The Immobilization of Enzymes onto Hydrolyzed Polyethylene-*g*-co-2-HEMA

C. G. BEDDOWS, *Department of Health and Applied Science, Leeds Polytechnic, Leeds LS1 3HE, United Kingdom,* M. H. GIL, *Department of Chemistry, University of Coimbra, Coimbra 3000, Portugal,* and J. T. GUTHRIE, *Department of Colour Chemistry, University of Leeds, Leeds LS2 9JT, United Kingdom*

Synopsis

A study of the immobilization of trypsin and other enzymes onto hydrolyzed poly(2-hydroxyethyl methacrylate)-*g*-co-polyethylene using hydroxyl and carboxyl activating agents has been undertaken. Some emphasis was placed on the immobilized trypsin system which involved examination of the variation of (i) the extent of hydrolysis of the graft copolymer, (ii) the concentration of activating agent, and (iii) the temperature of coupling. With the trypsin system, an increase in carbodi-imide concentration gave an increase in the amount of protein immobilized but a marked decrease in the retention of enzymic activity. Comparison of the behavior of the free with the immobilized enzyme showed that satisfactory yields were obtained and the immobilized system has an extended pH profile and good stability and thus would have broad applicability. The kinetic factors were examined further, and the role of the graft copolymer chains in the immobilized system is discussed.

INTRODUCTION

In a previous report, the systems polyethylene-*g*-co-2-hydroxyethyl methacrylate and polyethylene-*g*-co-2-hydroxypropyl acrylate were used for the immobilization of enzymes. A variety of methods of activating the hydroxyl groups were examined. It was found that high yields of coupling were obtained when the activation was achieved by using either *p*-benzoquinone or *p*-toluenesulphonyl chloride. However, the retention of the activity of the enzyme was low.[1]

It has been shown that the yield of coupling of the enzymes and the percentage of retention of activity may be increased when the enzyme is provided with a hydrophilic environment. In the present work, the hydrophilic character of polyethylene-*g*-co-2-hydroxyethyl methacrylate polymers was increased by partial hydrolysis with 1*M* NaOH, and the conditions for immobilization of enzymes onto the resulting copolymers were studied. The liberation of the carboxyl groups allowed for different activating agents to be examined.

EXPERIMENTAL

Reagents

Polyethylene was obtained from Telcon Plastics Ltd., Orpington, U.K. This was purified by Soxhlet extraction with absolute ethanol for 6 h before being

Journal of Applied Polymer Science, Vol. 35, 135–144 (1988)
© 1988 John Wiley & Sons, Inc.
CCC 0021-8995/88/010135-10$04.00

dried to constant weight, under vacuum at 313 K. Hydroxyethyl methacrylate (2-HEMA) was obtained from the Aldrich Chemical Co. Ltd., Gillingham, Kent, U.K. and was purified by initial treatment with solid KOH. This was followed by rapid distillation at atmospheric pressure over solid KOH. Such purification assists removal of inhibitor but does not necessarily remove other monomeric impurities such as ethylene glycol dimethacrylate which are commonly present in small amounts. 2-HEMA is recognized as being difficult to purify. However, despite some involuntary, thermally induced homopolymerization, sufficient purified monomer was produced in each stage to make this a viable procedure.

The enzymes, acid phosphatase (from wheat germ), alkaline phosphatase (from calf intestine), trypsin (from bovine pancreas), β-galactosidase (from *E. coli*), and glucose oxidase (from *Aspergillus niger*), 1-cyclohexyl-3-(2-morpholinoethyl) carbodi-imide metho-*p*-toluene sulfonate (CMC) and 1-ethyl-3(3-dimethylaminopropyl) carbodi-imide (EDAC) were obtained from Sigma Chemical Co., St Louis, MO. Other reagents used were of analytical quality and were obtained from BDH Ltd., Poole, Dorset, U.K.

