STUDIES IN POLYETHYLENEIMINE: MATRIX POLYMERISATION AND ENZYME IMMOBILIZATION

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FORM A

Certified that the work incorporated in the thesis **"STUDIES IN POLYETHYLENEIMINE: MATRIX POLYMERIZATION AND ENZYME IMMOBILIZATION"** submitted by **Mr. R. V. BAHULEKAR** was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

[Dr. N.R. AYYANGAR]

Research Guide

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CONTENTS

CHAPTER 2

EXPERIMENTAL

CHAPTER 3

RESULTS AND DISUSSION

SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 MATRIX POLYMERISATION

1.1.1 GENERAL INTRODUCTION

Polymerisation of monomers in presence of polymers (matrix) are termed as matrix polymerisations. The polymerisation of monomer occurs either partially or completely along the matrix chains at least over a short period. Free radical homo and copolymerisations generate stereochemically random atactic structures. This arises from inadequacy of precision in the propagation step and statistical nature of polymerisation reactions. It is highly stimulating to synthesise macromolecules with ordered and accurately predetermined structures. The randomness associated with the free radical polymerisation ought to be negated to derive structural order. Ordered homo and copolymers, having unique combination of properties and performances, may be synthesised in the following ways:

i. Selecting suitable catalysts (Ziegler-Natta) for polymerisation. This would introduce high degree of iso and syndio-tacticity.

ii. Polymerisation by step-growth techniques.[1]

Stereo-regular polymers cannot be synthesised by free radical polymerisation. In free radical polymerisations order can be induced by proper alignment of monomer molecules prior to the polymerisation. Ordered mono and multilayer arrays are observable in crystals and liquid crystals. Polymerisation of ordered monomer arrays have been documented in the literature.[2] Order in monomer units can be generated prior to free-radical polymerisation by the adding a suitable polymer to adsorb and to suitably orient the monomer molecules. The polymer added to induce such a topochemical effect is termed as "matrix" or "template" and the polymerisation is designated as matrix or template polymerisation.

The presence of a macromolecular matrix exerts numerous effects not observable in conventional free-radical polymerisations. The important changes brought in by the inclusion of a matrix are observable through:

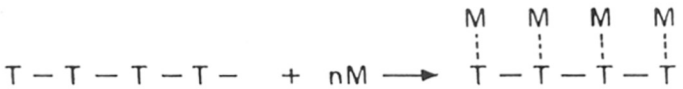
* Rate enhancements.
* Reaction mechanisms.
* Molecular weight and molecular weight distribution.
* Topochemistry and microstructure.
* Reactivity ratios in copolymerisation.

A simplistic mechanism of matrix polymerisation is pictorially depicted in *Figure 1*.

A sequence of reaction steps may be visualised. In the first step, monomer molecules are organised in the vicinity of the matrix as a linear array, by either adsorption or complexation. The organised monomer molecules are polymerised in the second step. The newly formed polymer may separate from the matrix which is then available for further polymerization. In *Figure 1* matrix is denoted by *T* and the monomer by *M*. Matrix is usually added as a preformed polymer and is termed "*parent polymer*". The newly formed polymer is often designated as "*daughter polymer*".

The concept underlying the matrix polymerisation has its origin in natural processes like self replication of DNA and biosynthesis of proteins. Biological polymerisations are unique and are impeccable manifestations of precisely organised systems. The comprehensive simulation of such systems is beyond the current art of matrix polymerisation. The first documented report to replicate nature came in 1956 through the pioneering work of Bamford *et al.*[3] Anionic ring opening polymerisation of the N-carboxy anhydride (NCA) of D,L-phenylalanine was examined in the presence of poly sarcosine. Bamford *et al.* christened this phenomenon as "*chain effect*". This gravitated into a major effort. Many research schools investigated number of different

STEP 1 : ASSOCIATION OF MONOMER MOLECULES WITH MATRIX



STEP 2 : POLYMERISATION OF ASSOCIATED MONOMER MOLECULES

STEP 3 : SEPARATION OF MATRIX FROM DAUGHTER POLYMER

FIG. 1 : SCHEMATIC REPRESENTATION OF MECHANISM OF MATRIX POLYMERISATION

systems. A number of buzzwords were invented for this unusual mode of polymerisation. A selected list from the major participants in this field and the terms customed are presented in Table 1.

Beside these, "*replica polymerisation*" is also used to imply some analogy to DNA replication.

1.1.2 CHARACTERISTICS OF MATRIX POLYMERISATION

The dominant features distinctive of matrix polymerisations are discussed in some detail below:

* **Rate enhancement**:

The polymerisation rate is enhanced by the presence of a matrix. There are multifarious scientific reports of reactions catalysed by polymers.[4,5] Matrix polymerisations belong to this class. The increased polymerisation rate is ascribed to the association of monomer molecules with the matrix. Rate enhancement is most pronounced when the matrix is saturated with the monomer. The magnitude of rate enhancement varies with the matrix-monomer combination. The nature of interaction between the matrix and monomer is the dominant facet. These are discussed in sections 1.1.6.1.1 and 1.1.6.2.1. A few cases of negative matrix effect have also been reported. In these systems the polymerisation rate decreases in the presence of a matrix.[6]

* **Topochemical effect**:

The structural and stereo-chemical attributes of the matrix are complemented in the daughter polymer. This is discussed in section 1.7.

The product of matrix polymerisation is a polycomplex of the matrix and daughter polymer. It is generally rather difficult to separate the individual polymers.

Table - 1

Various terms used for polymerisation of a monomer in presence of a polymer

CHAIN EFFECT	Bamford et al. (1956)
INTERACTIVE POLYMERISATION	Ferguson et al. (1968)
MATRIX POLYMERISATION	Kargin et al. (1969)
TEMPLATE POLYMERISATION	Challa et al. (1973)

1.1.3 GENERAL MECHANISM

Positive matrix effects are observable for matrix-monomer systems which generate polycomplex of matrix and daughter polymer. The organisation into polycomplex may ensue during matrix polymerisation by a variety of mechanisms. Tsuchida and Osada[7] proposed the ensuing mechanisms based on the relative interactive strengths between matrix-monomer and matrix-daughter polymer.

* **Case 1:**

Matrix has greater affinity for monomer than for the daughter polymer. The monomer molecules are preferentially adsorbed on to the matrix. The adsorption equilibrium constant K_m may be infinite.

Polymerisation of the adsorbed adjacent monomers proceed by either step-growth or chain-growth mechanism (Zip mechanism). After polymerisation, the daughter polymer is displaced by monomer molecules permitting further growth through matrix effect. This mechanism is depicted in *Figure 2a*. In this case, rate enhancement becomes independent or is less dependent on the relative concentrations of the matrix and monomer.

* **Case 2:**

The interaction between matrix and daughter polymer are stronger than that between matrix and monomer. In this case, the adsorption equilibrium constant K_m is zero. Here, the onset of matrix effect is observable only after a critical length of the growing polymer chain collapses onto the matrix. The reaction progresses by clasping monomer molecules from the encircling solution (pick-up mechanism). The final output is a matrix-daughter polymer complex. This mechanism is depicted in *Figure 2b*.

* Case 3:

The matrix has equivalent affinities for growing chains (daughter polymer) and monomer molecules. The polymerisation results in matrix-daughter polymer complex. This mechanism is schematically exemplified in *Figure 2c*.

It must be stressed that detailed mechanistic aspects of many "*matrix polymerisations*" are not fully understood as yet.

1.1.4 FACTORS OF MATRIX EFFECT

The following factors should be used as diagnostic tools to distinguish matrix polymerisations.

* The structural features of matrix and daughter polymer should be complementary.

* The matrix should alter the rate of polymerisation. In most cases the rate is enhanced by the presence of matrix.

* There ought to be some evidence for polycomplexation of matrix and daughter polymer.

1.1.5 TYPES OF INTERACTIONS

The unique mode of polymer chain propagation during matrix polymerisation are due to the following four types of specific interactions which operate between matrix and monomer or matrix and daughter polymer:

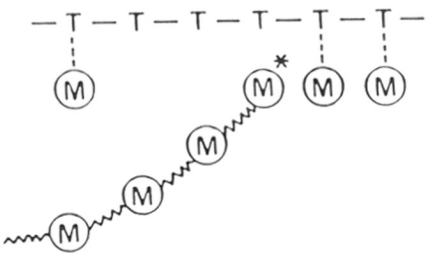
1.1.5.1 Van der Waals

1.1.5.2 Hydrogen bonding

1.1.5.3 Electrostatic

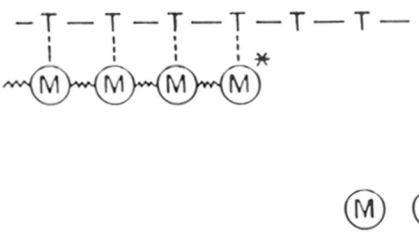
1.1.5.4 Hydrophobic

CASE 1 : MATRIX HAS GREATER AFFINITY FOR MONOMER



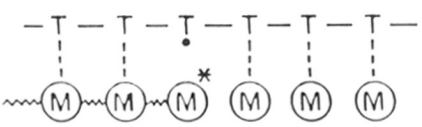
a

CASE 2 : MATRIX PREFERABLY INTERACTS WITH GROWING CHAIN



b

CASE 3 : MATRIX HAS EQUAL AFFINITY FOR GROWING CHAIN AND MONOMER



c

FIG. 2 : VARIOUS MECHANISMS OF MATRIX POLYMERISATION (TSUCHIDA AND OSADA)[7]

1.1.5.1 Van der Waals interactions

Polymerisation of methyl methacrylate [MMA] supported by the presence of stereoregular poly(methyl methacrylate) [PMMA] is due to Van der Waals forces. Melville *et al.*[8] were first to report the polymerisation of MMA by thoroughly purified PMMA. Styrene and vinyl acetate could not be polymerised by PMMA under analogous environment or by polystyrene and poly (vinyl acetate). Stereo-complexes are formed between iso- and syndiotactic PMMA in suitable solvents.[9] Challa *et al.*[10-12] extensively probed the free radical polymerisation of MMA in presence of stereoregular PMMA. The stereochemical polycomplexation during the polymerisation of MMA in N,N-dimethyl formamide (DMF) in presence of isotactic PMMA is appropriately confirmed through experimental observations.[13-15] Challa *et al.*[16] attributed the stereo associations to weak Van der Waals forces and to stereochemical fitting of the matrix and daughter polymers.

The investigation reveals that the matrix strongly influences the microstructure of the daughter polymer as well as the kinetics of polymerisation.[10] Polymerisation of MMA was examined in N,N-dimethyl formamide solution in the presence isotactic poly(methyl methacrylate) of varying molecular weights. The reaction was initiated by bis (p-tert butyl cyclohexyl) diperoxycarbonate at 25°C. The relative populations of isotactic, syndiotactic and heterotactic triads present in the daughter polymer was estimated by nuclear magnetic resonance (NMR) spectroscopy. The daughter polymer had a high concentration of syndiotactic triads, especially when isolated at low conversions. This tended to decrease with increasing conversion. The stereochemical effects were also found to be enhanced by higher molecular weight matrices. At higher conversions, isotactic daughter polymer was also observable. Thus, the daughter polymer formed at lower conversion acts as a matrix for further polymerisation. The final product was acetone-insoluble polycomplex of isotactic and syndiotactic PMMA. Another noteworthy element was the near equivalence between the molecular weight of matrix and the daughter.[10]

The polymerisation of MMA was also examined in the presence of syndiotactic PMMA.[11] The observations were similar. Isotactic PMMA was formed at lower conversions. The notable difference was that the syndiotactic matrix enhanced the rate less appreciably.

Some of the other important features of this system were as follows:

* The stereo-regulation was more prominent at lower temperatures.[11]

* Solvents effects were profound. Stereochemical complexation were most prominent in polar solvents such as N,N-dimethyl formamide (DMF), dimethyl sulphoxide (DMSO), and acetone. Non-polar organic solvents weakened while chlorinated solvents completely disrupted the association.[12]

One of the ramifications of matrix effect is enhancement in the polymerisation rate. Tacticity, molecular weight of matrix, solvent and temperature govern the magnitude of this acceleration.[17] Greatest enhancement was noted for polymerisation conducted, in presence of very high molecular weight isotactic PMMA matrix, at lower temperatures and in polar solvents fostering maximum stereochemical fitting of the two polymers. Gons *et al.*[18] studied the underlying elementary kinetic processes. The absolute rate coefficients of initiation (k_i), propagation (k_p) and termination (k_t) were determined by rotating sector technique. The rate enhancements were found to be related to a decrease in termination coefficient k_t. It was proposed that the segmental mobility of polymer radical stereo-associated with the matrix is decreased and consequentially the radical lifetime is increased. At a given rate of initiation, the steady state equilibrium concentration of radical is enhanced. This is reflected in the enhanced rate.

Challa *et al.*[19,20], proposed a mechanism to fit the experimental observations. The polymerisation is initiated in the solution. The growing polymer chain collapses onto the matrix after a critical chain length. This is reflected in entropy of the propagation step. The system conforms to case 2 proposed by Tsuchida and Osada[7] (see section 1.1.3).

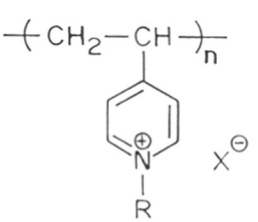
1.1.5.2 Hydrogen Bonding

The first report of Bamford *et al.*[3], polymerisation of N-carboxy-α-amino acid anhydrides (NCA) promoted by polysarcosine matrix, arises from hydrogen bonding. The system is pictorially depicted in *Figure 3*. The rate enhancement was related to increased concentration of NCA in the vicinity of matrix. Hydrogen bonding was confirmed by the shifts in *N-H* and *C=O* stretching vibrations.[21] The matrix effects in the polymerisations of N-vinyl pyrrolidone in presence of poly (methacrylic acid)[22,23] and of methacrylic acid by poly (N-vinyl pyrrolidone)[24] are also due to hydrogen bonding.

1.1.5.3 Electrostatic Forces

Electrostatic forces operate in the polymerisation of 4-vinyl pyridine (4-VP) on poly (styrene sulphonic acid) (PSSA)/ poly (vinyl sulphonic acid) matrices. Monomers are sequentially enveloped around the matrix by chemisorption, protonised and then polymerised. The reaction culminates in poly salt, as depicted in *Figure 4*.

Kabanov *et al.*[25-29] observed a rather intriguing phenomenon in the reaction between 4-vinyl pyridine and alkyl halide in an organic medium. Spontaneous polymerisation was observed instead of the expected Menshutkin reaction. The structure (I) was proposed for the polymer



(I)

Kabanov *et al.*[28] also reported the formations of corresponding poly (4-vinyl pyridinium) salts by reaction between 4-vinyl pyridine and sulphuric acid/ p-toluene sulphonic acid. The obser-

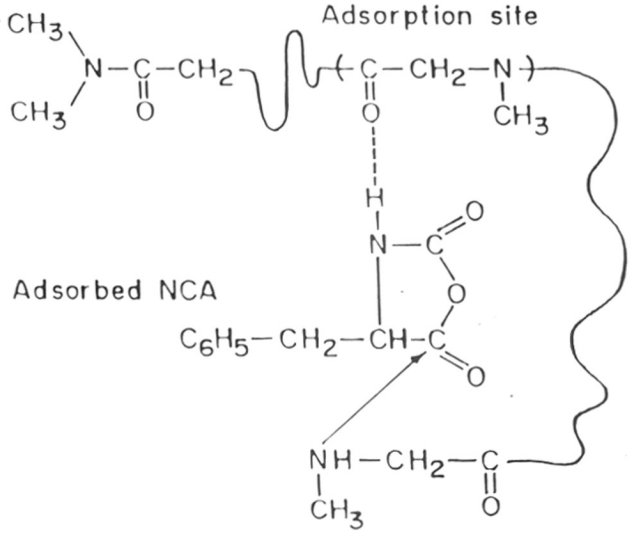


FIG. 3 : SCHEMATIC REPRESENTATION OF MATRIX EFFECT IN POLYSARCOSINE DIMETYL AMINE / PHENYL ALANINE NCA SYSTEM

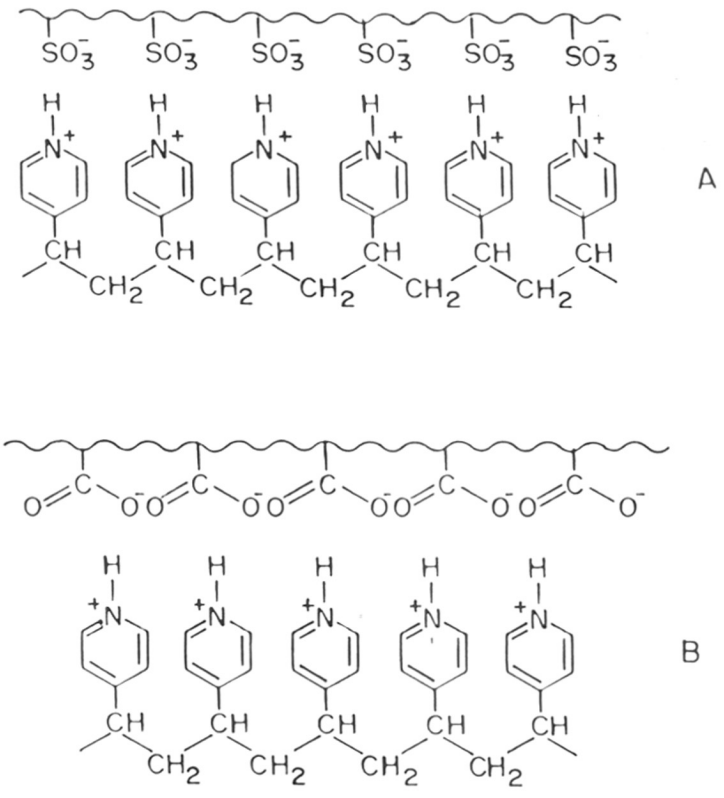
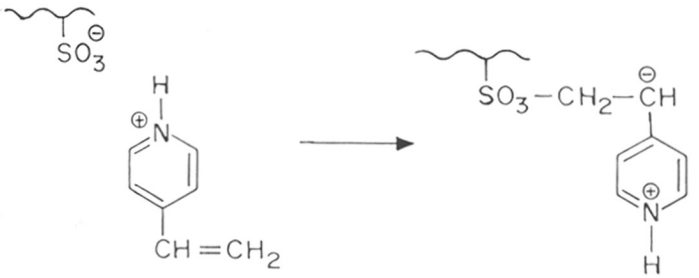


FIG. 4 : POLYSALT FORMATION BETWEEN (A) POLY (4-VINYL PYRIDINE) AND POLY (STYRENE-4-SULPHONIC ACID) POLY (VINYL SULPHONIC ACID) AND (B) POLY (4-VINYL PYRIDINE) AND POLY (ACRYLIC ACID)

vations were extended to the spontaneous uncatalysed reaction of 4-VP with poly (styrene-4-sulphonic acid) (PSSA) in methanol, which results in a precipitate.[27,28,30,31] The infra-red spectrum of the product was similar to that of 1:1 polysalt formed by mixing poly (4-vinyl pyridine) and poly (styrene sulphonic acid). Poly (acrylic acid) displays a similar behavior.[27]

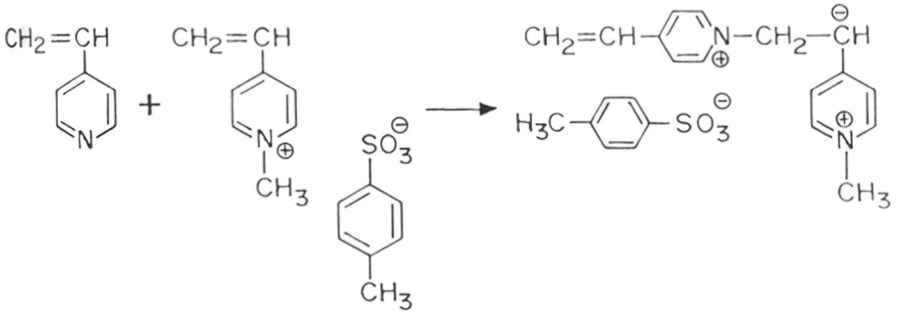
The morphology of the polysalt formed by matrix polymerisation is dictated extensively by the conformation of the matrix. The matrix, poly (styrene-4-sulphonic acid), exists in random coiled conformation in methanol due to marginal ionisation. The polysalt formed in methanol is globular. The matrix is ionised completely in water and exists in extended rod-like conformation. The polysalt formed in water has fibrillar conformation.[27,28]

Salamone *et al.*[32] examined the system in some detail since the weakly nucleophilic aromatic sulphonate ion could not possibly initiate anionic polymerisation of 4-vinyl pyridine as proposed in Equation (1) by Kabanov *et al.*[28]



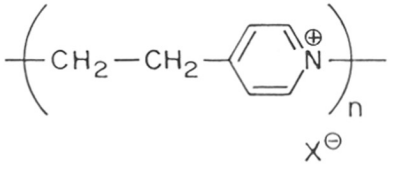
(1)

Salamone *et al.*[32] interpreted the results by recourse to nuclear magnetic resonance spectroscopy. The analysis reflect incomplete transformation of salt formed between 4-vinyl pyridine and toluene-4-sulphonic acid into polymer. The experimental evidence points to nucleophilic addition of 4-vinyl pyridine to 4-vinyl-N-methyl pyridinium-p-toluene sulphonate, as depicted in Equation (2)



(2)

In aqueous solutions, strong acids act as initiators in consonance with monomer and result in either ionene polymers or vinyl pyridinium salts (**II**).[33]



(II)

The propagation of 4-vinyl pyridinium ion anchored on poly (styrene-4-sulphonic acid) is accomplished by cationic species and not through zwitterion. These findings were confirmed by Kabanov *et al.*[34] The system comprises of vinyl pyridinium and ionene units as shown in *Figure* 5.

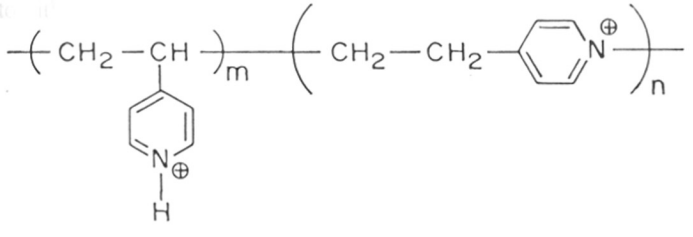
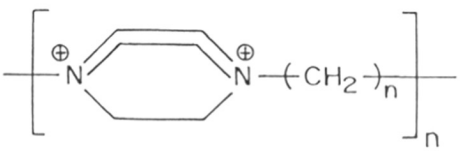


FIGURE-5

Polymerisation of p-styrene sulphonic acid (p-SSA) in presence of ionene matrix prepared by Blumstein *et al.*[35,36] also proceeds via electrostatic forces of interactions. The repeating unit of ionene was **(III)**



n = 4, 6 or 8

(III)

1.1.6 TYPES OF MATRIX POLYMERISATION

The large database generated over the years led Challa and coworkers[37] to classify matrix polymerisations into Type I and Type II systems. The mode of propagation, depicted schematically in *Figure 6.*, forms the basis for this evaluation. In Type I systems monomer molecules are a priori aligned along the matrix. In type II systems, polymerisation is initiated in solution and the growing polymer chain collapses on to the matrix and continues to grow by annexation of monomer molecules from the surrounding solution. The end result in both the cases is a polycomplex of daughter polymer and matrix. Many matrix polymerisations do not pertain ideally to either type I or type II behavior. These may exhibit some features common to both types.

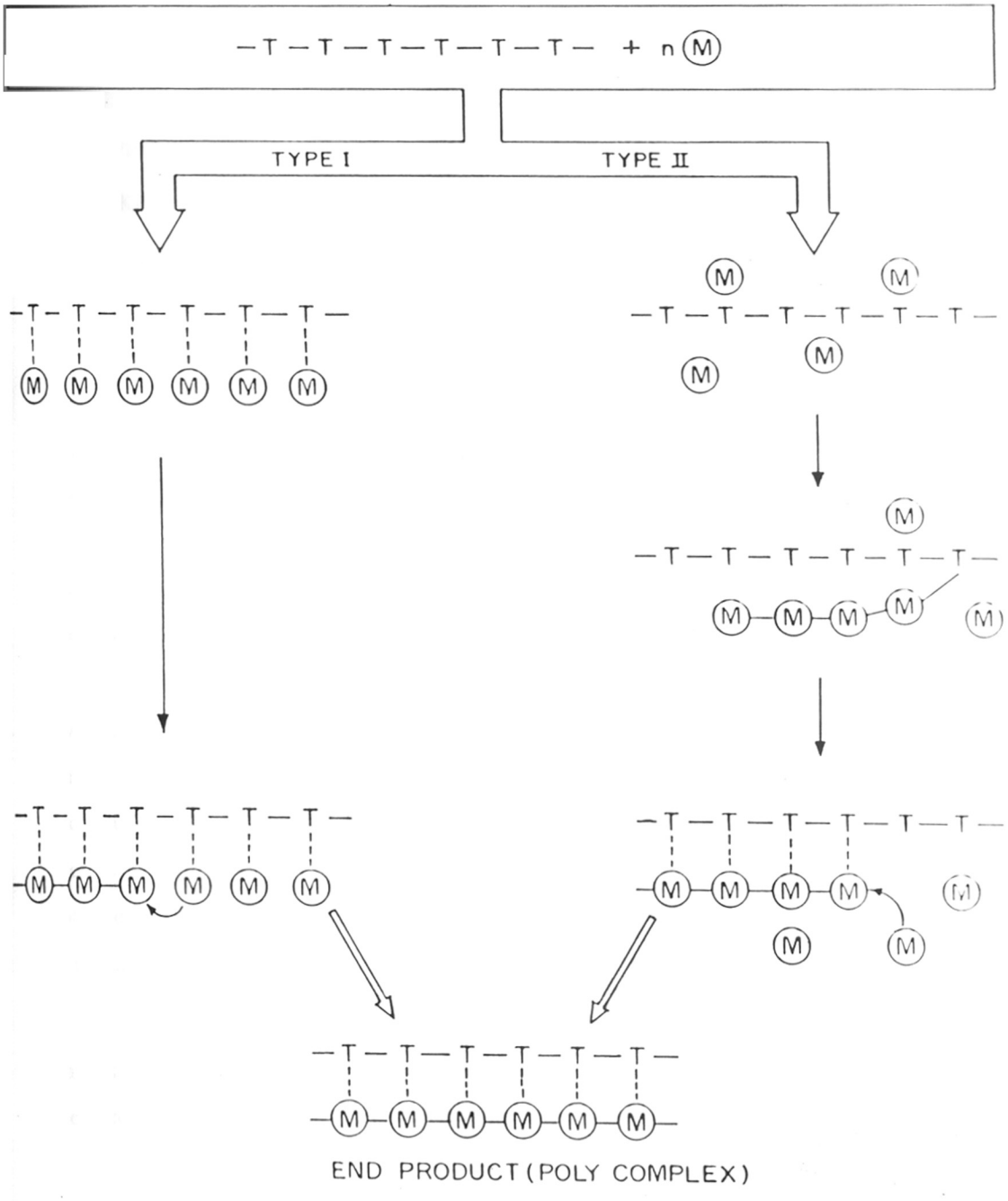


FIG. 6 : SCHEMATIC REPRESENTATION OF TWO TYPES OF MATRIX POLYMERISATIONS

1.1.6.1 Type I systems

1.1.6.1.1 Kinetic Behavior

Strong ionic interactions are prominent in these systems. The matrix increases the polymerisation rate by as high as 3 orders of magnitude. This is ascribed to increase in the rate of propagation. Blumstein *et al.*[35] investigated the polymerisation of styrene-4-sulphonate anion on (2.2.2),4 ionene cations and attributed this highly enhanced rate to increased local concentration of the monomer in the vicinity of the matrix. Other factors such as ordering of monomer arrays,[38,39] reduced repulsion of monomer[7] due to association with matrix and heterogeneity of the system[40] also contribute towards rate enhancement. Manning ion-condensation model was envoked to support the experimental observations.[41-44] The maximum in rate enhancement was observable at equimolar concentrations of the charges in the monomer and matrix due to complete saturation of the matrix with monomer molecules as counter ions. The matrix is not saturated at lower concentration of the monomer. The rate increases with the degree of saturation. Beyond complete saturation of monomer, the rate falls. The rate measured is the arithmetic mean of matrix and solution polymerisation rates. The polymerisations were conducted at high dilutions to exclude inter-matrix effects. The monomer ions are very mobile in the vicinity of the matrix and not fixed onto the matrix by either ion-atmosphere or site bindings.[43] The degree of polymerisation of the daughter corresponds to that of the matrix. Stereo-chemical effects were also noted.[44]

The overall rate is dependent on the first power of monomer and initiator concentrations. Termination is unimolecular. It probably proceeds by abstraction of labile hydrogen in the surrounding environment, most frequently the solvent molecules.

Another system which pertains closely to type I system is the polymerisation of methacrylic acid on ionene matrix, reported by Tsuchida *et al.*[7]. The discerned rate enhancement was ascribed to the deviation from solution polymerisation. In matrix polymerisation, electrostatic

repulsions between ionised propagating radicals and monomer are significantly reduced. Secondly, the intrinsic reactivity of monomer and polymer radical are enhanced by the adsorption onto the matrix. Many other systems can be identified as type I. A non exhaustive list is presented in Table 2.

1.1.6.1.2 Molecular Weight

In ideal cases, daughter polymer should have similar degree of polymerisation and molecular weight distribution corresponding to the matrix. Kammerer *et al.*[45,46] reported the polymerisation of methacrylic acid covalently linked to molecularly uniform polynuclear phenolic oligomer. Phenolic resin was reacted with methacryloyl chloride, dissolved in benzene and polymerised with azo-bis-isobutyronitrile. The chain length of daughter polymer was identical to the matrix. However, with very high molecular weight matrices (degree of polymerisation, P_n ~ 100) the molecular weight correlations disappear. Polowinski and coworkers[47] polymerised multimethacrylates on poly (vinyl alcohol). The molecular weight of daughter polymer was estimated to be three times larger. Other systems of similar nature were reported by Endo *et al.*[48] and Blumstein *et al.*[49]

1.1.6.2 Type II Systems

1.1.6.2.1 Kinetic Behavior

Type II behavior is displayed in systems arising from relatively weaker interactions between the matrix and monomer/daughter polymer. In these, rate enhancement is attributed to retardation of termination step. Gons *et al.*[18,50] investigated the underlying elementary processes in the polymerisation of methyl methacrylate in solution and in the presence of poly (methyl methacrylate), by rotating sector technique (see section 1.1.5.1). The rate of initiation of solution and matrix polymerisations were found to be similar. Radical lifetimes were graphically computed from the plot of normalised ratio, $V_{p,i}/V_{p,c}$, and log t. Here, $V_{p,i}$ and $V_{p,c}$ are the polymerisation

TABLE - 2

MATRIX POLYMERISATION SYSTEMS SHOWING TYPE I BEHAVIOR

Monomer	Matrix	Solvent	Initiator	Temp. °C	Ref.
Phenylalanine - NCA	Poly (Sarcosine - dimethyl amide	Nitrobenzene		25	3,61-64
Phenylalanine - - NCA	Poly (2-vinyl pyridine)	Nitrobenzene	Spont.	35	65, 66
4-vinyl pyridine	Poly (acrylic acid) Poly (L-glutamic acid) Poly (phosphoric acid)	Water	Spont.	25	67 - 72
4-vinyl pyridine	Poly (vinyl oxy-carbonyl phthalic anhydride)	Acetone, DMF	Spont.	50	73
4- vinyl pyridine 2-vinyl pyridine	poly (maleic anhydride)	DMF	Spont.	50	74,75
Propargyl chloride	Poly (4-vinyl pyridine)	Methanol	Spont.	50	76,69
N-β -methacryloyl oxyethyl of adenine (MAO - A)	Poly (MAO Thymine)	Pyridine	AIBN	20-70	77,78
Acrylic acid	Polyethyleneimine	Acetone - water	AIBN	25	39
4-styrene sulphonic acid	Poly (diazabicyclo octo -1-alkanes) [Ionene]	2-propanol-water	AIBN	70	2, 41-43
Acrylic acid	Polyethyleneimine	Water	$K_2S_2O_8$	30	60
Methacrylic acid, Acrylic acid	Poly (N,N,N',N'-tetramethyl-N-xylylene ethyl diammonium di-chloride [Ionene]	Water	$K_2S_2O_8$	50	7
Methacrylic acid	Poly (L-lysine)	Water	AIBN	80	79
Acrylic acid, 4-styrene sulphoonic acid	Poly (L-lysine)	Water	$K_2S_2O_8$ $NaHSO_3$	30	80
Acrylamide	Poly (4-styrene sulphonic acid)	Water	H_2O_2	80	81,82
Methacrylic acid	Poly (N-vinyl-5-methyl oxazolidone)	Water	$K_2S_2O_8$	60	83
N-vinyl oxazolidone	Poly (methacrylic acid)	Water	$K_2S_2O_8$	60	38

rates with intermittent and continuous exposure to radiation and t is flash time. The radical lifetimes for solution and matrix polymerisations were 8.3 and 64.0 sec. respectively. The data were utilised to compute k_p/k_t using the Equation (3)

$$\frac{k_p}{k_t} = \frac{2\tau_s V_{p,c}}{M} \qquad (3)$$

where, τ_s = Radical life time,

$V_{p,c}$ = Polymerisation rate with continuous radiation

[M] = Initial monomer concentration.

A 5-fold reduction in k_p and an 82-fold reduction in k_t were estimated in the presence of matrix. The retarded termination was due to the restricted mobility of the matrix associated polymer radicals. A detailed mechanism for the termination reaction has not been offered as yet. "Cross termination reaction" between non-associated (monomer) and associated polymer radicals cannot be ruled out. Some investigators[40] attribute retarded terminations to the heterogeneity of the system. The heterogeneous reaction traps the radical there by preventing termination.

Induction periods have been noted in a few matrix assisted polymerisations. These are attributed to impurities.[51-53] Ferguson *et al.*[54] observed the onset of induction period with the increase in molecular weight of poly (N-vinyl pyrrolidone) used as a matrix for the polymerisation of acrylic acid. This was estimated as the time for the inception of matrix activated polymerisation. Smid *et al.*[55] attributed the induction time, observed in the polymerisation of methacrylic acid on poly (2-vinyl pyridine) matrix, to the gradual build-up of matrix associated radical. Some type II systems are listed in Table 3. The rate enhanced even beyond the stoichiometric ratio of [monomer]/[matrix].

TABLE - 3

MATRIX POLYMERISATION SYSTEMS SHOWING TYPE II BEHAVIOR

Monomer	Matrix	Solvent	Initiator	Temp. °C	Ref.
N-vinyl pyrrolidone	Poly (methacrylic acid)	DMF	AIBN	60	22,52,57
Methacrylic acid	Poly (N-vinyl pyrrolidone)	DMF	AIBN	50	84
N-vinyl pyrrolidone	Poly (acrylic aicd)	DMF	AIBN	74	85
Methyl methacrylate	isotactic and syndiotactic poly (methyl meth-acrylate)	DMF	t-Butyl cyclohexyl peroxy di carbonate	25	50,86,87
Methyl methacrylate	isotatic and syndiotactic poly (methyl methacrylate)	Toluene	Grignard	-50	58
Methacrylic acid	Isotactic Poly (methyl methacrylate)	DMF Dioxane	t-Butyl cyclohexyl peroxydic carbonate	-10	88
Methacrylic acid	Poly (ethylene oxide)	Water	$K_2S_2O_8$	50	6,16
Methacrylic acid	Poly (vinyl alcohol)	Water	$K_2S_2O_8$	50	89
N-vinyl imidazole	Poly (methacrylic acid)	Water	AIBN	50	90

1.1.6.2.2 Effect on Molecular Weight

Type II systems exhibit direct dependence of molecular weight on rate constants k_p and k_t. Rate enhancement is accompanied by parallel increase in the molecular weight of daughter polymer.[53,56,37] In cases where in negative matrix effect are encountered,[6] lower molecular weights have not been reported as yet. There is no correlation between the molecular weight of matrix and daughter polymer. As a rule, the molecular weight of daughter polymer is higher.

1.1.7 TOPOCHEMICAL EFFECTS

The matrix influences the microstructure of the daughter polymer to an extent. The complementary stereochemical features in daughter polymer is significant factor in ascribing matrix effect. The stereo-regularity of matrix influences polymerisation rate as well. Maximum enhancement in the polymerisation rate of N-vinyl pyrrolidone was noted with syndiotactic poly (methacrylic acid) as matrix.[56,57] The microstructure of daughter differed from that of poly (N-vinyl pyrrolidone) prepared under identical conditions by solution polymerisation. The matrix polymerisation systems consisting of vinyl sulphonic acid/on (2.2.2),4-ionene matrix (type I)[44] and MMA/on isotactic-PMMA matrix (type II)[10,11] exhibit considerable stereochemical effects. Blumstein *et al.*[44,36] quantitatively separated daughter polymer from the matrix. An increase in hetero- and isotactic triad contents was reported. In the second system, MMA/i-PMMA, the daughter polymer was highly syndiotactic, particularly at low conversions. Miyamoto *et al.*[58] reported even more significant stereochemical effect in a similar system which proceeded by anionic polymerisation. Inaki *et al.*[59] observed stereochemical effects in the free radical polymerisation of methacrylamide derivatives of nucleic acid bases such as adenine and uracil (monomer) in presence of matrix comprising of polymethacrylamide with complementary bases.

1.1.8 POLYETHYLENEIMINE AS MATRIX

Ferguson *et al.*[60] and Bamford *et al.*[39] independently investigated the polymerisation of acrylic acid on polyethyleneimine matrix. The product was a polycomplex of polyethylenei-

mine and polyacrylic acid. Ferguson *et al.* investigated the system in aqueous medium at 30°C with potassium persulphate as initiator. The matrix, polyethyleneimine, was found to form a redox pair with potassium persulphate.[60] The polymerisation rate was found to increase with increase in matrix concentration, attained a maximum value and then dropped. The maximum was found to occur at less than stoichiometric equivalence of polyethyleneimine with respect to acrylic acid. The polycomplex precipitated from the reaction medium. The possibility of graft polymerisation was excluded. The individual components of the polycomplex could be separated by paper electrophoresis. The composition of the polycomplex was difficult to estimate. Ferguson *et al.* assigned the discerned rate enhancement to:

* Interaction between monomer and matrix, giving rise a high local concentration of the monomer,
* Enhancement in the rate of initiation, due to redox pair formation between polyethyleneimine (matrix) and potassium persulphate and
* Heterogeneous polymerisation, which causes a decline in the termination rate coefficient.

Bamford *et al.*[39] ascribed the rate enhancement to the adsorption of acrylic acid molecules on polyethyleneimine prior to polymerisation. This gives rise a high local concentration of monomer. Bamford *et al.* employed a mixture of acetone and water (2:1 v/v) as the reaction medium. The polymerisation was homogeneous in this medium. The equilibrium constant for adsorption of acrylic acid onto polyethyleneimine was estimated to be 2.0×10^{-2} mol^{-1} lit.

1.2 ENZYME IMMOBILISATION

1.2.1 INTRODUCTION

Enzymes are highly specific biocatalysts. The word "immobilisation" means physical confinement on a suitable solid support. Immobilisation of enzymes by such confinement allows economic utilisation of enzyme in commercial processes. The first documented report on enzyme immobilisation, by Nelson and Griffin[91] in 1916, was the adsorption of invertase on charcoal and alumina, with retention of activity. This research area remained dormant for over four decades. Finally, between 1954 and 1961 a number of investigators opened up the great potentials of immobilised enzymes through very well planned and executed experimentations. Mclaren *et al.*[92] investigated adsorption of enzymes on porous inorganic materials. Katchalski *et al.*[93] studied covalent binding of enzymes to synthetic organic polymers. Mitz[94] used cellulose derivatives to chemically bind enzymes. Thus, inorganic materials were used to adsorb the enzymes while attachment to organic carriers was by covalent linkages.

