Immobilization of enzymes by radiation-induced polymerization of glass-forming monomers: 1. Immobilization of some enzymes by poly(2-hydroxyethyl methacrylate)

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The immobilization of some enzymes has been studied by radiation-induced polymerization using 2-hydroxyethyl methacrylate as a glass-forming monomer. Radiation damage of enzymes was slight after irradiation at low temperatures. Moreover, the activity yield of immobilized enzymes increased markedly at polymerization temperatures below −24°C. The polymer formed was characterized by its porous structure which was studied in detail in relation to the activity yield and its activity retention with repeated use. It was deduced that enzymes were partly trapped at the pore surface within the polymer matrix and partly within the pores from which they were able to leak out with repeated use. Hence, the use of low temperature and super-cooled monomer was necessary for effective enzyme immobilization.

INTRODUCTION

Recently several studies have been reported on the immobilization of enzymes[1–4]. A number of methods has been proposed for immobilization. Amongst these the entrapping method is considered to be one of the most promising, because it can be most generally applied to the entrapping of any kind of enzyme, microbial cells and organelles with different sizes and properties, with little destruction of biological activity as compared with the covalent bonding method. Moreover, by using various techniques in polymer synthesis to control the polymer structure, enzymically stable and mechanically very firm entrapment conjugates might be possible. Such conjugates would compare very favourably with conjugates prepared by the adsorption method.

We have studied radiation-induced polymerization at low temperatures using glass-forming monomers[5], and have found in relation to its application that glass-forming monomers were very suitable as the entrapping matrix owing to their property of forming highly viscous liquids or amorphous solids on supercooling at low temperatures and also to their high polymerizability even at low temperatures. This method can be applied to the entrapping of any kind of biologically active substance for the purpose not only of immobilization, but also of controlled release[6]. In this report, we have tried to clarify the effects and characteristics of the new entrapping immobilization method using radiation-induced polymerization of a typical and moderately hydrophilic glass-forming monomer, 2-hydroxyethyl methacrylate.

MATERIALS AND METHODS

Materials

Bacillus subtilis liquefying α-amylase (Nagase Sangyo Ltd) and *Aspergillus niger* glucoamylase (NOVO Industry A.S., Denmark) were used as enzymes. Soluble starch (Katayama Chemical Ltd) and maltose (Tokyo Kasei Kogyo Co. Ltd) were used as substrates. 2-Hydroxyethyl methacrylate (HEMA) obtained from Mitsubishi Gas Chemical Co. Ltd was used as a carrier and purified using conventional methods[5].

Immobilization and enzyme assay

The immobilized enzymes were prepared as follows. The enzyme was dissolved in 0.02 M phosphate buffer solution (pH 6.9) in the case of α-amylase, and in 0.1 M acetate buffer solution (pH 4.5) in the case of glucoamylase. HEMA, acting as a carrier, was mixed with the above solution to a total volume of 1 ml. The enzyme–HEMA mixture was then charged into an 8 mm diam. glass ampoule and sealed off under a vacuum of 10^{-3} mmHg. γ-irradiation from a ^{60}Co source was carried out at various temperatures using various cryostats: liquid nitrogen (−196°C), dry ice–ethanol (−78°C), carbon tetrachloride (−24°C) and ice (0°C), respectively.

After irradiation, the immobilized enzymes were cut into 10 samples 8 mm diam. × 2 mm long and used to study leakage and activity yield. The polymer composites obtained using concentrations of HEMA in the ranges 10–40%, 50–80% and 90–100% were in the form of a sponge-like white gel, a hard sponge-like white gel, and a rigid transparent glass-like gel, respectively.

The examination of the leakage of the enzymes from the immobilized enzyme preparation was carried out by repeating the batch enzyme reaction. The batch enzyme assay was carried out under standard conditions with shaking for 60 min at 40°C using soluble starch substrate (pH 6.9) in the case of α-amylase and for 30 min at 45°C using maltose solution (pH 4.5) in the case of glucoamylase. After reac-

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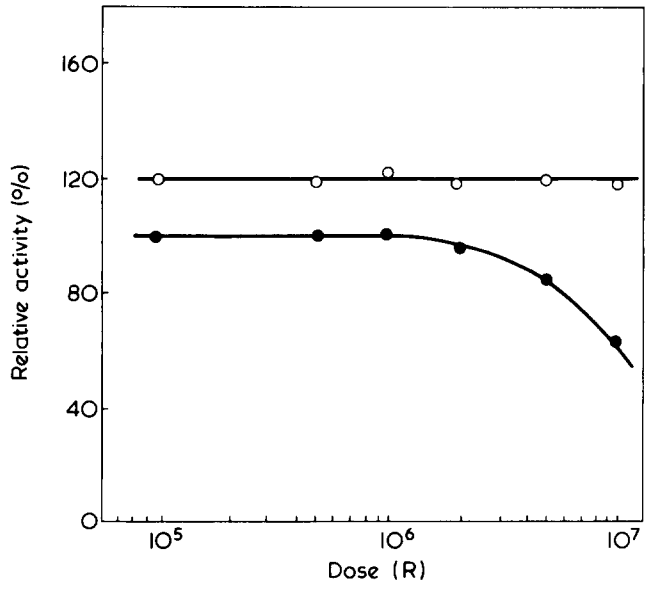