Procedures

Portions of polyethylene powder (2 g) were immersed in a HEMA solution (10% in methanol) (30 cm^3) and irradiated at 18.3 rad s^{-1} in a ^{60}Co source for 72 h at 298 K, in the presence of air. Idealized studies would require that grafting be carried out under vacuum conditions or via nitrogen-purged assemblies. This introduces a degree of ambiguity into identification of the nature of the reactive sites in grafting. This is not a disadvantage since irradiation in air does not give a significant reduction in radical yields. The major advantage of irradiation in air lies in the greater commercial viability of this procedure relative to vacuum-treated systems and nitrogen purged assemblies. We should also recall the wish to retard/inhibit homopolymerization of the monomer. Such retardation may be provided by the presence of oxygen in the systems. The bulk solutions and grafted polyethylene powder were transferred into a six times excess of methanol, filtered, and the solid washed thoroughly with methanol. Any homopolymer was removed by extraction with methanol. The graft copolymeric products were dried *in vacuo* at 313 K, to constant weight.

To transform the poly(2-hydroxyethyl methacrylate) branches on the graft copolymers into the corresponding poly(methacrylic acid), portions of the copolymer (1 g) were refluxed with 25 cm^3 of 1*M* NaOH for known intervals of time. The products were filtered, washed with water, with 1*M* HC1, and again with water. They were then dried to constant weight. The method used to determine the number of carboxylic acid groups present after hydrolysis, was as follows. Each hydrolyzed sample (50 mg) was treated for 30 min, at room temperature, with 10 cm^3 of 0.1*M* NaOH. Supernatant (5 cm^3) was back-titrated with 0.1*M* HC1 using phenolphthalein as indicator. The coupling of proteins was carried out using either the hydroxyl groups or the carboxyl groups of the hydrolyzed grafted polymers. For attachment through the hydroxyl groups *p*-benzoquinone was used.[1] For coupling through the carboxyl groups of the hydrolyzed graft copolymer, the activation was with CMC

whereby the copolymer (100 mg) was added to 10 cm^3 of buffer solution containing 40 mg of the carbodi-imide and 40 mg of the protein. After shaking the components for 18 h at 277 K, the solids were filtered and washed. The filtrate and washings were made up to 100 cm^3. The protein content was determined using the Lowry assay procedure,[2] and the amount of protein immobilized was found from the difference.

The activity of acid phosphatase was measured using *p*-nitrophenol phosphate (Bessey et al.[3]). β-Galactosidase was assayed following the method of Craven et al.[4] Trypsin was assayed using either benzoyl-D-L-arginine-*p*-nitroanilide (BAPNA)[5] or hemoglobin (Anson and Mirsky).[6] The glucose oxidase activity was assayed using the *o*-dianisidine procedure as described by Hugget and Nixon.[7]

RESULTS AND DISCUSSION

The introduction of carboxylic acid groups into the copolymer gives a more hydrophilic substrate than would otherwise be the case with unhydrolyzed copolymer or polyethylene alone. In the present work, coupling of the biocatalysts involved attachment to either the hydroxyl groups or the carboxyl groups. This provides two routes to immobilization. It also gives the basis for modified hydrophilicity within the grafted branch networks. A further important point involves modification of the microenvironment of the ultimately immobilized biocatalysts. The inertness of the polyethylene provides a polymeric backbone capable of providing limited adsorption of biocatalyst. Thus, any biocatalyst present in the immobilized assembly is highly likely to be covalently linked when the correct coupling procedures are used. Coupling to branches provides the biocatalyst in close proximity to the continuous medium.