In recent past, enzyme immobilisation has achieved immense importance since it offers innumerable advantages over soluble, free enzymes. A variety of techniques and carriers have been investigated in great detail for immobilising industrially attractive enzymes and cofactors. The immobilisation technique and the support of choice has to match the requirement of enzyme, processing details and end applications. There is no single universally preferred technique and support suitable for all enzymes. Presently, the annual technical out put in form of patents and papers has stabilized around 1200.[95]

1.2.1.1 Why Immobilisation

Enzymes are generally soluble in aqueous systems. The activity is affected irreversibly by small changes in the microenvironment such as pH and temperature. Additionally, enzymes can be used only once in a batch reactor. Separation of enzyme from the product stream entails an additional processing step. These disadvantages are circumvented by enzyme immobilisation.

1.2.1.2 Advantages of Immobilisation

Enzyme immobilisation enhances the scope to harness the complete potential of enzyme. Thus,

1. Enzymes can be used repetitively, till the activity of the enzyme decreases below economically viable levels. This offers considerable advantage for very expensive and rare enzymes.

2. The process details as well as post operations like separation are simplified. There is a greater control over the reaction. Reactions catalysed by free enzymes are terminated by altering the pH, substrate concentration or reaction temperature and enzyme cannot be reused. With immobilised enzyme, reaction can be stopped by simply modifying substrate flow rate through the immobilised system in plug flow reactor or by draining reactants and products in a stirred tank configuration.

3. Immobilised enzymes offer versatility in operation. Soluble enzymes can be used only in a batch mode but immobilised enzymes can be operated in different modes like plug flow, pack bed or continuously stirred tank reactors.

4. By immobilisation, the enzyme can be harnessed over a larger span of the catalytically active life. Additionally, half life of the enzyme ($T_{1/2}$) is enhanced. In many cases, thermal stability of the enzyme is also increased.

5. The reaction product is not contaminated with enzyme. This is a stringent requirement in food and pharmaceutical applications.

The methodology suffers from certain disadvantages. The limiting one is the loss in enzyme activity during immobilisation. This may decrease the economic viability. A number of immobilisation techniques are available. Each has its merits as well as demerits. They are discussed briefly under the respective headings.

1.2.1.3 Cell or Enzymes?

The choice between immobilisation of whole cell and cell free enzyme is dictated by process economics and end applications. Enzyme immobilisation is advantageous when:

i) The enzyme is extracellular.

ii) There is no significant loss in enzyme activity during immobilisation.

iii) The production process under consideration uses only one or two enzymes.

iv) Regeneration of the cofactors required for the process is easy.

v) The immobilised enzyme is stable under operating conditions.

Whole cell immobilisation is preferred when:

i) Enzyme is secreted within the cell (intracellular enzyme).

ii) The enzyme is unstable (when out of cell) during and after immobilisation.

iii) The substrate and products are not high molecular weight species.

Whole cell immobilisation is economical since enzyme need not be extracted and purified. Secondly, processes with multi step pathways may easily be conducted.

1.2.2 IMMOBILISATION STRATEGIES

A rich variety of immobilisation techniques are recorded in published scientific and patent literature. These may be grouped into the following categories.

1.2.2.1 Adsorption: Physical or Chemisorption on (a) rigid non porous surface or (b) Inside porous inorganic support.

1.2.2.2 Crosslinking of enzyme molecules with bifunctional reagents.

1.2.2.3 Covalent binding to carriers.

1.2.2.4 Entrapment method (microencapsulation).

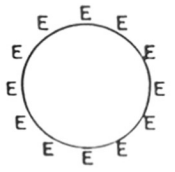
1.2.2.5 Immobilisation within semipermeable devices.

1.2.2.1 Adsorption

Adsorption on or inside porous inorganic support is the simplest method to immobilise enzymes (*Fig.* 7a & b) An aqueous solution of enzyme is contacted with the adsorbent. The resulting conjugate is washed to remove non-adhered enzyme. A wide variety of adsorbent have been investigated. The intensively investigated adsorbents are alumina, silica, controlled pore glass (CPG), carbon and kaolinite. The adsorption is mainly through cohesive forces. However, other forces like Van der Waals and hydrogen bonding also contribute. Adsorption depends on the ionic strength of the medium and on the nature of various types of interactions between the enzyme, the adsorbent and other solutes. The adsorption efficiency, enzyme accessibility to the adsorbent and stability of the resulting conjugate, depend upon surface area and on pore size and pore size distribution of the support. Whole cells, containing enzyme, can also be adhered to inorganic adsorbents.[96] This cell adhesion depends the nature of the interaction between the interface of cell wall and adsorbent. The adsorbed enzyme is crosslinked with bifunctional reagents like glutaraldehyde to enhance the immobilisation. The crosslinking generates an effective envelope and provides good conformational and operational stability to the enzyme. This technique was used to immobilise urease on kaolinite.[97]

Adsorption is a simple technique. The method is cheap. A variety of carriers are readily available. However, the method has a number of shortcomings. Adsorption is weak and

(a)

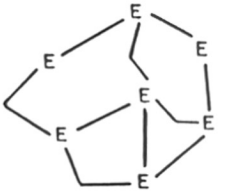


ADSORPTION ON NON POROUS MATRIX

(b)

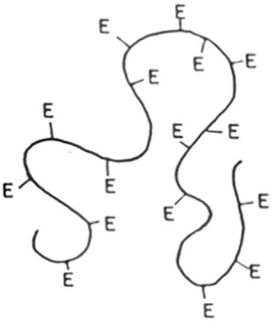
ADSORBED INSIDE THE POROUS SUPPORT MATRIX

(c)



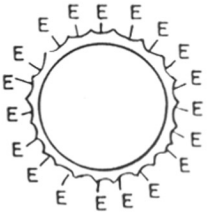
CROSSLINKING OF ENZYME TO ENZYME WITH BIFUNCTIONAL AGENTS AND WITHOUT SUPPORT

(d)



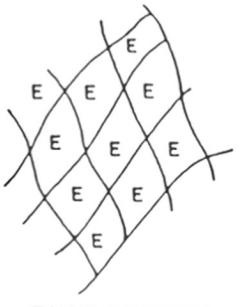
COVALENT BINDING TO POLYMER

(e)



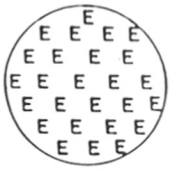
COVALENTLY BOUND TO THE POLYMER COATED ON INORGANIC SUPPORT

(f)



ENTRAPMENT

(g)



MICROENCAPSULATION

E-ENZYME

FIG. 7 : POSSIBLE MODES OF ENZYME IMMOBILISATION

leads to an easy loss of enzyme through desorption. The life of the support is very short. Changes in pH and ionic strength of the medium would result in easy desorption of the enzyme from the support.

1.2.2.2 Crosslinking of enzymes

The uses of multifunctional low molecular weight reagents employed in crosslinking of enzymes have been reviewed by Wold[98] and Fasold.[99] The multifunctional reagents used are presented in *Figure 8*. Crosslinking efficiency depends on concentrations of crosslinking reagent and reactive functional groups present in nonactive regions of the enzyme. Other important parameters are pH, ionic strength, reaction temperature and time. The technique is schematically depicted in *Figure 7c*. Glutaraldehyde is the most frequently employed bifunctional reagent. Jansen and Olsen[100] immobilised papin by this method.

The process is simple and inexpensive. The crosslinked enzyme is obtainable as gels, membranes or as spheres. However, the process may necessitate drastic conditions involving wide fluctuation in pH and temperature as well as high concentration of reacting components. This may result in considerable deactivation of the enzyme.

1.2.2.3 Covalent binding

This is probably the ideal technique to immobilise enzymes. Enzyme is attached to a solid, termed as "support", through strong covalent bonds (*Figure 7d*). The support could be natural/synthetic polymer or inorganic material. It must possess hydroxyl, carboxyl, amino or sulphydryl functional groups. Covalent binding overcomes major shortcomings in adsorption technique which originates from enzyme loss during repetitive use and results in product contamination. It is imperative that the tertiary structure of enzyme is unaltered to prevent loss of activity. The covalent binding reactions should be amenable in aqueous buffer, under very mild conditions of pH and temperature. Grubhoffer *et al.*[101] immobilised

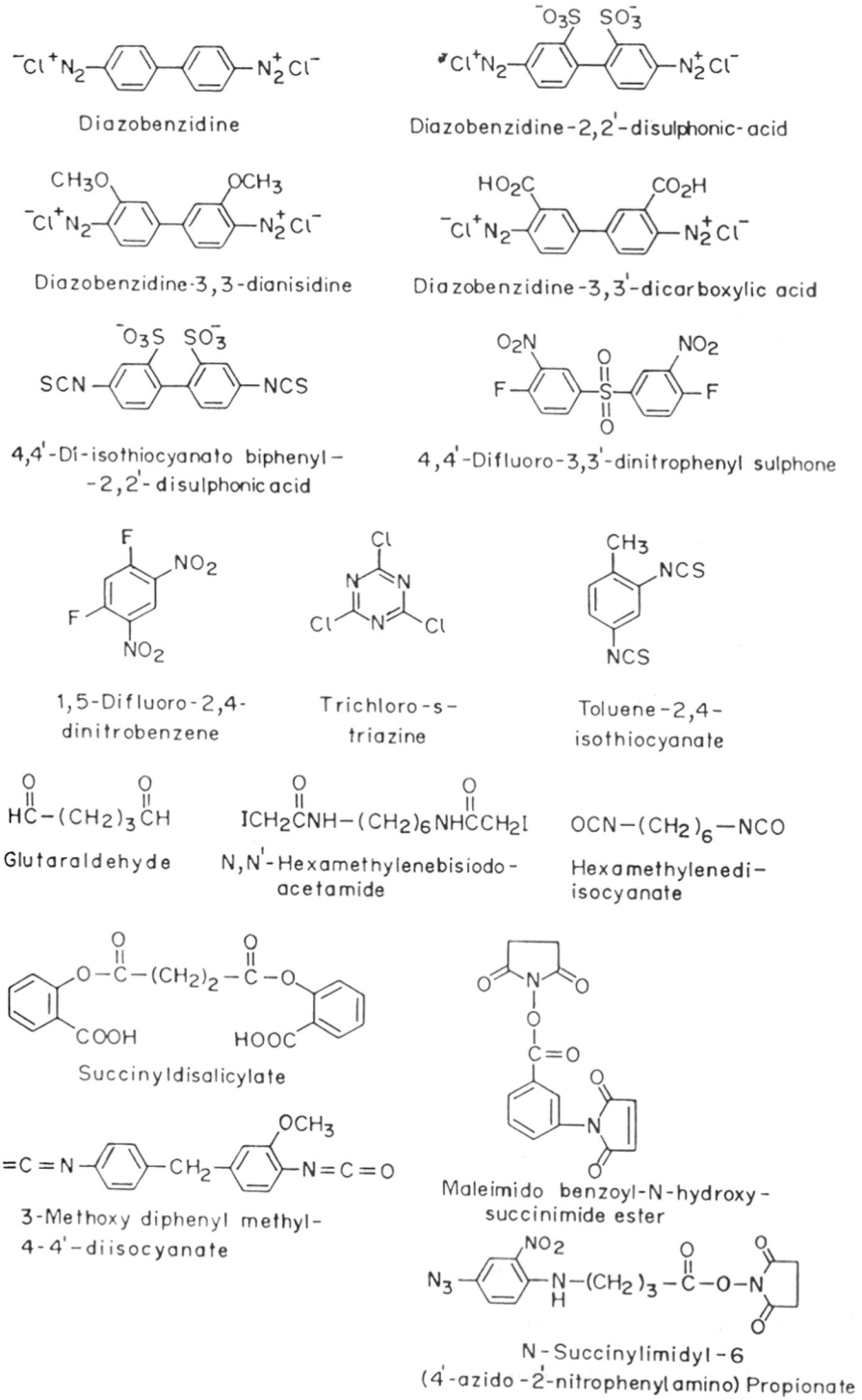


Fig. 8. Multifunctional Reagents Used for Crosslinking of Enzymes

α –amylase, pepsin, carboxypeptidase on diazotised poly (amino styrene), by this technique. Covalent binding can also be achieved through reactive polymer coated on to porous/non porous inorganic materials (*Figure 7e*).

Several functionalised carriers are commercially available in various physical and chemical forms. This technique may be used to generate immobilised system in forms required for a specific reactor.

During the covalent attachment the enzyme is contacted with chemicals, experiences fluctuations in pH and ionic strength which may alter the tertiary structure and hence the activity.

1.2.2.4 Entrapment method

Physical entrapment in porous polymeric supports is extensively employed for immobilising whole cells. In this method, a crosslinked polymeric network is formed around the enzyme. Enzyme molecules are trapped physically within the network. This prevents the leaking out of enzyme from the support, without posing any difficulty for the movement of substrate into and products out of the network. Enzyme may be entrapped within gel particles (gel entrapment), fibre (fibre entrapment) or in the pores of semipermeable polymeric membranes (microencapsulation). The pore dimensions in polymeric network and physico-chemical interactions between enzyme and polymeric network are factors which govern enzyme retention.

1.2.2.4.1 Gel entrapment

This involves the occlusion of enzyme within the crosslinked water-insoluble polymer gels (*Figure 7f*) Gels made from naturally occurring polymers such as calcium alginate, κ-carrageenan, collagen, gelatin and agar have been investigated. Gelation is induced by the incorporation of multivalent ions. Calcium ions decrease the solubility of alginates. Dropwise addition into a solution of calcium chloride transforms sodium alginate into calcium alginate beads. Further treatment with

Al^{+3} ions enhances the mechanical strength. N,N'-methylene bis acrylamide (BIS) crosslinked polyacrylamide, the most widely used synthetic gel, was first investigated by Bernfeld and Wan[102] for the entrapment of papin, trypsin and chymotrypsin. The gel block was dispersed into particles of required mesh size.

1.2.2.4.2 Fibre entrapment

In the fibre entrapment technique, enzymes are immobilised within micro-cavities of a synthetic fibre. This technique was first reported by Dinelli *et al.*[103] Aqueous enzyme solution is emulsified with polymer, dissolved in an organic solvent immiscible with water and extruded through spinnerets into a solvent to precipitate the polymer entrapped enzyme in fibre form.

The fibres are stable to high ionic strength and are resistant to weak acids and alkali. This technique can be applied for immobilising whole cells as well as enzymes. However, organic solvents used to solubilise the polymer and to precipitate it in fibre form may deactivate the enzyme. Cellulose acetate has been the primary choice due to low cost and good biological resistance. The technique has been used to immobilise D-glucose isomerase,[104] amino acylase[105] and penicillin amidase.[106]

1.2.2.4.3 Microencapsulation

In microencapsulation, enzymes are encased in semipermeable polymer membranes (*Figure 7g*). Enzymes, larger than the mean pore diameter of the membrane, are trapped within its walls. Substrate as well as product molecules, of smaller size, can easily diffuse in and out through the walls. This technique was first employed by Chang[107,108] in 1960 (See section 2.10.25). The two general methods used to prepare semipermeable microcapsules for enzyme immobilisation are based on: (i) Coacervation and (ii) **interfacial polymerisation.**

Microcapsules have been made by coacervation technique from collodion (cellulose nitrate), polystyrene and cellulose acetate/butyrate.

Enzymes have been entrapped in polyamide microcapsules by interfacial polymerisation of 1,6-diamino hexane and sebacoyl chloride in presence of the enzyme. The aqueous phase consists of enzyme and diamine. Sebacoyl chloride is dissolved in chloroform-cyclohexane mixed solvent and polymerisation occurs at the interface. Capsules are obtainable in a variety of bead sizes ranging from millimicron to few millimeter. Microencapsulation offers very large surface area for the bioconversion reactions. The technique is ideally suited for the simultaneous entrapment of several enzymes within a single microcapsule, to conduct a sequence of chemical transformations within a single reactor configuration. However, the technique is not suited to high molecular weight substrates. Enzyme loss by leakage is a distinct possibility, since entrapment within the microcapsule is only by physical confinement.

In general, entrapment causes very less disruption in the tertiary structure of enzyme. Since Enzyme does not contact chemical reagents, it can be immobilised with only marginal or no loss in its activity.

1.2.2.5 Immobilisation within semi-permeable devices

1.2.2.5.1 Dialysis membranes

An easy process of immobilisation is to pour enzyme/cell solution into a dialysis bag and to immerse the bag in substrate solution. Dialysis tubing are available commercially in different molecular weight cut offs. Substrate molecules diffuse to react with enzyme and the product formed diffuses out. However, the reaction rate is hampered by diffusional constraints.

1.2.2.5.2 Ultrafiltration Membranes

Enzyme solutions are purified and concentrated using ultrafiltration membranes. The repetitive concentration of enzymes was first studied in 1960. Entrapment within ultrafiltration membrane or hollow fibre membranes is by physical confinement. This technique of immobilisation was first reported by Rony.[109]

Enzyme solution is placed in cell cavity above the ultrafiltration membrane with a low molecular weight cut off. The cell is simultaneously pressurised and magnetically stirred. Sufficient time is provided for high conversions. The operating pressure is varied inversely with respect to the molecular weight of the substrate. The low molecular weight enzymes, which are too small to be retained by the membrane, may be chemically modified to be suitable for entrapment.

1.2.2.5.3 Hollow Fibre Devices

Hollow fibre devices offer an extremely large surface to volume ratio. This technique was simulated theoretically prior to the actual experimental studies. Enzyme may be incorporated either inside or outside the hollow fibre semipermeable membrane. The operative principle is similar to that in ultrafiltration membranes. The walls of the hollow fibres are permeable to low molecular weight substrate and products molecules but are impermeable to the larger enzyme molecules. Substrate solution passed around the outside of fibres, diffuses through the fibre wall and reacts with the enzyme. The reaction product diffuses back across the fibre wall into the fluid stream. This technique was first used to immobilise alkaline phosphatase and chymotrypsin.[110]

Entrapments in hollow fibre too is a non-chemical method of immobilisation. The immobilised enzymes retain the normal activity. Many enzymes can be simultaneously immobilised within a single system.

1.2.3 SUPPORTS

The supports used in immobilisation may be classified under three broad categories.

1.2.3.1 Natural Polymers

1.2.3.2 Synthetic Polymers

1.2.3.3 Inorganic Materials

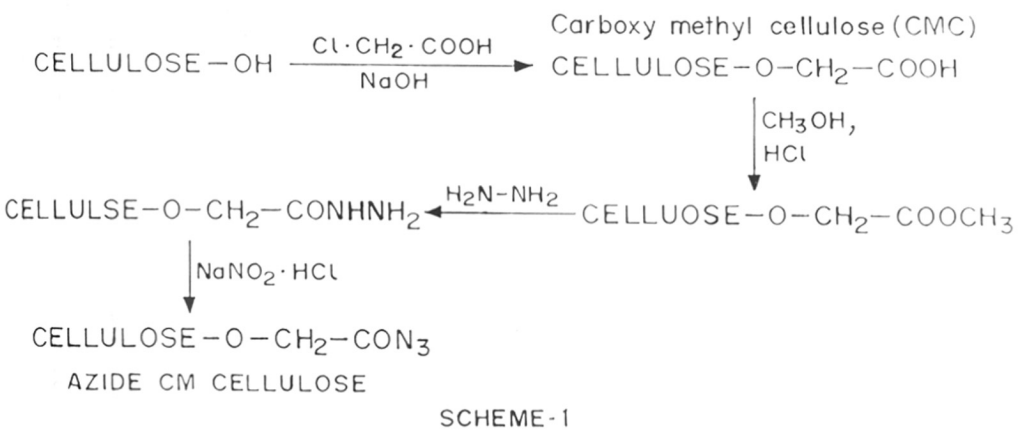
1.2.3.1 Natural Polymers

The natural polymers used extensively are: (i) Cellulose and its derivatives, (ii) Agarose and dextrans, (iii) Starch and its derivatives, (iv) Carageenan and (v) Chitin and chitosan.

1.2.3.1.1 Cellulose and its derivatives

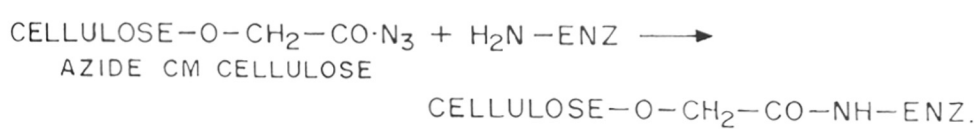
Carboxy methyl cellulose azide is the earliest example of semi-synthetic support used to covalently bind enzymes. It is generated by modifying the hydroxyl group of cellulose.

Sequential reactions are:



SCHEME-1

The enzyme attachment to this support is an easy one step process.



SCHEME-2

This immobilisation procedure was used by Mitz and Summaria[111] to bind chymotrypsin and trypsin.

1.2.3.1.2 Agarose and dextran derivatives[112,113]

Cyanogen bromide activated agarose and dextran are widely used. Spherical agarose gel particles are marketed as Sepharose.@ Dextran gel is commercially known as Sephadex.@ These are available in various grades, determined by the molecular exclusion limits.

Agarose is structurally more stable and less prone to microbial attack than dextran gels. Dextran and agarose are activated with cyanuric chloride and carbodiimide prior to immobilisation. Cyanuric chloride activates the hydroxyl groups and carbodiimide activates the carboxyl groups.

1.2.3.1.3 Starch derivatives[114]

Insoluble functionalised starch derivatives are prepared to covalently bind enzymes. Starch is converted into dialdehyde by oxidation, derivatised with 4,4'-diamino diphenyl methane to form insoluble azomethine derivatives which are reduced with sodium borohydride to generate primary amino groups. These are diazotised to facilitate covalent binding to enzymes. The methodology is presented in *Figure* 9.

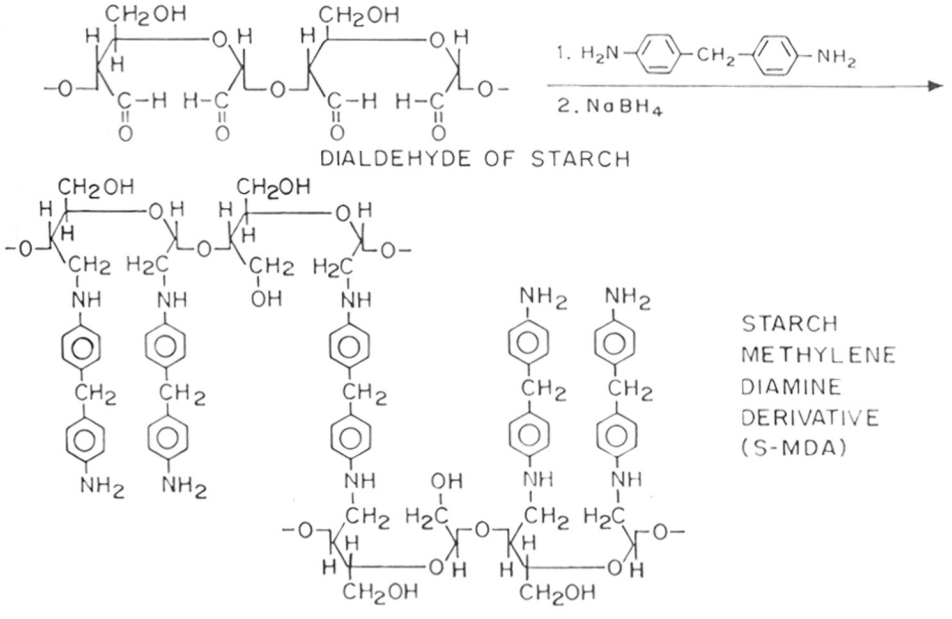
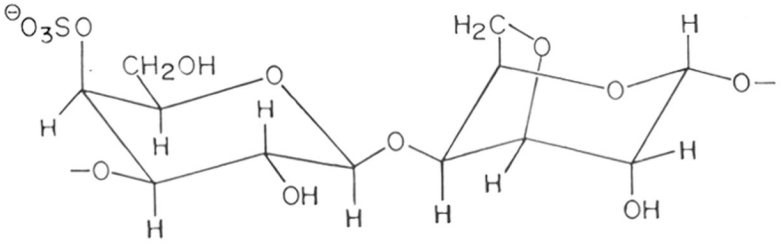


FIGURE-9

1.2.3.1.4 Carrageenan

Carrageenan consists of galactans extracted from red algae. The galactose units are partially esterified with sulphuric acid (IV).Chibata *et al.*[115] evaluated the relative performances of gamma, kappa and iota carrageenans. The performances of kappa and iota forms were

better. Of these, kappa form was found to be the superior support. Carrageenan is mechanically stable above pH 4.5. At lower pH, the weak 1,4-glycosidic linkage between galactose residues tends to break down.



(Ⅳ) k-CARRAGEENAN

1.2.3.1.5 Chitin and Chitosan[116,117]

Chitin, a by-product of fishing industry, is built-up β1,4-linkages of 2-acetamido-2-deoxy-D-glucopyranosyl units. Chitosan is similar. The repeating 2-amino-2-deoxy-D-glucose units are linked together by β 1,4-linkages. Hydrolysis of chitin with concentrated alkali solution generates chitosan. Chitin is treated with glutaraldehyde to covalently bind enzyme. Alternatively, enzyme may be adsorbed on to chitin and subsequently crosslinked using glutaraldehyde. Chitosan has also been found suitable as an immobilisation support in combination with glutaraldehyde.

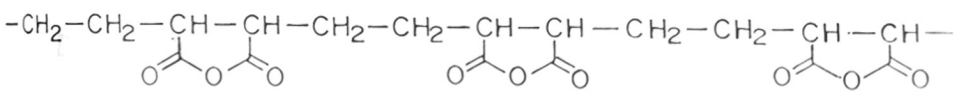
1.2.3.2 Synthetic Polymers

A variety of hydrophilic / hydrophobic, derivatised / functionalised reactive polymers have been investigated for their performances as suitable immobilisation supports. Among these, the polymers investigated in some detail are:

(i) Ethylene-maleic anhydride copolymer, (ii) Modified polyalkyl amides, (iii) Derivatives of polystyrene, (iv) Polyamines, (v) Polyamides, (vi) Silicones, (vii) Ion exchange resins and (viii) Epoxy polymers.

1.2.3.2.1 Ethylene-Maleic Anhydride copolymers[118]

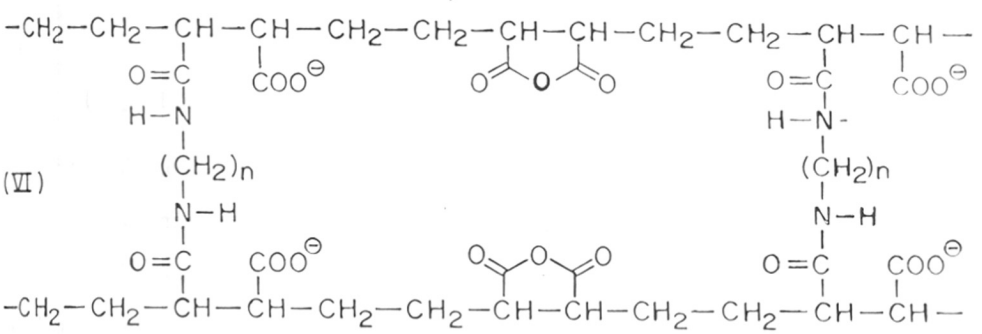
This support has been studied rather extensively since 1964 with a variety of enzymes. This commercial polymer is partially crosslinked with ethylene diamine / hexamethylene diamine, to render it water insoluble. Polymer has the following structures (Ⅴ, Ⅵ) prior to and after crosslinking:



(Ⅴ) UNCROSSLINKED POLYMER

+

$H_2N-(CH_2)_n-NH_2$ n = 2 or 6



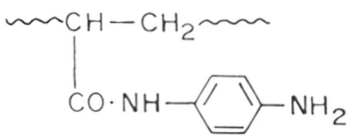
(Ⅵ)

The crosslinked polymer must possess free anhydride group to be suitable for covalent binding of enzymes.

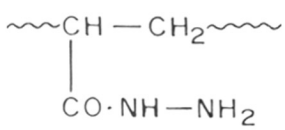
1.2.3.2.2 Modified Polyacrylamide[119-121]

A variety of water insoluble crosslinked polyacrylamides have been examined. Functionalised polyacrylamide derivatives are commercially marketed under the trade names Enzacryl® AA, Enzacryl® AH and Enzacryl® polythiol.

Enzacryl supports have the following structure:

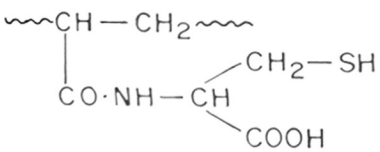


(VII) ENZACRYL AA



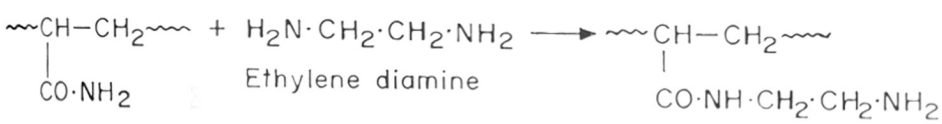
(VIII) ENZACRYL AH

Enzacryl AA and Enzacryl AH are activated by diazotisation of primary amino group



(IX) ENZACRYL POLYTHIOL

Crosslinked polyacrylamide gel beads are derivatised into reactive supports by additional chemical transformations. Such supports covalently bind enzymes under mild reaction conditions. The degree of crosslinking is controlled by varying the relative mole ratio of divinyl comonomer, N,N'-methylene bis acrylamide. The crosslinked supports offer uniform bead size / porosity and good chemical stability. The enzyme coupling is easily achieved. Polyacrylamide gel beads are also functionalised with ethylene diamine as depicted scheme 3 .



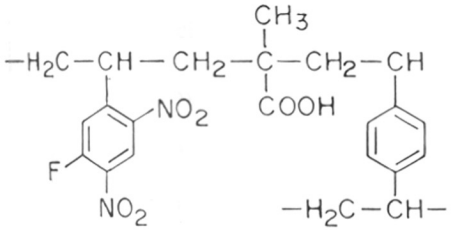
SCHEME-3

This reaction proceeds with ease at 50°C in an excess of ethylene diamine. Polyacrylamide gel beads can also be derivatised into polymers with aldehyde pendent groups, by reacting with excess of glutaraldehyde. This reaction proceeds with appreciable ease at 33°C below pH 6.9.

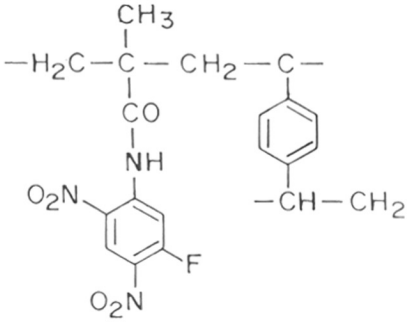
1.2.3.2.3 Polystyrenes

Functionalised hydrophilic/hydrophobic derivatives of polystyrene have also been investigated as suitable supports. Polystyrene is nitrated and reduced into poly (p-amino styrene). Reactions with nitrous acid, phosgene and thiophosgene transforms poly (amino styrene) to active diazonium, isocyanato and isothiocyanato derivatives suitable for enzyme binding.

The diazonium derivative has been used to immobilise α-amylase, pepsin, and ribonuclease.[101] Catalase has been immobilised using isocyanato derivative.[122] The hydrophobic nature of polystyrene limits its usefulness in binding hydrophilic enzymes. Thus, proteins cannot be loaded in sufficient quantities required in commercial processes. This drawback is circumvented by copolymerising styrene with hydrophilic comonomers such as acrylic and methacrylic acids. Nitrated terpolymers of 3-fluorostyrene, methacrylic acid and divinyl benzene as well as methacrylic acid-3-fluroanilide, methacrylic acid and divinyl benzene have been used successfully as supports.[123,124] The part structure (Ⅹ ,ⅪⅠ) of these polymers are:



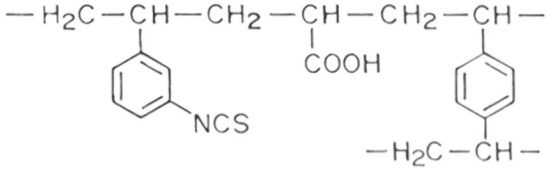
(Ⅹ)



(ⅪⅠ)

Porous beaded supports have been synthesised by terpolymerisation of 3-isothiocyanato styrene, acrylic acid, divinyl benzene. The part structure of this terpolymer is as follows (ⅫⅡ).

(ⅫⅡ)



The pore size and pore size distribution is controlled by varying the relative concentration of crosslinking agent, divinylbenzene.

1.2.3.2.4 Acrylic Polymers

Acrylic polymers are derivatised and used to entrap enzymes. The mechanical stability and resistance to microbial attack are good. A few polymers in this class can couple enzymes in underivatised form. Various acrylic polymers used in the immobilisation of enzymes are:

a) Polyacrylates and polymethacrylates: These are either homopolymers of acrylic acid, methacrylic acid or copolymers with acrylamide. These polymers need activation with carbodiimide. Copolymer of acrylic acid and acrolein, with pendent aldehyde and carboxyl groups, has also been used.[125] The covalent attachment of the enzyme occurs via the carbonyl pendent group.

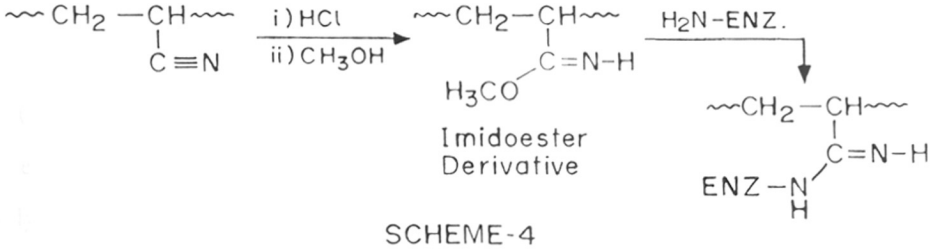
b) Poly (methacrylic anhydride): The anhydride linkages react directly with enzymes. The carboxylate anion generated by the coupling reaction shifts the pH downwards to lower values, which adversely affects the stability and activity of the immobilised enzyme.[126]

c) Poly (hydroxy alkyl methacrylates): These synthetic analogs of polysaccharides possess good mechanical strength and are resistant to the microbial attack. Porous spherical beads can be prepared by free radical suspension copolymerisation with a divinyl comonomer. Porosity can be controlled by regulating the amount of crosslinking reagent. Copolymers of 2-hydroxy ethyl methacrylate and ethylene dimethacrylate are widely investigated for immobilising a variety of enzymes.[127,128] The polymers can be modified by specific chemical transformations or by copolymerisation. Cyanogen bromide activation have also been recorded. Terpolymer of hydroxy alkyl methacrylate, a crosslinking agent and 4-acetamino phenyl ethoxy methacrylate directly couples the enzymes.[129]

d) Glycidyl methacrylate polymers: Oxirane (glycidyl) group can be used as is or easily modified to react very rapidly with enzyme under ambient conditions. The widely used polymers are crosslinked glycidyl methacrylate copolymers. The non-ionic oxirane doesn't shift the pH during immobilisation. The resultant bond is very stable.

The hydrophobic poly (glycidyl methacrylate) is copolymerised with water soluble monomers to enhance the hydrophilicity. Krammer[130] prepared suitable support by copolymerisation of glycidyl methacrylate and acrylamide. Internal crosslinking was prevented by synthesising at low temperatures and storing in cold. Svec and Kalal[131,132] reported the use of copolymers of glycidyl methacrylate and ethylene dimethacrylate as a support.

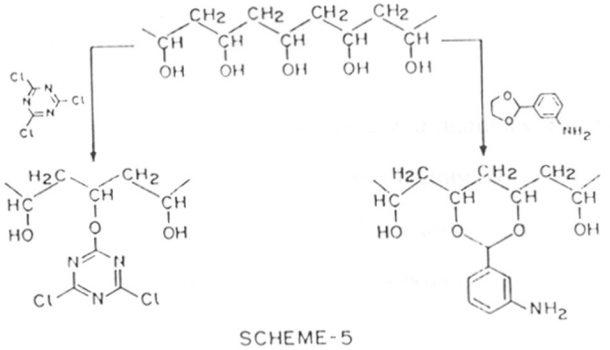
e) Acrylonitrile copolymers: These polymers are easily transformed to reactive imidoester derivative by reaction with hydrogen chloride gas in cool dry methanol (scheme 4), which covalently binds to amino group of enzyme.[133]



SCHEME-4

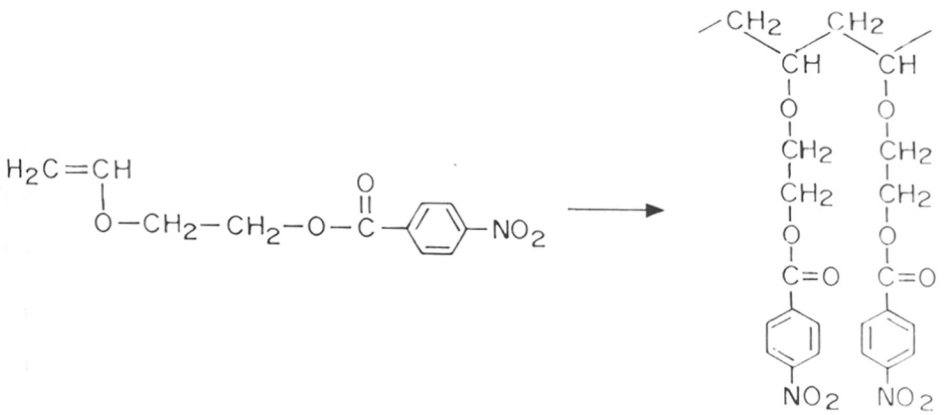
1.2.3.2.5 Polyols

Poly vinyl alcohol (PVA) and poly (allyl alcohol) are modified into water insoluble hydrophilic solids suitable for enzyme immobilisation. Thus, PVA, crosslinked with tere phthaldehyde, is activated with 2-(3-aminophenyl)-1,3-dioxolane, or cyanuric chloride for reaction with enzyme.[134] The reactions are depicted in scheme 5.



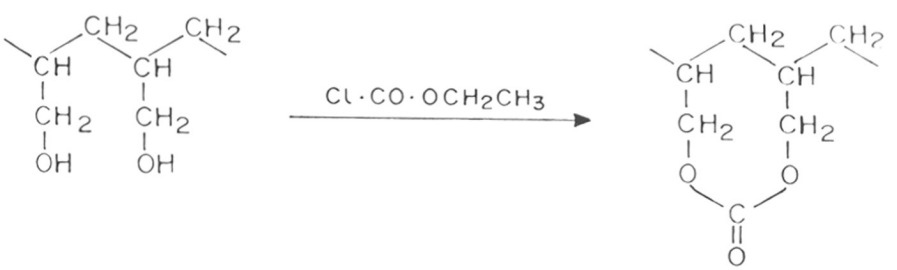
SCHEME-5

Reactive poly vinyl ethers are prepared by cationic polymerisation of 2-vinyl oxyethyl 4-nitro benzoate (scheme 6). The nitro groups on reduction to aryl amino groups are diazotised to generate reactive groups for enzyme coupling.