Figure 1 Effect of irradiation dose on activity of native enzymes. 1 ml enzyme solution (0.8 μg glucoamylase in acetate buffer solution, pH 4.5, or 200 μg α-amylase in phosphate buffer, pH 6.9) was poured into an ampoule and sealed off under a vacuum of 10^{-3} mmHg. The sealed ampoule was irradiated at a dose rate of 1 x 10^6Rad/h. The activity of native enzyme was taken as 100%. ○, α-amylase; ●, glucoamylase

tion, the maltose formed by the hydrolysis of starch was determined by means of 3,5-dinitrosalicylic acid[7]. The glucose formed by the hydrolysis of maltose was determined by measuring the absorption at 505 nm with a Shimazu Model QV-50 spectrophotometer, using 'GOD–PODLK' obtained from Nagase Sangyo Co. Ltd.

The activity yield of the immobilized enzymes may be represented as follows:

$$\text{Activity yield (\%)} = \frac{I_a}{N_a} \times 100 \qquad (1)$$

where I_a is the quantity of hydrolysis product formed by immobilized enzymes during each batch reaction and N_a is the quantity of hydrolysis product formed by native enzymes.

The pH of the reaction mixture was measured with a Toa Denpa Kogyo Co. Ltd, Model HA-5A pH meter.

Differential thermal analysis (d.t.a.)

The glass transition temperature (T_g) and melting point (T_m) were measured using the differential thermal analysis (d.t.a.) equipment described previously[5]. The phase diagram was also determined by d.t.a.

Determination of pore structure and water content of the polymerized composite

The polymerized composite was cut into slices 15–25 μm thick using a freezing-type microtome (Yamato Koki). The micrograph of its membrane (polymerized composite with a porous structure) was then taken using an Olympus Model FHF microscope with Olympus PM-10-M equipment.

The average area of a pore was read directly from the micrograph. The average diameter of a pore in the pore structure was determined from equation (2):

$$\text{Average diameter of pore, } \mu\text{m} = 2 \times \left(\frac{\text{Average area of a pore}}{\pi}\right)^{1/2} \qquad (2)$$

The porosity was defined as:

$$\text{Porosity (\%)} = \frac{\text{Total area of pores}}{\text{Total area of visual field in microscopy (pores and polymer matrix)}} \times 100$$

$$= \frac{\text{(Average pore diameter)} \times \text{(number of pores/cm}^2\text{)}}{\text{Total area of visual field in microscopy/cm}^2} \times 100 \qquad (3)$$

The water content in the polymerized composite was defined as:

$$\text{Water content (\%)} = \frac{(A+B)}{(A+B)+C} \times 100 \qquad (4)$$

where A is the weight of water in the saturated porous structure of the polymerized composite before (in the case of wet composite), or after (in the case of dried composite) the drying treatment; B is the weight of water absorbed in the saturated polymer matrix itself before or after the drying treatment; and C is the weight of the polymer composite after the drying treatment. In this case, B takes a constant value and a water content of 25–30 wt % of the pure polymer is found.

RESULTS AND DISCUSSION

Effect of irradiation on enzymes

When irradiation is used for immobilization, it is important to know the effect of irradiation on the activity of the native enzyme. *Figures 1* and *2* show the result of investigations into the relation between the irradiation conditions and the relative activity of native enzymes. According to these results, radiation damage to enzymes can be consi-

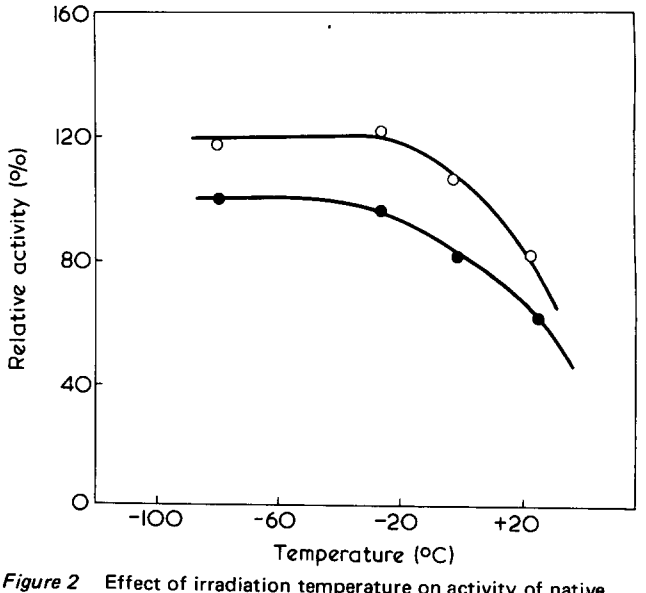

Figure 2 Effect of irradiation temperature on activity of native enzymes. 1 ml enzyme solution (0.8 μg glucoamylase in acetate buffer solution, pH 4.5, or 200 μg of α-amylase in phosphate buffer solution, pH 6.9) was poured into an ampoule and sealed off under a vacuum of 10^{-3} mmHg. The sealed ampoule was irradiated for 1 h at a dose rate of 1 x 10^6 Rad/h. The activity of native enzyme was taken as 100%. ○, α-amylase; ●, glucoamylase