A hydrolyzed sample of polyethylene-*g*-co-2-hydroxyethyl methacrylate (PE/HEMA) (127% graft–52%hydrolyzed) was used to immobilize acid phosphatase and trypsin. Coupling through the hydroxyl groups was carried out after initial activation of the copolymer using *p*-benzoquinone. Good coupling was obtained with good retention of activity (Table I). When *p*-toluenesulfonylchloride was used as the activating agent, 73 mg of trypsin coupled

TABLE I
Immobilization of Enzymes onto PE/2-HEMA (127% Graft, 52% Hydrolysis) in 0.1*M* $NaHCO_3$ at 277 K for 18 h

	—OH Activation		—COOH Activation			
	p-Benzoquinone		CMC		EDAC	
Enzyme	Coupled[a]	Activity[b]	Coupled[a]	Activity[b]	Coupled[a]	Activity[b]
Acid phosphatase	20	26	20	6	20	6
Trypsin	50	11	220	11	70	13
Alkaline phosphatase	—	—	10	0	110	2
β-Galactosidase	—	—	40	12.5	—	—
Glucose oxidase	—	—	0	0	0	0

[a] Coupled = mg protein immobilized/g of copolymer
[b] Activity expressed as % retention of enzymic activity assuming all the protein is enzyme.

per g of copolymer with 22% of the enzymic activity being retained. These results are promising, considering that the system also has the desired physical characteristics for use in a commercial process. The activity was much better than that obtained with the unhydrolyzed system which suggests that the carboxyl-containing copolymer offers an environment that is more sympathetic to maintaining the immobilized protein's conformation.

Immobilization through the carboxyl groups present in the hydrolyzed polymers could offer advantages as carbodi-imides could be used.[8] Good levels of immobilization were obtained except for glucose oxidase (Table I). Both β-galactosidase and trypsin had good retention of activity compared to other graft copolymer systems.[8] With the unhydrolyzed copolymer, the best results had been with trypsin when 81 mg of enzyme coupled per g of copolymer with only 1.5% of retention of activity.[1]

The improvement with the hydrolyzed system may be explained as follows:

(i) When the grafted chains are based only on 2-hydroxyethyl methacrylate (2-HEMA), the presence of the bulky —$COOCH_2CH_2OH$ groups may cause some steric hindrance and not all the —OH groups would be available for coupling. On hydrolysis of the 2-HEMA, a number of —COOH groups is formed, and consequently the degree of steric hindrance should be reduced, making it easier for the protein to react with the —COOH groups.

(ii) After hydrolysis, the presence of the —COOH groups renders the medium more polar, and the grafted chains may be able to disperse more extensively into the aqueous phase.

Another advantage of using this type of graft copolymer is that by controlling the extent of hydrolysis, activation of the copolymer can arise using either the hydroxyl groups or the carboxyl groups.

In general, good results were obtained when trypsin was immobilized onto the hydrolyzed graft PE/2-HEMA copolymer. Hence this system was studied in more detail.

The Effect of Hydrolysis on the Coupling of Trypsin to PE-*g*-co-2-HEMA (127% Graft)

The graft copolymer PE/2-HEMA (127% graft) was hydrolyzed with 1*M* NaOH over various intervals of time. The resulting samples were activated with either CMC or *p*-benzoquinone, and then treated with trypsin in 0.1*M* $NaHCO_3$ at 277 K. The amount of trypsin immobilized, using CMC as activating agent, increased as the concentration of the carboxyl groups increased (Table II). The retention of enzyme activity reached a maximum at about 40–50% of hydrolysis. These results suggest that the hydrophilic environment is suitable for the coupling of these enzymes since good retention of activity was obtained. However, maximum coupling was attained with the copolymer having 30% of the 2-HEMA groups hydrolyzed. In general, the yield of coupling was lower than when the CMC method was used; this was anticipated because of the decrease in the number of hydroxyl groups available for coupling.

Activation using *p*-benzoquinone was not dissimilar to the CMC in that the optimum for protein immobilization was at approximately 30% hydrolysis and, for activity, was at 40–50% hydrolysis, but the levels were different for the two.