SCHEME-6

Poly (allyl alcohol) are transformed into reactive cyclic carbonate derivatives, by reaction with ethyl chloroformate (scheme 7) , which are very reactive and bind enzymes efficiently.[135,136]



SCHEME-7

1.2.3.2.6 Polyamides

Polyamides, the condensation products of diacid and diamines, can be made in various physical forms like hollow fibres, foils, tubes, membranes and powders with good mechanical strength and hydrophilic character. These are resistant to microbial attack. The only binding sites in polyamides are terminal carboxyl and amino groups. Additional

binding sites need to be generated to bind sufficient concentration of enzyme. Polyamides are partially hydrolysed with acid. The resulting amino or carboxyl groups are activated by alkylation either at nitrogen (N-alkylation) or at oxygen (O-alkylation) atom (See section 1.2.10.2.3).

1.2.3.3 Inorganic Materials

Porous and non-porous alumina, column grade silica, controlled pore glass beads (CPG), carbon (charcoal), Kaolinite, controlled pore titania, zirconia, nickel oxide, bentonite, fuller's earth, diatomaceous earth, and wollastonite have been used as supports for enzymes.

Inorganic carriers are very cost effective supports for cheaper enzymes. The advantages of these supports are:

* High mechanical strength and good rigidity
* Resistance to microbial attack
* Insolubility
* Thermal stability and stability to organic solvents.
* Excellent shelf-life
* Easy regeneration
* Structural stability

The adsorbed enzyme may further be crosslinked. Inorganic supports can be porous and non porous. Porous supports offer a high surface area per unit weight. Larger amounts of enzyme are adsorbed, within the pores, well protected from the external environment. The support must ensure easy diffusion of substrate and product. The ideal pore diameter would differ with the enzyme.

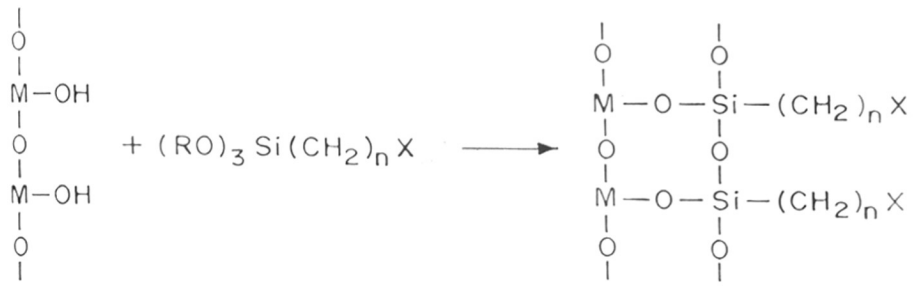
Controlled pore glass (CPG), a macroporous borosilicate glass, has been extensively investigated.[137] Controlled pore glass is unstable in alkaline pH. It is brittle and suffers from attrition problems. A zirconia coating is applied to increase the durability of the support.[138] The high cost

of CPG renders it unsuitable for industrial applications. Other porous inorganic supports used are silica, alumina and titania. Alumina and titania are suitable for applications involving alkaline pH and silica is good for acidic solutions. Naturally occurring porous materials such as kieselghur,[139] pumice stone,[140] sand[141] and bentonite[142] have also been investigated. Very wide pore size distribution is the major disadvantage.

Non-porous inorganic supports have low surface area. Enzyme loading is marginal and is localised on the surface. However, diffusional constraints are eliminated. Decreasing the particle size, with the objective of increasing the surface area, results in a unfavourably large drop in pressure. Simultaneously, adsorption of product becomes a major problem. Sand is most attractive from the process economics perspectives. Other non-porous supports such as magnetic oxide, nickel and stainless steel are found to be suitable for fluidised bed enzyme reactors.

Enzyme binding efficiencies are enhanced by coating porous/non-porous inorganic supports with a thin layer of polymer. This layer is functionalised / derivatised to incorporate reactive groups to bind enzymes. This strategy has been used to immobilise glucose isomerase on to porous alumina coated with polyethyleneimine / poly (amino styrene). The process details are presented in section 1.2.10.2.1

Covalent binding of enzymes directly to the inorganic supports is very difficult though not impossible. Weetal[143,144] achieved covalent binding through silanisation. Trialkoxy silane reagents condense with hydroxyl group of oxidised inorganic carrier to form strong and stable metal-oxygen-Si linkage (scheme 8).



SCHEME-8

where X = - NH_2,  , -I, -Br, -Cl

-CHO, -CH-$(OC_2H_5)_2$, M - metal

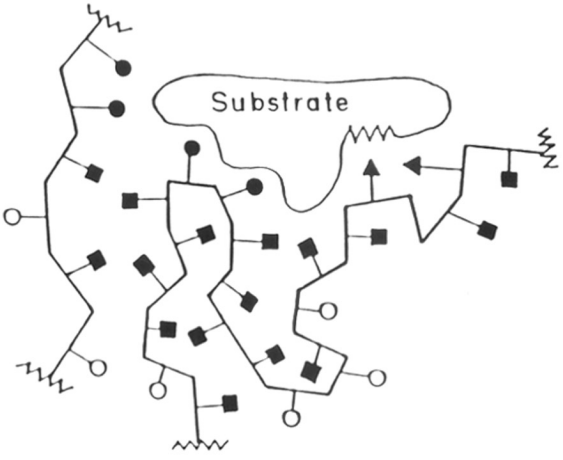
3-aminopropyl triethoxy silane, the widely used reagent gives alkylamine derivative. Amino alkylated controlled pore glass, activated with glutaraldehyde, was introduced by Corning Glass Works, USA to bind enzyme at neutral pH. Glycidoxy propyl trimethoxy silane, another derivatised CPG support, has been used to immobilise hexokinase with a higher retention of activity.

1.2.4 ACTIVE SITE OF ENZYME

One of the unique properties of enzymes is their specificity in catalysing chemical transformations. This arises from the presence of substrate specific active sites in enzymes, as schematically represented in *Fig*. 10 . According to Koshland,[145] enzyme molecules comprise of four different types of amino acid residues.

* The non essential ones located at the surface of an enzyme. These have no direct or indirect contribution towards catalytic activity. These may be cleaved off without loss in catalytic activity.

* Residues which help in retaining the tertiary structure intact. Perturbations in these residues disrupts the active site resulting in loss of activity.



●:BINDING SITES , ▲ : CATALYTIC SITES , ww:SUBSTRATE BOND,
O:NON ESSENTIAL AMINO ACID RESIDUES, ■ : AMINO ACID RESIDUES HELP TO MAINTAIN TERTIARY STRUCTURE

FIG. 10 : SCHEMATIC REPRESENTATION OF THE ACTIVE SITE

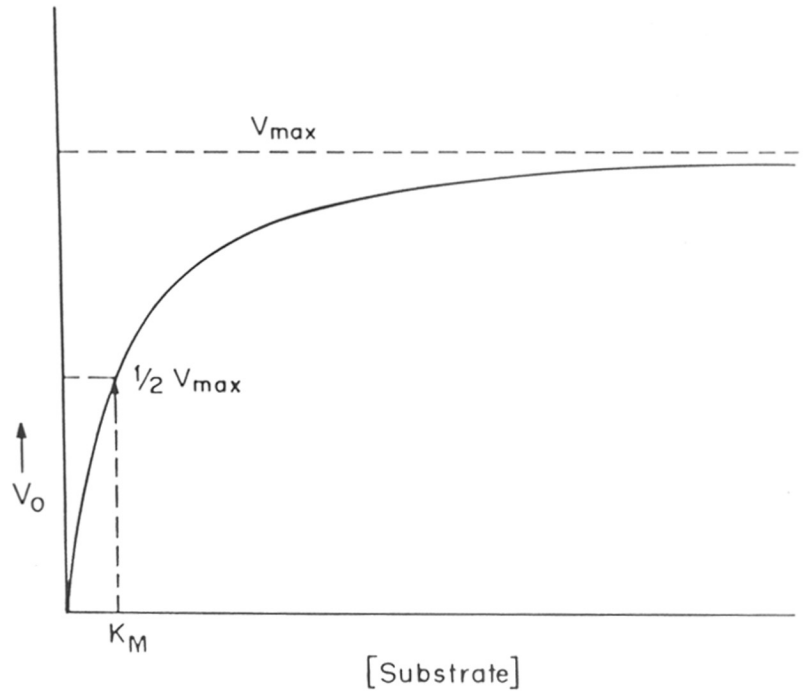


FIG. 11 : EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF ENZYME CATALYSED REACTION

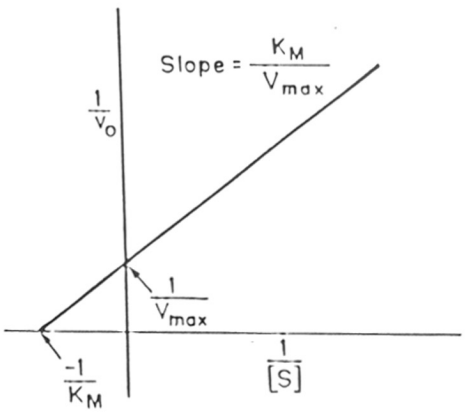


FIG. 12 : LINEWEAVER-BURK PLOT

Binding residues. These hold the substrate molecules in position and orient them towards the active site (catalytic residues).

Catalytic residues. These are the most critical domains. Damage to these amino acid residues lead to complete enzyme inactivation, since these are actually involved in the catalytic chemical transformations.

Enzymes are identified by their classification number. The number is assigned on the basis of the reaction they catalyse. Enzyme Penicillin G acylase (PGA) or Penicillin amidase, used in the present investigation has classification number EC 3.5.1.11 where, EC stands for Enzyme Commission. The number comprises of four digits separated by points. The first digit (3) denotes the class to which this enzyme belongs to (Hydrolases). The second digit (5) ihdicates sub-class (Enzyme acting on C-N bonds). The third digit (1) indicates sub-subclass (Enzyme acting on linear side chain amide bonds) and fourth digit (11) designates the serial number of enzyme in its sub-subclass.

1.2.5 KINETICS OF ENZYME CATALYSED REACTIONS

Enzyme-catalysed reactions follow the general principles of a chemical-reaction kinetics. However, enzyme catalysed reactions exhibit an unique feature of substrate saturation. The effect of substrate concentration on the rate of a simple (one substrate) enzyme catalysed reaction is depicted in *Figure*. 11 . At low substrate concentration, initial reaction rate ("V_o") is directly proportional to substrate concentration. The reaction shows a first order dependence with respect to substrate. The initial rate tends to decrease with increase in substrate concentration and the reaction rate is no longer proportional to substrate concentration. The reaction has a mixed order in this concentration regime. The reaction rate is unaffected by further increase in substrate concentration. The reaction becomes essentially zero order with respect to substrate. The enzyme is saturated with the substrate at this concentration. This effect is observed with all enzymes, but at differing substrate concentrations, which are unique to each enzyme.

The earliest general theory for simple enzymatic reactions involving only one substrate was developed in 1913 by Michaelis and Menten. This forms the basis of quantitative analysis of all aspects of enzyme kinetics and inhibition. The basic assumption was that enzyme E combines with substrate S to give enzyme-substrate complex, ES, which breaks down in second step to generate free enzyme and product P.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \qquad (1)$$

$$ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E + P \qquad (2)$$

These reactions were assumed to be reversible.

The Equation gives a mathematical relationship between initial rate of enzyme-catalysed reaction, concentration of a single substrate and certain characteristics of the enzyme.

Initial rate is the rate at which enzyme-substrate [ES] complex breaks down to the products. According to Equation (2), the first order rate expression may be written as

$$V_o = k_{+2}[ES] \qquad (3)$$

Rate cannot be evaluated from Equation (3) since neither k_{+2} nor [ES] can be determined directly. A second order rate expression for formation of ES from E and S can be written from Equation (1)

$$\frac{d[ES]}{dt} = k_{+1}([E_T] - [ES])[S] \qquad (4)$$

where $[E_T]$ = Total enzyme concentration (free + combined form)

[ES] = The concentration of enzyme-substrate complex

[S] = Substrate concentration (assumed to be far greater than [E])

k_{+1} = Second order rate constant

The expression for the breakdown of ES is

$$-\frac{d[ES]}{dt} = k_{-1}[ES] + k_{+2}[ES] \quad (5)$$

since [ES] breaks down in two different steps, one into product and other into E + S (i.e reverse reaction or Equation 1).

Under steady state conditions

Rate of formation of [ES] = Rate of disappearance of [ES]

$$k_{+1}([E_T] - [ES])[S] = k_{-1}[ES] + k_{+2}[ES] \quad (6)$$

Rearranging Equation (6)

$$\frac{[S]([E_T] - [ES])}{[ES]} = \frac{k_{-1} + k_{+2}}{k_{+1}} = K_m \quad (7)$$

The constant K_m, written for

$\frac{k_{-1} + k_{+2}}{k_{+1}}$ is called Michaelis-Menten constant

Equation (7), can be solved for [ES] to compute the steady-state concentration

$$[ES] = \frac{[E_T][S]}{K_m + [S]} \quad (8)$$

from Equation (3), the initial rate V_o is

$$V_o = k_{+2}[ES] \quad (9)$$

substituting the value of the $V_o$in Equation (8)

$$V_o = k_{+2}\frac{[E_T][S]}{K_m + [S]} \quad (10)$$

When the enzyme is saturated with substrate, such that all enzyme molecules are transformed into enzyme-substrate complex, initial velocity reaches a maximum given by

$$V_{max} = k_{+2}[E_T] \quad (11)$$

Substituting V_{max} for k_{+2} $[E_T]$ in Equation (10)

$$V_o = \frac{V_{max}[S]}{K_m + [S]} \quad (12)$$

Equation (12) is the rate expression for enzyme-catalysed reaction involving one substrate, called as Michaelis-Menten Equation.

When initial rate is one-half of the maximum velocity (V_o = 1/2 V_{max}), Equation (12) takes the form

$$\frac{V_{max}}{2} = \frac{V_{max}[S]}{K_m + [S]} \quad (13)$$

Dividing both sides by V_{max},

$$\frac{1}{2} = \frac{[S]}{K_m + [S]} \quad (14)$$

On rearranging, Equation (14) takes the form

$$K_m + [S] = 2[S] \quad (15)$$

$$K_m = [S] \quad (16)$$

From Equation (16) it becomes apparent that K_m is the substrate concentration at which initial rate is one-half of the maximum rate

Determination of K_m:

After transformation, the Michaelis-Menten expression (Equation 12) is utilised for plotting the experimental data and to compute K_m

Taking the reciprocal of both sides, equations (12) can be written as

$$\frac{1}{V_o} = \frac{K_m + [S]}{V_{max}[S]} \qquad (17)$$

After rearrangement

$$\frac{1}{V_o} = \frac{K_m}{V_{max}[S]} + \frac{[S]}{V_{max}[S]} \qquad (18)$$

Equation (18), takes the final form of

$$\frac{1}{V_o} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (19)$$

This Equation (19) is termed as Lineweaver-Burk Equation. The plot of $\frac{1}{V_o}$ against 1/[S], Lineweaver-Burk plot (*Figure*.12), is a straight line of slope K_m/V_{max} and intercept of $1/V_{max}$. K_m calculated is expressed in molarity, the units of substrate concentration. K_m may increase, decrease or remain unaltered on immobilisation, depending on the nature of support and the procedure adopted for immobilisation. Evaluation of K_m is of intrinsic significance since immobilisation results in concurrent shift in the enzyme conformation. This is manifested in the enzyme-substrate affinity. K_m is indicative of the attraction of an enzyme towards the substrate.

1.2.6 EFFECT OF pH

The catalytic activity of every enzyme is most active at a pH specific to it, termed as the optimum pH. A typical profile of activity against pH is depicted in *Figure* 13 . The enzyme becomes less active/inactive at other pH due to disruption of tertiary structure of the active site. This leads to a reduction in the rate of catalysis or to total inactivation of the enzyme. This is ascribed to effect of pH on ionisations of acidic and basic groups at the active site.

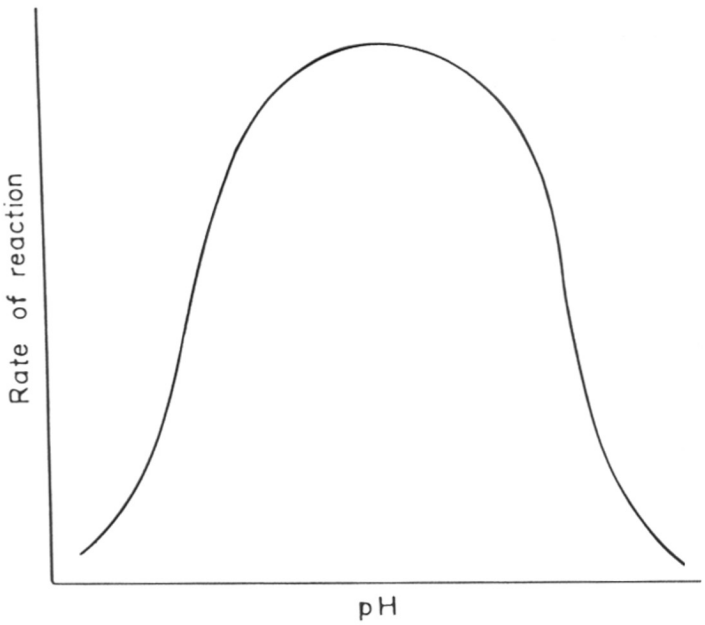


FIG. 13: A TYPICAL pH-ACTIVITY PROFILE

After immobilisation, the optimum pH either remains unaltered or shifts to lower/higher value. The pH activity profile also exhibits a similar trend. The extent to which shift occurs, depends on hydrophilic / hydrophobic nature and charge on the support and/or enzyme.[146,147] The pH-activity profile can be narrower or wider for immobilised enzyme, depending upon the nature and type of support. A downward shift of 0.5 pH unit was noted for aminoacylase[148,149] and invertase[150] immobilised by ionic binding to diethylamino ethyl (DEAE)-cellulose. This was attributed to charges in the polycationic support. Positive charge on the support increase pH in the micro-environment of the immobilised enzyme. The tertiary structure is stabilised at a lower pH, as observable by the lower pH optima. Trypsin and chymotrypsin[151] exhibit an upward shift in pH optima when immobilised on carboxy methyl cellulose (CM-cellulose) azide. The negative carboxyl groups of CM-Cellulose make the micro-environment of immobilised enzyme more acidic. In some enzyme-support systems the pH optima is unaltered after immobilisation. This behavior is observed in the immobilisation of chymotrypsin,[152] trypsin[153] and papin[154] on diazotised amino acid copolymer.

1.2.7 EFFECT OF TEMPERATURE

The enzyme activity increases till an optimum temperature. Beyond this temperature the activity tends to decrease and disappear above a certain temperature due to disruption of the tertiary structure (denaturation). The temperature at which maximum activity is displayed is termed as optimum temperature.

Immobilisation may or may not alter the optimum temperature. The alterations are dependent on nature of the support and immobilisation methodology. Immobilisation of trypsin and chymotrypsin[151,155] on CM-cellulose shifted the temperature optima upwards by 5-15°C. Similar shifts in temperature optima have also been reported for enolase[156] and amino acylase[157] immobilised within polyacrylamide gel. A lowering of temperature optima was observed for immobilised invertase.[158] The temperature shift, from 55°C (for native enzyme) to 30-40° C, was dependent on the support

and method of immobilisation. No shift in temperature optima were observable for glucose isomerase[159] immobilised by diazo coupling to porous glass, chymotrypsin[160] immobilised onto cyanogen bromide (CNBr) - activated cellulosic membrane and trypsin[161] immobilised with CNBr-activated agarose.

1.2.8 STABILITY AND ACTIVITY OF IMMOBILISED ENZYME

Immobilisation decreases the optimum activity of the enzyme. The tertiary structure of free enzyme is altered on immobilisation. Also, the additional steric factors arising from restricted accessibility of substrate to the bound enzyme contribute to this effect.[162] Conformational perturbations originating from the strains imposed on tertiary structure have been well investigated.[163,164] The catalytic activity towards high molecular weight substrates are reduced due to the inability of substrate to approach the active site effectively. Electrostatic repulsions between charged supports and substrates reduce the estimated activity considerably.

Immobilisation enhances the thermal stability of the enzyme and offers the enzyme protection from denaturing agents[165,166] by stabilising the tertiary structure. Enzyme becomes less flexible due to the strong ionic interactions with and/or covalent binding to rigid support. A few instances of decreased or unaltered stability have also been observed.[167] The enzyme may be locked-in in a less stable conformation of tertiary structure. Thus, immobilisation exerts a influence on the conformation, which may or may not affect the overall stability and catalytic activity of enzymes.

1.2.9 EFFECT ON KINETIC PROPERTIES

Immobilisation alters the physicochemical and kinetic properties of an enzyme. The extent of change in pH optima and the resistance to proteolytic and thermal inactivation depends on the immobilisation technique used. Some techniques alter not only the specificity and affinity to substrate, but also thermodynamic parameters of the catalysed reactions.[168,169] These changes may be attributed to

* Conformational perturbations of enzyme molecules

* Heterogeneity of the enzyme resulting in differing substrate concentration, products and other cofactors from that in solution.

The reasons for changes in the micro-environment in the vicinity of bound enzyme have been investigated in some detail.[167,170] These are due to

(i) Partitioning effects.

(ii) Diffusional constraints.

(i) Partitioning effects are related to the hydrophilic/hydrophobic nature of support. Electrostatic interactions between support and substrate / cofactors also contribute towards partitioning. The cumulative effect of these factors leads to the unequal distribution of substrate, product and cofactors between the micro-environment and surrounding solution (macro-environment). At hydrophobic surface, concentration of non-polar species will be more than that of polar species. A concentration gradient is developed between the immobilised enzyme and surrounding solution. The magnitude depends essentially on the extent of hydrophobic / hydrophilic character of the support and on the chemical entities under consideration.

(ii) Diffusional limitations of substrates and products also leads to the build up of concentration gradient. This effect is more pronounced with porous supports. Larger molecules will have greater diffusional resistance. Diffusional limitation effects dictate the selection of proper support and immobilisation technique. Thus, enzymes which act on high molecular weight substrates should be surface bound and not entrapped within pores and enzymes acting on low molecular weight substrates can be immobilised by simple entrapment.

In short, many factors contribute to the changes observed on immobilisation. The cumulative effect of these changes contribute to the altered physical and kinetic properties of an enzyme on immobilisation. The behavior of an immobilised enzyme is governed by constraints imposed on

the tertiary structure, steric factors, diffusional and partitioning effects and mass transfer limitations. Some of these factors have an adverse effect on the performance of an immobilised system. However, globally immobilisation is advantageous due to greater versatility and the reusability of enzyme.

1.2.10 ENZYME IMMOBILISATION WITH POLYETHYLENEIMINE

1.2.10.1 MODIFICATION OF AMINO GROUP

A number of strategies have been worked out to convert polyethyleneimine into suitable support for enzyme immobilisation. The major approach has been to derivatise the amino groups by glutaraldehyde treatment. This generates reactive aldehyde pendant groups, fairly well removed from the polymer back-bone, which couple with enzymes under mild conditions. In general, the amino groups can be partially/completely modified with phosgene, thiophosgene, cyanuric chloride and glutaraldehyde into highly reactive polyfunctional moieties. Reactions of the amino group with the reagents are depicted in *Figure* 14. Amino group is also activated *insitu* by four component condensation (4CC) Ugi reaction.[171] The reaction was optimised for proteins.[172] Amide bond is formed between amino group of polymer and carboxyl group of the enzyme. Aldehyde and isocyanide components are then incorporated sequentially.

Enzymes have also been entrapped in composite membranes of polyethyleneimine with other polymers. Polyethyleneimine is also used to strengthen the walls of alginate beads. Rigid inorganic materials like alumina are coated with polyethyleneimine to generate carriers. These strategies are presented in the following sections.

1.2.10.2 IMMOBILISATION METHODOLOGIES

Polyethyleneimine is used in a variety of ways with/without other synthetic/natural polymers. As minor constituent, it imparts hydrophilic character and mechanical strength to immobilised cell preparations. It is extensively used in crosslinked / derivatised forms as the primary constituent of the carrier.

The literature on polyethyleneimine may be subclassified on the basis of methodologies into:

1.2.10.2.1 Coating on inorganic materials.

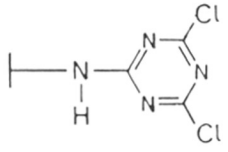
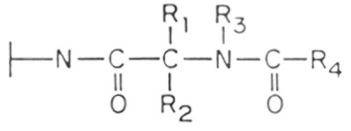
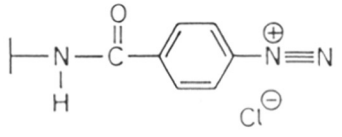
POLYMER HAVING $-NH_2$ GROUP	REACTION	REACTIVE DERIVATIVE OF POLYMER
$\vdash\ddot{N}H_2$	CARBAMYLATION	$\vdash N=C=O$
	THIOCARBAMYLATION	$\vdash N=C=S$
	S — TRIAZYNYL	
	GLUTARALDEHYDE	$\vdash N=CH-(CH_2)_3-CHO$
	UGI REACTION (4 CC REACTION)	
	DIAZO COUPLING REACTION	

FIG. 14 : VARIOUS REACTIONS FOR MODIFICATION OF $-NH_2$ GROUP CONTAINING POLYMERS

1.2.10.2.2 Immobilised cell systems.

1.2.10.2.3 Crosslinked / derivatised polyethyleneimine.

1.2.10.2.4 Macromolecularised cofactors.

1.2.10.2.5 Membranes.

1.2.10.2.1 Coating on Inorganic Materials

Polyethyleneimine is coated onto inert inexpensive rigid inorganic beaded/fibrous supports. Trypsin (EC 3.4.21.4) immobilised in silica-polyethyleneimine composite beads[173] had good activity retention, rigidity and durability. The technique was also used[174] to immobilise lactate dehydrogenase (EC 1.1.1.27), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1). The systems displayed a small shift in K_m, pH optima and excellent operational stability in a stirred flow through reactor.

Wasserman *et al.*[175,176] used polyethyleneimine coated non-porous glass micro beads (particle size 13-44 μm) for immobilising glucose oxidase (EC 1.1.3.4), catalase (EC 1.11.1.6) and glucoamylase (EC 3.2.1.3). The enzymes were covalently bound by glutaraldehyde treatment. The polyethyleneimine treatment resulted in better activity retention and thermal stability compared with aminopropyl derivatisation.

Shumel[177] synthesised water insoluble enzyme carrier. Diatomaceous earth, perlite, cellulose and plastics were coated with 0.01-10 weight percent polyethyleneimine and insolubilised with glutaraldehyde. The preparations were used to immobilise Penicillin amidase (EC 3.5.1.11), amino acylase (EC 3.5.1.14), amyloglucosidase (EC 3.2.13), lactase (EC 3.2.1.23), invertase (EC 3.2.1.26), phenol oxidase (EC 1.14.18.1), inulase (EC 3.2.1.7) and glucose isomerase (EC 5.3.1.18).

Dekker[178] reported a magnetic immobilised lactase (EC 3.2.1.23) formed by treating magnetite with polyethyleneimine and crosslinking with glutaraldehyde. The enzyme lost

80 percent of lactase activity on immobilisation. However, in a stirred batch reactor the immobilised enzyme completely hydrolysed lactose in shorter time at 55°C and displayed greater stability.

Novo industri[179] described a process for immobilising urease (EC 3.5.1.5), lactase (EC 3.2.1.23) and inulinase (EC 3.2.1.7). Sand particles were coated with gelatin, treated with glutaraldehyde (0.1 weight percent), mixed with urease and 3 mL of 10 weight percent polyethyleneimine (mol. weight 40,000-60,000). These were gelled with 25 weight percent glutaraldehyde to generate a product which retained 27 and 36 percent of activity in the size range 300-700 and 700-1000 microns respectively. Glucoamylase (EC 3.2.1.3) was immobilised on diatomaceous earth (particle size 0.74- 0.84 mm) coated with polyethyleneimine and derivatised with glutaraldehyde.[180] The preparation was used to produce high dextrose syrup (96.6 percent dextrose) from enzyme liquefied starch.

DeFilippi *et al.*[181,182] immobilised glucose isomerase (EC 5.3.1.18) on to a porous inorganic material. Porous gamma-alumina, of 60-80 mesh size, 300 - 2000 A°, pore diameter and surface area 150 m^2/gm, was coated with 5 weight percent polyethyleneimine solution, treated with 2.5 weight percent glutaraldehyde solution at room temperature for 1.5 hours and washed. The support matrix was contacted for 48 hours at 2-6°C with glucose isomerase in 0.04 Molar phosphate buffer of pH 7.4 and washed to remove free enzyme. The preparation showed good activity retention in column mode at 60°C in continuous flow technique. The half life was estimated to be 18- 22 days. The activity and half life were enhanced by magnesium ions. The support could be regenerated[183] to load fresh enzyme.

Similarly, Rohrbach[184-187] immobilised glucose isomerase (EC 5.3.1.18) on larger porous gamma alumina particles (25/35 mesh size) impregnated with polyethyleneimine and glutaraldehyde, for bioconversions in batch and continuous modes (*Figure* 15). A drying step after

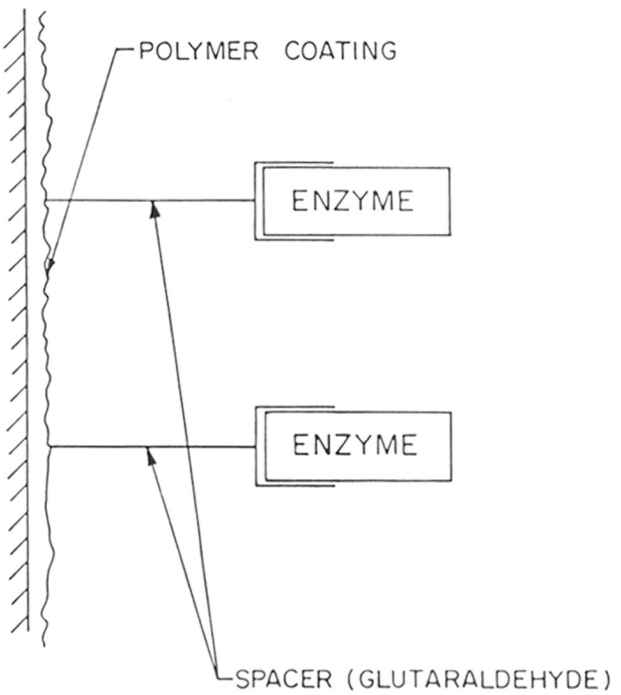


FIG. 15 : SCHEMATIC REPRESENTATION OF IMMOBILISED SYSTEM.

impregnation with polyethyleneimine increased activity by 14 percent. Magnesium ions (0.13 m mole per gram of support) enhanced the half life by 34 percent.[188] Polyethyleneimine enhanced the operational stability[189] and half life from 1900 to 2500 hours

Solid electrode with immobilised peroxidase (EC 1.11.1.7) and cytochrome b2 (EC 1.1.2.3) were modified by treatment with polyethyleneimine.[190] The electrode was used to generate current by reduction of hydrogen peroxide and oxidation of lactate. Polyethyleneimine treatment enhanced limiting biocatalytic oxidation of lactate 4.5-32.0 fold while hydrogen peroxide reduction was decreased. Glucose oxidase (EC 1.1.3.4) was adsorbed on compacted carbon powder disc coated with polyethyleneimine and crosslinked with glutaraldehyde.[191] Hexamethylene diisocyanate, hexamethylene diisothiocyanate and N,N'-ethylene bis maleimide were also studied as crosslinking reagents. Glucose sensors were prepared by this easy and firm immobilisation process. DeFilippi and Malloy[192] immobilised glucose oxidase (EC 1.1.3.4) on porous refractory oxide (delta alumina) coated with carbonaceous pyropolymer, impregnated with polyethyleneimine and treated with excess of glutaraldehyde. The system generated higher current in presence of glucose. The process was also suitable for immobilising methanol dehydrogenase. Alcohol oxidase (EC 1.1.3.13) was encased in polyethyleneimine crosslinked with glutaraldehyde and used as ethanol sensor.[193] A similar immobilisation of glucose oxidase (EC 1.1.3.4) is also reported.[194]

Glucoamylase (EC 3.2.1.3) was immobilised on granular activated carbon coated with polyethyleneimine (4 weight percent) and derivatised with glutaraldehyde (1 weight percent) solution.[195] The carbon was of particle size 12-40 mesh, pore radius 35 - 1000 A° and surface area 200-600 m^2/gm Glucoamylase (EC 3.2.1.3) immobilised on a composite of polyethyleneimine and epoxy polymer (Araldite 6010) coated on solid glass fibre, charcoal or ion exchange resin[196] converted 75 percent starch to glucose at 60°C. Nonporous glass fibres offer facile and inexpensive means to immobilise glucoamylase[197] (EC 3.2.1.3). The fibres were coated with

a mixed solution of enzyme, polyethyleneimine and substrate (optional) and insolubilised with 1 weight percent glutaraldehyde solution. The preparation was highly active, with half life of 40 days at 60 °C.

Swann and Nolf[198] immobilised a high concentration of *E.coli* cells, with good retention of aspartase (EC 4.3.1.1) activity. The system was prepared by mixing *E.coli* cell paste with aqueous polyethyleneimine solution, coating on twisted glass fibre, drying and treating with 5 weight percent aqueous glutaraldehyde solution. It effectively converted ammonium fumarate to L-aspartic acid and displayed excellent durability, strength and stability.

Yeast invertase (EC 3.2.1.26) was adsorbed onto a solid support and treated to generate thin film ultraporous membrane diffusion barriers.[199] Enzyme solution (12 weight percent) was mixed at room temperature for two hours with Brunswik Technetics BT S555, treated sequentially with polyethyleneimine solution (0.5 weight percent) and toluene diisocyanate (0.1 weight percent, in cyclohexane) for 30 seconds, washed and dried. The performance was evaluated at ambient temperature, 4.5 pH, in a cross-flow reactor by pumping sucrose solution across the face of the thin film diffusion barrier. The system exhibited a specific conversion rate of 95 mole sucrose/minute for 24 hours, with no change in activity.

1.2.10.2.2 Immobilised Cell Systems

Novo industri[200] immobilised whole cells with penicillin V acylase (EC 3.5.1.11) activity. Aqueous polyethyleneimine (45 mL, 10 weight percent) and glutaraldehyde (30 mL, 25 weight percent) were added to a 1.5 litre fermentation broth of bacterial strain NRRL 11240 (activity 0.68 units/ml) maintained at 4°C. After stirring for 1 hour at a reduced speed, multicell particles formed were filtered, washed with phosphate buffer (pH 7.0) and air dried. The activity of the yield was 30 percent.

Vojtisek *et al.*[201] immobilised *E.coli* cells with penicillin acylase (EC 3.5.1.11) activity. The cells were crosslinked with 25 weight percent glutaraldehyde solution, permealised with butyl

acetate and a surfactant. The cell mass was treated with polyethyleneimine/glutaraldehyde, frozen and crushed to 0.30-0.45 mm particle size. In a similar process,[202] *E.coli* cells with penicillin acylase (EC 3.5.1.11) activity were treated for 10 minutes with 3.3 weight percent polyethyleneimine solution. The resulting paste was added to cellulose acetate solution (10 weight percent in acetone), extruded and dropped into a water bath to form small globules. This system retained 60 percent of its original activity after 20 cycles.

Antigens and antibodies used in enzyme immunoassay were immobilised on spherical copolymer of ethyleneimine and methyl methacrylate. The amino groups on the surface were modified with glutaraldehyde.[203]

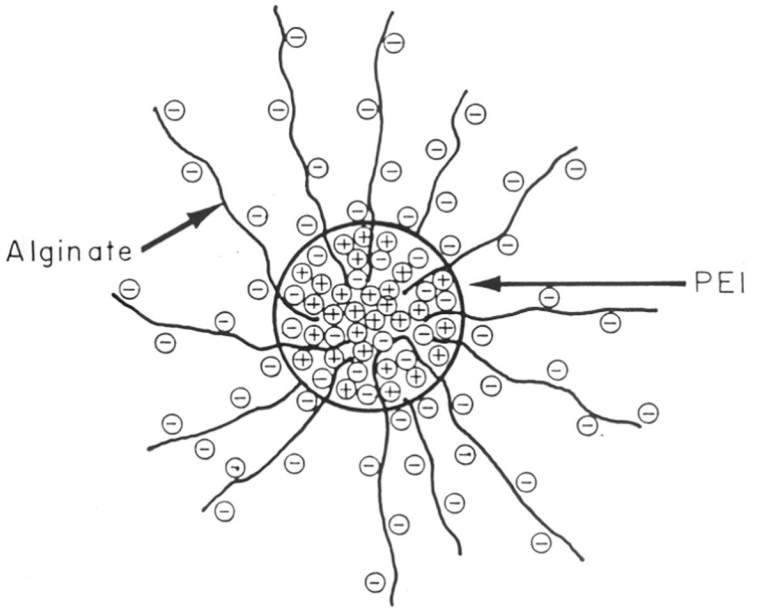
Chao *et al.*[204] used linear and branched polyethyleneimines to stabilise, to increase abrasion resistance and to conduct bioconversions at elevated temperatures. Spherical beads, formed by dispersing a warm carrageenan-cell suspension in oil, were allowed to gel by gradual cooling and cured with linear/branched polyethyleneimine. *B.stearothermophilus* cells with lactase (EC 3.2.1.23) activity, *Saccharomyces cerevisiae* cells having glucose isomerase (EC 5.3.1.18) and invertase (EC 3.2.1.26) activity were immobilised by this technique. Baltzer[205] immobilised *A. niger* cells by mixing with 2 weight percent solution of k-carrageenan and injecting into a flowing stream of mineral oil. The droplets formed were cooled into beads and gelled with polyethyleneimine / epichlorohydrin modified polyethyleneimine. *Streptomyces olivaceus*[206] and *Brevibacterium flavum* cells[207] were immobilised in a similar manner. *Brevibacterium flavum* cells ATCC 14067 (8 gm), with fumarase (EC 4.2.1.2) activity, were suspended in physiological saline solution (8 ml), mixed with carrageenan solution (30 ml, 5 weight percent), added to polyethyleneimine solution (4 ml, 1.5 weight percent) maintained at 50°C, gelled by cooling to room temperature and cut into 3 mm cubes. Whole cells of *Protaminobacter rubrum*, with sucrose mutase activity, were similarly immobilised.[208]

Whole cells/enzyme were adsorbed onto vermiculite and treated with crosslinked polyethyleneimine to form an effective envelope.[209] Thus, *E.coli* cells having aspartase (EC 4.3.1.1) activity were immobilised to convert fumarate into aspartate in a column at 37°C, 8.5 pH.

Polyethyleneimine treatment stabilised living *Saccharomyces cerevisiae* cells entrapped in alginate beads[210] and imparted strength without affecting the fermentation activity. Bajpai *et al.*[211] reported a positive stabilisation effect by polyethyleneimine-glutaraldehyde hardening of calcium alginate beads used in the immobilisation of *Kluyveromyces marxianus* cells having inulinase (EC 3.2.1.7) activity. The stability of *Saccharomyces cerevisiae / Zymomonas mobilis* cells, entrapped in calcium alginate beads and used for ethanol production, was improved by polyethyleneimine treatment.[212] The "leak-proof" system exhibited no loss in activity of the entrapped cells.