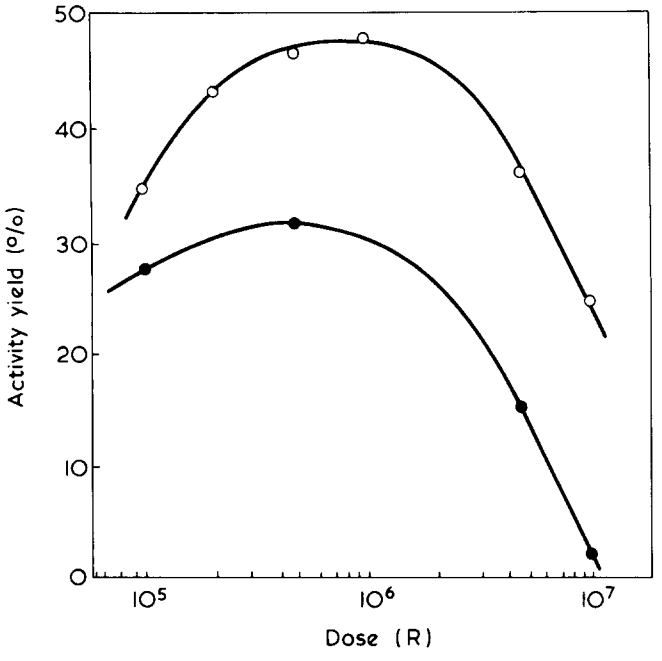

Figure 3 Relationship between the irradiation dose and the activity yield of immobilized enzymes. Immobilized α-amylase: 200 μg enzyme, 1 ml 50% HEMA in buffer (pH 6.9), and 5 x 10^5 Rad/h at −24°C, *in vacuo*; immobilized glucoamylase: 0.8 μg of enzyme, 1 ml 50% HEMA in buffer (pH 4.5), and 5 x 10^5 Rad/h at −78°C, *in vacuo*; substrate: 6 ml 2% starch solution in the case of α-amylase, 5 ml 1% maltose solution in the case of glucoamylase. Batch enzyme reaction was carried out repeatedly up to 20 times. ○, α-amylase; ●, glucoamylase

derably avoided by selecting conditions of relatively low temperature and small irradiation doses. The activity of native enzymes decreased markedly with increasing irradiation temperature above −20°C. The same tendency has generally been observed for other enzymes[8]. Clearly then, immobilization by low temperature polymerization is desirable.

Effect of the polymerization conditions on the activity of immobilized enzymes

2-Hydroxyethyl methacrylate was polymerized according to the method described above.

The relationship between the polymerization conditions and the activity yield of immobilized enzyme is shown in *Figures 3* and *4*. These results show that the activity yield was optimal in a certain dose range; the activity yield decreased due to radiation damage in a dose range in excess of this and also decreased by enzyme loss due to lower polymer conversion in the much smaller dose range. The polymer conversion in a suitable dose range was shown to be almost 100%. Activity yields were highly dependent on irradiation temperature, as shown in *Figure 4*, and decreased sharply with increasing temperature above 0°C. This abrupt decrease (*Figure 2*) could not be explained by radiation damage of the enzyme alone.

The phase diagram of this monomer–water system was then investigated, as shown in *Figure 5*, to identify the immobilization mechanism. This phase diagram was characterized by the fact that the melting peak of the eutectic composition at the eutectic point (above −24°C) could hardly, or only slightly, be distinguished and that the change of apparent specific heat corresponding to glass transition temperature could clearly be observed. This fact proved that in the temperature region below −24°C, most of the water has crystallized to ice, while all eutectic compositions consisting mainly of monomer with a small amount of water have undergone supercooling. That is, the monomeric system is a suspension of ice in supercooled monomer, acting as the dispersion medium at low temperatures. Consequently, the polymerization phase, the mechanism and the structure of the polymerized composite were completely different at temperatures above and below −24°C. In aqueous solution above −24°C, heterogeneous precipitation polymerization occurred forming a polymer which was isolated from the monomeric phase due to the lower solubility of polyHEMA