TABLE II
Effect of Degree of Hydrolysis of PE/2-HEMA (127% Graft) on the Immobilization of Trypsin Using Either CMC or *p*-Benzoquinone in 0.1*M* $NaHCO_3$ for 18 h at 277 K

Time of hydrolysis (h)	—COOH (mmol/g)	Hydrolysis (%)	—COOH Activation CMC Coupled (mg/g)	—COOH Activation CMC Activity (%)	—OH Activation *p*-Benzoquinone Coupled (mg/g)	—OH Activation *p*-Benzoquinone Activity (%)
0	0	0	0	0	0	0
0.5	0.58	14	20	11	42	12.8
1	1.32	31	30	8	66	15.4
2	1.77	41	60	7	58	16.3
4	2.07	48	120	25	50	16.4
18	2.24	52	150	24	50	11.0
30[a]	3.44	80	180	22	—	—

[a] The sample adsorbed 100 mg of trypsin/g copolymer which retained 55% of the activity.

The Effect of the CMC Concentration on the Immobilization of Trypsin to PE-*g*-co-2-HEMA (127% Graft–52% Hydrolyzed)

The graft copolymer PE-*g*-co-2-HEMA (127%graft–52% hydrolyzed) was used for the coupling of trypsin using different concentrations of CMC at 277 K over 19 h. Table III shows that good yields of coupling were obtained and that these yields increased as the concentration of CMC increased but the percentage retention of activity decreased. The latter could be due to various factors including: (i) the increased likelihood that each enzyme molecule could couple to more than one —COOH group on the support; (ii) the enzyme molecules condensing with each other; or (iii) distortion of the enzyme due to the greater likelihood of interaction between activated carboxyl groups of the extended copolymer chains with those residues on the protein that are important for maintaining the structure. If the steric hindrance is such that the grafted chains interact with the active site of the enzyme, then this can be investigated kinetically, as the affinity of the enzyme would be affected.

Attempts were made to rationalize the decrease in the percentage of retention of activity which arose when more than 40 mg of CMC g^{-1} copolymer were used. Thus, two approaches were adopted each involving the sample of PE-*g*-co-2-HEMA (127% graft–52% hydrolyzed). The procedures are given here for ease of replication. In the first approach, the graft copolymer (50 mg) was treated with a trypsin solution (18 mg) containing 5 mg of CMC.

TABLE III
Coupling of Trypsin to the Graft Copolymer PE/2-HEMA (127% Graft—52% Hydrolysis) Using Different Concentrations of CMC in 0.1*M* $NaHCO_3$ at 277 K for 18 h

mg CMC g^{-1} copolymer	mg Enzyme coupled g^{-1}	% Retention of activity
40	20	66
50	60	50
100	90	49
200	150	24
400	220	11
600	230	6

It was found that 90 mg of enzyme coupled g^{-1} copolymer, with 40% of retention of activity. To see if the enzyme coupled further with the support, the copolymer–enzyme composite was treated again with 5 mg of CMC in $0.1M$ $NaHCO_3$, and the activity of the enzyme was measured. It was found that the retention of the activity was 38%. Thus, the enzyme was not deactivated by the increase in the CMC concentration. In another experiment, the polymer–enzyme composite was treated with an extra 9 mg of trypsin and 5 mg of CMC. No more coupling occurred; thus the enzyme did not couple with itself.

In the second approach, we wished to ascertain if multiple coupling of the enzyme with carboxyl groups of the support, occurred. Here, CMC was added slowly, in aliquots of 1 mg (10 aliquots) to the copolymeric support in the presence of trypsin over 5 and 18 h. It was thought that, by adding the CMC in portions, the activation step would proceed slowly and multiple coupling could be rendered less likely to occur or avoided altogether. It was found that only 90 mg of enzyme coupled g^{-1} copolymer (compared to 150 mg obtained when the CMC was added in one stage) and the retention of activity was the same (24%). These experiments suggest that when high levels of coupling are achieved, distortion of the protein molecules occurs, which could possibly be due to steric hindrance of the enzyme. If the grafted chains interact with the active site of the enzyme, the chains will affect the attraction between the enzyme and the reactant. This can be measured kinetically by determining