Yeast cells (0.3 gm wet weight) from *Saccharomyces* were immobilised by mixing with polyethyleneimine (0.7 ml, 30 weight percent), sodium alginate (25 ml, 4 weight percent) and carboxy methyl cellulose (1 gm) and adding dropwise to calcium chloride solution (0.1 mole) at room temperature.[213] Beads were obtained on stirring for one hour. The mechanical strength was enhanced by treatment with aluminium sulfate solution. The mechanical strength of thus immobilised glucoamylase[214] (EC 3.2.1.3) and cells/enzymes for continuous fermentation of ethanol[215] were enhanced in the presence of polyethyleneimine. In a related process, aminoacylase (EC 3.5.1.14) was mixed with sodium alginate, polyethyleneimine and added to calcium chloride to obtain granular beads. These were surface modified into semipermeable polyamide membrane[216] by sequential soaking in hexamethylene diamine and sebacoyl dichloride solutions. The polyethyleneimine treatment enhanced the enzyme retention within the membrane.

A novel polymeric matrix for the immobilisation of growing yeast cells was developed by Joung and coworkers for the continuous fermentation to generate ethanol.[217] It was based on the reversible gelation of alginate in the presence of polyethyleneimine, as presented in *Figure* 16. . The cell growth dissolved the surrounding gel matrix which gelled again in presence of calcium



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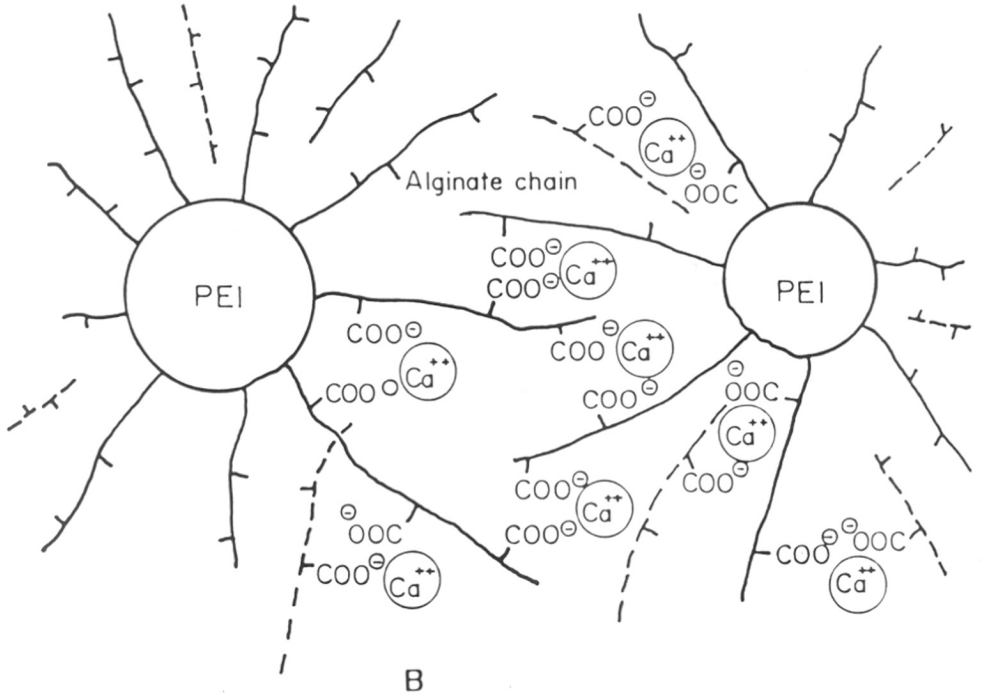


B

FIG. 16 : A) INTERACTION OF POLYETHYLENEIMINE WITH ALGINATE
B) REVERSIBLE GELATION IN PRESENCE OF Ca^{++} IONS

ions to retain the growing cells. A minimum of 500 mg/litre of calcium chloride was required to prevent rapid and irreversible dissolution of carrier. The matrix could be expanded ten fold. Polyethyleneimine modification enhanced the mechanical strength, improved the cell retention, increased catalyst life and imparted bacteriostatic stability to the beads. An efficiency of 94 percent was achieved. The catalyst life was 3 months. Yeast cells have also been immobilised using commercial flocculant Sedipur CL-930 (polyethyleneimine) and glutaraldehyde by Smolik *et al.*[218]

Whole cells, with glucose isomerase (EC 5.3.1.18) activity, were mixed with polyethyleneimine and chitosan. Immobilisation was accomplished by crosslinking with glutaraldehyde.[219] The entrapped cell mass was extruded through a 1 mm die and ground to 30-40 mesh size.

Schutt[220] immobilised *Bacillus subtilis* ATCC 6633 cell suspension by entrapment in crosslinked gels prepared from polyethyleneimine, glutaraldehyde and tannin. The system was used to cleave 3-acetoxy group (protecting group) of 7, β-acylamido cephalosporonic acids.

The aspartase (EC 4.3.1.1) activity of *E.coli* cells, entrapped in cellulose acetate treated cotton cloth, were stabilised by polyethyleneimine and glutaraldehyde treatments in the presence of 0.1 molar sodium dithionite.[221] Invertase (EC 3.2.1.26) immobilised on cotton cloth with polyethyleneimine/glutaraldehyde[222,223] was highly active and exhibited efficient sucrose hydrolysis in continuous mode for 3 months. The process was also suitable for immobilising lactase (EC 3.2.1.23) and lipase (EC 3.1.1.3) used in food processing. D'souza *et al.*[224,225] immobilised urease, yeast cells with invertase activity and co-immobilised glucose oxidase (EC 1.1.3.4) and catalase[226] (EC 1.11.1.6) by adsorbing on polyethyleneimine-cotton cloth and crosslinking with dimethyl suberimidate. The immobilised urease showed a shift in pH optimum towards the acidic side without substantial change in temperature optimum and thermostability. The immobilised yeast was operated repeatedly in a specially fabricated 2-3 litre capacity frame reactor for the inversion of sucrose. The system retained 80 percent of initial activity after 16 batches.

Yeast cells formed a strongly adhering monolayer on adsorption onto polyethyleneimine treated glass. This could not be desorbed under extreme conditions of pH, ionic strength and on washing under running tap water.[227] The immobilised cells permitted repeated use.

Miksite *et al.*[228] reported enhancements in the L-lysine amidase and L-amino caprolactam hydrolase activities of *Cryptococus* species No. 112 cells by inclusion into agar-polyethyleneimine gel matrix. Dow chemical Co.[229] developed a process for the simultaneous flocculation and encapsulation of microbial *Ampullariella* cells with glucose isomerase (EC 5.3.1.18) activity using partially carboxymethylated polyethyleneimine. Half life of cells was 1484 hours as against 669 and 310 hours for unmodified and fully carboxymethylated polyethyleneimine respectively.

Ikeda *et al.*[230] immobilised invertase (EC 3.2.1.26) using polyethyleneimine in combination with cellulose and glutaraldehyde under neutral and basic conditions. The invertase retained 26 percent of its initial activity on immobilisation. Polyethyleneimine was used to provide additional structural rigidity to the walls of *E.coli* cells, with penicillin acylase (EC 3.5.1.11) activity, entrapped in cellulose diacetate beads.[231] The cells could be entrapped in geometries such as beads, filaments, membranes etc.. The immobilisation offered additional advantages such as low cost, simplicity, and operability in fluidised bed reactors.

Katsuyuki *et al.*[232] immobilised bacteria (denitrifying, nitrifying, methane fermentation and dephosphate bacteria) using polyethyleneimine, sodium alginate and carrageenan. Vojtisek *et al.*[233,234] immobilised a variety of enzymes in polyethyleneimine crosslinked with glutaraldehyde. *Saccharomyces cerevisiae* cells thus immobilised were treated with a commercial poly (2,6-dimethyl phenyl oxide). Amyloglucosidase (EC 3.2.1.3) pretreated with mycelium of *Penicillium chysogenum*, was immobilised[235] on polyethyleneimine crosslinked with 25 weight percent glutaraldehyde solution. *E.coli* β- galactosidase (EC 3.2.1.23) (30 mg protein/mL) was immobilised[236] in polyethyleneimine crosslinked with 1 weight percent glutaraldehyde.

Heat and operational stabilities of *Brevibacterium flavum* cells, with fumarase (EC 4.2.1.2) activity, were enhanced by treatment with 3 weight percent polyethyleneimine.[237-240] The increased heat stability permitted prolonged operation in the temperature range of 50-55°C and resulted in a two-fold increase in the L-malic acid productivity. The immobilised cells produced one ton of malate per day on a 1000 litre column. Whole broth, with Penicillin V acylase (EC 3.5.1.11) activity, immobilised by polyethyleneimine and glutaraldehyde treatments[241] was separated, extruded and dried to circumvent separation and purification processes. The immobilised enzyme could be used 15-30 times. It required activation between batches with buffer containing mercapto ethanol.

Water was freed from dissolved fertilizers (denitrification) by presaturating with air and treating in stirred tank/continuous flow reactor with active sludge cells bound to polyethyleneimine.[242] The biocatalyst concentration was 50 gm/litre and operating temperature was 21.5 °C. It was used in both batch and continuous modes to reduce nitrate ion.

E.coli cells with penicillin amidase (EC 3.5.1.11) activity,[243] yeast cells with invertase (EC 3.2.1.26) activity[244] and denitrifying microorganisms[245] were immobilised in ethyleneimine-glutaraldehyde adduct in presence of animal, plant or microbial protein. Egg protein treatment[244] enhanced activity and stability as well as mechanical strength, sedimentation, separability and service life of the preparation.

Miles Laboratories[246] reported immobilisation of *Sporobolomyces roseus* cells, containing phenyl alanine ammonialyase (EC 4.3.1.5), by suspending in polyethyleneimine (mol. weight 60,000) and treating with dilute solutions of chitosan and glutaraldehyde in acetic acid. The performance was evaluated in column reactor by passing 1 weight percent solution of cinnamic acid.

Manecke *et al.*[247,248] immobilised *Arthrobactor simplex* whole cells in glutaraldehyde/polyacrolein crosslinked polyethyleneimine to transform cortisol to prednisolone. Best results were recorded for the system with 80 percent crosslink density. The activity half life was six

days. *E.coli* cells rich in aspartate phenylalanine transaminase were immobilised in polyazetidine.[249] The immobilised preparation was used in the production of L-phenyl alanine from phenyl pyruvate.

1.2.10.2.3 Crosslinked / Derivatised Polyethyleneimine

Kazanskaya *et al.*[250] covalently coupled trypsin (EC 3.4.21.4) to polyethyleneimine through glutaraldehyde. The binding was effected by the initial modification of either the enzyme or the polymer. The performance of immobilised trypsin was unaltered by the coupling mode. The modified trypsin derivatives obtained were resistant to the autolysis and acquired stability against thermal denaturation.

Kuniak *et al.*[251] developed a water insoluble polyethyleneimine - isothiocyanate support by crosslinking polyethyleneimine with epichlorohydrin in presence of sodium hydride and subsequent reaction with aqueous thiophosgene. A single step preparation of polyethyleneimine-isothiocyanate support by reacting polyethyleneimine with thiophosgene for immobilisation of enzymes having sulphhydryl groups[252] was also described.

Glucose oxidase (EC 1.1.3.4) was immobilised by Zemek and Kuniak.[253] An aqueous suspension of polyethyleneimine, glucose oxidase and bovine serum albumin were stirred in presence of 2- chloromethyl oxirane (crosslinking agent). The gel formed was disintegrated, washed and dried to get immobilised protein.

Imperial Chemical Industries[254] described a process for immobilisation of *Pseudomonas putide* cells having D-2-monochloro- propionic acid dehydrogenase on acrylic copolymer/styrene-butadiene rubber latex particles in combination with polyethyleneimine.

Sulphonated macroporous styrene-divinyl benzene cation exchange beads were treated with polyethyleneimine to prepare hydrophilic electrostatic polycomplex support to immobilise invertase (EC 3.2.1.26).[255] Aspartase (EC 4.3.1.1) was mixed with succinic acid modified

polyethyleneimine and crosslinked with 1-ethyl-3,3- dimethylaminopropyl carbodiimide.[256] The resulting gel was ground to small pieces and used in the conversion of ammonium fumarate to aspartic acid.

Freedman[257] immobilised glucose oxidase (EC 1.1.3.4) in water swellable gels prepared by crosslinking polyethyleneimine with hexamethylene diisocyanate. Carboxy-methylated purifloc C-31 (a polyamine) was used by Cheng *et al.*[258] to stabilise glucose isomerase (EC 5.3.1.18). Half life of glucose isomerase was enhanced by this stabilisation.

Polyethyleneimine, in combination with sorbitol-(ethylene oxide)$_{80}$ surfactant, has been used for immobilising lipase (EC 3.1.1.3), alkaline protease and dispersed microorganisms.[259] The surfactant was reacted with epichlorohydrin. Pancreas lipase, tris buffer and polyethyleneimine were then mixed to obtain the immobilised system as a gel.

Glucose oxidase (EC 1.1.3.4) was immobilised on surface modified nylon and tested for reagentless automated analysis of glucose.[260] Polyethyleneimine was linked covalently to nylon tube activated by alkylation and reacted with glutaraldehyde/ethyl adipimidate. This technique was also used to immobilise invertase[261] (EC 3.2.1.26), pigeon liver nicotinamide adenine dinucleotide kinase[262] (EC 2.7.1.23) as well as mixed enzyme systems consisting of alkaline phosphatase (EC 3.1.3.1)/adenosine deaminase (EC 3.5.4.4) and rabbit muscle pyruvate kinase (EC 2.7.1.40)/lactate dehydrogenase (EC 1.1.1.27).[263] Immobilisation of NAD kinase altered K_m, and activation energy but not the pH dependence.

Macroporous styrene-divinyl benzene beads were modified with polyethyleneimine and derivatised with glutaraldehyde for immobilising α- chymotrypsin (EC 3.4.21.1) and pepsin[264] (EC 3.4.23.1). The immobilisation of α-chymotrypsin proceeded with marginal loss in activity. Solomon *et al.*[265] studied the coupling of acetate kinase (EC 2.7.2.1) onto Polyacrylate beads (Martex 101, Amicon) coated with polyethyleneimine and derivatised with glutaraldehyde in presence of glycine.

Trypsin (EC 3.4.21.4) and pepsin (EC 3.4.23.1) have been immobilised through isothiocyanato linkages to crosslinked polyethyleneimine.[266] The enzymes were modified with 3-isothiocyanato-1-propyl isocyanate and covalently bound to amino groups of polyethyleneimine with good retention in activity.

Urease (EC 3.5.1.5) was immobilised on polyethyleneimine modified into benzene diazonium salt.[267] Polyethyleneimine was reacted with p-nitro benzoyl chloride, reduced to amino groups and diazotised to generate reactive support for immobilisation. Manecke *et al.*[268] described the synthesis of highly reactive immobilisation supports by derivatising linear/crosslinked polyethyleneimine with 4-nitrophenyl isothiocyanate, 4-nitrophenyl isocyanate or ethyl chloroacetate. The binding efficiencies of papin (EC 3.4.22.2), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and urease (EC 3.5.1.5) were tested. The immobilised enzymes were evaluated for enzymic activity, storage and operational stability. Zemek and coworkers[269] derivatised polyethyleneimine with epichlorohydrin and activated with thiophosgene/succinic anhydride for enzyme immobilisation. Excellent binding of D-glucose oxidase (EC 1.1.3.4), glucoamylase (EC 3.2.1.3), acetyl choline esterase (EC 3.1.1.7) and butyl choline esterase (EC 3.1.1.8) were observed at low levels of derivatisation.

The effects of immobilisation on the kinetic parameters of lactate dehydrogenase[270] (EC 1.1.1.27) has been studied. The activation observed was related to association with polyethyleneimine. α-amylase (EC 3.2.1.1) was immobilised on polyethyleneimine grafted with 1-chloro acetaldehyde modified poly (vinyl alcohol).[271]

Ohshiro[272] reported the synthesis of biomedical materials with high antithrombogenecity consisting of Urokinase (EC 3.4.99.26) immobilised onto derivatised polyethyleneimine. Urokinase was treated with polyethyleneimine, maleic anhydride-methyl vinyl ether copolymer and dicyclohexyl carbodiimide.

1.2.10.2.4 Macromolecularised Cofactors

Zappelli *et al.* effectively recycled cofactor systems by immobilisation. The cofactors such as nicotinamide proteins (NAD^+ and $NADP^+$) and flavo proteins (FAD) were coupled to hydrosoluble polymers. The modification did not alter the coenzymic activity. The molecular size was increased to prevent diffusional losses. By this technique, NAD^+ and FAD were modified with 3,4-epoxy butanoic acid at N^1 site of adenine moiety, converted by Dimroth rearrangement to N^6 alkylated derivative and immobilised onto polyethyleneimine to form soluble macromolecularised cofactors.[273-276] The coenzymic efficiency of the macromolecularised NAD^+ derivative was tested with rabbit muscle lactate dehydrogenase (EC 1.1.1.27), yeast alcohol dehydrogenase (EC 1.1.1.1) and *B. subtilis* alanine dehydrogenase (EC 1.4.1.1). The method was also applied to modify $NADP^+$.[277] Similar investigations have also been reported by Wykes and coworkers.[278]

Bueckmann[279] bound coenzymes, with adenine ring systems, to polyethyleneimine. The coenzyme was alkylated at 1 position of the adenine ring moiety with an alkylating agent capable of binding covalently to polyethyleneimine and transformed by Dimroth rearrangement to N^6 form of alkylation. Nicotinamide adenine dinucleotide was hooked by this method.

Gulio *et al.*[280] immobilised oxidoreductases together with macromolecularised NAD^+/formyl-polyethyleneimine-NAD^+ derivatives onto cellulose triacetate fibres in the presence of polyethyleneimine. Immobilisation facilitated the conversion of NAD^+ to NADH. Thus, the following oxidoreductases were immobilised:

(1) Lactate dehydrogenase (EC 1.1.1.27) and alanine dehydrogenase (EC 1.4.1.1)

(2) 3 α 20β-hydroxy steroid dehydrogenase, alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3)

(3) β-hydroxy steroid dehydrogenase and diaphorase (EC 1.6.4.3).

1.2.10.2.5 Membranes

Goldberg[281,282] immobilised a variety of enzymes in membranes to be suitable for cross-flow / flow-through reactors. Hydrated silica (Hi Sil 233) and poly (vinyl chloride) were dry blended, stirred in cyclohexane/water and extruded into sheets having micropores in the range 0.15 to 0.25 μ These sheets were cut into discs and modified with polyethyleneimine and glutaraldehyde for enzyme binding. The method was successfully applied to the immobilisation of glucose isomerase (EC 5.3.1.18), glucose oxidase (EC 1.1.3.4), lactase (EC 3.2.1.23), sucrase (EC 3.2.1.26) and alcohol dehydrogenase (EC 1.1.1.1). The immobilised glucose isomerase was used in a flow-through type reactor and immobilised lactase was used in a spiral reactor.

Franklin[283-285] encapsulated living mammalian or bacterial cell useful in isolation and separation of cell metabolic products such as hormones, lymphokines, interferon and antibodies. Insulin was isolated from the pancreatic Islet of Langerhans cells encapsulated using sodium alginate, calcium chloride solution, 2- (cyclohexyl amino) ethane sulphonic acid, poly (lysine) and polyethyleneimine.

Goto[286] described immobilisation of urease (EC 3.5.1.5) by sequentially soaking albumin-polyethyleneimine membrane in glutaraldehyde and urease solutions. The system displayed good mechanical strength and had high retention of activity. An urea sensor was also developed[287] by chemical vaporization plasma etching of a p-type wafer plate, coating with porous cellulose triacetate membrane and treating sequentially with polyethyleneimine, urease and toluene diisocyanate solutions to immobilise urease on the surface.

Glucoamylase (EC 3.2.1.3) was immobilised in the pores of ultrafiltration / dense filtration membranes[288] by circulating gluco- amylase-polyethyleneimine complex. α-amylase (EC 3.2.1.1) was immobilised in hollow fibre membranes.[289,290] Polyethyleneimine solution was passed through porous polysulphone hollow fibres under pressure and treated with glutaraldehyde/diisocyanate solution. α-amylase was immobilised in the membrane under pressure. The membrane could be regenerated by treating with sodium hypochlorite solution and 0.01 Normal

hydrochloric acid.[291] Nitto Electric Industrial Co.[292,293] immobilised glucose isomerase (EC 5.3.1.18) in porous membranes. Porous polyamide hollow fibre was treated with polyethyleneimine and glutaraldehyde solutions to create a crosslinked gel on the fibre surface. Enzyme was bound on this surface.

Nakao *et al.*[294] immobilised enzymes in porous polyamide membrane modified with polyethyleneimine. Polyethyleneimine was adsorbed on poly (m-phenylene iso phthalamide), transformed into a hollow ultrafiltration membrane and treated with 2.5 weight percent glutaraldehyde solution. Glucoamylase (EC 3.2.1.3) was immobilised in the membrane by penetration under pressure.

Glucose oxidase (EC 1.1.3.4) was immobilised by Norihiko[295] in a porous cellulose membrane treated with polyethyleneimine and crosslinked with glutaraldehyde. Alcohol oxidase (EC 1.1.3.13) was entrapped in membrane by Tanaka *et al.*[296] Polyethyleneimine solution was mixed with alcohol oxidase and glutaraldehyde solution. The mixture was allowed to stand at 0-0.5 °C for 2 hours and spread as a membrane (2-5 μm thick). The activity of immobilised alcohol oxidase was stable for over 1000 hours.

Aisina *et al.*[297] reported the microencapsulation of chymotrypsin in polyethyleneimine modified polyamide semipermeable membranes formed by reacting 1,6-hexamethylene diamine with sebacoyl chloride.

Chang and coworkers described a multienzyme / cofactors system immobilised with polyethyleneimine and found suitable as artificial cells.[298] NAD^+ was coupled to dextran and encapsulated within the artificial cells. The research group[299-302] developed immobilisation of multienzyme/soluble dextran NAD^+ in nylon-polyethyleneimine semipermeable microcapsules for the continuous recycling of dextran-NAD^+ derivatives. Yeast alcohol dehydrogenase (ADH) (EC 1.1.1.1), malic dehydrogenase (EC 1.1.1.37) and soluble dextran-NAD^+ were immobilised in the presence of substrates such as ethanol (for yeast alcohol dehydrogenase)

and oxaloacetic acid (for malic dehydrogenase), as schematically depicted in *Figure* 17. Dextran-NAD^+ was recycled within the microcapsules at a rate of 72 cycles per hour by the sequential reactions with the enzymes. The microcapsules exhibited good storage stability.

Lipid-polyamide membrane microcapsules were prepared in the presence of polyethyleneimine (50 weight percent solution),[303-305] to simultaneously immobilise glutamic dehydrogenase (EC 1.4.1.2), alcohol dehydrogenase (EC 1.1.1.1), urease (EC 3.5.1.5), cofactors NAD^+ /NADH and the substrate α- ketoglutarate. The NAD^+ retained in the microcapsules was efficiently recycled. By this technique enzymes and cofactors required for multistep enzymatic transformations could be occluded in the same microcapsule. The method did not involve covalent binding. Hence, the entrapped enzymes, cofactors and substrate could interact freely. Diffusional restrictions or steric hindrances do not arise within the microcapsules. The process offered high surface area for the reaction.

Glucose oxidase (EC 1.1.3.4) was immobilised in polyamide/ polyacrylonitrile membranes treated with polyethyleneimine/poly (acrylic acid) for use in semiautomatic glucose analyzer.[306] The immobilised enzyme preparation was evaluated for kinetic parameters such as Km, optimum pH and temperature. The immobilised glucose oxidase retained 60-80 percent of its original activity after one year at 40°C.

Sun *et al.*[307] used a combination of alginate-polylysine- polyethyleneimine to encapsulate the islet cells in form of biocompatible semipermeable membranes. The cells remained viable in culture for four months. The encapsulated cells can be transplanted into the animal body and survive for several months. Such microencapsulated cell systems have tremendous clinical application in the treatment of diabetes and other hormone or enzyme replacement therapies. Anzai *et al.*[308,309] have reported the construction of enzyme-immobilised Langmuir-Blodgett (LB) membranes for biosensor applications. The LB membrane was coated on ion sensitive

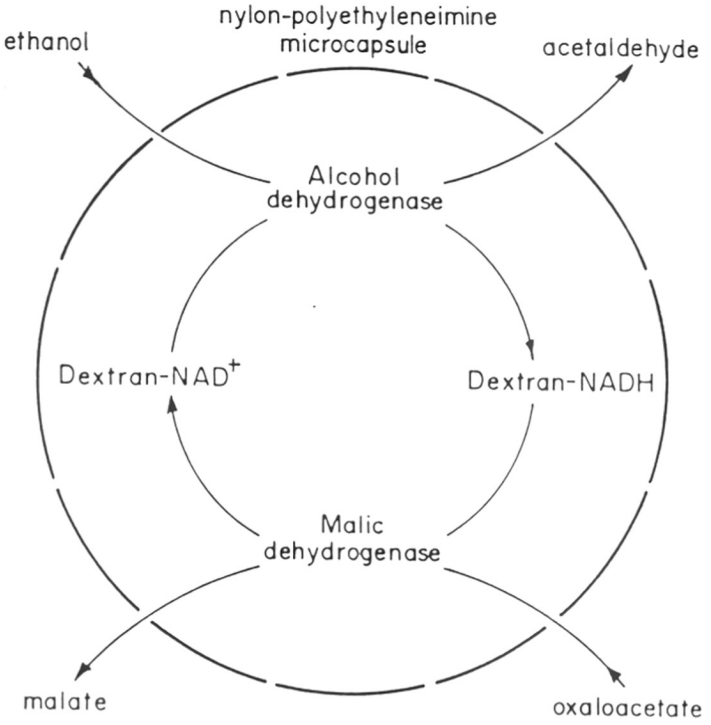


FIG. 17: ARTIFICIAL CELL CONTAINING ALCOHOL DEHYDROGENASE, MALIC DEHYDROGENASE, AND NAD^+-DEXTRAN.

field effect transistors (ISFET), treated with branched polyethyleneimine followed by glutaraldehyde and used for immobilising urease and α-chymotrypsin. The performance characteristics of these urea and ester sensitive FET sensors have been reported with respect to pH response, response time and long term stability.

Gianfreda *et al.*[310] described microenvironmental effect of polyelectrolytes in stabilising enzymes in ultrafiltration (UF) membranes. Entrapment of invertase within either polyethyleneimine (PEI) or poly (acrylic acid) (PAA) shifted the pH optima towards acid or alkaline values respectively. This indicates that two enzymes having distinct optimal pH values, in alkaline and acidic region, on immobilisation within a layer of PAA or PEI respectively could be operated with a single feed stream at an intermediate pH.

1.3 SCOPE OF WORK

1.3.1 Enzyme Immobilisation

The last two decades has seen a phenomenal increase in the use of immobilised enzymes as biological catalysts to replace conventional chemical processes. Immobilised enzymes have become an important aspect of the biotechnological revolution. The merits of immobilisation have been well documented. The use of enzymes in commercial processes was restricted mainly due to their high cost, scarce availability and inability to be used repeatedly. These are circumvented by immobilisation. The first industrial processes, introduced in 1969, were the immobilised glucose isomerase[311] and amino acid acylase.[312] Glucose isomerase is used in production of high fructose corn syrup (HFCS) by the isomerisation of glucose. Amino acid acylase is used for the optical resolution of amino acids. Around the same time, semi-commercial processes were established for the conversion of penicillin G to 6-amino penicillanic acid (6-APA) using immobilised penicillin G acylase.[313] 6-amino penicillanic acid is an important β-lactam intermediate in the manufacture of semi-synthetic penicillins. The annual production of 6-APA is currently estimated to be over 8,000 tons. The commercial interest in penicillin G acylase has continued to increase. There have been untiring efforts to explore newer supports for immobilising penicillin G acylase, primarily to enhance the performance of immobilised penicillin G acylase.

The research on developing suitable supports for the immobilisation of penicillin G acylase was undertaken with the following objectives:

1) Synthesis of porous beaded polymers.

2) Functionalising the beaded polymers.

3) Explore cheaper supports like porous alumina.

4) Evaluate performance of immobilised enzyme (IME) with respect to effect of pH, temperature, and number of cycles of repeated use.

1.3.2 Matrix Polymerisation

The most attractive feature of polymerisation in biological systems is the order and precision in sequential addition of monomeric units. Examples are the self replication of DNA and biosynthesis of proteins. Extensive efforts have been made in the past to achieve a similar degree of order in synthetic polymers. Matrix polymerisation provides a means to mimic the natural process and to synthesise the macromolecules with a ordered structural features. In matrix polymerisation, monomer is polymerised in presence of a polymer. Addition of polymer (usually termed as *matrix or template*) to the reaction medium influences the kinetics, topochemistry of the polymerisation and permits The synthesis of polycomplexes not possible by conventional means. These *in situ* polycomplexes generally have a more ordered structure than those formed by mixing two polymers, after polymerising them individually. The *in situ* polycomplexes can be utilised to generate ultra-filtration (UF) membranes of controlled and predetermined pore sizes.

A wide variety of vinyl monomers have been polymerised in the presence of templates/matrices.[314] There have been only a few reports on free radical polymerisation in the presence of branched polyethyleneimine as matrix. Polymerisation of acrylic acid in presence of polyethyleneimine was investigated by Ferguson *et al.*[60] and Bamford *et al.*[39] A literature survey reveals that polymerisation of methacrylic acid has been investigated in the presence of poly (N-vinyl pyrrolidone) (PVP).[84] The mirror system i.e. polymerisation of N-vinyl pyrrolidone in presence of poly (methacrylic acid) has also been reported.[22,52,57] However, there has not been a single report on the polymerisation of methacrylic acid using branched polyethyleneimine as a matrix.

It was, therefore, considered appropriate to investigate methacrylic acid - polyethyleneimine system. The doctoral research work on matrix polymerisation was undertaken with a view of following objectives:

* To study the kinetic features and to compare the kinetics with solution polymerisation of methacrylic acid.

* To investigate the dual role of polyethyleneimine as a matrix and as part of the redox initiator system with potassium persulphate.

* Characterisation of the polycomplex by differential scanning calorimetry (DSC), X-ray , C-13 nuclear magnetic resonance (NMR) and infra-red (IR) spectroscopy. This was essential, since the most difficult task in matrix polymerisation is the isolation and characterisation of the newly formed polymer (often termed as *daughter polymer*).

CHAPTER 2

EXPERIMENTAL

EXPERIMENTAL

2.1 MATERIALS

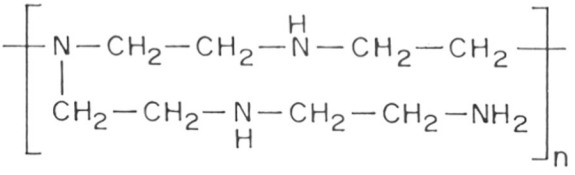
(i) Branched Polyethyleneimine:

Empirical formula $(C_2H_4N)_n$

Molecular weight per repeat unit

Molecular weight ~ 20,000

Chemical structure



Branched polyethyleneimine (Polymin) was obtained from BASF, (Germany) as a 100 percent solution in water. It was used diluted to the required concentrations with deionised water, as reported in the ensuing sections. The relative ratios of primary:secondary:tertiary nitrogens were estimated in glacial acetic acid. (See section 2.2.3)

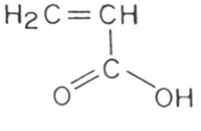
(ii) Acrylic Acid

Empirical formula $C_3H_4O_2$

Molecular weight 72.06

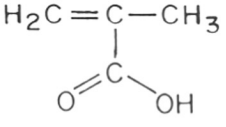
Density 1.051 gm/mL

Chemical structure



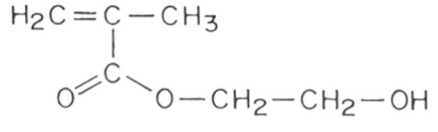
Acrylic acid (propenoic acid) was obtained from Fluka A/G, Switzerland. It was freed from the inhibitor by distillation at reduced pressure, under nitrogen blanket. Freshly distilled acrylic acid was used in all the matrix and solution polymerisation experiments.

(iii) Methacrylic acid

Empirical formula	$C_4H_6O_2$
Molecular weight	86.09
Density	1.015 gm/mL
Chemical Structure	

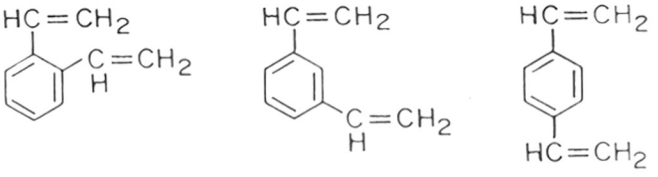
Methacrylic acid (2-methyl propenoic acid) was obtained from Fluka A/G Switzerland. It was purified by distillation at reduced pressure under nitrogen blanket. Freshly distilled methacrylic acid was used as a monomer in matrix and solution polymerisation experiments.

(iv) Hydroxyethyl methacrylate

Empirical formula	$C_6H_{10}O_3$
Molecular weight	130.14
Density	1.034 gm/mL
Chemical structure	

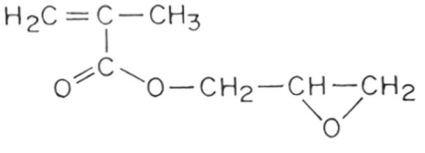
Hydroxyethyl methacrylate (HEMA) was obtained from Fluka A/G, Switzerland. It was used as received for preparing macroporous beaded HEMA-DVB copolymer supports, by suspension polymerisation, for the immobilisation of penicillin G acylase.

(v) Divinyl benzene

Empirical formula	$C_{10}H_{10}$
Molecular weight	130.19
Density	0.914 gm/mL
Chemical structure	

Divinyl benzene (DVB) was obtained, as a mixture of all the three isomers, from Fluka A/G, Switzerland. The active concentration of divinyl benzene was 60 weight percent. It was used as received to prepare macroporous beaded HEMA-DVB copolymers as well as GMA-HEMA-DVB terpolymers, used as supports for the immobilisation of penicillin G acylase.

(vi) Glycidyl Methacrylate

Empirical formula	$C_7H_{10}O_3$
Molecular weight	142.15
Density	1.042 gm/mL
Chemical structure	

Glycidyl methacrylate (GMA), also known as 2,3-Epoxypropyl methacrylate, was obtained from Fluka A/G, Switzerland. It was used in the preparation of macroporous GMA-HEMA-EGDM, GMA-MMA-EGDM and GMA-HEMA-DVB terpolymers. These terpolymers were evaluated for binding penicillin G acylase.

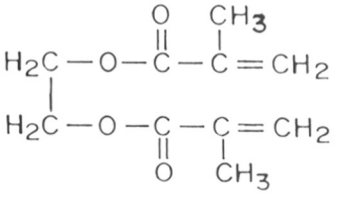
(vii) Ethylene glycol dimethacrylate

Empirical formula $C_{10}H_{14}O_4$

Molecular weight 198.22

Density 1.051 gm/mL

Chemical structure



Ethylene glycol dimethacrylate (EGDM), also known as 1,2-ethane diol dimethacrylate, was obtained from Fluka A/G, Switzerland. It was used as received to prepare macroporous GMA-HEMA-EGDM and GMA-MMA-EGDM terpolymers.

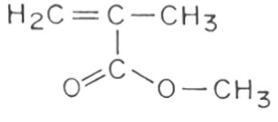
(viii) Methyl methacrylate

Empirical formula $C_5H_8O_2$

Molecular weight 100.12

Density 0.936 gm/mL

Chemical structure



Methyl methacrylate (MMA), was obtained from Fluka A/G, Switzerland. It was used in the preparation of macroporous GMA-MMA-EGDM terpolymers. These were evaluated for binding penicillin G acylase.

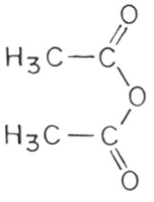
(ix) Acetic anhydride

Empirical formula $C_4H_6O_3$

Molecular weight 102.09

Density 1.082 gm/mL

Chemical structure



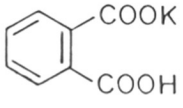
Analytical reagent grade acetic anhydride was obtained from M/S. Loba Chemie, Bombay (India). It was used to cap the primary and secondary amino groups in the estimation of tertiary amino groups in polyethyleneimine by perchloric acid.

(x) Potassium hydrogen phthalate

Empirical formula $C_8H_5O_4K$

Molecular weight 204.23

Chemical structure



Potassium hydrogen phthalate was obtained from M/S.. Loba Chemie, Bombay (India). It was used as the primary standard to estimate the normality of perchloric acid used in the analysis of the distributions of primary:secondary:tertiary nitrogen atoms.

(xi) Perchloric acid

Empirical formula $HClO_4$

Molecular weight 100.46

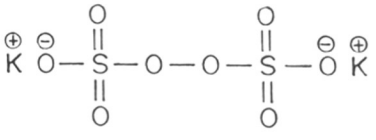
Perchloric acid was obtained as 70 weight percent solution from S.D.Fine Chem Pvt. Ltd., Boisar (India). It was used to estimate the distributions of primary:secondary:tertiary nitrogens in branched polyethyleneimine.

(xii) Potassium persulphate

Empirical formula $K_2S_2O_8$

Molecular weight 270.33

Chemical structure



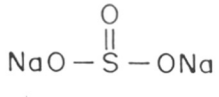
Analytical reagent grade potassium persulphate was obtained from M/S. Loba Chemie, Bombay (India). It was used, as received, as the initiator for the solution and matrix polymerisations of acrylic and methacrylic acids.

(xiii) Sodium sulphite

Empirical formula Na_2SO_3

Molecular weight 126.04

Chemical structure



Analytical reagent grade sodium sulphite was obtained from M/S. Loba Chemie, Bombay (India). It was used, as received, as a component of the redox initiator system for solution polymerisation of acrylic and methacrylic acids.

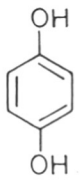
(xiv) Hydroquinone

Empirical formula $C_6H_6O_2$

Molecular weight 110.11

M.P 173°C

Chemical structure



Hydroquinone was obtained from M/S. Loba Chemie, Bombay (India). It was used to terminate the solution and matrix polymerisations. It was also employed as stabiliser for 4-(dimethylamino) benzaldehyde, used to estimate the activity of immobilised enzyme.

(xv) Aluminium nitrate nonahydrate

Empirical formula $Al(NO_3)_3.\ 9\ H_2O$

Molecular weight 375.14

Analytical reagent grade aluminium nitrate nonahydrate was obtained from local suppliers. It was used as received to form macroporous alumina, used as a support for enzyme immobilisation.

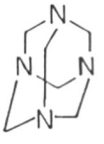
(xvi) Hexamethylene tetramine

Empirical formula $C_6H_{12}N_4$

Molecular weight 140.19

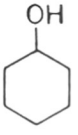
M.P. 280°C

Chemical structure



Hexamethylene tetramine (HMTA) was obtained from M/S.Loba Chemie, Bombay (India). It was used as pore generating agent to form macroporous alumina from aluminium nitrate.

(xvii) Cyclohexanol

Empirical formula	$C_6H_{12}O$
Molecular weight	100.16
B.P.	160°C
Density	0.963 gm/mL
Chemical structure	

Cyclohexanol was obtained from M/S. Aldrich Chemical Co. (USA). The 99 percent pure reagent was used as one of the two pore generating solvents in the synthesis of macroporous beaded supports by suspension polymerisation.

(xviii) Lauryl alcohol

Empirical formula	$C_{12}H_{26}O$
Molecular weight	186.34
B.P.	260°C
Density	0.820 gm/mL
Chemical structure	$H_3C-(CH_2)_{11}-OH$

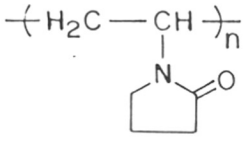
Lauryl alcohol (1-dodecanol, dodecyl alcohol) was obtained from M/S. Aldrich Chemical Co. (USA). It was used as a pore generating solvent in the synthesis of macroporous beaded supports by suspension polymerisation.

