomer solution was 100 mg/ml.

TABLE I: Incorporation of Proteins Copolymerized in Polyacrylamide Microparticles with Different Mean Pore Radius.[a]

		Protein Immobilized		
T-C (%)	Mean Pore Radius of the Gel[b] nm, p	Bovine β-lacto-globulin (%) (a = 2.67 nm)	Human Serum Albumin (%)(a = 3.34 nm)	Human Immuno-globulin G (%) (a = 4.92 nm)
15-5	0.85	4.2	8.2	23.4
8-5	1.62	3.6	6.8	24.2
8-25	3.90	4.2	7.2	15.5, 17.5

[a] The microparticles were prepared as described under Materials and Methods. The protein concentration in the water phase was, in all cases, 100 mg/ml. The protein content of the particles was determined according to Eskamani et al., 1974. [b] Taken from Fawcett and Morris, 1966.

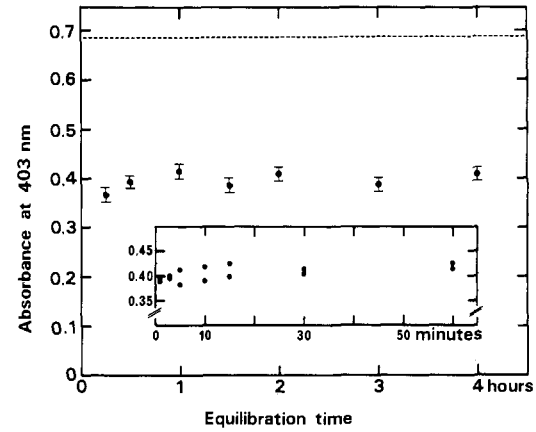

FIGURE 3: The rate of uptake of bromocresol green (BCG) in microparticles (T-C = 8-25%) containing human serum albumin (2.5%). Microparticles were added to a solution of BCG (A_{403nm} = 0.68, dotted line) in 0.1 M KCl and 0.005 M phosphate buffer, pH 7.4. At the times stated, the suspension was centrifuged and the decrease of the absorbance of BCG in the supernatant was determined.

concentration of the monomer (T) was 4, 6, 8, 10, or 12%, with 15% cross-linking (C) in all cases. In the other series, C was varied between 10, 15, 20, 25, and 30% within a total monomer concentration (T) of 8%. With the concentrations used, no systematic difference could be detected for the incorporation of protein, which in all cases amounted to about 3% of the total particle weight. When not otherwise stated in the following, the microparticles were prepared from monomer solutions with T-C = 8-25. The effect on the incorporation of increasing the protein concentration in the monomer solution was followed with albumin. As seen in Figure 1, the incorporation increases proportionally up to about 30% at a protein concentration of 200 mg/ml. The maximal amount of albumin which could be trapped corresponded to about 35% of the dry weight of the microparticles (T-C = 8-25%).

The influence of the size of the macromolecules on the incorporation was studied with lactoglobulin, albumin, and human IgG. The Stoke's radius (a) of lactoglobulin was calculated from $s^\circ_{20,w}$ (Timasheff and Townend, 1961) and the partial specific volume estimated from the amino acid composition (Piez et al., 1961), and of albumin and IgG from the frictional ratios and partial specific volumes (Oncley et al., 1947). The results are shown in Table I and compared with the mean pore radius, p, obtained from Fawcett and Morris (1966). As is evident from the table, a higher percentage of protein was incorporated with increasing protein size. Moreover, particles with small pore radius were more efficient in binding IgG, having the largest Stoke's radius. Particles with T-C = 15-5 also bound albumin more efficiently. In Figure 2, the results are related to the molar concentrations. In the macroporous particles with T-C = 8-25, the molar concentration was the same, about 1.05 μmol/g dry weight, regardless of the size of the macromolecules; i.e., the number of macromolecules incorporated was directly proportional to the concentration (on a weight basis) in the water phase. With lactoglobulin the incorporation was the same with all gels, but with the larger molecules the incorporation was more efficient in the gels with smaller pores.

Unspecific Binding to the Microparticles. The unspecific binding of small molecules to the microparticle network was specifically tested with [^{14}C]salicylic acid. Increasing amounts of microparticles not containing any entrapped molecules were added to a buffer solution with the salicylic acid. Essentially no radioactivity was lost to the particles from the supernatant after centrifugation. In the same way, [^{14}C]warfarin and bromocresol green have not shown any tendency to unspecific binding to the microparticles.