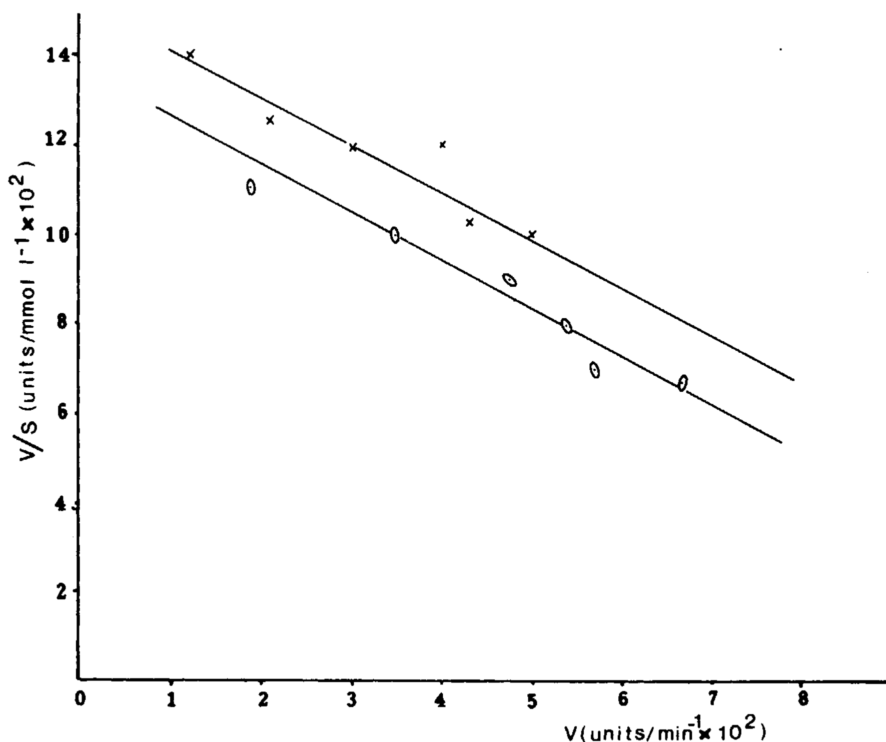

Fig. 1. Determination of the Michaelis Menten constant: BAPNA used as substrate: (X) free enzyme; (O) immobilized enzyme.

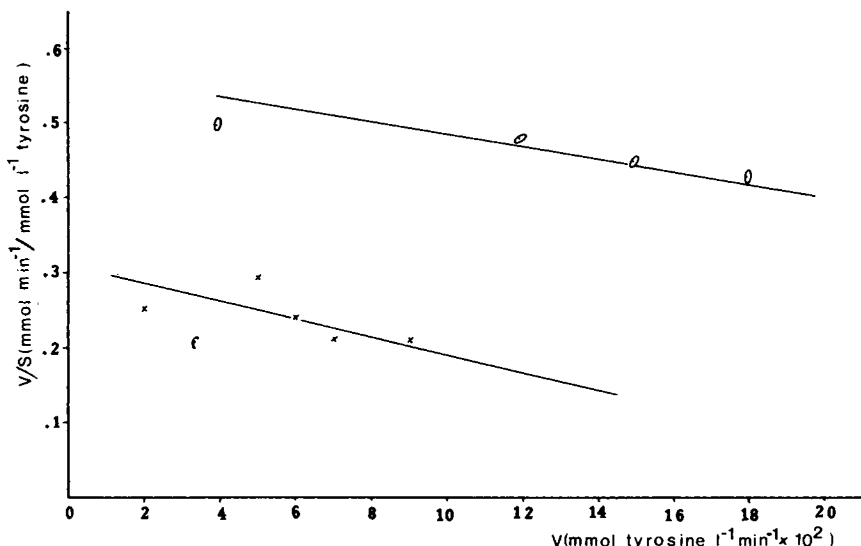

Fig. 2. Determination of K_m: hemoglobin used as substrate: (X) free enzyme; (O) immobilized enzyme.

the Michaelis constant (K_m) for the free enzyme using both BAPNA (Fig. 1) and hemoglobin (Fig. 2). The results (Table IV) show that they are of the same order for both substrates for both the free and immobilized enzymes. Thus the grafted chains are not taking up a steric configuration that is interfering with the active site.