(xix) Poly (vinyl pyrrolidone)

Empirical formula $(C_6H_9NO)_n$

Molecular weight 3,60,000

Chemical structure



Poly (vinyl pyrrolidone), K-90, was obtained from M/S. Polysciences, USA. It was used as protective colloid in the suspension polymerisations to generate macroporous supports.

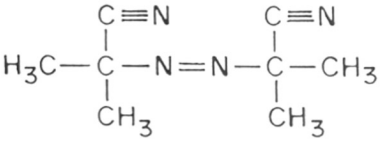
(xx) **Azo bis (isobutyro nitrile)**

Empirical formula $C_8H_{12}N_4$

Molecular weight 164.21

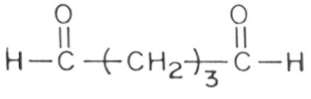
M.P. 103°C

Chemical structure



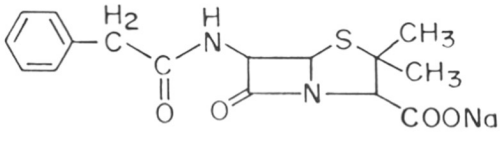
Azo bis (isobutyro nitrile), [AIBN], was obtained from M/S. SISCO, Bombay, India. It was used as the initiator for the suspension polymerisation to generate macroporous supports.

(xxi) Glutaraldehyde

Empirical formula	$C_5H_8O_2$
Molecular weight	100.12
B.P.	103°C
Chemical structure	

Glutaraldehyde, 50 weight percent solution in water, was obtained from M/S. Fluka A/G , Switzerland. It was used to form supports with aldehyde pendant groups used for binding Penicillin G acylase.

(xxii) Penicillin G Sodium salt

Empirical formula	$C_{16}H_{17}N_2O_4SNa$
Molecular weight	356.38
Chemical structure	

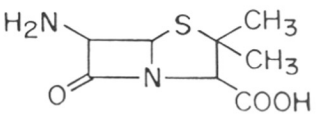
Penicillin G was obtained from the production line of M/S. Hindustan Antibiotics Limited, Pimpri (India). It was used as received as substrate to estimate the activity of immobilised and free penicillin G acylase.

(xxiii) Ammonium sulphate

Empirical formula	$(NH_4)_2SO_4$
Molecular weight	132.14
M.P	235°C
Density	1.769 gm/mL

Ammonium sulphate was obtained from M/S. Loba Chemie, Bombay (India). It was used in the partial purification of penicillin G acylase by precipitation.

(xxiv) 6-Amino Penicillanic acid

Empirical formula	$C_8H_{12}N_2O_3S$
Molecular weight	216.26
M.P.	207°C
Chemical structure	

6-Amino Penicillanic acid (6-APA) was obtained from the production line of M/S. Hindustan Antibiotics Limited, Pimpri (India). It was used as received in the assay method to estimate the activity of free and immobilised Penicillin G acylase.

(xxv) Methanol

Empirical formula	CH_4O
Molecular weight	32.04
B.P.	64°C
Density	0.791 gm/mL

Analytical reagent grade methanol was obtained from Qualigens, Bombay (India). It was used for preparing 4-(Dimethyl amino) benzaldehyde (PDAB) reagent solution.

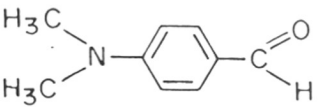
(xxvi) 4-(Dimethyl amino) benzaldehyde

Empirical formula $C_9H_{11}NO$

Molecular weight 149.19

M.P. 74°C

Chemical structure



4-(Dimethyl amino) benzaldehyde (PDAB) was obtained from M/S. Aldrich Chemical Co. (USA). It was used as the reagent for the initial standardisation of 6-amino penicillanic acid as well as that generated by the immobilised enzyme.

(xxvii) Bovine serum albumin (BSA)

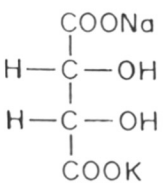
Crystallised bovine serum albumin was obtained from Sigma chemical company, USA. It was used for constructing the standard plot for protein estimation.

(xxviii) Sodium potassium tartarate tetrahydrate (Rochelle salt)

Empirical formula $C_4H_4O_6NaK.\ 4H_2O$

Molecular weight 282.23

Chemical structure



Analytical reagent grade sodium potassium tartarate tetrahydrate was obtained from M/S. Loba Chemie, Bombay (India). It was used as 2 weight percent aqueous solution in the protein estimation.

(xxix) Copper sulphate pentahydrate

Empirical formula $CuSO_4.5H_2O$

Molecular weight 249.68

Melting point 200°C

Analytical reagent grade copper sulphate pentahydrate was obtained from M/S. Loba Chemie, Bombay (India). It was used as 1 weight percent aqueous solution in protein estimation.

(xxx) Sodium carbonate

Empirical formula Na_2CO_3

Molecular weight 105.99

Analytical reagent grade sodium carbonate was obtained from M/S. Loba Chemie, Bombay (India). It was used as 2 weight percent solution in 0.1 molar sodium hydroxide, for protein estimation.

(xxxi) Folin and Ciocalteu's phenol reagent

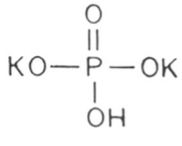
Folin and Ciocalteu's phenol reagent was obtained as 2 molar solution from M/S. Loba Chemie, Bombay (India). It was used for protein estimation by diluting in 1:1 proportion with deionised water.

(xxxii) Dipotassium hydrogen phosphate

Empirical formula K_2HPO_4

Molecular weight 174.18

Chemical structure



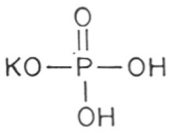
Analytical reagent grade dipotassium hydrogen phosphate was obtained from M/S. Loba Chemie, Bombay (India). It was used to prepare penicillin G sodium salt solution. This solution was used in the preparation of buffer used to assay the activity of immobilised and free penicillin G acylase.

(xxxiii) Potassium dihydrogen phosphate

Empirical formula KH_2PO_4

Molecular weight 136.09

Chemical structure



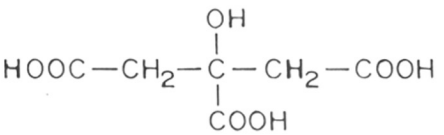
Analytical reagent grade potassium dihydrogen phosphate was obtained from M/S. Loba Chemie, Bombay (India). It was used as a buffer in the preparation of penicillin G sodium salt solution. This solution was used to assay the activity of immobilised and free penicillin G acylase.

(xxxiv) Citric acid

Empirical formula $C_6H_8O_7.H_2O$

Molecular weight 210.14

Chemical structure



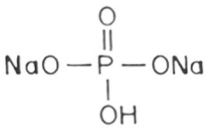
Analytical reagent grade citric acid monohydrate was obtained from M/S. Loba Chemie, Bombay (India). It was used as a buffer to quench the enzymatic conversion of penicillin G to 6-amino penicillanic acid.

(xxxv) Disodium hydrogen phosphate

Empirical formula Na_2HPO_4

Molecular weight 141.96

Chemical structure



Analytical reagent grade disodium hydrogen phosphate was obtained from M/S. Loba Chemie, Bombay (India). It was used as a buffer in conjunction with citric acid to quench the enzymatic conversion of penicillin G to 6-amino penicillanic acid.

2.2 ANALYSIS OF B-PEI

A non aqueous millivolt titration was used to establish the tertiary nitrogen concentration in branched polyethyleneimine (B-PEI). The method consisted of blocking primary and secondary amino groups with acetic anhydride and subsequently estimating the tertiary nitrogen with perchloric acid. The basic and valid premise is that during polymerisation of ethyleneimine primary and tertiary nitrogens emanate from the secondary amino groups by back-biting reaction. Thus, the concentrations of primary and tertiary nitrogens will be similar. The ratio of primary:secondary:tertiary nitrogens can be established from the quantitative estimation of one of these three species.

2.2.1 Stock Solutions

(i) B-PEI Solution: 5.81 gm of 100 percent B-PEI (11.62 gm for 50 weight percent aqueous samples) was weighed into 100 mL volumetric flask and made up to the mark with glacial acetic acid (stock solution A). This was diluted five fold by pipetting 20 ml into another 100 mL volumetric flask and making up to the mark with glacial acetic acid (stock solution B).

(ii) Perchloric acid: The 70 weight percent solution was diluted with glacial acetic acid to prepare

the stock solution. Specifically, 1.2 mL was diluted to 250 mL with glacial acetic acid. The normality of this solution was estimated using potassium hydrogen phthalate as basic standard. (See section 2.2.2)

(iii) Potassium hydrogen phthalate: A 0.1 molar stock solution was prepared by weighing 5.1058 gm into 250 mL volumetric flask and making up to the mark with glacial acetic acid.

2.2.2 Perchloric acid standardisation

The standardisation was conducted by millivolt titration. 25 mL of perchloric acid stock solution was titrated against the standard solution of potassium hydrogen phthalate. The millivolt signal and incremental increase corresponding to the volume of potassium hydrogen phthalate added were recorded. A "S" shaped curve results. The end point, corresponding to 11.95 mL, was obtained from the intersection of the two tangents at inflection point (*Figure* 18). The data is presented in Table 4. The exact normality of perchloric acid was estimated as 0.0478 N.

2.2.3 Analysis of B-PEI

5.0 mL of the B-PEI stock solution B was pipetted into a 100 mL stoppered Erlenmeyer flask. 7.0 mL of acetic anhydride was added to the flask, allowed to react for 48 hours at room temperature and then titrated against the standardised perchloric acid solution in millivolt mode. The data is presented in Table 5. The end point was computed graphically (*Figure* 19) as 7.36 mL. The normality of B-PEI in terms of tertiary nitrogen is 0.0736 N. The weight of PEI in 1000 ml of the stock solution B was 5.81 x 2 = 11.62 gm. The formula weight per repeat unit of B-PEI is 43.0662. The normality of the stock solution based on repeat unit was 0.2698 N. Thus, the percentage of tertiary nitrogen atoms in B-PEI was

$$\frac{0.07036}{0.2698} x\ 100 = 26.08\%$$

Thus, as discussed in section 2.2, the relative ratio of primary:secondary:tertiary nitrogens in B-PEI are 1:2:1.

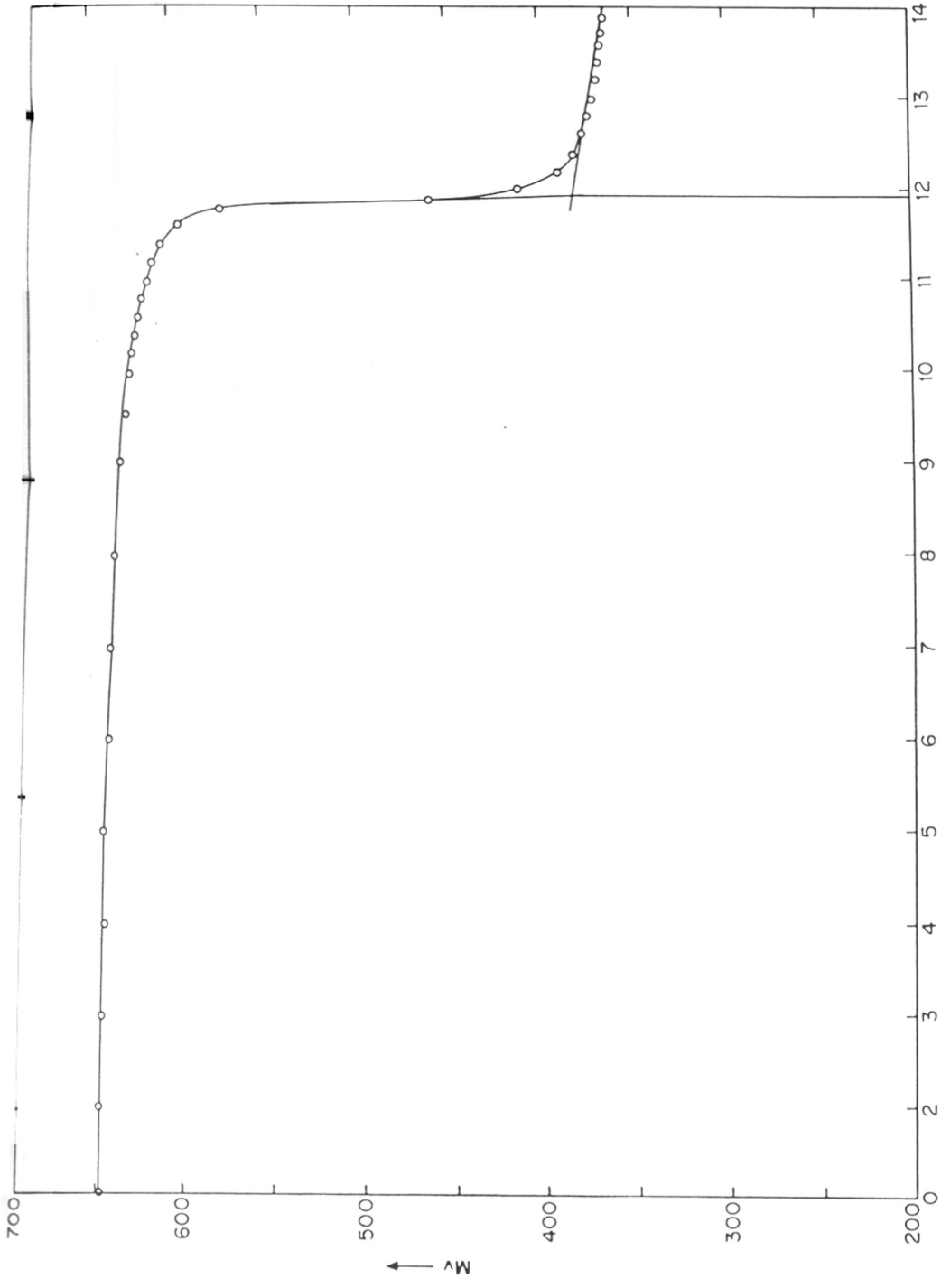


FIG.18: TITRATION CURVE FOR STANDARDISATION OF PERCHLORIC ACID

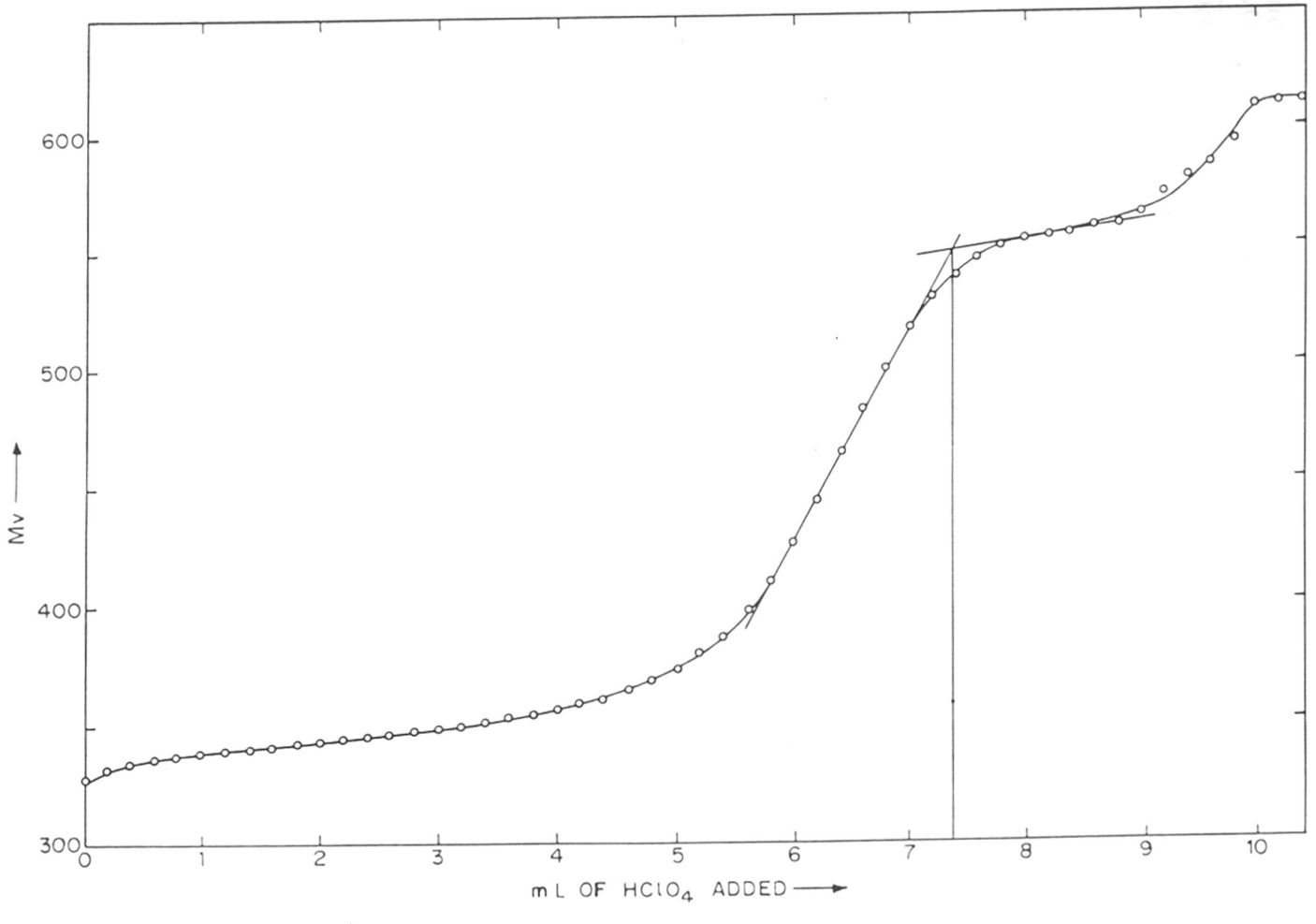


FIG.19 : TITRATION CURVE FOR ESTIMATION OF TERTIARY NITROGEN IN B-PEI

Table - 4

Standardisation of $HClO_4$ with 0.1 N KHP
25 mL OF $HClO_4$ in beaker. KHP added from burette

KHP mL	mV	mV	V mL x 10	mV/V
0.0	649	-	10	0.0
1.0	648	1	10	0.1
2.0	646	2	10	0.2
3.0	644	2	10	0.2
4.0	643	1	10	0.1
5.0	642	1	10	0.1
6.0	639	3	10	0.3
7.0	638	1	10	0.1
8.0	635	3	10	0.3
9.0	631	4	10	0.4
10.0	625	6	10	0.6
10.2	624	1	2	0.5
10.4	622	2	2	1.0
10.6	619	3	2	1.5
10.8	617	2	2	1.0
11.0	614	3	2	1.5
11.1	612	2	1	2.0
11.2	611	1	1	1.0
11.3	608	3	1	3.0
11.4	606	2	1	2.0
11.5	601	5	1	5.0
11.6	596	5	1	5.0
11.7	588	8	1	8.0
11.8	573	15	1	15.0
11.9	459	114	1	114.0
12.0	411	48	1	48.0

12.1	397	14	1	14.0
12.2	390	7	1	7.0
12.3	385	5	1	5.0
12.4	381	4	1	4.0
12.5	378	3	1	3.0
12.6	376	2	1	2.0
12.7	374	2	1	2.0
12.8	373	1	1	1.0
12.9	371	1	1	1.0
13.0	370	1	1	1.0
13.1	369	1	1	1.0
13.2	369	0	1	0.0
13.3	368	1	1	1.0
13.4	367	1	1	1.0
13.5	366	1	1	1.0
13.6	366	-	1	0.0
13.7	365	1	1	1.0

Table - 5

Estimation of tertiary nitrogen in B-PEI

5 mL [B], 7 mL Acetic anhydride, 10 mL Glacial acetic acid and titration against $HClO_4$

$HClO_4$ mL	mV	mV	V mL x 10	mV/V
0.0	327	-	1	2.5
0.2	332	5	2	1.0
0.4	334	2	2	0.5
0.6	335	1	2	0.5
0.8	336	1	2	1.0
1.0	337	2	2	0.5
1.2	338	1	2	0.5
1.4	339	1	2	0.5
1.6	340	1	2	0.5
1.8	341	1	2	0.5
2.0	342	1	2	0.5
2.2	343	1	2	0.5
2.4	344	1	2	0.5
2.6	345	1	2	0.5
2.8	346	1	2	0.5
3.0	347	1	2	0.5
3.2	348	1	2	0.5
3.4	349	2	2	0.5
3.6	351	2	2	1.0
3.6	353	1	2	1.0
3.8	354	2	2	0.5
4.0	356	3	2	1.0
4.2	359	2	2	1.5
4.4	361	4	2	1.0
4.6	365	4	2	2.0

4.8	369	5	2	2.0
5.0	374	6	2	2.5
5.2	380	7	2	3.0
5.4	387	10	2	3.5
5.6	397	13	2	5.0
5.8	410	15	2	6.5
6.0	425	18	2	7.5
6.2	443	20	2	9.0
6.4	463	18	2	10.0
6.8	481	19	2	9.0
6.8	500	16	2	9.5
7.0	516	13	2	8.0
7.2	529	9	2	6.5
7.4	538	7	2	4.5
7.6	545	5	2	3.5
7.8	550	3	2	2.5
8.0	553	1	2	1.5
8.2	554	2	2	0.5
8.4	556	2	2	1.0
8.6	558	1	2	1.0
8.8	559	5	2	0.5
9.0	564	8	2	2.5
9.2	572	8	2	4.0
9.4	580	5	2	4.0
9.6	585	9	2	2.5
9.8	594	6	2	4.5
10.0	609	10	2	3.0
10.2	610	-1	2	5.0
10.4	609	1	2	---
10.6	610	0	2	0.5
10.8	610	0	2	---

2.3 POLYMERISATIONS

The following polymerisations were investigated: (i) Solution polymerisations of acrylic and methacrylic acids in water using persulphate-sulphite redox initiator [See section 2.3.2], (ii) solution polymerisations of acrylic and methacrylic acids using persulphate-polyethyleneimine redox initiator [See section 2.3.3] and (iii) Polymerisations of acrylic and methacrylic acids initiated by persulphate in presence of polyethyleneimine matrix. In the matrix polymerisation experiments, kinetic features such as (a) effect of monomer to matrix ratio on the polymerisation rate [See section 2.3.4.1] and (b) effect of initiator concentration on the rate [See section 2.3.4.2] were estimated. Deionised water was used as the reaction medium and for preparing stock solutions.

2.3.1 Stock Solutions

All the reactants (monomers, matrix, initiators and inhibitor) were accurately pipetted out from the prepared stock solutions, to avoid weighing errors.

(i) Acrylic acid: A 4 molar stock solution was prepared by weighing out 72.06 gm of acrylic acid and making up to 250 mL with deionised water.

(ii) Methacrylic acid: A 4 molar stock solution was prepared by weighing out 86.09 gm of methacrylic acid and making up to 250 mL with deionised water.

(iii) Polyethyleneimine: (a) Matrix: A 4 molar stock solution of polyethyleneimine was prepared by weighing out 43.0662 gm of B-PEI and making up to 250 mL with deionised water. (b) Initiator: A 1 molar stock solution was prepared by weighing 43.0662 gm of B-PEI and making up to 1 litre with deionised water. 3.5 mL of this stock solution was diluted to 100 mL to make a 3.5×10^{-2} molar initiator stock solution.

(iv) Potassium persulphate: A 3.5×10^{-2} molar stock solution was prepared by weighing 2.3654 gm into 250 mL volumetric flask and making up to mark with deionised water.

(v) Sodium sulphite: A 3.5×10^{-2} molar stock solution was prepared by weighing 1.1029 gm into

250 mL volumetric flask and making up to mark with deionised water.

(vi) Hydroquinone: A 7.0 x 10^{-2} M stock solution was prepared by dissolving 1.9269 gm in 250 ml of deionised water in a volumetric flask.

2.3.2 Solution Polymerisation

The reaction vessel used for conducting polymerisation is depicted in *Figure* 20.The isothermal reactions were conducted in a thermostated water bath maintained at 30.0 $\pm$ 0.1°C. The monomer concentration was kept at 1 molar. The concentration of the persulphate-sulphite initiator concentration was 3.5 x 10^{-3} molar. In a typical experiment, 12.5 mL of monomer (acrylic/methacrylic acid) stock solution was buretted out into the reaction vessel. 27.5 mL of deionised water was then added. Purified nitrogen was bubbled through the reaction vessel for 10 minutes. 5.0 mL each of persulphate and sulphite stock solutions were then added sequentially in rapid succession. The reaction vessel was stoppered and transferred to the thermostated water bath. After a predetermined time the vessel was opened and the polymerisation was terminated by the addition of 10.0 mL of hydroquinone stock solution. A number of experiments were run for each monomer, keeping all the variables excepting the reaction time constant.

The polymer was quantitatively isolated by precipitation. The reaction mixture was poured into 1 litre of 80/20 V/V acetone/pet-ether mixed non-solvent system. The precipitated polymer was quantitatively transferred into weighed G-4 sintered glass crucible and dried to constant weight in a vacuum desiccator, over phosphorous pentoxide, at room temperature. The percent conversion was estimated. The experimental data for the acrylic acid and methacrylic acid are presented in Table 6 and 7 respectively. The percentage conversion vs time curves for the two solution polymerisations are presented in *Figure* 21.

2.3.3 Induced Decomposition

The rates of polymerisation of acrylic and methacrylic acids initiated by the persulphate in the presence of polyethyleneimine, in equivalent concentration, were determined. The conditions

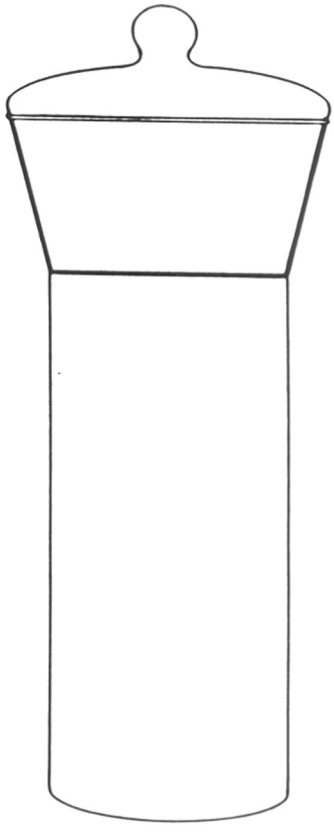


FIG.20: REACTION VESSEL USED FOR CONDUCTING SOLUTION AND MATRIX POLYMERISATIONS

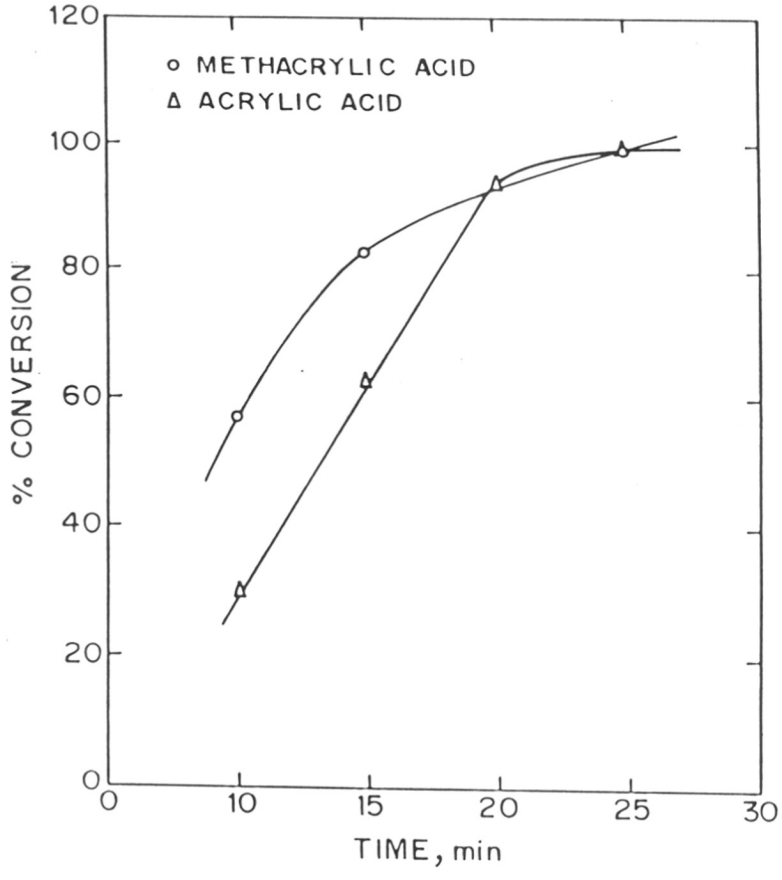


FIG. 21: PERCENTAGE CONVERSION Vs. TIME CURVES FOR THE POLYMERISATION OF METHACRYLIC AND ACRYLIC ACID IN ABSENCE OF B-PEI

Table - 6

Experimental data of solution polymerisation of acrylic acid

Time, minutes	Polymer yield, gm	% Conversion
10	1.0604	29.43
15	2.1407	59.41
20	3.3500	92.98
25	3.5529	98.61

Table - 7

Experimental data of solution polymerisation of methacrylic acid

Time, minutes	Polymer yield, gm	% Conversion
10	2.4344	55.55
15	3.5488	82.44
25	4.2521	98.78

were maintained identical to that presented in section 2.3.2 to make a comparative evaluation with respect to the persulphate-sulphite redox system. This was to determine whether poly-ethyleneimine forms a redox pair with persulphate and to estimate the efficiency of this redox pair. The experimental details such as concentrations of monomer, initiator and inhibitor as well as the work-up procedure were identical to that presented in section 2.3.2. The experimental details are tabulated in Tables 8 and 9 respectively.

2.3.4 Matrix Polymerisations

The matrix polymerisations of acrylic and methacrylic acids were investigated. Branched polyethyleneimine, of molecular weight 20,000, was used as the matrix.

2.3.4.1 Monomer-Matrix Ratio

A series of matrix polymerisations of acrylic/methacrylic acids were conducted, wherein the monomer-matrix ratio were varied. Within each series, the monomer-matrix ratio was maintained constant while the reaction times were varied. The polymerisations were investigated in the following relative monomer-matrix ratios: 0.4:1, 0.6:1, 0.8:1, 1:1, 1.5:1 and 2:1. In all the experiments the concentration of the matrix was 1.0 molar with respect to the amino group. The monomer concentration was varied to get the respective monomer-matrix ratios.

In a typical 1:1 monomer-matrix ratio series, 12.5 mL of polyethyleneimine stock solution was buretted into the reaction vessel. Next, 12.5 mL of monomer stock solution was buretted into the reaction vessel. 20.0 mL of deionised water was buretted in. Purified nitrogen was bubbled through the reaction vessel for 10 minutes and polymerisation was initiated by the addition of 5.0 mL of potassium persulphate stock solution. The other pertinent experimental details were the same as that presented in section 2.3.2.

Table - 8

Experimental details of polymerisation of acrylic acid initiated by $Na_2SO_3/K_2S_2O_8$ and B-PEI/$K_2S_2O_8$

Redox polymerisation of acrylic acid in water at 30°C ± 0.1°C.

Acrylic acid = 1.0 M; $K_2S_2O_8$, Na_2SO_3, B-PEI = 3.5 X 10^{-3} M.

Initiating system	Rate X 10^{-3} moles.lit-1. sec.
B-PEI/$K_2S_2O_8$	1.6390
$Na_2SO_3/K_2S_2O_8$	1.6420

Table - 9

Experimental details of polymerisation of methacrylic acid initiated by $Na_2SO_3/K_2S_2O_8$ and B-PEI/$K_2S_2O_8$

Redox polymerisation of methacrylic acid in water at 30°C ± 0.1°C.

Methacrylic acid = 1.0 M; $K_2S_2O_8$, Na_2SO_3, B-PEI = 3.5 X 10^{-3} M.

Initiating system	Rate X 10^{-3} moles.lit-1. sec.
B-PEI/$K_2S_2O_8$	1.6655
$Na_2SO_3/K_2S_2O_8$	1.6195

2.3.4.2 Monomer-Initiator Ratio

The overall rate expression for the matrix polymerisation of methacrylic acid was estimated by studying the effect of initiator concentration on the initial rate. The monomer:matrix ratio was maintained at 1:1. The monomer and matrix concentrations in the reaction mixture were 1 molar. The rate was estimated for the following three initiator concentrations: 3.5×10^{-4}, 1.17×10^{-3} and 3.5×10^{-3} molar.

The experimental details were similar to that presented in section 2.3.4.1.

2.4 SUPPORT PREPARATION

The following supports were prepared for immobilising Penicillin G Acylase. (i) Macroporous alumina, (ii) Hydroxyethyl methacrylate-divinyl benzene (HEMA-DVB) copolymers, (iii) Glycidyl methacrylate-hydroxyethyl methacrylate-ethylene glycol dimethacrylate (GMA-HEMA-EGDM) terpolymers, (iv) Glycidyl methacrylate-methyl methacrylate-ethylene glycol dimethacrylate (GMA-MMA-EGDM) terpolymers and (v) Glycidyl methacrylate-hydroxyethyl methacrylate-divinyl benzene (GMA-HEMA-DVB) terpolymers. The preparation of these polymer supports and their modification with polyethyleneimine are presented below.

2.4.1 Macroporous alumina

Macroporous alumina, of differing micro-pH and pore size, were synthesised from aluminium nitrate. Magnesium nitrate was added in varying amounts to alter the micro-pH. The pore size was controlled by changing the relative amount of the pore generating agent, hexamethylene tetramine (HEXA), with respect to aluminium and magnesium nitrates.

Aluminium nitrate nonahydrate (500 gm) was dissolved in minimum amount of distilled water at room temperature. Required amount of hexamethylene tetramine and magnesium nitrate were added. The experimental compositions are presented in Table 10.The pH was adjusted to 10 by the addition of ammonium hydroxide under stirring to ensure complete precipitation as mixed aluminium-magnesium hydroxides. The precipitate was separated by centrifugation, dried at

Table - 10

Experimental composition for preparation of porous alumina samples

Sample Code	HMTA** content, weight %	MgO content, weight %	Pore radii (Å)
AL 1	12	0.84	56.05
AL 2	12	1.5	46.94
AL 3	12	2.5	46.73
AL 4	12	3.8	47.94
AL 5	20	0.84	60.60
AL 6	20	1.5	47.45
AL 7	20	2.5	51.70
AL 8	20	3.8	49.07
AL 9	24	0.84	45.06
AL 10	24	1.5	50.12
AL 11	24	2.5	50.29
AL 12	24	3.8	49.85

** Hexamethylene tetraamine

100°C under normal pressure. The dried mass was calcined for 3 hours at 450°C to decompose aluminium-magnesium hydroxides into alumina-magnesia. HEXA decomposed during the calcination and generated the porous alumina-magnesia. The calcined product was sieved. Porous beads in the 40-60 mesh size (0.25 - 0.42 mm) were used for enzyme adsorption studies. Pore size distribution was studied by mercury intrusion porosimetry using Quantachrom mercury porosimeter No. 33.

2.4.1.1 Polyethyleneimine Modification

The porous alumina-magnesia were contacted with 4 weight percent aqueous solution of polyethyleneimine (20 mL per gm of alumina) for 24 hours at room temperature. Vacuum was applied initially for 1 hour to facilitate polyethyleneimine coating inside the pores. The coated alumina was recovered by filtration, treated with 25.0 mL of 2.5 weight percent glutaraldehyde solution (per gm of alumina) at room temperature for 24 hours. Excess glutaraldehyde was decanted off and the support was washed repeatedly with distilled water till the filtrate was free from glutaraldehyde. The details are presented in Table 11.

2.4.2 HEMA-DVB Beaded Supports

The reactor assembly used for the synthesis of macroporous HEMA-DVB beads is depicted in *Figure 22*.

Suspension polymerisation was conducted in this 1 litre double walled cylindrical reactor under nitrogen blanket. Continuous phase comprised of 2 weight percent poly (vinyl pyrrolidone) (PVP) in water. The discontinuous organic phase consisted of HEMA, DVB, polymerisation initiator AIBN and a mixture of cyclohexanol and lauryl alcohol used as pore generating solvents. The ratio of aqueous to organic phase was set at 2.8572:1. The discontinuous organic phase was introduced into the aqueous phase, stirring was set at 300 rpm and the temperature was raised to 65 $\pm$ 0.1°C by circulating hot water. The polymerisation was allowed to proceed for 3 hours.

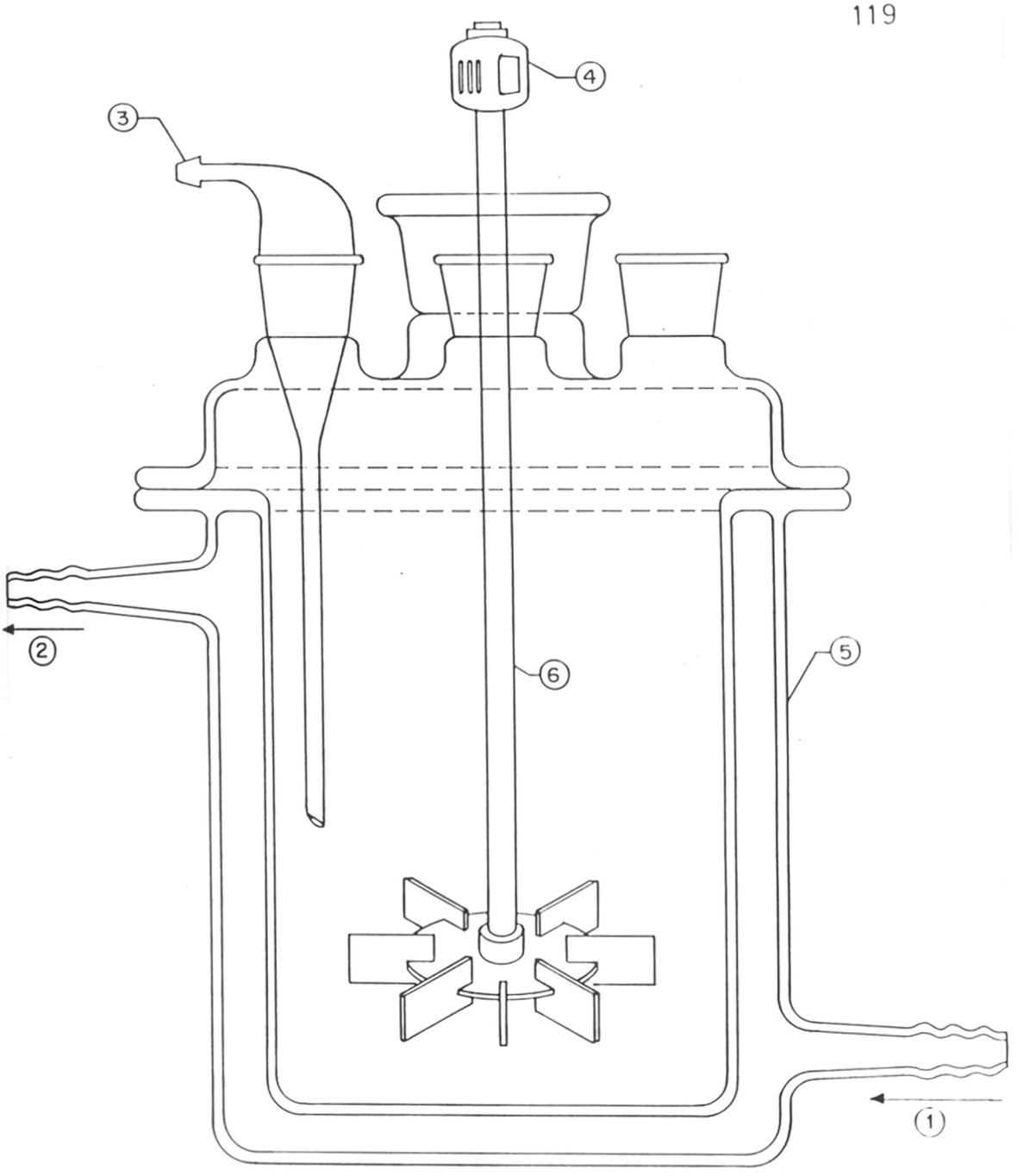
Table - 11

Modification of Alumina (AL 11) with B-PEI and Glutaraldehyde.