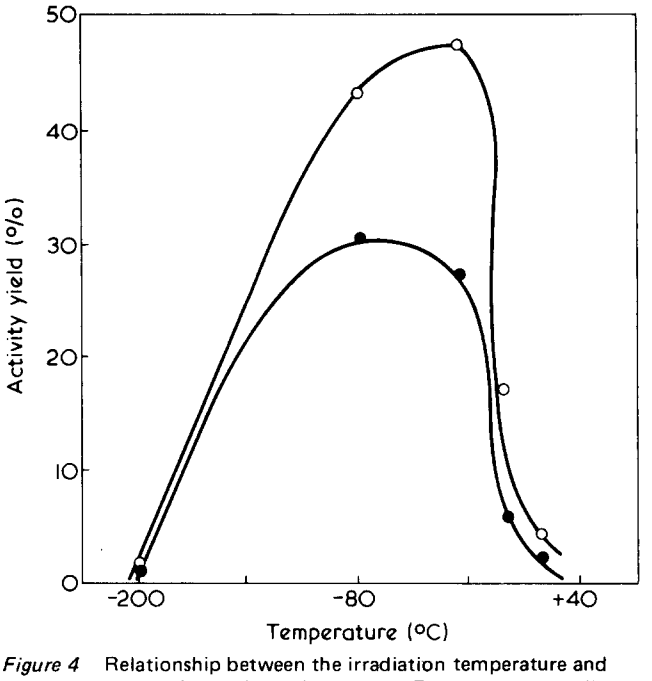

Figure 4 Relationship between the irradiation temperature and the activity yield of immobilized enzymes. Experimental conditions were same as those in *Figure 3* except for irradiation dose which was; 1 x 10^6 Rad in the case of α-amylase, 5 x 10^5 Rad in the case of glucoamylase. ○, α-amylase; ●, glucoamylase

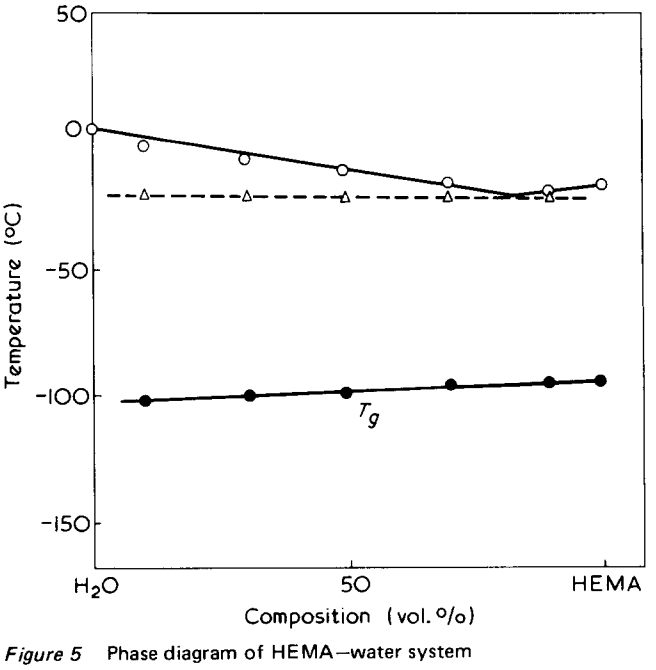

Figure 5 Phase diagram of HEMA–water system

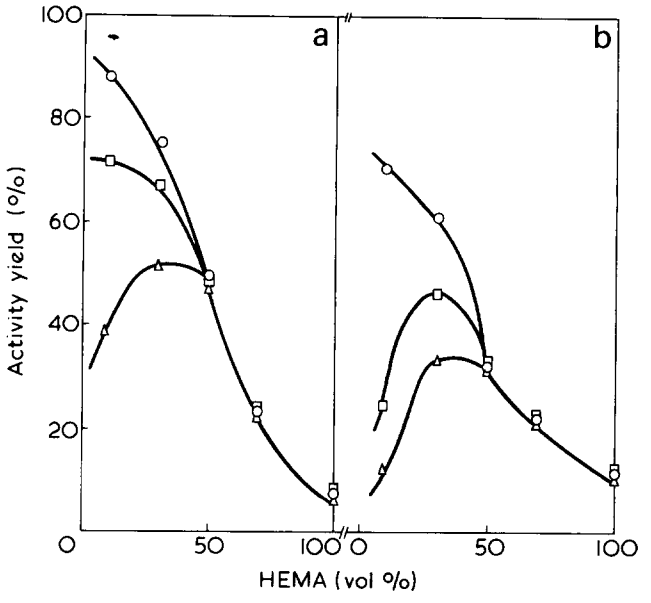

Figure 6 Relationship between the HEMA concentration and the activity yield of immobilized enzymes. (a) Immobilized α-amylase: 200 μg enzyme, 1 ml HEMA in buffer (pH 6.9), and 1 x 10^6 Rad at −24°C, *in vacuo*, (b) immobilized glucoamylase: 0.8 μg enzyme, 1 ml HEMA in buffer (pH 4.5), and 5 x 10^5 Rad at −78°C, *in vacuo*; substrate: 6 ml 2% starch in the case of α-amylase, 5 ml 1% maltose solution in the case of glucoamylase. Batch enzyme reaction (times); ○, 1; □, 5; △, 20