Binding Properties of Proteins Entrapped in the Microparticles. The macroporous structure of the microparticles allows small molecules to penetrate easily the particles. This

TABLE II: Partition of [^{14}C]Warfarin between Microparticles and Supernatant.[a]

Experiment No.	Albumin in Microparticles (nmol)	Warfarin: Total Amount (μmol/ml)	Warfarin: Amount Free in Supernatant (μmol/ml)	Albumin Added to Supernatant (nmol)	Warfarin: Amount Bound in Microparticles (μmol/ml)	Warfarin: Amount in Supernatant (μmol/ml)	Warfarin: Theoretical Amount Bound in Supernatant (μmol/ml)
1	71.73	1.1447	0.1096	0	1.0217	0.1011	0
2	74.00	1.1447	0.1076	62.87	0.6067	0.5166	0.5155
3	80.24	0.9536	0.0986	0	0.8425	0.0914	0
4	70.95	0.9536	0.1077	62.87	0.4887	0.4434	0.4331

[a] Microparticles (25 mg) containing about 20% human serum albumin are suspended in 1.0 ml of 0.1 M KCl and 0.005 M phosphate buffer, pH 7.4, with different amounts of [^{14}C]warfarin. After centrifugation, 0.2 ml of the supernatant is withdrawn and the amount of warfarin is determined. The aliquot withdrawn is replaced by 0.2 ml of either buffer or human serum albumin in buffer and the distribution of warfarin between microparticles and supernatant is determined.

is illustrated in Figure 3, which shows how albumin trapped in particles rapidly binds bromocresol green added to a particle suspension. Indeed, the equilibrium between the solution and albumin in the microparticles is attained so rapidly that the time taken to separate the microparticles by centrifugation is too long to allow the initial rate of uptake in the particles to be studied.

It is easy to show that the equilibrium with the microparticles is reversible. In Table II, the binding of [^{14}C]warfarin to albumin in microparticles and in free solution is studied. As is quite evident, [^{14}C]warfarin, initially bound in microparticles, is released out to the medium, to which albumin is added. From the relative amounts of albumin in supernatant and in microparticles and the amount of warfarin initially bound in the microparticles, the theoretical amount of warfarin bound to albumin in the supernatant was calculated. As seen, the warfarin is released into the supernatant to account for the theoretically bound *and* the free fraction in the supernatant.

The quantitative binding to albumin in microparticles was studied with bromocresol green, salicylic acid, and warfarin, and compared with results obtained from equilibrium dialysis. Scatchard's plots of the binding are shown in Figure 4a,b. In all cases, there was good agreement for the binding constants determined with the microparticles and by equilibrium dialysis, as well as with constants given in the literature. The calculations were based on the amount of albumin in the microparticles determined from amino acid analysis. (The amount of albumin immobilized ranged to maximally 32% of the dry weight.) The results clearly indicate that the number of sites on albumin available for binding was not changed when albumin was incorporated in the microparticles The binding of salicylic acid and warfarin, thus, closely followed the results earlier obtained in our laboratory (Sjöholm et al., 1976), and the results with bromocresol green show that albumin has one primary binding site with $K_a = 1.7 \times 10^6$ M^{-1} and three secondary sites with $K_a = 1.4 \times 10^5$ M^{-1}. These figures coincide well with those presented by Rodkey (1964).

The complete, nonperturbed, and rapid interaction with proteins incorporated in the microparticles is best explained by the macroporous structure of the polyacrylamide network. The porosity, detected by scanning electron microscopy (Figure 5) and also shown by Rüchel and Brager (1975), ensures an easy diffusion of small molecules into the polymeric lattice and permits a rapid equilibrium with the bound macromolecules to take place.

The Density of Microparticles. The density of the microparticles will depend on the cross-linking (C) and the amount of protein incorporated, as found by ultracentrifugation, in a colloid silica sol (Ludox^R) in water. However, the total concentration of monomer (T) did not affect the density. The results are summarized in Figure 6, which indicates that the lightest particles are obtained with C = 10%. By increasing C over 25%, particles can be prepared with densities exceeding those of biological cells. High concentrations of protein in particles will decrease the density. Thus, particles with T-C = 8–25% have a density of 1.077 ± 0.002 g/cm^3 and similar particles with 30% albumin have a density of 1.057 ± 0.002 g/cm^3.