Amount of support taken = 1.0 gm

Sample code	B-PEI* Molecular Weight	B-PEI,(4 %, wt./vol.), mL	Time, h	Glutaraldehyde, (2.5 % wt./vol.), mL
AL-11	Nil	Nil	Nil	Nil
AL-13	1,300	20.0	24	25.0
AL-14	2,000	20.0	24	25.0
AL-15	20,000	20.0	24	25.0
AL-16	6,50,000-10,000,00	20.0	24	25.0

* Branched polyethyleneimine



(1) HOT WATER INLET (2) HOT WATER OUTLET (3) NITROGEN BUBBLER
(4) STIRRER MOTOR (5) REACTOR FLASK (6) STIRRER

FIG.22: LABORATORY REACTOR ASSEMBLY FOR SUSPENSION POLYMERISATION

The copolymer was obtained in beaded form. It was separated by decantation, washed with methanol, water and dried at room temperature under vacuum. The compositions of the synthesised copolymers are presented in Table 12.

2.4.2.1 PEI Modification of copolymers

The polymer beads were sieved. Those passing through 40 mesh and retained on 80 mesh were set aside for enzyme adsorption studies. 1 gm of beads from the composition showing greatest adsorption of enzyme was coated under vacuum for 18 hours with 25 mL of 4 weight percent aqueous solution of branched polyethyleneimine (B-PEI) of molecular weight 2,000. The coated support was filtered, washed with deionised water and treated with an excess of 25 weight percent aqueous glutaraldehyde solution on a New Brunswick Scientific shaker at 200 rpm for 2 hours. The beads were filtered and washed repeatedly with deionised water till free from glutaraldehyde. The beads were air dried and evaluated for enzyme binding.

2.4.3 Glycidyl methacrylate terpolymers

The following three terpolymer series were synthesised in beaded macroporous form: (i) Glycidyl methacrylate-hydroxyethyl methacrylate-ethylene glycol dimethacrylate (GMA-HEMA-EGDM) terpolymers, (ii) Glycidyl methacrylate-methyl methacrylate-ethylene glycol dimethacrylate (GMA-MMA-EGDM) terpolymers and (iii) Glycidyl methacrylate-hydroxyethyl methacrylate-divinyl benzene (GMA-HEMA-DVB). Within each series the crosslink density was kept constant. The relative amounts of the other two comonomers were varied to generate a number of polymers. Suspension polymerisation was conducted as described earlier in section 2.4.2. Cyclohexanol was used as the pore generating solvent The reaction temperature was 70 $\pm$ 1°C. The feed compositions are described in Tables 13-15.

Table - 12

Composition of hydroxyethyl methacrylate (HEMA) - divinyl benzene (DVB) copolymers and their verage pore size.

Poly (vinyl pyrrolidone) [PVP] = 2 weight percent in water

Temperature = 65 $\pm$ 0.1°C

AIBN = 6.0897 X 10^{-3} moles

Polym. code	HEMA (Mole fraction)	DVB (Mole fraction)	Water (mL.)	Average pore size, $\overset{o}{(A)}$
HD 1	0.5047	0.4953	257.7	54.90
HD 2	0.4541	0.5459	265.7	53.22
HD 3	0.4053	0.5947	273.4	62.81
HD 4	0.3579	0.6421	279.0	59.05
HD 5	0.3123	0.6877	285.7	67.73
HD 6	0.2390	0.7610	292.4	68.71
HD 7	0.2256	0.7744	300.5	71.65
HD 8	0.1848	0.8152	307.3	77.17
HD 9	0.1454	0.8546	311.7	95.55

Table - 13

Glycidyl methacrylate (GMA) - Hydroxyethyl methacrylate (HEMA)- Ethylene glycol dimethacrylate (EGDM) terpolymers:

Composition and average pore radii values

Polymer No.	GMA (Moles)	HEMA (Moles)	EGDM (Moles)	Average Pore radii, (Å)
P 1	0.1078	-----	0.0779	58.0
P 2	0.1051	0.0027	0.0779	56.4
P 3	0.1024	0.0054	0.0779	60.6
P 4	0.0970	0.0108	0.0779	62.3
P 5	0.0889	0.0189	0.0779	55.5
P 6	0.0809	0.0270	0.0779	53.5
P 7	0.0674	0.0404	0.0779	48.6
P 8	0.0539	0.0539	0.0779	53.5
P 9	0.0270	0.0809	0.0779	53.5

Table - 14

Glycidyl methacrylate (GMA) - Methyl methacrylate (MMA) - Ethylene glycol dimethacrylate (EGDM) terpolymers:

Composition and average pore radii values

Polymer No.	GMA (Moles)	HEMA (Moles)	EGDM (Moles)	Average Pore radii, Å
M 1	0.1051	0.0027	0.0779	61.0
M 2	0.1024	0.0054	0.0779	59.6
M 3	0.0970	0.0108	0.0779	54.9
M 4	0.0889	0.0189	0.0779	60.7
M 5	0.0809	0.0270	0.0779	58.1
M 6	0.0674	0.0404	0.0779	54.5
M 7	0.0539	0.0539	0.0779	55.9
M 8	0.0270	0.0809	0.0779	52.6

Table - 15

Glycidyl methacrylate (GMA) - Hydroxyethyl methacrylate (HEMA) - Divinyl benzene (DVB) terpolymers:

Composition and average pore radii values

Polymer No.	GMA (Moles)	HEMA (Moles)	DVB (Moles)	Average Pore radii, Å
DVB 1	0.1078	-----	0.1303	66.2
DVB 2	0.1051	0.0027	0.1303	59.3
DVB 3	0.1024	0.0054	0.1303	62.2
DVB 4	0.0970	0.0108	0.1303	59.0
DVB 5	0.0889	0.0189	0.1303	63.2
DVB 6	0.0809	0.0270	0.1303	55.0
DVB 7	0.0674	0.0404	0.1303	62.2
DVB 8	0.0539	0.0539	0.1303	58.5
DVB 9	0.0270	0.0809	0.1303	67.1

2.4.3.1 PEI modification of terpolymers

The glycidyl terpolymers were modified to varying degrees with polyethyleneimine, by contacting the beads with polyethyleneimine solution in distilled water for 24 hours. The concentration of polyethyleneimine was varied to control the extent of modification between 3 and 50 percent of oxirane (glycidyl) groups present in the beads. The experimental data is represented in Table 16.

2.5 ENZYME PREPARATION

Enzyme penicillin G acylase used in this study was isolated from *Escherichia coli* ATCC 11105. The strain was obtained from the National Collection of Industrial Microorganism, Pune, India. *E. coli* was grown in shake flasks at 26°C in 2 weight percent soyabean casein digest medium, with 0.1 weight percent phenylacetic acid as inducer. Cells were harvested after 24 hours by centrifugation as 10,000 g and stored at - 20°C. Isolation and purification steps were conducted at 4°C. Cells were suspended in 0.05 M potassium phosphate buffer, pH 7.8, sonicated for 3 minutes in Biosonic III sonic oscillator and centrifuged at 10,000 g. The cell debris were discarded. The supernatant was treated with streptomycin sulfate (1.4 gm/100 mL) and the precipitate was removed by centrifugation (10,000 g, 30 minutes). The clear supernatant was fractionated with ammonium sulfate. The penicillin G acylase precipitating between 0.3 and 0.8 saturation was collected by centrifugation (10,000 g, 20 minutes). The precipitate was dissolved in minimum volume of 0.05 molar potassium phosphate buffer, pH 2.8, dialysed and clarified. The activity of native (free) penicillin G acylase towards benzylpenicillin sodium salt was estimated by 4-(dimethylamino) benzaldehyde (PDAB) method as presented in section [2.6 (vii)].

2.5.1 Buffer Stock Solutions

i) Phosphate buffer, 0.1 M, pH 7.8 : Phosphate buffer was prepared by mixing 86.0 mL of 1 molar dipotassium hydrogen phosphate (K_2HPO_4) and 14.0 mL of 1 molar potassium dihydrogen phosphate (KH_2PO_4) and diluting the resulting solution to 1000 mL with deionised water in a

Table - 16

Modification of P-1 support (oxiranyl groups) by polyethyleneimine (Molecular weight 2000)

Amount of support taken = 1.0 gm

Polym. No.	Percent modification	PEI solution, molar	Volume of PEI solution, mL	Time, h
P 1	----	Nil	Nil	Nil
PGMA 1	3.13	0.015	10.0	24
PGMA 2	6.25	0.029	10.0	24
PGMA 3	12.50	0.059	10.0	24
PGMA 4	25.00	0.12	10.0	24

volumetric flask. This buffer was used in the immobilisation of Penicillin G acylase and to assay the activities of free and immobilised Penicillin G acylase.

ii) Citrate-phosphate buffer, 0.25 M, pH 2.5 (CPB) : Citrate-phosphate buffer was prepared by dissolving 56.0 gm of citric acid monohydrate and 17.5 gm of disodium hydrogen phosphate dodecahydrate in 1.0 litre of deionised water. This buffer was used to quench the penicillin G to 6-amino penicillanic acid reaction and to estimate the amount of 6-amino penicillanic acid formed.

2.6. ENZYME ACTIVITY ESTIMATION

(i) Principle: Penicillin G acylase catalyses the cleavage of side chain linear amide bond in the penicillin G molecules to produce 6-amino penicillanic acid (6-APA). The reaction scheme is depicted in *Figure* 23.

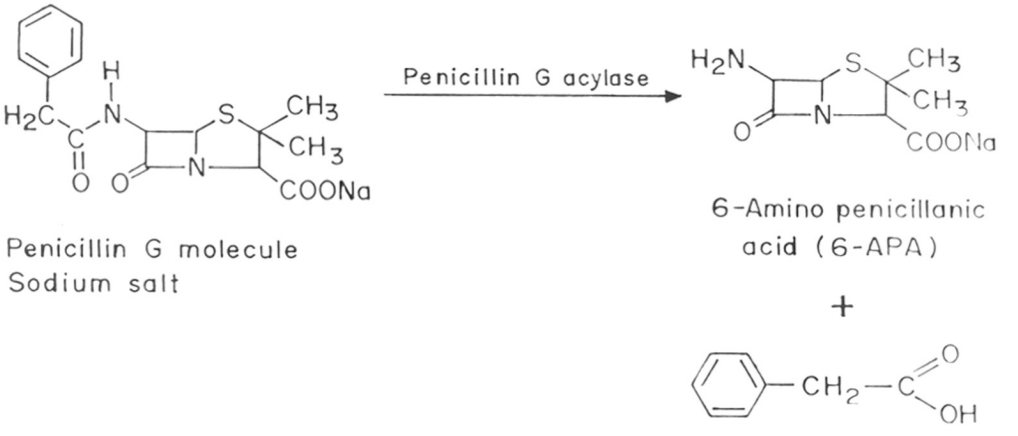


FIGURE. 23

(ii) 4-(dimethylamino) benzaldehyde reagent : The reagent solution was prepared by dissolving 0.2 gm in 34.0 mL of analytical reagent grade methanol. Few grains of hydroquinone was added to impart stability to the reagent.

(iii) Definition of Unit: One International Unit (IU) of penicillin G acylase is defined as the quantity of enzyme required to liberate 1 µmole of 6-amino penicillanic acid (6-APA) in one minute under the assay conditions.

The activity was estimated in units which is quantified by the amount of 6-amino penicillanic acid produced under assay conditions. 6-amino penicillanic acid reacts with 4-(dimethylamino) benzaldehyde to form yellow coloured complex at low pH (pH 2.5). This complex has absorbance maximum at 415 nm.

(iv) Standard plot of 6-amino penicillanic acid : Stock solutions of various concentrations (µmole/mL) of 6-APA were prepared in citrate-phosphate buffer. 1.0 mL of 6-APA solution was mixed at room temperature with 2.0 mL of PDAB reagent. After exactly 3.0 minutes the absorbance was read at 415 nm against the blank prepared under identical conditions by mixing 1.0 mL of citrate-phosphate buffer and 2.0 mL of PDAB reagent solution. The standard plot is shown in *Figure* 24.

(v) Standard plot of bovine serum albumin (BSA) for protein estimation : Stock solutions of various concentrations (µgm/mL) of BSA were prepared in deionised water. 1.0 mL of BSA solution was mixed at room temperature with 5.0 mL of freshly prepared reagent (50.0 mL 2 weight percent sodium carbonate in 0.1 molar NaOH + 0.5 mL copper sulphate + 0.5 mL sodium potassium tartarate). After 10.0 minutes 0.5 mL of Folin and Ciocalteu's phenol reagent was added and after 30 minutes absorbance was read at 500 nm on spectrophotometer. The standard plot is shown in *Figure* 25.

(vi) Penicillin G sodium salt : A 4 weight percent (40 mg/mL) solution was prepared in 0.1 molar phosphate buffer of pH 7.8 for estimating the activity of free and immobilised Penicillin G acylase.

(vii) Penicillin G acylase activity estimation : 0.1 mL of the solution, in which penicillin G acylase activity is to determined, was incubated with 1.0 mL penicillin G sodium salt substrate

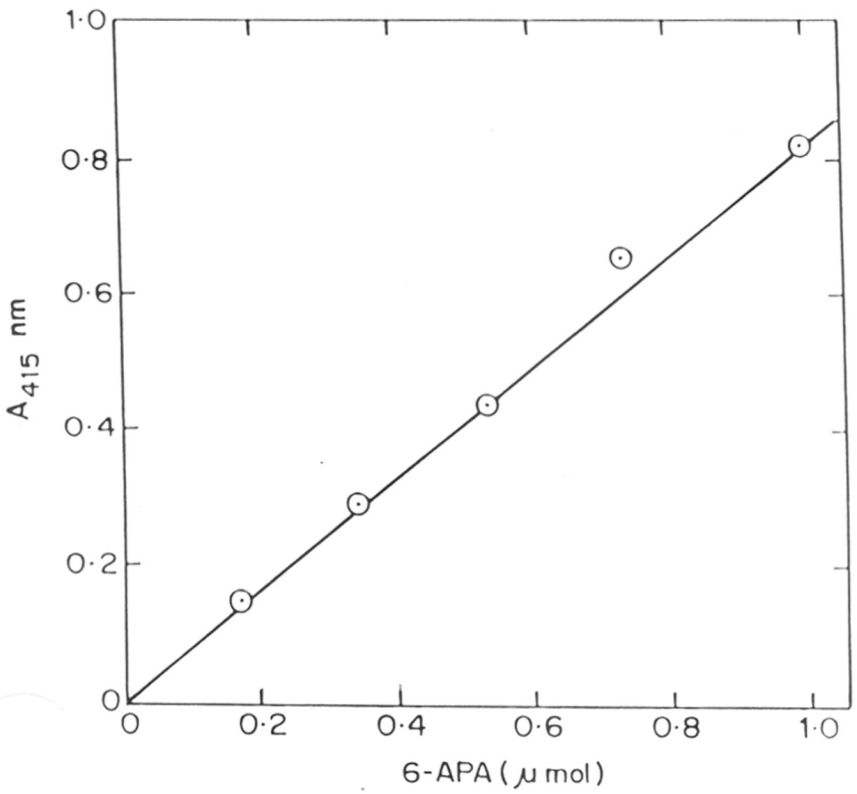


FIG.24 :STANDARD PLOT OF 6-APA. ESTIMATIONS WERE CARRIED OUT WITH p-DIMETHYLAMINOBENZALDEHYDE AS DESCRIBED IN THE TEXT

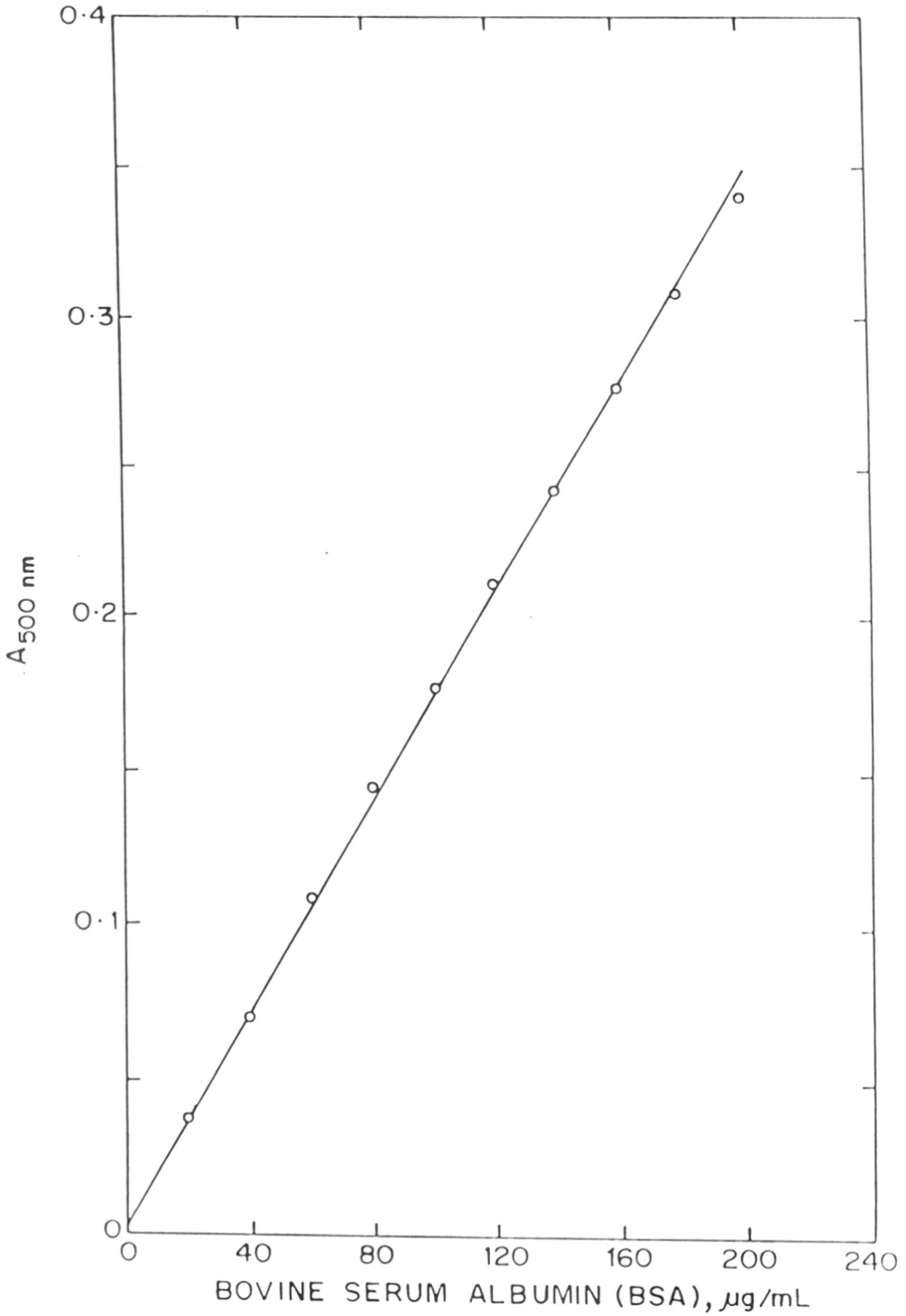


FIG. 25 : STANDARD PLOT FOR PROTEIN ESTIMATION

solution at 40°C for 15 minutes. Then, 0.1 mL aliquot was withdrawn, added to 1.9 mL of citrate-phosphate buffer to quench the reaction. 2.0 mL of PDAB reagent stock solution was added and the absorbance was measured at 415 nm, after 3.0 minutes against deionised water. A blank was prepared under identical conditions by mixing 2.0 mL of citrate-phosphate buffer with 2.0 mL PDAB reagent solution.

From the standard plot

1 μ mole of 6APA = 0.81 OD.

0.81 OD = 1 μ mole of 6-APA

'X' OD = X x 0.81 μ mole of 6-APA

Since 0.1 mL aliquot was withdrawn, and total volume was 1.1 mL, the amount of 6-APA formed in 1.1 mL is

0.1 mL = X x 0.81 μ mole of 6-APA

$$1.1\ mL = \frac{1.1xXx0.81}{0.1}\ \mu mole\ of\ 6-APA\ formed$$

In 1.1 mL the enzyme taken was 0.1 mL

Hence 0.1 mL of enzyme = $\frac{1.1xXx0.81}{0.1}\ \mu mole of 6-APA$

1.0 mL of enzyme = $\frac{1.1xXx0.81}{0.1x0.1}\ \mu mole\ of\ 6-APA formed$

2.7 ENZYME IMMOBILISATION

2.7.1 Immobilisation on Porous Alumina

(i) Immobilisation onto alumina : 5.0 gm of untreated porous alumina, taken in a 250 mL stoppered Erlenmeyer flask, was contacted with 1160 IU of penicillin G acylase in 100 mL of 0.05 molar phosphate buffer (pH 7.5). The flasks were incubated at room temperature for 18 hours with shaking (100 rpm). The enzyme in the supernatant was assayed.

(ii) Immobilisation onto PEI treated alumina : 5.0 gm of alumina modified with B-PEI, taken in 250 mL stoppered Erlenmeyer flask, was contacted with 1160 IU of penicillin G acylase in 100 mL of 0.05 molar phosphate buffer, pH 7.5. It was incubated on a rotary shaker (100 rpm) at room temperature for 18 hours. After the enzyme binding, the preparation was washed with deionised water and stored in 0.05 molar phosphate buffer, pH 7.5 at 5°C. The supernatant was assayed for unbound enzyme.

2.7.2 Immobilisation on HEMA-DVB Copolymers

0.20 gm of support was weighed into 50 mL Erlenmeyer flask and 7.0 mL of soluble enzyme in 0.1 molar phosphate buffer, pH 7.5 (16·39 IU) was added. Enzyme was bound by shaking at 200 rpm for 42 hours at 26°C. The immobilised enzyme was filtered. The units of free enzyme as well as the protein content in the supernatant were estimated. The amount of penicillin G acylase bound on the support was the difference between the units of enzyme added and that remaining in the supernatant.

2.7.3 Immobilisation on Glycidyl Terpolymers

Polymer beads (5 gm) were suspended in 100 mL of 0.05 molar phosphate buffer, pH 7.5 containing 1160 IU of penicillin G acylase. The flasks were incubated in a rotary shaker (100 rpm) at room temperature for 48 hours. The beads were washed with distilled water and stored in 0.05 molar phosphate buffer, pH 7.5 at 5°C. The supernatant was assayed for unbound enzyme.

Quantity of enzyme loaded and amount of enzyme remained in supernatant as unadsorbed. Immobilised enzyme beads were processed for activity determination. The expression of the adsorbed enzyme is defined as the activity of the immobilised enzyme as compared to that of the enzyme bound on the matrix.

2.7.4 Assay of immobilised enzyme

Penicillin acylase activity of immobilised enzyme (IME) was assayed at 40°C in a jacketed vessel under stirring. The assay medium was identical to that used for free enzyme, as described in section 2.6. The volume of the reaction mixture was 20 mL. 0.1 mL aliquots were withdrawn after 15 minute intervals to estimate the 6-APA formed, as described in section [2.6 (vii)].

2.8 CHARACTERISATION

2.8.1 Matrix Polymerised Polycomplex

The polycomplex formed by matrix polymerisation was characterised by (i) Differential scanning calorimetry, (ii) X-ray diffraction, (iii) C-13 nuclear magnetic resonance spectroscopy and (iv) Infra-red spectroscopy.

(i) Differential Scanning Calorimetry : The thermodynamics of phase transitions of matrix polymerised polycomplex was investigated with Mettler TA 4000 coupled with DSC 30 cell. Sample was heated at 10°C/minute.

(ii) X-ray : The wide angle x-ray diffraction patterns of powdered matrix polymerised polycomplex was obtained at room temperature at a scanning rate of 4000 counts per second (cps) between the 2 θ range of 5 to 40°, on a Phillips PW 1730 X-ray diffractometer using CuK_{α} target and nickel filter.

(iii) C-13 NMR : The solid state spectra of synthetic mixture of polyethyleneimine-poly (methacrylic acid) as well as matrix polymerised polycomplex of similar composition were recorded on Bruker MSL-300 FT-NMR spectrometer.

(iv) Infra-red spectroscopy : The infra-red spectra of the polycomplexes were recorded on a

Shimadzu IR-470 dispersive spectrophotometer. The samples were ground to a fine powder, 4 mg was mixed with 100 mg of finely ground KBr and pressed under 10 ton pressure to form the pellets. The spectra were recorded in the range 4000 cm^{-1} to 400 cm^{-1}.

2.8.2 Immobilisation Supports

The porous beaded supports were analysed for (i) Pore size and its distribution and (ii) Surface morphology.

(i) Pore size : Pore size distribution in resin was evaluated by mercury intrusion porosimetry using Quantachrome mercury porosimeter model No.33. The pore size distribution was between 32 and 2000 $\mathring{A}$

(ii) Surface Morphology : The surface morphology of porous beaded HEMA-DVB copolymer support exhibiting the highest adsorption of Penicillin G acylase was examined on Cambridge Stereoscan Model 120 Scanning Electron Microscope. The operating voltage was 15 KV.

CHAPTER 3

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 PENICILLIN G ACYLASE IMMOBILISATION

3.1.1 IMMOBILISATION ONTO MACROPOROUS ALUMINA

Porous alumina have been studied in some detail for catalytic applications.[315-318] The pore size and its distribution is controlled by adding reagents which decompose during calcination. The calcination temperature determines the crystal form of the alumina generated. Beads are formed by dropping into hot oil.[319] Cylinders can be formed by extrusion.[320] Alumina is acidic. The pH can be controlled by incorporating basic alkaline earth metal ions.[321] Thus, the shape, size, pore size distribution, surface pH are easily controllable factors. In addition, the support is easy and cheap to prepare using standard techniques and is rigid. These parameters have attracted the attention of enzymologists, as seen from the numerous fundamental and patent literature relating to the use of inorganic supports for enzyme immobilisation (See section 1.2.10.2.2)

Enzymes are shear sensitive. The catalytic activity of enzymes can be harnessed only over a narrow range of pH and temperature. This is the range over which the tertiary structure of the specific enzyme is stable. Design of proper support to anchor enzymes by adsorption or by covalent linkage should not disrupt this tertiary structure drastically and must protect the enzyme from shear fields encountered in stirred batch processes. Thus, design of porous supports, to adsorb enzymes within the pores and to provide ideal surface pH which stabilises the tertiary structure, would vary with the enzyme under investigation. In addition, the pore size and its distribution should be large enough for the enzyme to penetrate. The enzyme should be anchored close to the surface to prevent diffusional constraints for the reactants. The globally decreased activity profiles evaluated for immobilised enzymes are attributed to

(i) Decrease in activity due to disrupted tertiary structure arising from specific affinity between enzyme and support.

(ii) Involvement of the catalytic sites in the binding process.

(iii) Diffusional limitations which prevent substrate from effectively reaching the catalytic site.

Macroporous alumina of varying (i) pore volume and pore size as well as (ii) surface pH were synthesised to anchor penicillin G acylase enzyme. Pore volume was altered by incorporating differing amounts of the pore generating agent, HEXA prior to the calcination step. The surface pH was altered by incorporating magnesium oxide. The adsorption and expression (activity) of immobilised penicillin acylase are related to these synthesis variables.

Beaded porous alumina supports differing in average pore radii, pore volume and acidity/basicity were synthesised as presented in Table 10. The pore volume was altered by changing the HEXA loading onto aluminium hydroxide during the calcination. In the three series, the HEXA content were 12, 20 and 24 weight percent of aluminium hydroxide. The acidity/basicity within each series were controlled by incorporating differing amounts of magnesium oxide. The Magnesium oxide content and corresponding pH of the carrier is presented in Table 17. The pH at the surface increases with magnesium oxide.[321] The average pore radii does not follow any trend relative to either HEXA loading or magnesium oxide content in the supports.

Penicillin G acylase was adsorbed and covalently bound onto the supports by treatment with glutaraldehyde. Globally, the adsorption efficiency increases while the percent expression decreases with increasing magnesium oxide content. Adsorption onto alumina is a physical phenomenon. The degree of adsorption depends on the affinity between alumina and the enzyme. Since affinity is related to hydrophilicity, hydrophobicity and electrostatic interactions, these should be optimised for effective adsorption and expression of the enzyme. On alumina Al 11 support a maximum adsorption efficiency of 46.5 percent was achieved (Table 18). However, only 8.2 percent of the adsorbed enzyme retain the activity towards phenyl acetamido group in penicillin G. This could be due to steric hindrance arising from the hydrophobicity of alumina which affects the interaction between the bound enzyme and penicillin G molecule. Hydrophobicity of alumina was

Table - 17

Variation of pH of porous alumina supports with MgO content

No.	MgO content, Weight percent	pH of the carrier
1	0.84	7.0
2	1.50	7.5
3	2.50	8.2
4	3.80	8.4

Table - 18

Adsorption and expression of penicillin G acylase onto porous alumina samples

Enzyme loaded, (IU/gm)	Adsorption (IU/gm)	% Adsorption (IU/gm)	IME Assay*	
			Activity (IU/gm)	% Expression
232	76.9	33.16	14.9	19.4
232	96.6	41.60	11.4	11.8
232	90.6	39.0	12.8	14.1
232	103.7	44.7	11.1	10.7
232	86.5	37.3	14.6	16.9
232	96.6	41.6	13.1	13.6
232	81.1	35.0	12.4	15.3
232	100.8	43.4	7.8	7.7
232	70.4	30.3	10.3	14.6
232	64.4	27.8	14.7	22.8
232	108.0	46.5	8.9	8.2
232	90.0	38.8	5.4	6.0

* Immobilised enzyme assay

altered by treating it with polyethyleneimines of different molecular weight. The results are summarised in Table 19 . Increasing the hydrophilicity of alumina increased the expression of penicillin G acylase by 106 percent but reduced extent of adsorption by 62 percent. Treatment with polyethyleneimine of molecular weight 1,300 increased the expression by 63 percent and decreased the adsorption by 40 percent. Thus, hydrophobicity of the alumina plays an important role and should be balanced and/or compromised for adsorption and expression of penicillin G acylase.

3.1.2 IMMOBILISATION ON HEMA-DVB COPOLYMERS

Requirements of synthetic polymer supports used for immobilisation are dependent on the nature of enzyme and conditions under which enzyme-support system has to operate. Polymer can be tailor made with specific:

* Hydrophilic / hydrophobic balance
* Porosity
* Reactive groups for anchoring enzymes

Additionally, synthetic polymer supports have good chemical and mechanical stability and good resistance to biodegradation.

A series of hydroxyethyl methacrylate-divinyl benzene copolymers were synthesised to anchor enzymes. The objective was to vary pore size and its distribution and hydrophilic/hydrophobic character and to evaluate their ability for immobilising penicillin G acylase. The affinity of an enzyme to a support is primarily related to the hydrophilic/hydrophobic balance in the support. The hydrophobic residues in penicillin G acylase, provide specific adsorption sites.[322] Pore size and pore volume also play a decisive role in amount of enzyme entrapped by the support. Divinyl benzene imparts hydrophobic character to the supports. In certain cases binding efficiency may be more but the expression of the activity of bound enzyme may be poor. In case of penicillin G acylase, phenyl acetic acid is liberated during the catalytic conversion of penicillin G to 6-amino penicillanic acid (6-APA). If the support has affinity for phenyl acetic acid, there will be a lowering of pH in

Table - 19

Effect of polyethyleneimine modification of alumina on binding and expression of penicillin G acylase.

Sample code	Penicillin G acylase				
	Loaded (IU/gm)	Adsorbed (IU/gm)	Adsorption %	IME++ activity (IU/gm)	Expression %
AL-11	232	108	46.5	8.9	8.2
AL-13	232	65	28.0	8.7	13.4
AL-14	232	70	30.2	7.0	9.9
AL-15	232	53	22.7	7.7	14.5
AL-16	232	41	17.8	7.0	16.9

++ Immobilised Enzyme Activity

the microenvironment which will either reduce the catalytic efficiency or totally deactivate the enzyme. Due to the hydrophobic nature of phenyl acetic acid, it gets adsorbed onto the HEMA-DVB copolymers and hence reduces the catalytic efficiency.

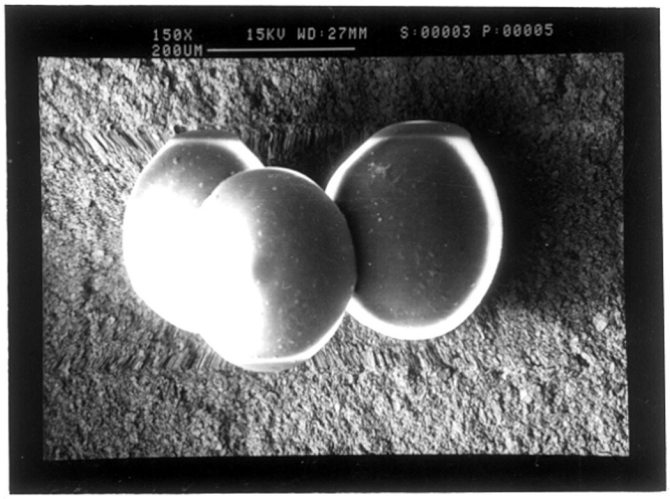
The HEMA - DVB macroporous beaded copolymers presented in Table 12 were synthesised by suspension polymerisation. The relative mole ratio of DVB was varied between 0.98 (HD 1) and 5.88 (HD 6) and the reaction were taken to virtual completion (> 98%). The reactivity ratios of HEMA and DVB were not estimated. Since these would differ, the copolymers formed at different conversions would widely vary in compositions. The beads would differ in cross-link densities, and hence in pore size. However, global comparisons of the relative performances of the copolymers can be made. A mixed solvent system consisting of cyclohexanol (mole fraction 0.95) and lauryl alcohol (mole fraction 0.05) was used to generate the internal pores in all the copolymers. The average size of pores estimated by mercury porosimetry are presented in Table 12. The estimated average pore size increases with the DVB content in the copolymers. Similar observations are reported in the literature. The size of the beaded particles was between 177 and 420 microns. A representative distribution is presented in Table 20.

The surface morphology of a representative copolymer is presented in *Figure 26*. The beads are spherical (*Figure 26a*). Cross section of the beads (*Figure 26b*) shows that the pores are present as irregular holes of varying dimensions. Penicillin G acylase was adsorbed on the copolymers under identical conditions. The results are presented in Table 21. Adsorption affinity increases from HD 1 to HD 6 and then falls. The factors contributing to enzyme adsorption are size of pores and hydrophobicity/hydrophilicity balance in the beads. In this series, the pore size and hydrophobicity increases with DVB content while hydrophilicity decreases. It is difficult to quantify the relative contributions of these factors to the adsorption and entrapment of enzyme within the beads. Optimal pore size is probably the most important of these factors. The distribution in size of pores in copolymer HD 6 is presented in *Figure 27*.

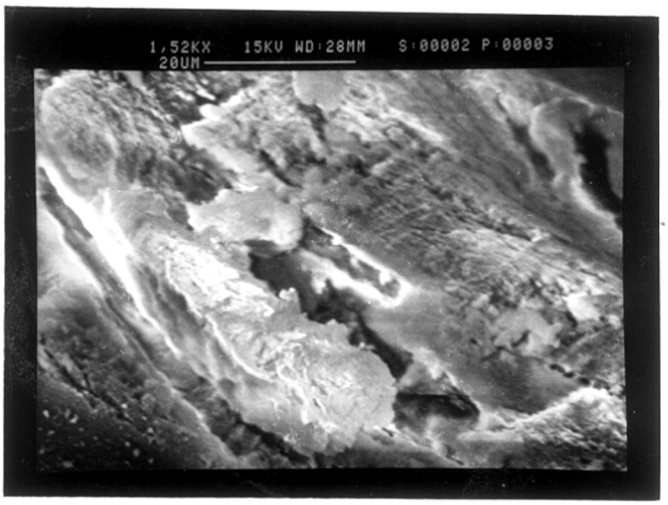
Table - 20

Particle size distribution data of HD series.

Particle size, (microns)	Percentage
420	40.54
250	55.74
177	3.72



(a)



(b)

FIG. 26 SEM MICROGRAPH OF COPOLYMER HD 6.(a) SURFACE and (b) CROSS SECTION.

Table - 21

Percentage adsorption of penicillin G acylase on HEMA-DVB copolymers

Amount of support taken = 0.2 gm

Total IU loaded in each case = 16.39

Total protein loaded = 76.8 mg

Polym. code	IU adsorbed	% Adsorption	Protein adsorbed (mg)	% Protein Adsorbed
HD 1	0.11	0.67	37.8	49.22
HD 2	3.89	23.73	18.0	23.44
HD 3	7.0	42.71	33.0	42.97
HD 4	6.79	41.43	39.6	51.56
HD 5	6.53	39.84	24.8	45.31
HD 6	10.16	61.99	51.0	66.41
HD 7	3.53	21.54	43.8	57.03
HD 8	Nil	Nil	33.6	43.75
HD 9	Nil	Nil	33.0	42.97

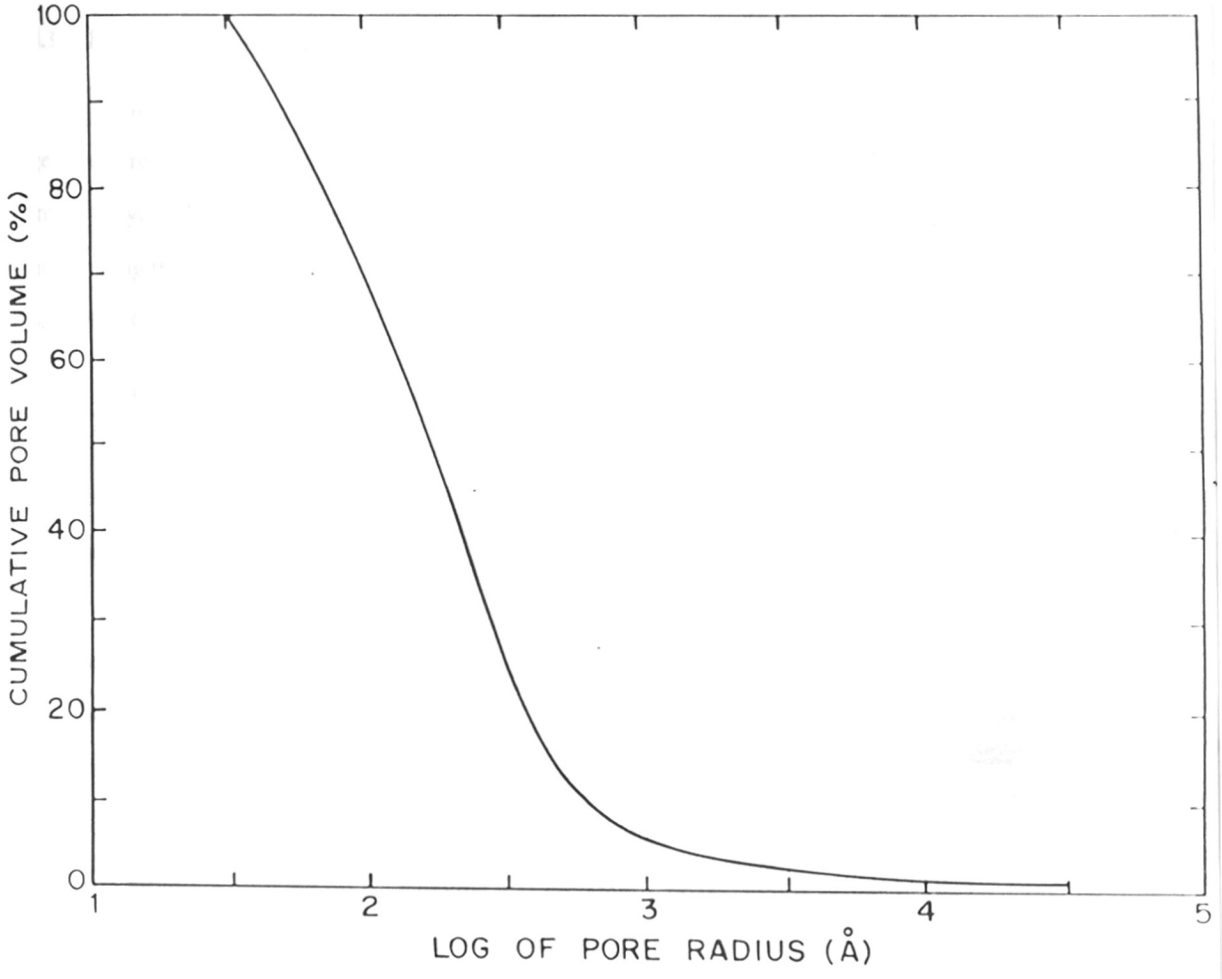


FIG. 27 : PORE SIZE DISTRIBUTION IN COPOLYMER HD 6

3.1.2.1 Modification of HEMA-DVB copolymer with polyethyleneimine

The modification of HEMA-DVB copolymer with polyethyleneimine and glutaraldehyde generates reactive aldehyde groups for covalent binding of penicillin G acylase. Polyethyleneimine imparts hydrophilic character and the introduction of spacers in form of glutaraldehyde helps in minimising the steric hindrance. This increases the accessibility of penicillin G molecule for bound penicillin G acylase and improves the catalytic efficiency.