It is far more likely that with the increase in the CMC concentration, the chains are activated and becoming more mobile. Hence, they are able to interact more freely with the immobilized proteins. This effect is to be investigated further as it could lead to a major advancement in the understanding of what is happening in such systems at the graft copolymer surface.

The Effect of Time of Treatment with *p*-Benzoquinone on the Amount of Enzyme Attached

Portions of the graft copolymer were treated with *p*-benzoquinone for different intervals of time. Table V shows that a maximum in attachment of *p*-benzoquinone is obtained after 2 h, under the chosen conditions. The maximum yield of coupling of the enzyme was obtained with samples which had received 1 h of activation. The percentage retention of activity after immobilization was good.

TABLE IV

K_m for the Free and Immobilized Trypsin Using Either BAPNA or Hemoglobin as Substrate

Sample	Substrate	K_m (mmol dm^{-3})
Free enzyme	BAPNA	1.10
Free enzyme	Hemoglobin	0.96
Immobilized enzyme	BAPNA	1.05
Immobilized enzyme	Hemoglobin	0.96

TABLE V
Coupling of Trypsin to the Graft Copolymer PE/2-HEMA (127% Graft–52% of Hydrolysis) Using *p*-Benzoquinone as the Activating Agent

Time of activation (h)	mmol *p*-Bq attached g^{-1} copolymer	mg E coupled g^{-1} copolymer	% Activity
1	0.15	50.4	11
2	0.29	41.5	11
3	0.28	24.0	16
4	0.32	32.0	15

TABLE VI
Coupling of Trypsin to the Graft Copolymer PE/2-HEMA (127% Graft–52% of Hydrolysis) with Different Concentrations of *p*-Benzoquinone

mg *p*-Benzo quinone g^{-1}	mmol *p*-Benzoquinone coupled g^{-1}	mg E g^{-1}	% Activity
100	0.08	25	25
200	0.15	27	17
270	0.15	50	11
400	0.19	46	12
500	0.31	51	13

The amount of *p*-benzoquinone retained in the sample isolated, after activation for four hours and treatment with trypsin, was determined; 0.13 mmol of *p*-benzoquinone g^{-1} copolymer were still present and presumably still potentially active. Only 40% of the activated groups reacted with the enzyme.

In another experiment, aliquots of the graft copolymer were treated with different amounts of *p*-benzoquinone for 1 h. The results (Table VI) show that maximum coupling was obtained when the concentration of *p*-benzoquinone was 500 mg g^{-1} copolymer. However, the activity was little affected.

Characteristics of Trypsin, Immobilized on Hydrolyzed PE-*g*-co-2-HEMA (127% Graft–52% Hydrolysis)

When trypsin was immobilized onto the hydrolyzed PE-*g*-co-2-HEMA, a good yield of coupling was obtained. The retention of activity of this immobilized trypsin was considered satisfactory. It was found that when the immobilized enzyme was stored in distilled water at 277 K over 6 months, 83% of the initial activity was retained. The influence of temperature, and pH (from pH = 3 to 10.5), on the activity of the free and the immobilized trypsin was studied. Samples of the immobilized enzyme were prepared containing 140 mg of protein coupled g^{-1} copolymer, having 18% retention of the activity (using BAPNA as substrate) or 6% retention of the activity (using hemoglobin as substrate); this activity difference probably arises from the relative accessibilities of the two substrates to the immobilized enzyme sites.