in water; a continuous pore structure in the polymer composite was formed. On the other hand, at lower temperatures, homogeneous, almost bulk polymerization took place in the supercooled phase due to good compatibility of polyHEMA with monomer, forming an almost independent pore structure due to the dispersed ice. It is natural to expect that the great differences in the polymerization phase and polymer structure have an important effect on immobilization. In polymerization by precipitation, the enzyme is concentrated and remains in the water phase (monomer phase) without effectively being tapped by the polymer. On the other hand, in a cooled system, the enzyme is apt to become isolated from crystallized water, to shift and be dispersed on the surface of the supercooled viscous monomer phase and then effectively to be trapped by homogeneous polymerization. Moreover, excess continuous pore structure in solution polymerization is disadvantageous in preventing enzyme leakage, though it is advantageous for the release of low molecular weight substances[6]. These are probably the other important reasons for the sharp change in activity between the temperatures above and below −24°C seen in *Figure 4*.

Dependence of activity yield on monomer concentration and the change in activity yield with repeated use

The formation of a pore structure due to ice is one of the most characteristic features of polymers obtained by the present method, and the key factor in determining activity. The monomer concentration should have a direct effect on porosity and activity. *Figure 6* shows the dependence of activity yield on monomer concentration.

The *Figure* shows that the activity yield decreases steadily with increasing monomer concentration after a small number of batch reactions. However, when a steady state is reached after repeated use in the enzyme assay, the activity yield curve shows an optimum at a certain monomer concentration. From an analysis of the reacted solution, it was shown that the initial decrease in activity in the low monomer concentration system could be attributed mainly to leakage of enzyme due to an excess of large pores. On the other hand, in the high monomer concentration system there was very little enzyme leakage from the polymer; but activity decreased because the dense polymer matrix caused an increase in diffusion resistance to the substrate. Moreover, the proportion of deeply embedded enzyme, not contributing to the enzyme reaction, increased with increasing monomer concentrations. This probably explains the peak in *Figure 6*. The relationship between activity change and repeated use is shown in *Figures 7* and *8* for α-amylase and glucoamylase respectively. These results support the above mechanism; that is, leakage behaviour varies with monomer concentration owing to the difference in the pore structure resulting from the presence of ice. The limiting condition for

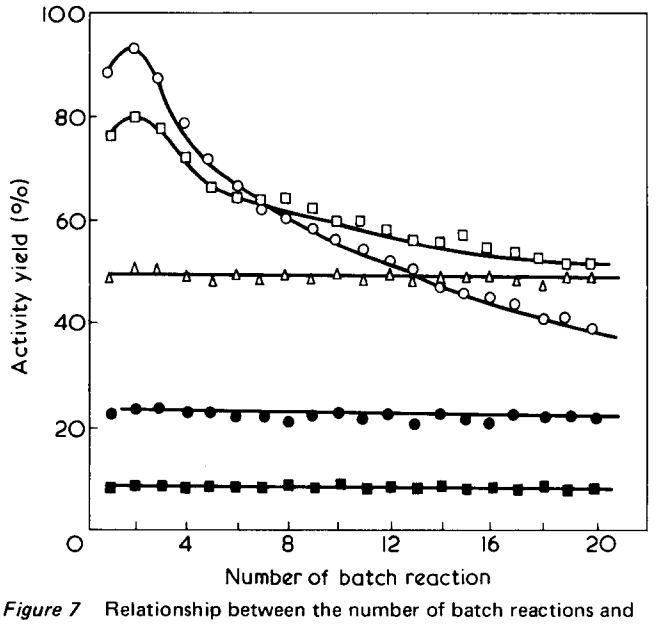

Figure 7 Relationship between the number of batch reactions and the activity yield of immobilized α-amylase as a function of HEMA concentration. Experimental conditions were the same as those in *Figure 6*. HEMA concentration: ○, 10%; □, 30%; △, 50%; ●, 70%; ■, 100%

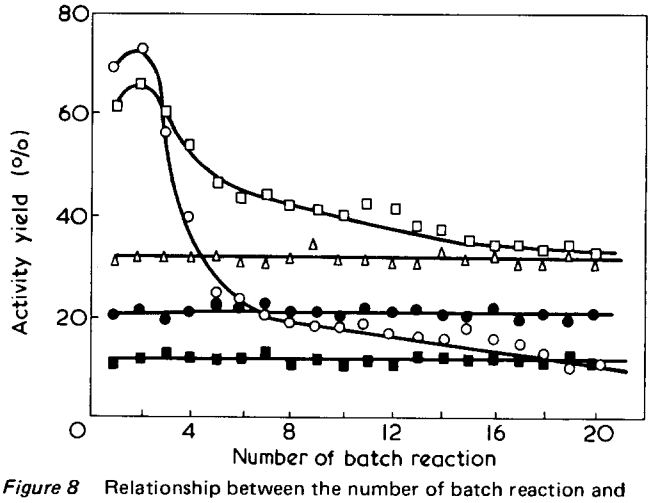

Figure 8 Relationship between the number of batch reaction and the activity yield of immobilized glucoamylase as a function of HEMA concentration. Experimental conditions were same as those in *Figure 6*. HEMA concentration: ○, 10%; □, 30%; △, 50%; ●, 70%; ■, 100%