Discussion

The bead polymerization technique, used to prepare polyacrylamide particles from different monomeric acrylic derivatives, offers a convenient method for the immobilization of different macromolecules. It fulfills a number of requirements, which are significant in several applications. Thus, we have shown with albumin that the biological activity of the immobilized compounds is preserved, partly due to the fact that no covalent coupling methods are needed. The method is simple, and high concentrations of the immobilized substances can be obtained (up to 35% with albumin in 8–25% particles). The fraction of the macromolecules not entrapped in the particles can be regained from the water phase and the washings during the isolation of the particles. Moreover, the particles consisting of cross-linked polyacrylamide will show very good mechanical stability and will resist bacterial degradation (Johansson and Mosbach, 1974; Mori et al., 1974), which also holds true for the macromolecules trapped inside the particles. Finally, the size of the spherical beads can be conveniently varied according to special needs by changing the homogenizing conditions. Generally, a mean diameter of the particles around 200 μm has been used, to ascertain good flow when they are packed in columns (Wada and Kishizaka, 1968; Nilsson et al., 1972). However, in other applications, several advantages are obtained by preparing smaller particles with an average diameter between 1 and 10 μm. The biological activity of the entrapped molecules is better utilized and is less affected by restricted diffusion, which can be seen with larger particles (O'Driscoll et al., 1972; Mori et al., 1974). The microparticles are also easier to dispense uniformly, e.g., by pipetting. The immobilized molecules will be randomly distributed throughout the

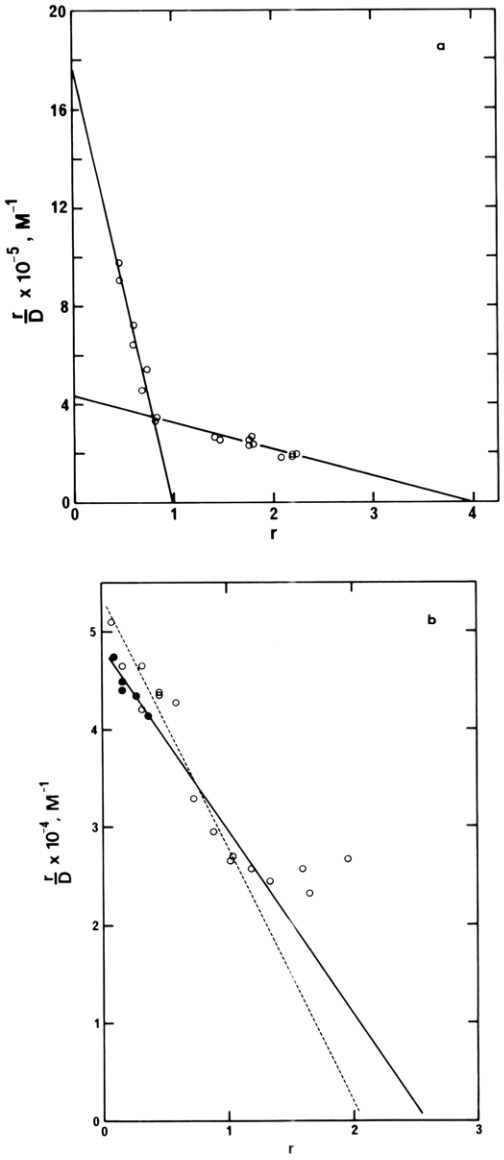

FIGURE 4: (a) Binding of bromocresol green to microparticles (T–C = 8–25%) with human serum albumin (2.5% of the particle dry weight) in 0.1 M KCl and 0.005 M phosphate buffer, pH 7.4. (b) Binding of [^{14}C]salicylic acid to microparticles (T–C = 8–25%) with human serum albumin (2.5% of the particle dry weight) in 0.1 M KCl and 0.005 M phosphate buffer, pH 7.4. The binding of salicylic acid (dotted line) is compared with the results obtained earlier (Sjöholm et al. 1976) by equilibrium dialysis with isolated albumin (unbroken line).

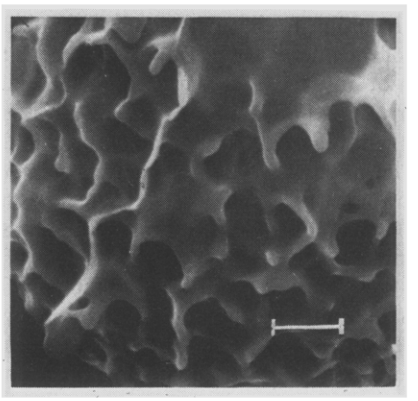

FIGURE 5: Scanning electron micrograph (5 000×) of polyacrylamide particle. The bar indicates 1 μm.