The free and immobilized systems did not show any marked differences with respect to temperature. The optimum pH was the same for both the free and immobilized systems. The latter, however, had an enhanced pH profile which showed that it was still quite active at pH 10, having 80% of optimum

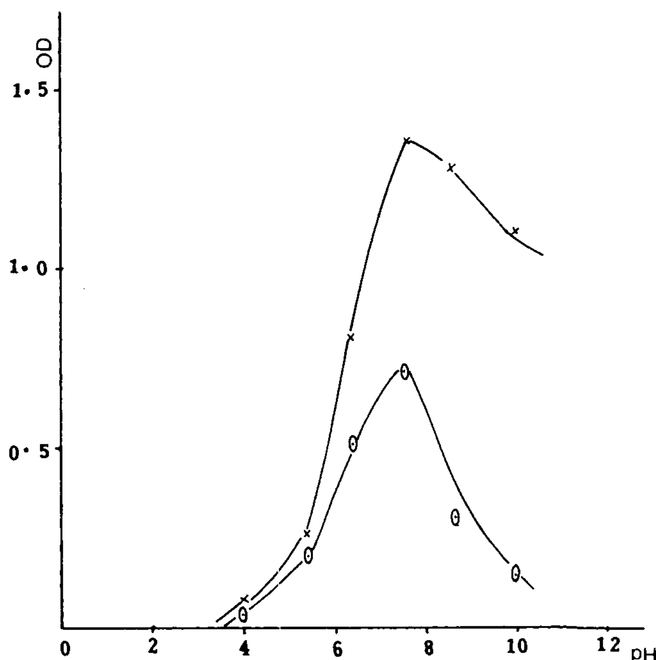

Fig. 3. Variation of the activity of the free and the immobilized trypsin with pH using hemaglobin as substrate: (X) immobilized enzyme; (O) free enzyme.

activity whereas the free enzyme had only 10% (Fig. 3). Thus the carboxyl groups of the grafted chains appear to have a stabilizing effect.

CONCLUSION

It was found that good yields of coupling of enzymes, particularly trypsin, were obtained, with good retention of activity, when the immobilization was carried out onto hydrolyzed polyethylene-*g*-co-hydroxyethyl methacrylate. This system offers potential as both a general and a specific model, and it could be used for many applications. An investigation into the relationship of the degree of hydrolysis to the coupling ability has given a valuable insight into the interaction that takes place at the graft copolymer surface in an immobilized enzyme system. While we cannot ignore the fact that some volumetric grafting could occur, the impervious nature of the polyethylene together with the water/2-HEMA medium would indicate that surface grafting is likely to dominate. A further point needs to be emphasized. In view of the inherent presence of monomeric impurities, albeit in small quantities (such as the aforementioned ethyleneglycol dimethacrylate), we cannot rule out the possibility of low levels of crosslinking between (intermolecular) and within (intramolecular) the branches. Our inclination is that grafting dominates the polymerization events. However, it can be argued that the development of crosslinking is not necessarily a disadvantage, provided that the occluded material does not migrate from the surface. However, these points require clarification and are the subject of current study. Details related to the application of the immobilized trypsin system will be made available in due

course. Our initial interest has been in the treatment of milk and its components,[9] though other avenues are currently being investigated.

We gratefully acknowledge the assistance provided by JNICT, Lisbon, the ORS and the CVCP to MHG throughout the duration of this program.

References

1. M. Alves da Silva, C. G. Beddows, M. Helena Gil, and J. T. Guthrie, Proceedings of the 9th Iberoamerican Symposium on Catalysis, Lisbon, July 1984, p. 358.

2. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

3. P. A. Bessey, O. H. Lowry, and M. J. Brook, *Biol. Chem.*, **164**, 321 (1946).

4. G. R. Craven, E. J. Steers, and C. B. Anfinsen, *J. Biol. Chem.*, **240**, 2468 (1965).

5. B. F. Erlanger, N. Kokowsky, and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271 (1961).

6. M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.* **17**, 151 (1933).

7. A. G. Huggett and D. A. Nixon, *Lancet*, **2**, 368 (1957).

8. F. I. Abdel-Hey, C. G. Beddows, M. H. Gil, and J. T. Guthrie, *J. Polym. Sci., Polym. Chem. Ed.*, **21**, 2463–2472 (1983).

9. C. G. Beddows, M. H. Gil, and J. T. Guthrie, Proceedings of the International Conference on Radiation Processing for Plastics and Rubber II, March 1984, University of Kent, Canterbury, U.K.

Received October 31, 1986
Accepted April 30, 1987