Copolymer HD 6 was modified for covalent binding of penicillin G acylase by coating with polyethyleneimine and derivatising with glutaraldehyde. Two approaches were investigated. (i) The enzyme was first adsorbed onto the support. The trapped enzyme was encased forming a thin membrane on the surface of the beads by polyethyleneimine and glutaraldehyde treatments. The expression of enzyme immobilised by this technique was very poor and the approach was abandoned. The poor expression may be attributed to the inaccessibility of the substrate to the enzyme encased within the membrane.

(ii) An alternative approach was adopted to overcome this difficulty. The copolymer was first coated with polyethyleneimine and treated with an excess of glutaraldehyde solution to generate pendent aldehyde groups within the pores. Enzyme was covalently bound to this support. The introduction of spacers between the support and enzyme was beneficial in minimising the diffusional and steric effects. This gave a better expression for the immobilised enzyme.

3.1.2.2 Studies with immobilised penicillin G acylase

The affinity of substrate molecules for an enzyme may be high, low or remain unaltered after immobilisation. This is evaluated by determining the Michaelis-Menten constant, K_m for free and immobilised enzyme under identical conditions of pH, temperature and substrate concentration. In case of free enzyme, sometimes all catalytically active sites may not exposed to the substrate molecules. Catalytically active sites are present in the inner most region of enzyme molecule. For this reason enzyme display a higher K_m value. For free penicillin G acylase from *E.coli* K_m is 30.1 μ mole/mL. After immobilisation the exposure to catalytically active centre is better. This leads to

a drop in K_m as compared to free enzyme. The K_m value towards benzyl penicillin at pH 7.8 in 0.1 molar phosphate buffer is 12.8 μ mole/mL for penicillin G acylase immobilised on polyethyleneimine treated, glutaraldehyde derivatised copolymer support (Table 22). The K_m is inversely related to the substrate affinity and is indicative of relative affinity of an enzyme for that substrate. Immobilisation methodology and nature of support are the two main governing factors contributing towards the shift in K_m.

Approximately 2.3 fold decrease in K_m was observed on covalent binding. Due to the introduction of spacer in form glutaraldehyde, penicillin G Acylase is immobilised on the surface and therefore it is freely available for catalysis. Carleysmith *et al*[323]. however, had observed enhancement in the K_m value of glutaraldehyde modified enzyme compared to the native enzyme. The expression of this IME preparation was also higher compared to the previous approach.

Immobilised enzyme may also display shift in other two experimental parameters such as pH and temperature. Free penicillin G acylase from *E.coli* has a optimum pH and temperature of 7.8 and 40°C respectively. The support plays a vital role in altering the pH optima of the immobilised system. In some cases the shift in optimal pH is marginal, but the pH-activity profile may differ from free enzyme. Broadening in the pH-activity profile due to immobilisation is indicative of good stability for bound enzyme over a wider pH range. Temperature optima also follows a similar trend. Penicillin G acylase isolated from *E.coli* has optimum temperature of 40°C. An increase in temperature optima, indicates increase in the resistance of immobilised enzyme against temperature and other denaturing agents. The tertiary structure of enzyme becomes less flexible and more rigid by association with support.

3.1.2.3 Effect of pH

Immobilisation marginally altered the pH optima, from 7.8 to 7.0 - 7.5 as presented in *Figure 28*. The broadening of the pH profile curve in case of IME is due to the stability of IME over a wider pH range compared to the native enzyme.

Table - 22

Michaelis-Menten constants (K_m) of native and immobilised penicillin acylase.

Enzyme was immobilised on PEI treated and glutaraldehyde derivatised HD 6 copolymer

Preparation	K_m (μmole)
Native enzyme	30.1
Immobilised (Bound) enzyme	12.8

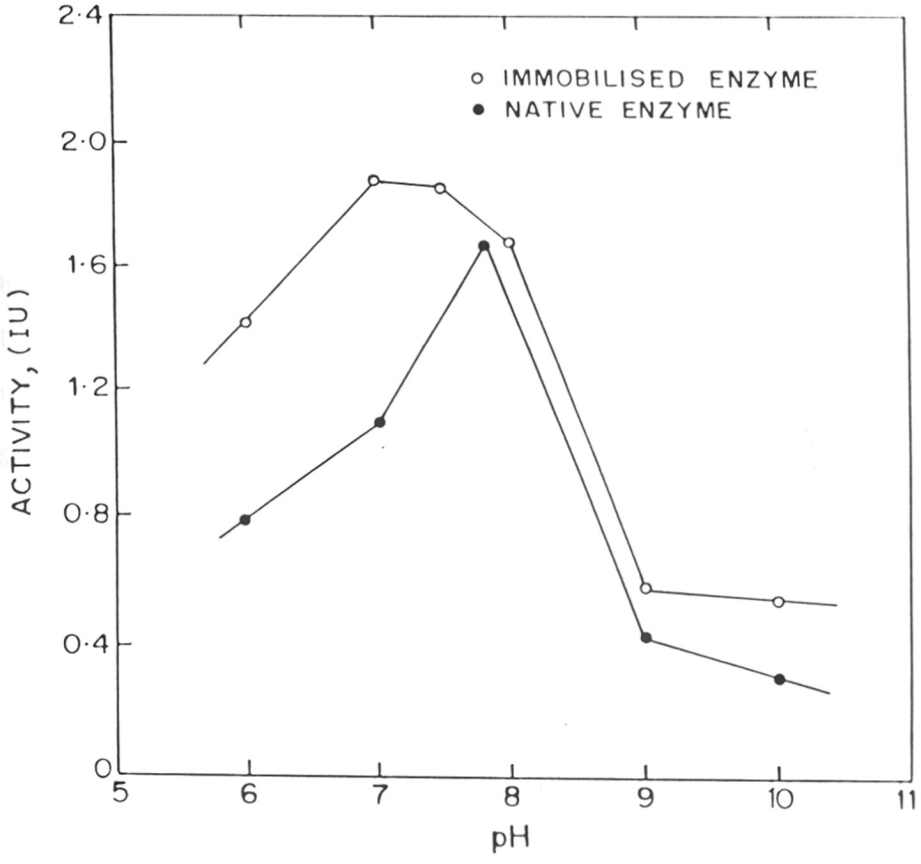


FIG. 28 : EFFECT OF pH ON ENZYME ACTIVITY OF NATIVE (•) AND IMMOBILISED (o) PENICILLIN G ACYLASE

3.1.2.4 Temperature effect

The effect of temperature after immobilisation is shown in *Figure 29*. The optimal temperature of immobilised penicillin acylase is 57°C. The temperature activity profile of the native and immobilised enzyme are very distinct. Broadening of the temperature activity profile in case of bound enzyme near its optimum activity indicates that the bound enzyme is stable over a wider temperature range compared to the native enzyme.

3.1.2.5 Thermal stability

The temperature stability of the penicillin acylase was improved considerably after immobilisation. No significant inactivation of IME was observed after incubation in 0.1 molar phosphate buffer at 57°C for 2 hours The loss in initial activity was 10 percent vis - a - vis 95 percent for native enzyme.

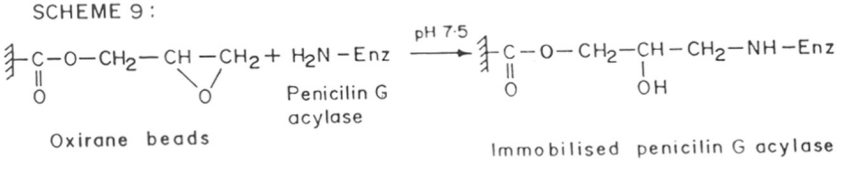
3.1.2.6 Operational stability

Operational stability was evaluated in a batch mode. 20 ml of the reaction mixture was used. The final substrate concentration was set at 4 percent weight/volume. The reaction mixture was stirred mechanically while incubating at 40°C, pH 7.8. The 6-APA formed was estimated as described earlier by withdrawing 0.1 mL aliquots of the reaction mixture at regular intervals, and adding immediately to 1.9 mL 0.25 molar citrate-phosphate (CPB) buffer, pH 2.5, to arrest the reaction. After each cycle (about 95 percent conversion) the beads were washed thoroughly with buffer. No detectable loss in activity was observable after 32 work cycles (*Figure 30*).

3.1.3 IMMOBILISATION ONTO GLYCIDYL TERPOLYMERS

Oxiranyl (epoxy) groups react rapidly with amino groups at ambient temperature and neutral pH. Polymer with oxiranyl groups would be ideally suited to covalently bind biomolecules with amino pendent groups (lysine units). Covalent binding occurs through the oxirane group and a number of functional groups, mainly primary amino groups, present in the enzyme molecule

SCHEME 9 :



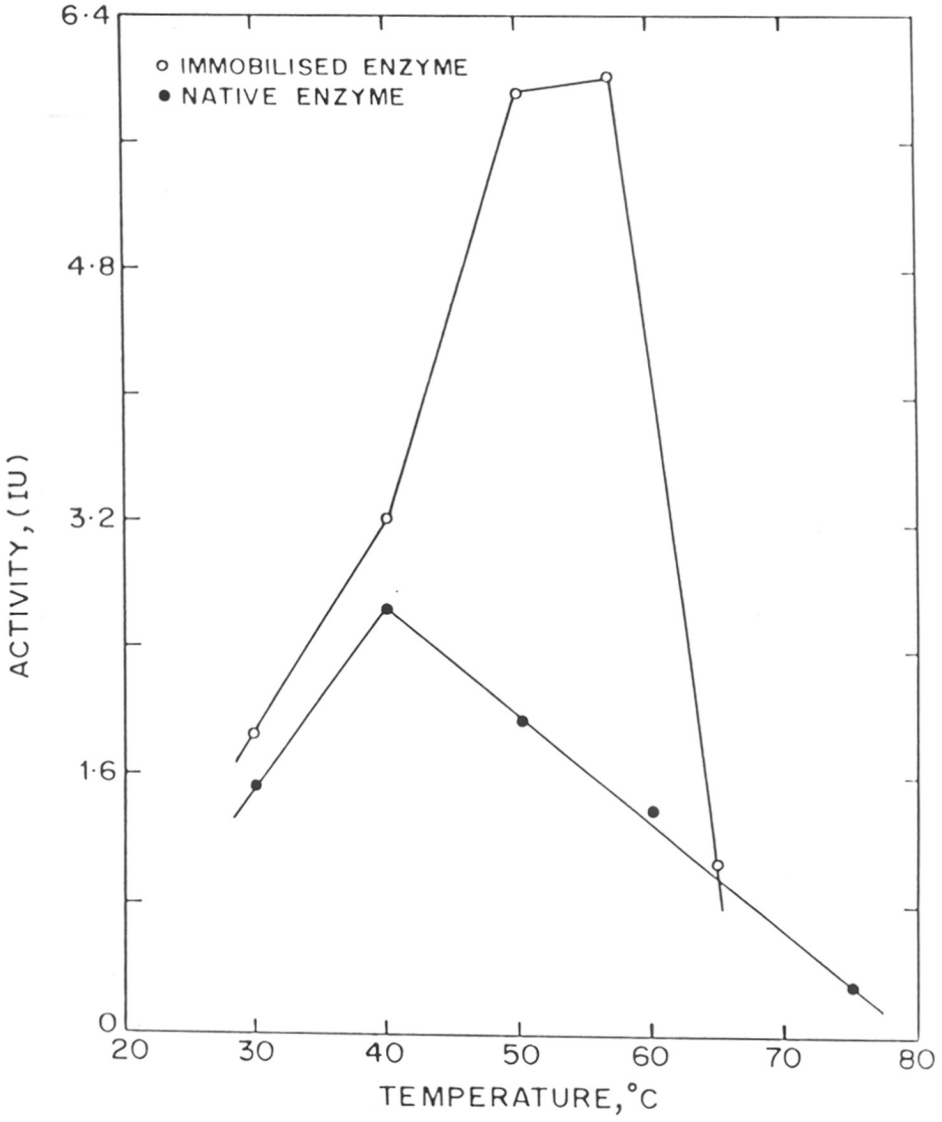


FIG. 29: TEMPERATURE EFFECT ON THE ENZYME ACTIVITY OF NATIVE (•) AND IMMOBILISED (o) PENICILLIN G ACYLASE

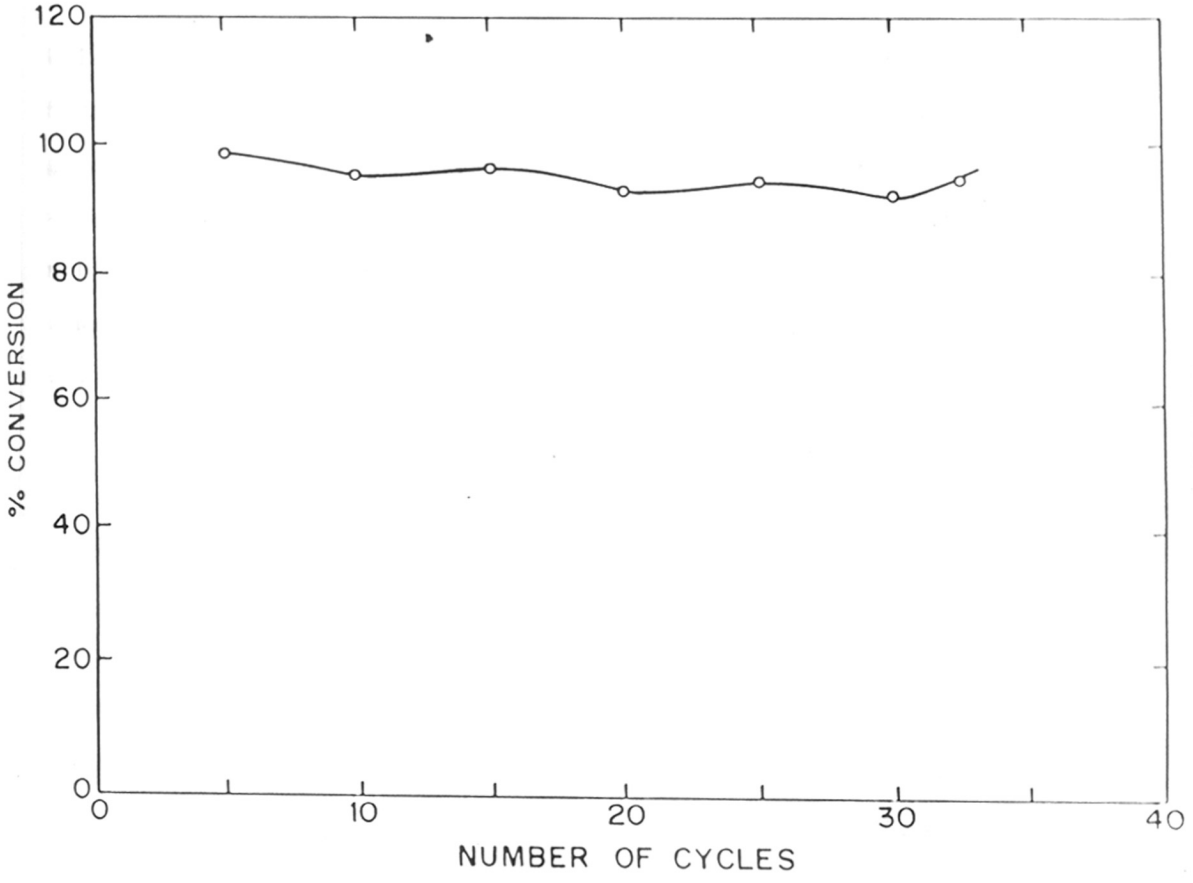


FIG. 30: OPERATIONAL STABILITY OF PENICILLIN G ACYLASE IMMOBILISED ON MACROPOROUS HD 6 COPOLYMER BEADS, TREATED WITH POLYETHYLENEIMINE AND DERIVATISED WITH GLUTARALDEHYDE

In this study, a variety of macroporous beaded oxiranyl polymers were synthesised and evaluated for their relative efficiencies to immobilise penicillin G acylase. Three types of terpolymers were generated. Glycidyl methacrylate was incorporated in all polymerisation recipes. The crosslinking agent used were divinyl benzene and ethylene glycol dimethacrylate. Two comonomers, methyl methacrylate and hydroxyethyl methacrylate were incorporated to impart hydrophobic/hydrophilic character. Hydrophobicity was enhanced in the presence of methyl methacrylate and hydrophilicity by hydroxyethyl methacrylate. A more hydrophobic support consisting of glycidyl methacrylate and hydroxyethyl methacrylate with divinyl benzene as a crosslinking agent was also prepared. Penicillin G acylase was immobilised on these supports. The activity of the immobilised enzyme were evaluated.

The polymer variables are (i) composition (ii) bead size and its distribution (iii) pore size and its distribution (iv) pore volume and (v) the concentration of oxirane groups at the surface. These are related to reactivity ratios of comonomers and other process variables such as ratio of continuous to discontinuous phases, molecular weight and concentration of protective colloid, pore generating solvent and its volume, reaction temperature and initiator concentration. The volume and nature of pore generating solvent will affect pore volume, pore size and its distribution. Beads with very large pore volume would tend to float and will be mechanically weak resulting in attrition problems in a stirred tank reactor or will not be suitable for column in packed bed reactor.

In suspension polymerisation monomers, initiator and pore generating solvent form the dispersed phase, as droplets. The continuous aqueous phase acts as an efficient heat transfer medium. High rate of polymerisation can be maintained to achieve complete conversion, in relatively short periods. Ratio of continuous to discontinuous phase is an important parameter. An increased in the discontinuous phase concentration leads to agglomeration. Initially formed droplets consisting of partially polymerised monomers are gradually hardened during the progress of polymerisation. The droplets in this stage should be prevented from coalescence, otherwise it would lead to the coagulation of entire bulk polymerised phase. Mass coagulation is prevented by increasing the interfacial

viscosity which helps in keeping the droplets away from each other. Poly (vinyl pyrrolidone) is added to the continuous phase to act as a protective colloid and to prevent agglomerisation of partially polymerised droplets. With higher molecular weight poly (vinyl pyrrolidone), a low concentration is sufficient to prevent agglomerisation. Increase in protective colloid concentration and stirring speed decreases the bead size. Beads of uniform size are obtained only at a constant rate of stirring. Bead size plays an important role in adsorption as well as in expression of immobilised enzyme system. Adsorption of enzyme should not be at the surface or very deep inside the pores.

Suspension polymerisations were conducted at constant speed (300 rpm) to maintain the uniformity of beads. The internal pores were generated using a solvent freely miscible with monomers but was a non-solvent for the polymer. This pore generating solvent is dispersed uniformly in the discontinuous phase with monomers. During chain growth the pore generating solvent is rejected out as droplets within the discontinuous phase. This leads to pore generation. The droplet size, which determines the pore size and its distribution is governed by (i) rate of polymerisation and (ii) the rate of aggregation of pore generating solvent. Macropores are generated at low rates of polymerisation by large differences in the solubility parameters of copolymer and the pore generating solvent. Polymerisation temperature is another important factor, which influences the pore size and its distribution. The formation of micropores is predominant with solvents having solubility parameter equivalent to the copolymer, especially at high rates of polymerisation. The divinyl comonomer offers supporting bridges to stabilise pores. Homopolymerisation in presence of pore generating solvent would initially generate pores. These would collapse in the absence of supporting bridges provided by the crosslinking agent and would result in non porous beads.

The reactivity ratios of the comonomers used, play important role in determining the composition as well as pore size distribution. The reactivity ratios of following binary polymerisation are reported in the literature.[131,324,325]

(i) Glycidyl Methacrylate (M_1) : $r_1 = 0.98$

Ethylene glycol dimethacrylate (M_2) : $r_2 = 1.00$

(ii) Glycidyl Methacrylate (M_1) : $r_1 = 1.05$

Methyl methacrylate (M_2) : $r_2 = 0.80$

(iii) Methyl methacrylate (M_1) : $r_1 = 0.41$

Divinyl benzene (M_2) : $r_2 = 0.61$

In methacrylate terpolymers the composition would be predominantly governed by the feed ratios. Hence pore size and its distribution will be independent of conversion. In terpolymers with divinyl benzene as a constituent, the divinyl monomer will be consumed preferentially in the early stages of the polymerisation. Therefore, this system would intrinsically display a wide distribution in pore size. The enzyme molecule would diffuse into those pores which are larger than its dimensions.

Ideal supports) should be inert and provide reactive functional groups to bind enzyme effectively under mild conditions. Oxiranyl polymers offer facile reactive groups for covalent binding of enzyme molecules. The covalent binding occurs through the oxirane group and a number of functional groups present in the enzyme molecule. Other structural moieties in the polymer backbone contribute to the enzyme binding by providing suitable topochemical environment and/or by increasing the affinity of the polymer to the enzyme. Optimising microenvironment and modifying hydrophilic/hydrophobic character form the integral part of the overall strategy in the identification and design of ideal polymer supports for a specific enzyme.

Three series of terpolymers were synthesised by suspension polymerisation technique. The polymers were obtained in spherical "beaded" form. Six of the seven process variables were unaltered, to generate terpolymers of differing hydrophilic/hydrophobic character. The variables maintained at constant levels were: relative volumes of the continuous (water) and discontinuous (organic) phases, the amount of protective colloid [poly (vinyl pyrrolidone), K-90], the volumes of pore generating solvent and crosslinking agents (ethylene glycol dimethacrylate/divinyl benzene), the initiator concentration and reaction temperature. Thus, the relative performances of the terpolymers could be related to the hydrophilic/hydrophobic balance in the polymers.

3.1.3.1 Effect of hydroxyethyl methacrylate

In the first series, P, 1 copolymer and 8 terpolymers were synthesised by an interplay of the relative mole ratios of hydroxyethyl methacrylate and glycidyl methacrylate at a constant crosslink density, provided by incorporating ethylene glycol dimethacrylate. The data is presented in Table 13. Hydrophilicity is increased from P-1 to P-9 due to the introduction of polar -OH groups. The polymers were all macroporous with a distribution of pores in the range 32-1000 A°. The average size of the pores, presented in Table 13, is between 48.6 to 62.3 A°.

The penicillin G acylase binding characteristics are listed with a few representative polymers. The adsorption and expression of bound enzyme are presented in Table 23. The binding of penicillin G acylase decreases with increasing HEMA content of the polymer. The percent expression of the bound enzyme was not measurably altered at different levels of HEMA. Thus, increasing the hydrophilicity decreases the adsorption of enzyme on the matrix, a desired prerequisite for binding, resulting in lesser covalent binding of penicillin G acylase.

3.1.3.2 Effect of divinyl benzene

In the second series, 1 copolymer and 8 terpolymers were synthesised. In this series GMA was replaced to varying degrees with HEMA .Divinyl benzene (DVB) was used as crosslinking agent instead of ethylene glycol dimethacrylate. The degree of crosslinking was maintained constant. The divinyl benzene used was composed to 60 percent DVB and 40 percent 4-ethyl styrene. The composition and crosslink density of the polymers were similar to those in the P series. Incorporation of DVB imparts greater hydrophobicity to these polymers. The composition and average pore radii are presented in Table 15. The average pore radii varied between 55.0 and 67.1 A°.

The relative abilities to bind penicillin G acylase are presented in Table 24. Relatively high concentration of the enzyme was bound to the polymer matrix. Increase in hydrophilicity had practically no effect on the binding characteristics. At very high levels of HEMA (Polymer DVB-9) the binding efficiency dropped by 24 percent. The bound penicillin G acylase does not effectively

Table - 23

Effect of terpolymer composition on binding and expression of penicillin G acylase.

GMA-HEMA-EGDM series

Polymer No.	Enzyme loaded, (IU/gm of polymer)	Enzyme Adsorbed		IME Assay*	
		(IU/gm)	% Adsorption	Activity (IU/gm)	% Expression
P 1	232	194	83.6	113	58.2
P 3	232	146	62.9	64	43.8
P 4	232	141	60.7	62	43.9
P 6	232	48	25.0	25	43.1
P 8	232	44	18.9	20	45.4
P 9	232	7	3.0	4	57.1

* Immobilised enzyme assay

Table - 24

Effect of terpolymer composition on binding and expression of penicillin G acylase

GMA-HEMA-DVB series

Polymer No.	Enzyme loaded, (IU/gm of polymer)	Enzyme Adsorbed		IME Assay*	
		(IU/gm)	% Adsorption	Activity (IU/gm)	% Expression
DVB 1	232	185	79.7	25	13.5
DVB 2	232	166	71.5	21	12.6
DVB 3	232	189	81.4	35	18.5
DVB 4	232	188	81.0	28	14.9
DVB 5	232	188	81.0	21	11.1
DVB 6	232	173	74.5	35	20.2
DVB 7	232	188	81.0	32	17.0
DVB 8	232	190	81.9	29	15.2
DVB 9	232	131	56.4	21	16.0

* Immobilised enzyme assay

promote the hydrolysis of penicillin G to 6-amino penicillanic acid. It appears that the aromatic groups of DVB either disrupt the tertiary structure of the bound penicillin G acylase or hinders the interaction between penicillin G acylase and penicillin G molecules. This decreases the catalytic efficiency.

3.1.3.3 Effect of methyl methacrylate

In this M series 2.5 to 75.0 mole percent of glycidyl methacrylate (GMA) was substituted with more hydrophobic but non reactive methyl methacrylate (MMA). The crosslinking agent was ethylene glycol dimethacrylate. The compositions of the macroporous beaded terpolymers are presented in Table 14. The average pore radii in this series varies between 52.6 and 61.0 A°.

The binding efficiencies of polymer supports in this series are presented in Table 25. The binding efficiency decreases initially and goes through a minima corresponding to 25 mole percent of MMA (polymer M-6). The acetate pendent group of MMA is relatively less hydrophobic than benzene ring in the DVB series. This hydrophobic character of MMA is inadequate to effectively adsorb penicillin G acylase. At higher more fraction of MMA (polymer M 7 and M 8) the binding efficiency increases. However, the bound enzyme is either inaccessible to penicillin G or loses its catalytic activity due to structural reorganisation.

3.1.3.4 Effect of polyethyleneimine

The oxirane group in the polymer P 1 were partially derivatised with low molecular weight branched polyethyleneimine (Mol. Wt. 2,000), to increase the hydrophilicity without altering the porosity of the beads. The oxirane groups were quantified by sodium thiosulphate method.[326] The beads have reactive oxirane groups in more hydrophilic microenvironment. The data is presented in Table 26. The unmodified polymer matrix had an overall immobilisation efficiency of 48.2 percent. The binding efficiency was 82.7 percent and expression was 58.3 percent. On modification with PEI, binding and expression of penicillin G acylase are markedly decreased. The binding efficiency dropped by 38 percent at as low a modification of oxirane group as 3.13 percent. Thus,

Table - 25

Effect of GMA-MMA-DVB terpolymer composition on binding and expression of penicillin G acylase.

GMA-MMA-EGDM series

Polymer No.	Enzyme loaded, (IU/gm of polymer)	Enzyme Adsorbed		IME Assay*	
		(IU/gm)	% Adsorption	Activity (IU/gm)	% Expression
M 1	232	83	35.7	20	24.1
M 2	232	52	22.4	8	15.4
M 3	232	48	20.7	9	18.7
M 4	232	46	19.8	10	21.7
M 5	232	22	9.5	11	50.0
M 6	232	61	26.3	13	21.3
M 7	232	94	40.5	12	12.7
M 8	232	115	49.5	11	9.5

* Immobilised enzyme assay

Table - 26

Effect of polyethyleneimine (Molecular weight 2000) modification on binding and expression of Penicillin G acylase

Polym. No.	Enzyme loaded (IU/gm of polymer)	Enzyme Adsorbed		IME Assay*	
		(IU/gm)	% Adsorption	Activity (IU/gm)	% Expression
P 1	232	190.0	81.80	112.0	57.8
PGMA 1	232	104.6	45.08	31.7	30.3
PGMA 2	232	98.2	42.32	26.7	27.1
PGMA 3	232	82.9	35.73	18.8	22.6
PGMA 4	232	56.0	24.13	8.7	15.5

Immobilised enzyme assay.

hydrophilic microenvironment hinders the interaction of penicillin G acylase with the polymer desired for immobilisation and the interactions of penicillin G acylase with the substrate penicillin G during the catalytic reaction.

3.2 MATRIX POLYMERISATION

3.2.1 Induced Initiation

Aqueous solution polymerisation of acrylic acid and methacrylic acid was investigated at 30 ± 0.1°C using (i) potassium persulphate, (ii) potassium persulphate/sodium sulphite and (iii) potassium persulphate/polyethyleneimine initiator systems. No polymerisation was observed with potassium persulphate initiator. Homogeneous polymerisations were observed with the other two initiator systems. The experimental data is presented in Table 9. The initial rate of polymerisations computed for the two initiator systems are the same within experimental errors. The rate of decomposition of persulphate induced by the reducing agents sodium sulphite and polyethyleneimine are the same. Microanalysis of the polymer samples revealed presence of sulphur, while nitrogen was absent. The polymerisations in both initiator systems were initiated by sulphate radical ion.

3.2.2 Matrix Effects

The matrix polymerisations were conducted at 30 ± 0.1°C with potassium persulphate initiator. The monomer to matrix mole ration were varied as 0.4:1.0, 0.6:1.0, 0.8:1.0, 1.0:1.0, 1.5:1.0 and 2.0:1.0. In type I systems where-in the association between the matrix and the monomer are strong due to electrostatic interactions, the rate of polymerisation goes through a maximum corresponding to 1.0:1.0.[327] Conductivity measurements reveal that the matrix is partially saturated with monomer at lower monomer concentrations.[328] The saturation increases with increase in relative monomer concentration. This is reflected in the rate of polymerisation. When the monomer concentration exceeds the matrix concentration normal polymerisation of free monomer in the bulk of the medium proceeds simultaneously. The overall rate observed kinetically is an arithmetic average of the two rates. Since the rate of solution polymerisation is much lower, the overall rate is lower than that at 1.0 : 1.0 composition. In type II systems, the polymerisation starts in the bulk of the medium. At a critical degree of polymerisation, the growing polymer chain collapses onto the matrix due to co-operative hydrogen bonding between the two. In such systems, the rate continues to

increase with increase in monomer concentration even beyond the stoichiometric monomer-matrix complex.[329] The experimentally determined rate of polymerisation is presented in Table 27. The polymerisation rate was enhanced even at a relatively low monomer concentration. Rapid phase separation was observed after one minute. The polymerisation rate was found to be linear upto 70 percent conversion. This is typical of matrix polymerisations. The rate of matrix polymerisations were 2-10 times faster than redox polymerisation of methacrylic acid under identical conditions (Table 9). In type I matrix polymerisations rate enhancements to the tune of 2-3 order are observed. In the present system rate enhancement observed were much lower. The initial rate of polymerisation continued to increase beyond the stoichiometric 1.0:1.0. This points to weaker interactions between monomer and matrix. This could be hydrogen bonding rather than electrostatic interactions. Thus, the polymerisation is initiated in the bulk and the growing poly (methacrylic acid) chain collapses onto the polyethyleneimine matrix generating the turbidity after a time lapse of one minute. The matrix-daughter polymer complex would not be an exact one.

A comparison of the methacrylic acid data with that for matrix polymerisation of acrylic acid (Table 28) under identical conditions, reveals that the hydrophobic methyl group does not contribute significantly to the rate. The solubility characteristics of the two polycomplexes are found to be different. The polycomplex of polyethyleneimine-poly (acrylic acid) was soluble in 50 weight percent aqueous formic acid solution. Polyethyleneimine-poly (methacrylic acid) complex was not soluble in this solvent system.

3.2.3 Effect of Monomer Concentration

The concentration of polyethyleneimine (matrix) was kept constant at 1 molar and methacrylic acid concentration was varied with respect to polyethyleneimine. The log/log plot of rate versus monomer concentration is shown in *Figure 31*. From the slope of the plot the order with respect to monomer was found to be 1.0. This is typical of free radical polymerisation. Polyethyleneimine forms a redox pair with persulphate, when present in stoichiometric amounts (3.5×10^{-3} molar). At higher concentration, when it is added as a matrix (1 molar), polymerisation rate is

Table - 27

Experimental data of polymerisation of methacrylic acid (MAA) in presence of branched polyethyleneimine (B-PEI).

B-PEI = 1.0 M, $K_2S_2O_8$ = 3.5 X 10^{-3} M

Temperature = 30 $\pm$ 0.1°C

[MAA] to [B-PEI] ratio, M	Rate X 10^{-3} moles.lit^{-1} sec.
0.4	3.23
0.6	4.79
0.8	6.36
1.0	7.98
1.5	11.80
2.0	15.90

Table - 28

Experimental data of polymerisations of methacrylic acid (MAA) and acrylic acid (AA) in presence of branched polyethyleneimine.

Comparison of rate values.

Monomer to Matrix ratio, molar	MAA Rate x 10^{-3}, moles.lit^{-1}.sec.	AA Rate x 10^{-3}, moles.lit^{-1}.sec.
0.4	3.23	2.96
0.6	4.79	4.67
0.8	6.36	6.24
1.0	7.98	7.88
1.5	11.80	12.10
2.0	15.90	16.30

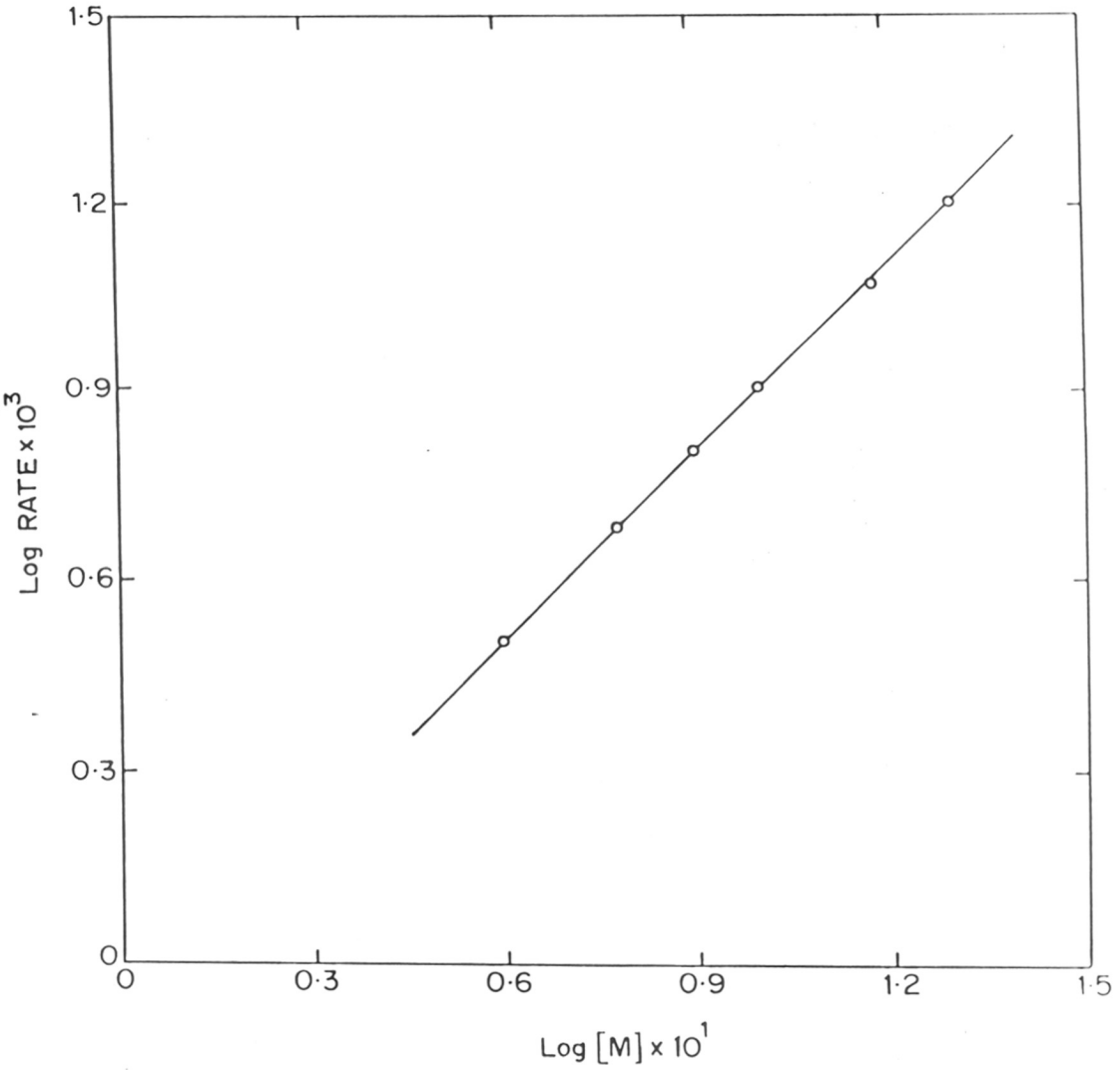


FIG.31: EFFECT OF MONOMER (METHACRYLIC ACID) CONCENTRATION ON THE RATE

enhanced. This could be attributed to higher rate of decomposition of persulphate induced by polyethyleneimine. This is discounted by the rate expression with respect to monomer, of 1.0. In case the enhanced rate was due to increased initiation rate, the monomer exponent in the rate expression would have been 1.5. Thus, polyethyleneimine forms a redox pair with persulphate and simultaneously acts as a matrix to promote the polymerisation of acrylic and methacrylic acids.