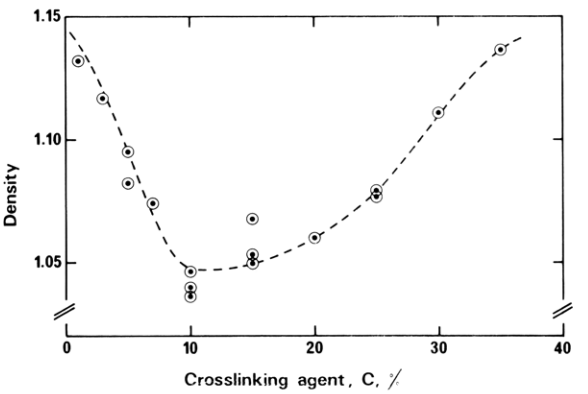

FIGURE 6: The density of microparticles of polyacrylamide, prepared as described under Materials and Methods from monomer solutions containing different amounts of cross-linking agent (C). The total amount of monomer (T) was in all cases 8%.

particle, which means that the smaller the particles are the larger will be the proportion exposed at the surface (Ekman and Sjöholm, 1975). Consequently, the microparticles are of great potential value in cell studies to detect specific cell surface structures and to separate such cells from a mixture, e.g., by centrifugation (I. Ljungstedt, B. Ekman, and I. Sjöholm, unpublished results).

The total concentration of monomer derivatives (T) and the proportion of the divalent cross-linking agent, e.g., Bis (C), will highly influence the physical properties of the microparticles. The polymer forms a network with macropores, which can be detected by the electron microscope. The size of the macropores depends mainly on C as shown empirically in gel filtration studies on proteins (Fawcett and Morris, 1966) and RNA (Richards and Lecanidou, 1974). The gel filtration behavior of macromolecules can be related to the available volume in the gel for the molecules, which in turn can be estimated from Ogston's theory for the arrangement of the polymeric network in the gel (Ogston, 1958), as advanced by Laurent and Killander (Laurent and Killander, 1964). According to the theories, the solid network is formed by threads with diameter $2r$ and the concentration (length), L, expressed as cm/cm^3. Then the fraction, f, of the particle available to a sphere of radius R, can be expressed as

$$f = e^{-\pi L(R+r)^2} \qquad (2)$$

assuming a random distribution of the threads forming the network. The Ogston theory can satisfactorily explain the permeability changes of gels with different T and C. Thus, it has been experimentally shown (Nilsson et al., 1972; Richards and Lecanidou, 1974; Laurent and Killander, 1964) that L is directly proportional to T at constant C. When changing C, at constant T, the changes of the gel permeability, and thereby also the volume available for macromolecules within the gel, will pass through a minimum at C = 5–10% and then paradoxically increase significantly with increasing C. This phenomenon is best interpreted as a linear increase of the diameter of the threads, r, with C and an initial increase of the length, L, up to C values of about 5% with a subsequent rapid decrease at higher C values (Fawcett and Morris, 1966). Perceptibly, the changes of the gel permeability can be explained by the fact that Bis can also participate in the formation of the polymeric chains in addition to its function as the cross-linking agent.

A relation similar to that between f and C exists between the density of the microparticles and C. The density will show a minimum of about 1.05 g/cm^3 at C values ranging from 8–10%. Our results indicate that the limit value, when C approaches 0, will reach about 1.15 g/cm^3, which then is the value of the linear polyacrylamide. A similar value was reached with C = 35%, at which situation the gel threads have a large diameter (r) and are relatively short. Large amounts of protein within the microparticles will decrease the density. The effect on the density is, however, relatively limited and C will be the main factor determining the density. Lymphocytes, for instance, have a density around 1.06 g/cm^3. Heavier microparticles can easily be prepared with incorporated specific antigens or reagents directed towards a specific group of cells by a proper choice of C. Cells reacting with the particles can then be isolated from the rest of the cell population by density gradient centrifugation. The concentration of the monomers, both T and C, will also be of importance for the mechanical stability of the particles obtained. Our experience is that heavy microparticles preferably are prepared with large C and moderate T, as these will show good resistance in the centrifuge and are easy to handle. High concentrations of the monomers (T) may denature the proteins (Degani and Miron, 1970).

Our results show that two mechanisms are responsible for the incorporation of the macromolecules in the microparticles. In particles made up of a polyacrylamide network with large mean pore radius, the number of molecules bound is only dependent on the quantity present in the monomer solution, regardless of the molecular size. This phenomenon seems to be best explained as a fixation of a part of the macromolecule in the threads formed during the polymerization. Thus, also molecules with a Stoke's radius smaller than the mean pore radius of the gel will be incorporated. With the gels used in this study, the incorporation degree was also constant, as long as the mean pore size was not too small compared to the size of the macromolecule. However, when a tighter gel is used, a second mechanism, an entrapment, within the *network* of the gel, is also responsible for the binding. IgG, for instance, was bound 50% more efficiently in the 10–5 and the 8–5% particles than in the 8–25% particles. Also, albumin was better incorporated in the 15–5% particles. However, it is, generally, not necessary to use the tighter gel, as the incorporation degree in the more macroporous gels is also high. Preliminary experiments even indicate that particles with large pore radius are advantageous, as the macromolecules are very stably bound in them and will interact very rapidly with ligands.

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