enzyme leakage can be determined as a function of both the monomer and enzyme concentrations as shown in *Figure 9*. The leakage takes place at higher enzyme concentration and smaller monomer concentration (above the limiting curve) and no enzyme leakage occurs in the region below the curve.

Estimation of porosity by microscopy and water content and its effect on activity

Microscopic observation was adopted for the estimation of the porous structure. The micrographs obtained at various monomer concentrations are shown in *Figure 10*. It can clearly be seen that the pore structure becomes richer with a decrease in monomer concentration. The average pore diameter and number of pores per unit surface area of the sample in the microscopic visual field were read and estimated. The porosity can be compared with water content, because the water content (subtracting the water absorption of matrix itself at 100% HEMA monomer concentration) is equal to the amount of water occupying the pores, and thus increases in a manner proportional to the porosity.

These pore factors are plotted against irradiation temperature and monomer concentration in *Figures 11* and *12*. The distribution of pore diameters at various monomer concentrations is shown in *Figure 13*. Pore diameter decreased steadily but the number of pores reached a maximum with an increase of monomer concentration as shown in *Figure 12*. The decrease in the number of pores at lower monomer concentrations can be attributed to the mutual joining of very large pores. The porosity also decreased steadily with the increase in monomer concentration. The trend in porosity obtained by microscopic estimation agreed with that observed by water content measurements.

The pore factors exhibited a discontinuous change over the temperature region between 0° and −24°C, as shown in *Figure 11*. This fact can be related to the change in polymerization phase and mechanism as already stated. The

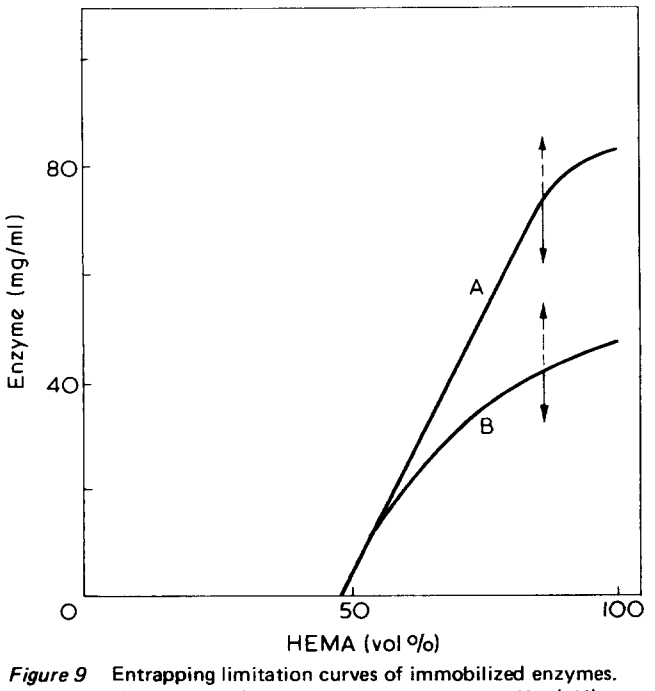

Figure 9 Entrapping limitation curves of immobilized enzymes. Immobilized α-amylase; 1 ml the HEMA–enzyme–buffer (pH) 6.9) mixture was irradiated for 1 h at a dose rate of 1 x 10^6 Rad/h at −24°C, *in vacuo*; immobilized glucoamylase: 1 ml the HEMA–enzyme–buffer (pH 4.5) mixture was irradiated for 1 h at a dose rate of 5 x 10^5 Rad/h at −78°C, *in vacuo*; substrate: 20 ml 10% starch solution in the case of α-amylase and 20 ml 20% maltose solution in the case of glucoamylase. Batch enzyme reaction was carried out repeatedly up to 20 times, – – – →, Decrease of activity occurred with repeating the batch reaction; ↑, no decrease of activity occurred with repeating the batch reaction. Enzyme: A, glucoamylase; B, α-amylase

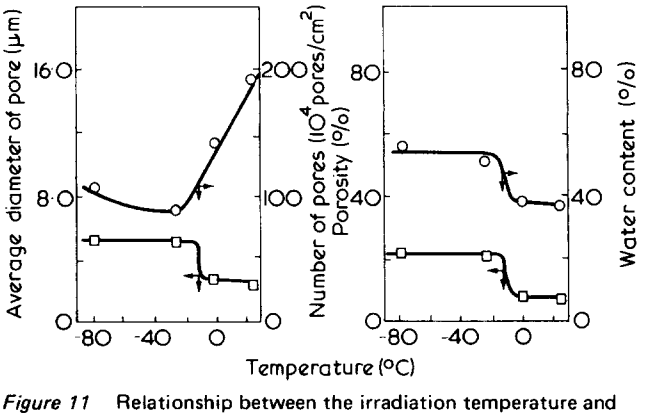

Figure 11 Relationship between the irradiation temperature and the physical properties of pore structure such as average pore diameter, pore number, porosity and water content of polymer composite. Experimental conditions were same as those in *Figure 4* except for irradiation temperature. The activity yield is shown in *Figure 14* as a function of pore factors, in the case of immobilized glucoamylase

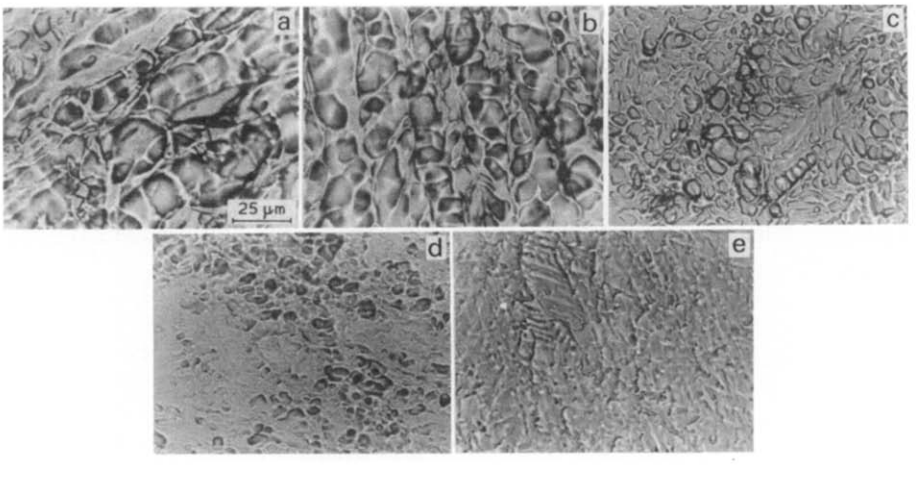

Figure 10 Optical microphotographs of pore structure in polymerized composite obtained in various HEMA concentrations. Experimental conditions were same as those in *Figure 8*. HEMA concentration: (a), 10%; (b), 30%; (c), 50%; (d), 70%; (e), 100%

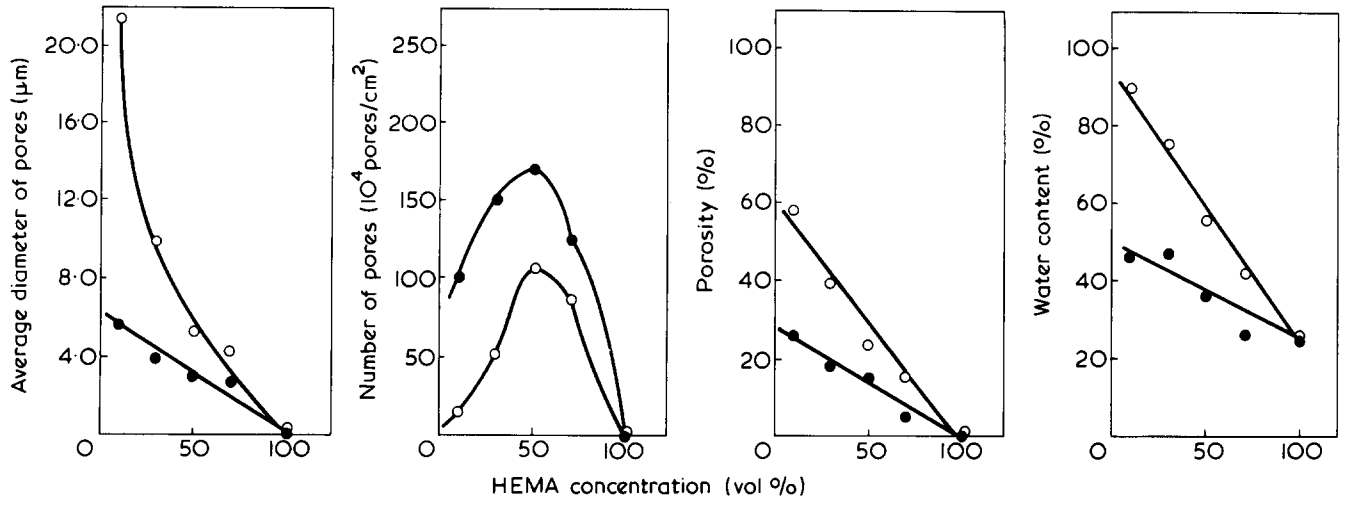

Figure 12 Relationship between the HEMA concentration and the physical properties of pore structure such as average pore diameter, pore number, porosity, and water content of polymer composite. Experimental conditions were same as those in *Figure 8*. Polymer composite: ○, as-polymerized; ●, after drying treatment

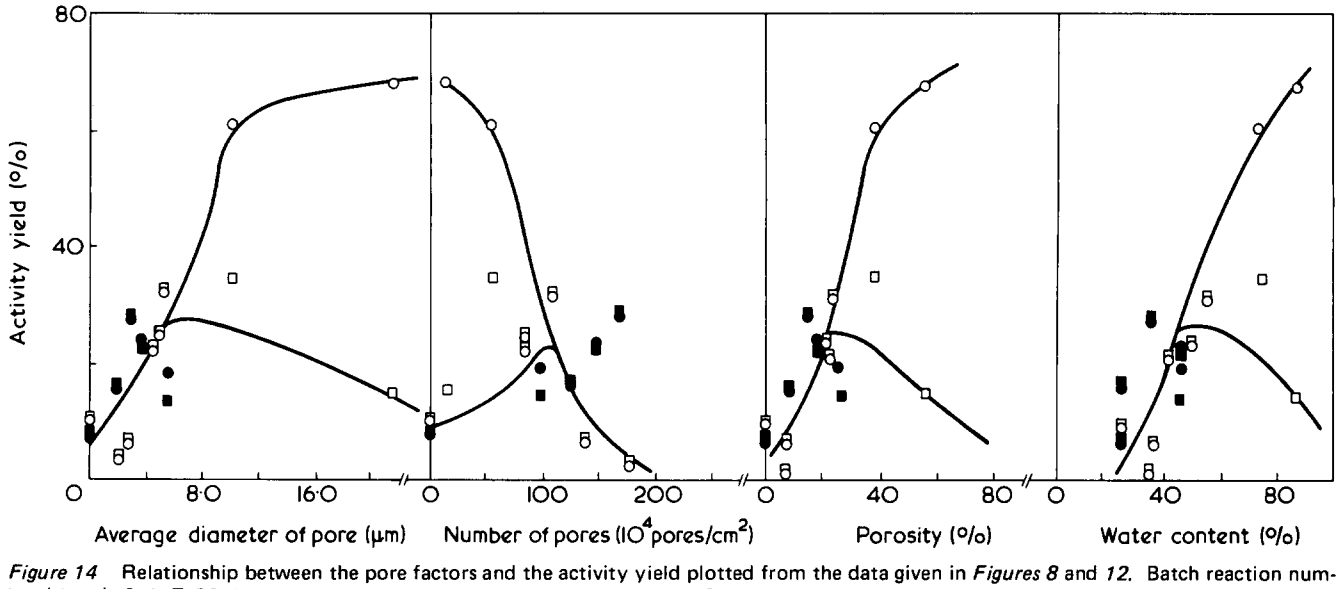

Figure 14 Relationship between the pore factors and the activity yield plotted from the data given in *Figures 8* and *12*. Batch reaction number (times): ○, 1; □, 20: in the case of as-polymerized polymer composite: ●, 1; ■, 20, in the case of polymer composite after drying treatment

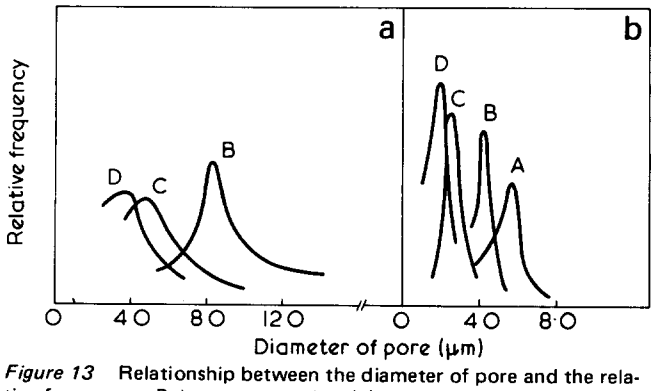

Figure 13 Relationship between the diameter of pore and the relative frequency. Polymer composite: (a) as-polymerized; (b) after drying treatment. HEMA concentration: A, 10%, B, 30%;C, 50%; D, 70%

activity yield was studied in relation to the pore factors as shown in *Figure 14*; it increased with an increase in porosity at an early stage of repeated use, but it showed a maximum value at a certain porosity at a later stage of repeated use for the same reason described in the interpretation of *Figure 6*.

The effect on the porous structure of drying the as-polymerized polymer was also investigated and plotted in *Figure 12*. Generally, the pore structure was reduced in size and porosity decreased by drying treatment. However, the degree of shrinkage was rather limited and the pore structure was still present after drying, because of the less hydrophilic properties of the polyHEMA matrix. That is, permanent pores could be obtained in spite of the drying treatment. The activity yield in the dry polymer also showed the same dependence on the porosity as in the wet system in *Figure 14*.

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