3.2.3 Effect of Initiator Concentration

The initiator concentration was varied by an order of a magnitude. The ratio of [MA]:[PEI] was held constant at 1:1 molar. From the slope of, log <rate> against log [initiator] plot (*Figure 32*) the value of initiator exponent was found to be .02. Earlier Ferguson *et al.*[85] reported anomalous kinetic behavior for polymerisation of N-vinyl pyrrolidone in presence of poly (acrylic acid) in organic (DMF) medium. The order of reaction with respect to initiator was found to be different in early and late stages. The order fell from 0.65 to zero in later stages of reaction. In solution free radical polymerisations a value of 0.5 is observed for the initiator exponent. This indicates a termination step involving two radicals. Order less than 0.5 for initiator has been theoretically predicted and experimentally demonstrated by Deb *et al.*[330] During polymerisation of methacrylic acid in presence polyethyleneimine, the system becomes heterogeneous. A lower initiator exponent could be due to the heterogeneous nature of the system. Radical termination becomes diffusion control. Primary radical termination[331,332] may be other factor responsible for the observed deviation from the normal kinetic behavior. It must be added that conclusive evidence needs to be obtained from the radical life time studies.

3.3 CHARACTERISATION OF POLYCOMPLEX

3.3.1 Differential scanning calorimetry (DSC)

Physical or chemical changes occurring in a polymer sample when subjected to a programmed heating or cooling rate, can be monitored by DSC. The method is quantitative as the thermogram (output of the technique) can be directly related to the thermodynamic parameters such

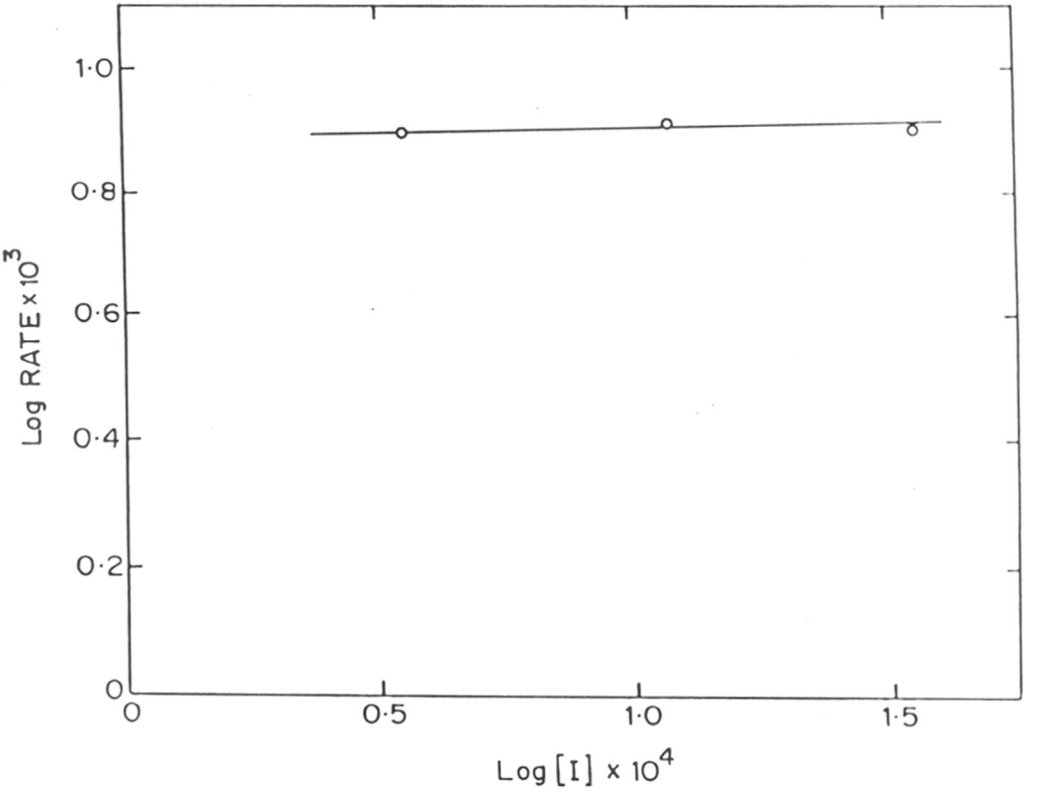


FIG.32: EFFECT OF INITIATOR ($K_2S_2O_8$) CONCENTRATION ON THE RATE

as changes in specific heat, enthalpy or entropy of transition. The polymer sample and a reference material (usually aluminium) are separately but simultaneously subjected to a programmed heating. The thermogram is representative of the excess differential power necessary during a transition to keep the sample and the reference material at identical temperatures, as the temperature is increased or decreased at a constant rate.

The melting of semicrystalline polymers are first order transitions. The peaks are usually not sharp as sample melts over a range of temperature, due to polydispersity. The amorphous polymers display only glass transition temperature (T_g). The first order transitions are affected by

* Degree of crystallinity

* Crystal size and imperfection

* Molecular weight and its distribution of sample

The DSC thermogram of a representative poly (methacrylic acid)- polyethyleneimine complex synthesised by matrix polymerisation, dried to constant weight, is presented in *Figure 33*. Polycomplex was thoroughly dried before loading. Two first order transitions were noted, at 101°C and 227°C. As two first order transitions were noted in DSC investigation, it was inferred that polycomplex may have crystalline character. X-ray study revealed amorphous character of the polycomplex. Thus, the former corresponds to bulk unbound water. The latter at 227°C probably corresponds to water bound in the hydration shell of the free ionic groups present as a result of only partial neutralisation between the carbonyl groups of poly (methacrylic acid) and amino groups of polyethyleneimine. The δH values for 101°C and 227°C were 289.4 J/g and 231.3 J/g respectively.

3.3.2 X-ray diffraction

X-ray of wavelength 1.542 $\mathring{A}$ is suitable for investigation of diffraction of organic polymers. X-rays are generated by the electron bombardment of a copper target in an evacuated tube. The

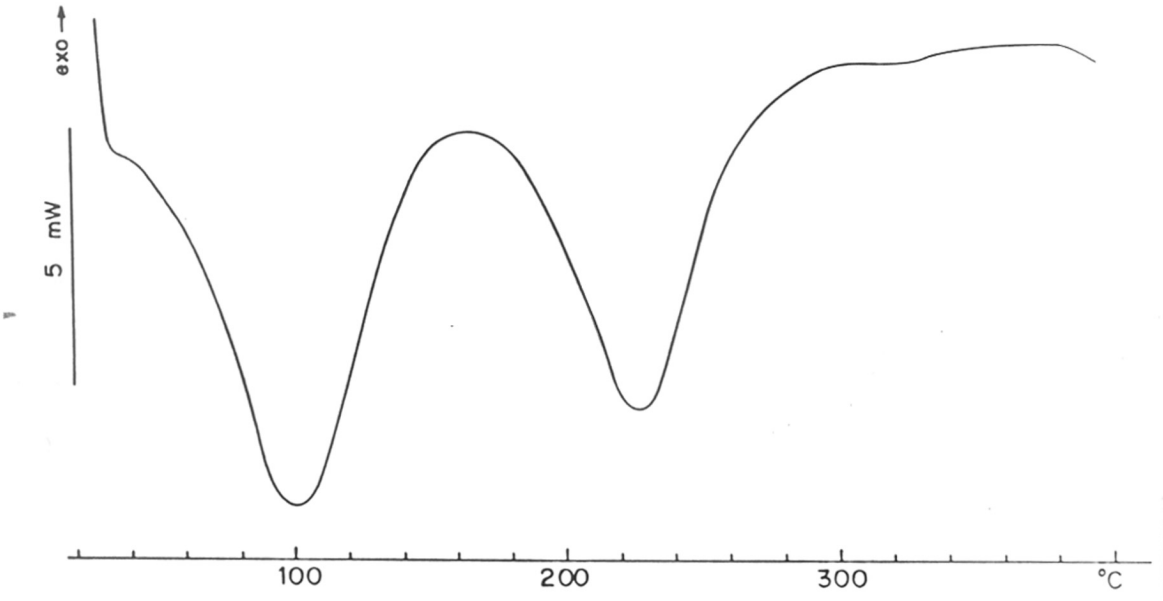


FIG.33: DSC THERMOGRAM OF POLYCOMPLEX SYNTHESISED BY MATRIX POLYMERISATION

resulting X-rays are filtered using a nickel filter. Normally, the wavelength is held constant and 2 θ is varied. The X-ray scans are obtained as a plot of 2 θ versus scattered intensity. The intensities are obtained from X-ray counters.

X-ray spectrograph in case of polymers can either be recorded in powder, film or fibre form. The diffraction could either be amplified and recorded by electron X-ray detectors or be photographed.

X-ray diffraction studies are useful in determining the percentage of crystalline and amorphous regions in the semicrystalline polymers. Polycomplexes formed by free radically induced matrix polymerisations are known to exhibit some crystallinity.[333] This is generally interpreted in terms of interaction between the pendent groups of the parent and daughter polymers. A typical X-ray diffraction scan indicating crystalline and amorphous scattering is depicted in *Figure 34*.

The wide angle X-ray scattering patterns of polycomplexes synthesised by matrix polymerisation in as-precipitated state were obtained at room temperature. The polycomplexes were in powder form.

The X-ray diffraction pattern of poly (methacrylic acid)-polyethyleneimine complex generated by matrix polymerisation is presented in *Figure 35*. A single amorphous hump is noted in the X-ray spectrograph. It reveals that the polycomplex is amorphous.

3.3.3 Solid state C-13 NMR

High resolution solid-state C-13 NMR of crystalline polymers display narrow and well defined lines. Substantial broadening occurs in case of amorphous linear polymers and polymers having three-dimensional network. The broadening may be due to a variety of conformations and environments or arise from the chemical disorder existing in the system under investigation.

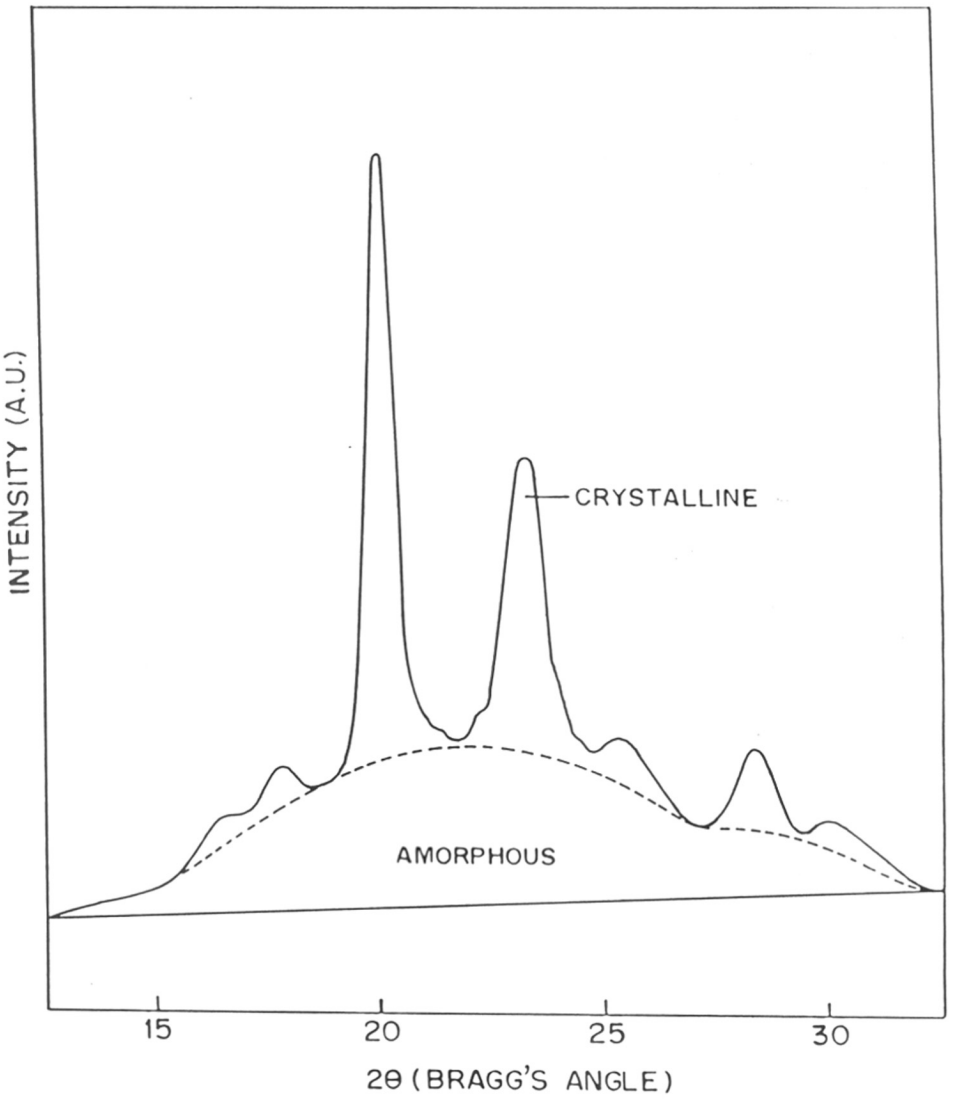


FIG. 34 : A TYPICAL X-RAY DIFFRACTION PATTERN

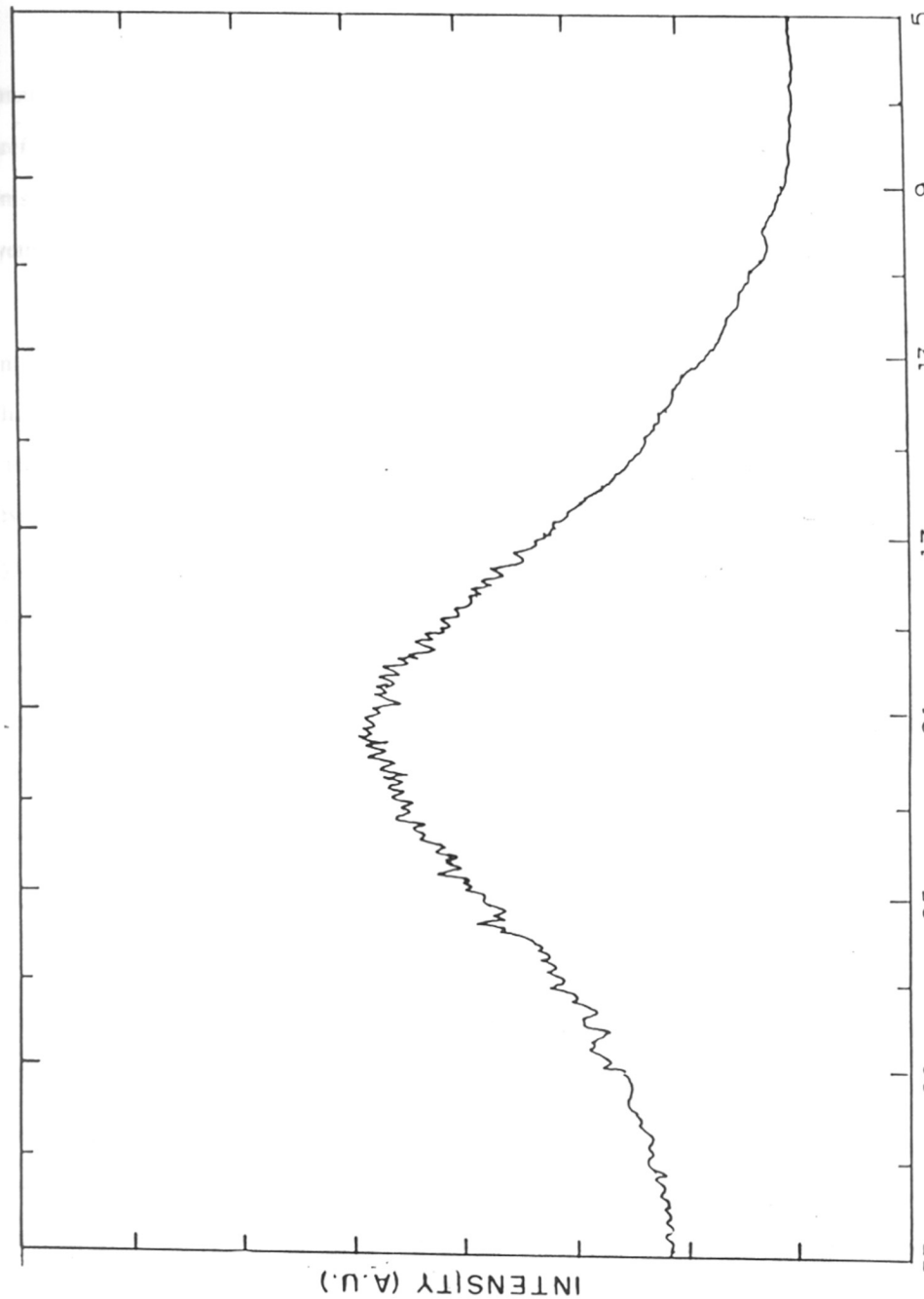


FIG.35: X-RAY DIFFRACTION PATTERN OF MATRIX POLYMERISED POLYCOMPLEX

In many cases spectra of solid polymers are more informative due to the ability of conformational resolution. In solution, the conformations are in rapid exchange with each other and therefore are motionally averaged. Well known cases of distinct chemical shift differences of C-13 atoms in crystalline and amorphous regions are polyethylene,[334] polyoxymethylene[335] and polyoxyethylene.[336] Chemical shifts are also affected by the modes of inter chain packing.

The C-13 NMR spectra of matrix polymerised polycomplex (polycomplex I) and polycomplex of identical composition prepared by mixing together aqueous solutions of poly (methacrylic acid) and polyethyleneimine (polycomplex II) are presented in *Figure 36*. The two spectra are identical. Scrambled polysalts are formed by mixing two oppositely charged polyelectrolytes. These arise from random acid-base neutralisation reactions. The polycomplex is disordered. Matrix polymerisation of methacrylic acid on polyethyleneimine results in a disordered complex. Polyethyleneimine used as matrix is highly branched with a statistically average of one branch point for every three repeat units. Thus, the polycomplex formed by the matrix polymerisation is also disordered. Ordered polycomplex may be generated only by using linear polyethyleneimine (L-PEI) as the matrix.

3.3.4 INFRA-RED CHARACTERISATION

3.3.4.1 Curve Resolution Technique

Infra-red spectra of polymers are composed of extensively overlapping peaks. This makes quantitative analysis difficult. A schematic diagram of the various methods currently employed in the analysis of a complex spectra is depicted in *Figure 37*.

Curve fitting techniques using computer, permits one to greater structural detail from a complex infra-red spectrum. Curve fitting of individual bands in a composite spectrum provides an attractive method for quantitative calculations. Parameters of a typical infra-red band in absorbance mode are presented in *Figure 38*.

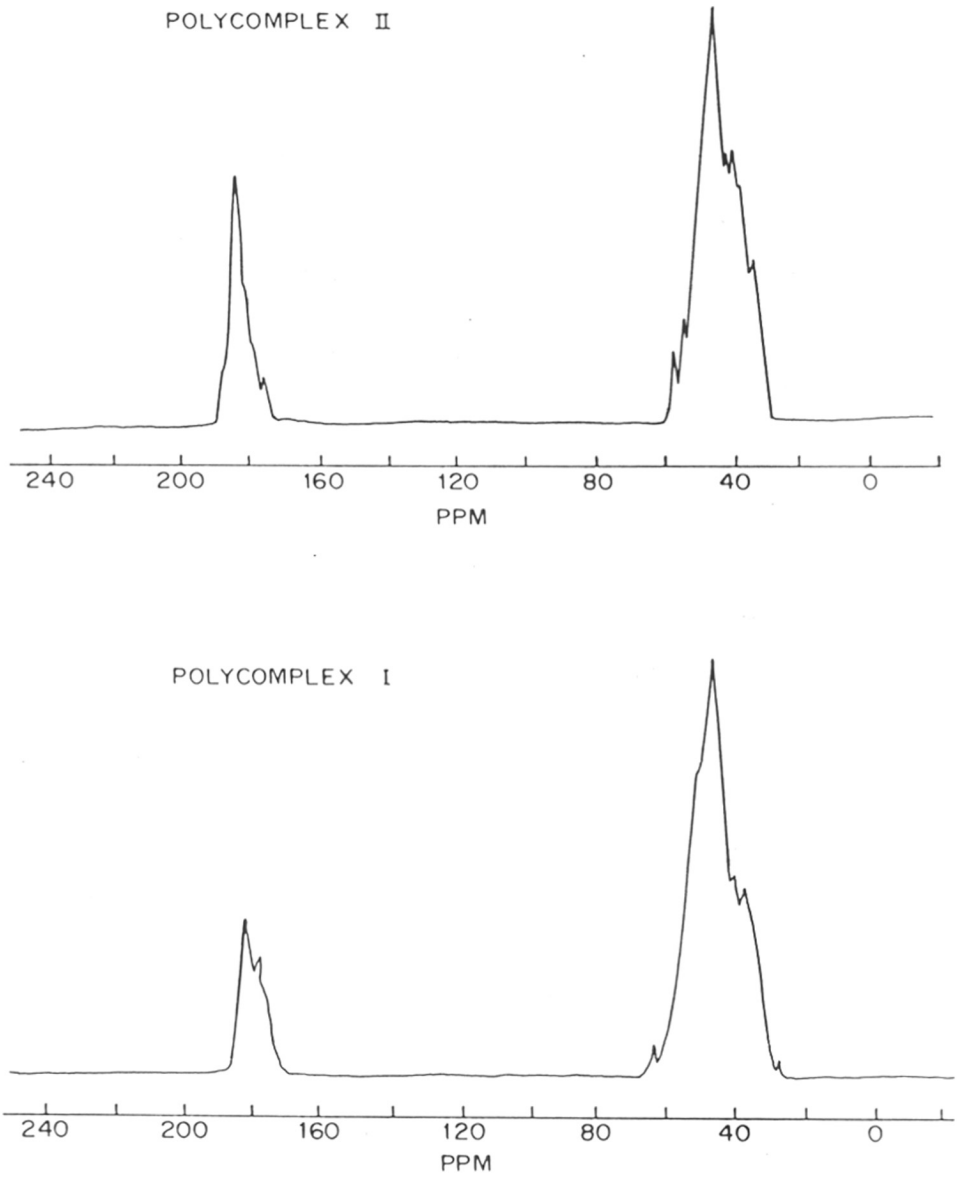


FIG.36:C-13 NMR SPECTRA OF TWO POLYCOMPLEXES

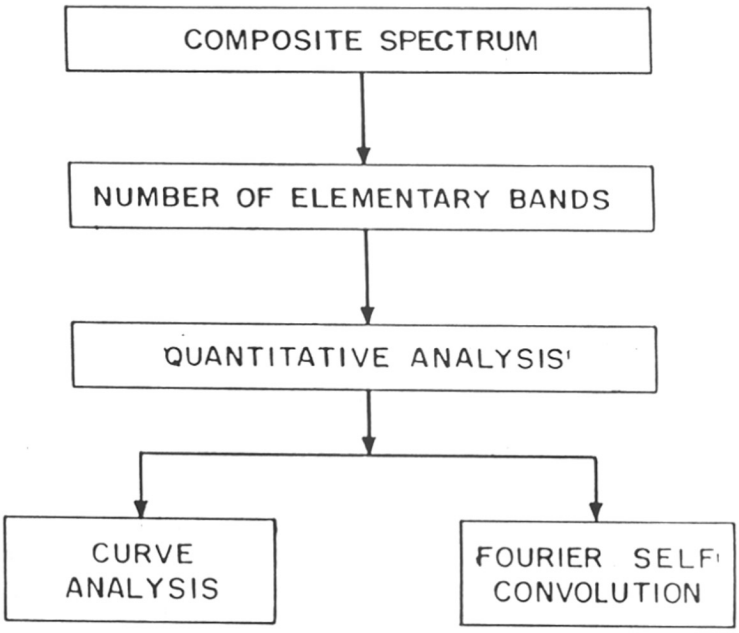


FIG. 37: METHODS ROUTINELY USED FOR THE ANALYSIS OF OVERLAPPING ABSORPTION BANDS

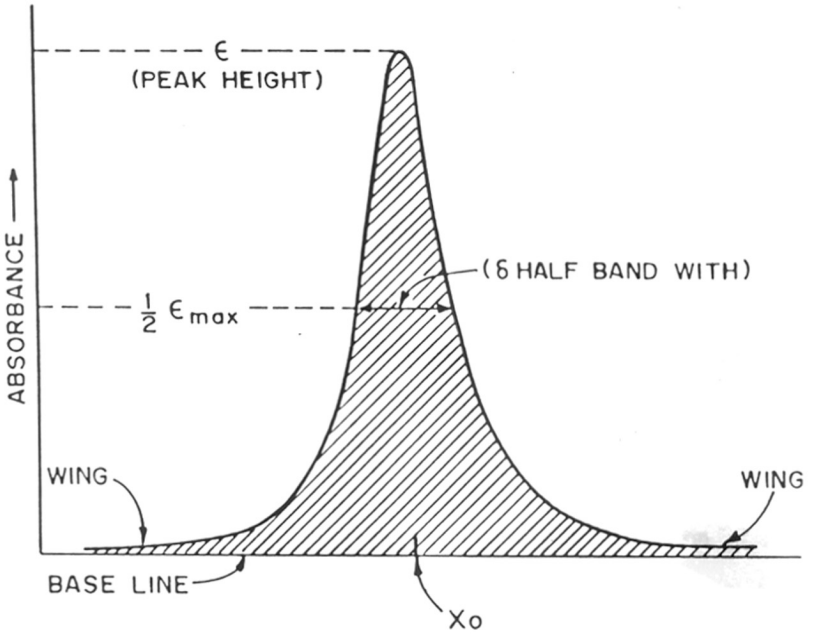


FIG.38: PARAMETERS OF A TYPICAL INFRARED BAND IN ABSORBANCE MODE

3.3.4.2 Theoretical Background

Two most commonly encountered line shapes in various branches of spectroscopy are the Lorentzian and Gaussian. As a general rule, the spectra recorded in vapor phase or in liquid state give rise to peaks (bands) which are well approximated by Lorentzian function. In such cases collision broadening is the main factor which determines the half band width. In case of solid samples, the degrees of freedom are less and the relative orientations and positions of the interacting species do not change with time. Spectral peak shapes of such materials can be well approximated by a Gaussian profile.

Mathematically a single Lorentzian spectral line can be expressed as

$$y = \varepsilon\left[\left(\frac{x - x_o}{\delta}\right)^2 + 1\right]^{-1} \qquad (1)$$

where 'y' represents measure of absorption or emission intensity at the position 'x'

ε = peak height

x_o = The position of the center of peak

δ = Half band width

The area beneath the Lorentzian curve is given by $\pi\,\varepsilon\,\delta$

Similarly a single Gaussian spectral line is represented by

$$y = \varepsilon 2^{-\left(\frac{x - x_o}{\delta}\right)^2} \qquad (2)$$

The area beneath the Gaussian curve is given by $\varepsilon\,\delta\sqrt{\pi/\ln 2}$

Significant differences between the two line shapes are as follows:

Lorentzian is a sharper, narrower function towards the centre. Unlike Gaussian it does not fall off abruptly towards the wings. Gaussian has the maximum slope of the absorption close to the half intensity mark whereas for Lorentzian the maximum slope is approximately at the three-quarter mark of the maximum intensity. A plot of Lorentzian as well as Gaussian profile is depicted in *Figure 39*. The discussion about the line shapes and curve fitting procedures is well documented in the literature.[337-339]

3.3.4.3 Band shapes of infra red spectral lines

The band shapes in the infra-red spectra are usually intermediate between Lorentzian and Gaussian profile. In order to deal with such profiles a linear combination (Equation 3) of the functions given in Equation 1. (i.e. Lorentzian) and Equation 2 (i.e. Gaussian) is generally employed.[340,341]

$$y = \varepsilon\left\{\left[(F)\left(2^{-\left(\frac{x-x_o}{\delta}\right)^2}\right)\right] + (1-F)\left[\left(\frac{x-x_o}{\delta}\right)^2+1\right]^{-1}\right\} \qquad (3)$$

Here, a new term 'F' is introduced. F takes any value between 0 to 1 including both 0 and 1. As the value of F varies from 0 to 1 the profile varies smoothly from Lorentzian to Gaussian. The band shapes for values F = 0, 0.5 and 1 are depicted in *Figure 40*. A suitable value for F can be given by trial and error to get the best fit.

An iterative curve fitting analytical procedure was adopted to resolve the overlapping bands in the infra-red spectra of polycomplexes of poly (methacrylic acid)-polyethyleneimine. A program is written in BASIC@. Line width at half height (half band width) and 'F' were varied to get best fit between experimental and simulated spectrum. In simulated spectrum intensities were normalised to one.

3.3.4.4 Polycomplex characterisation

Polycomplexes formed during matrix polymerisation were studied using the polycomplexes formed by mixing the aqueous solutions of poly (methacrylic acid) (PMAA) and branched polyethyleneimine (B-PEI) as model compounds. The latter polycomplexes obtained in form of

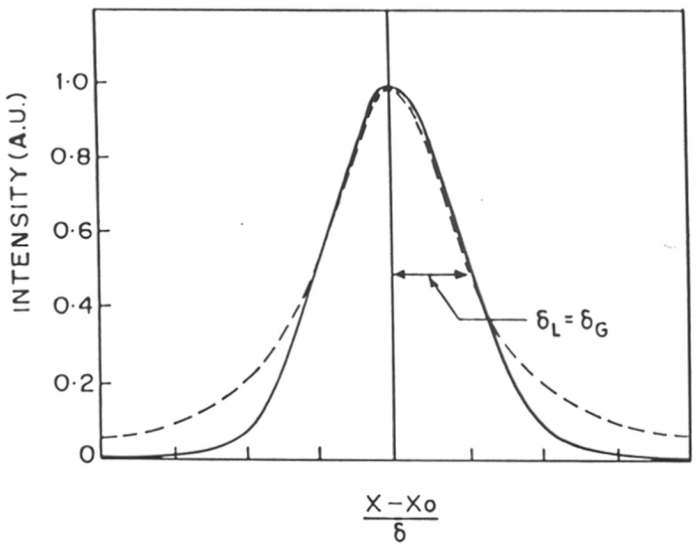


FIG. 39: PLOT OF LORENTZIAN (----) AND GAUSSIAN (—) PROFILES

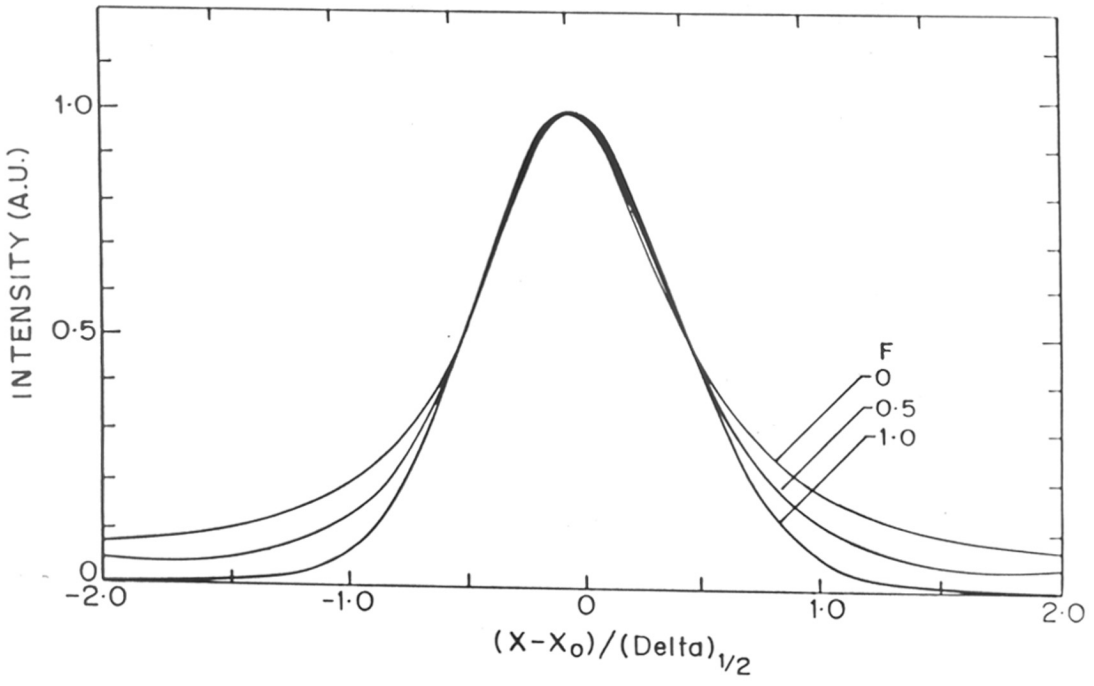


FIG. 40 : BAND SHAPES GENERATED BY USING A LINEAR COMBINATION OF LORENTZIAN AND GAUSSIAN FUNCTION F=0 REPRESENTS EQUATION (1) AND F=1 REPRESENTS EQUATION (2)

powder were not soluble in any solvent and therefore could be analysed in solid state by infra-red spectroscopy. Since B-PEI contained various basic sites (primary:secondary:tertiary in the ratio 1:2:1, (See section 2.2.3)) it was important to make sure which sites were involved in complexation. Pioneering studies of Barrow *et al.*[342] with simple acid and a variety of bases have shown that there is possibility of charge transfer complex between simple carboxylic acids and tertiary amines. Formation of polyelectrolyte complexes between polyethyleneimine and polycarboxylic acid have been investigated in the past.[343-346]

Infra-red spectra of PMAA (solid) and B-PEI (viscous liquid) are presented in *Figure 41*. The prominent peak at 1715 cm^{-1} in PMAA is characteristic of carboxylic group. Similarly in B-PEI medium band at 1602 cm^{-1} is characteristic of deformation of $-NH_2$/-NH groups. Infra-red spectra of precipitated polycomplexes (Polycomplex II), obtained by mixing the aqueous solution in various proportions (Table 29) are given in *Figure 42* and *43*. A quick examination of these spectra indicate substantial changes in the region 1800 to 1500 cm^{-1}. The most prominent of these are the appearance of a new band at 1542 cm^{-1} which is accompanied by a shift of carboxylic band of PMAA to 1699 cm^{-1}. The band at 1542 cm^{-1} can only be ascribed to COO^- (carboxylate ion) arising from the interaction between tertiary amino groups (of B-PEI) and carboxyl groups of PMAA. A band in this region has been reported in the charge transfer complex, formed between simple carboxylic acid (benzoic, acetic acid) and a tertiary base like triethyl amine, in a high dielectric constant solvent like dimethyl sulphoxide.[347] The charge transfer complex between B-PEI and PMMA can be represented as shown in *Figure 44*.

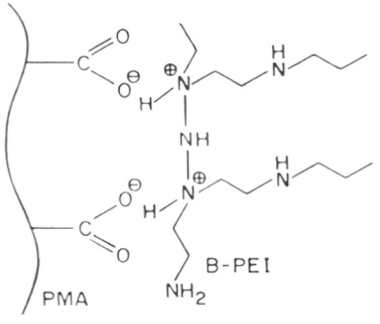


FIG. 44 : COMPLEXATION BETWEEN PMA AND B-PEI

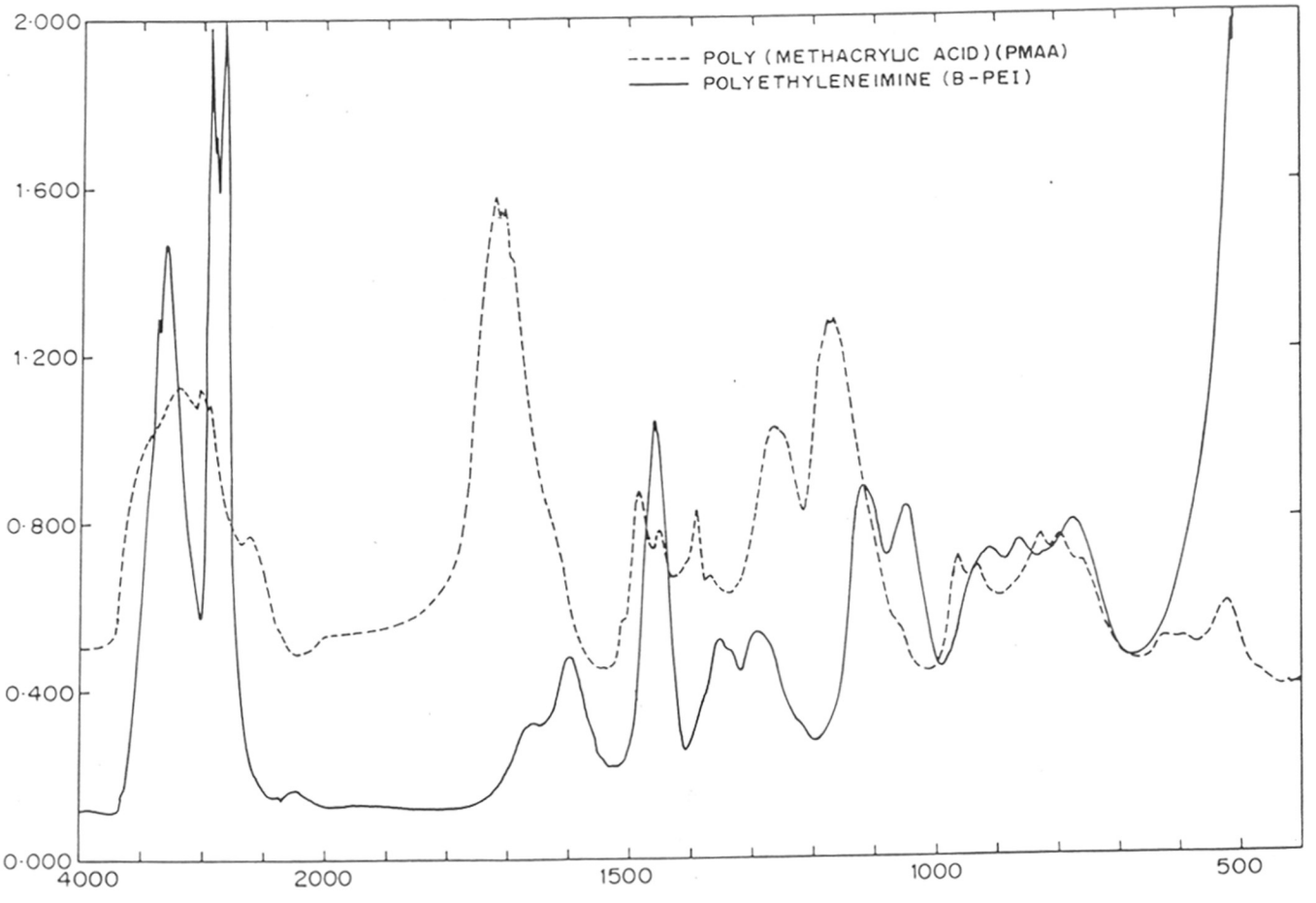


FIG. 41 : INFRA-RED SPECTRA OF TWO INDIVIDUAL HOMOPOLYMERS

Table - 29

Composition of polycomplexes formed by mixing the aqueous solutions of poly (methacrylic acid) [PMAA] and branched polyethyleneimine [B-PEI].

Polym code	B-PEI (0.25 molar), mL	PMA (0.25 molar), mL	Wt. of B-PEI gm	Wt.of PMAA gm	B-PEI (tert. N):PMAA proportion, molar	Wt. of polyc omplex obtained gm
MPPC 2	16.0	4.0	0.1722	0.0861	0.25:0.25	0.2637
MPPC 3	14.0	6.0	0.1507	0.1291	0.25:0.43	0.2893
MPPC 4	12.0	8.0	0.1292	0.1722	0.25:0.67	0.3276
MPPC 5	10.0	10.0	0.1077	0.2152	0.25:1.0	0.2994
MPPC 6	8.0	12.0	0.0861	0.2583	0.25:1.5	0.2616
MPPC 7	6.0	14.0	0.0646	0.3013	0.25:2.3	0.1926
MPPC 8	4.0	16.0	0.0431	0.3444	0.25:4.0	0.1358

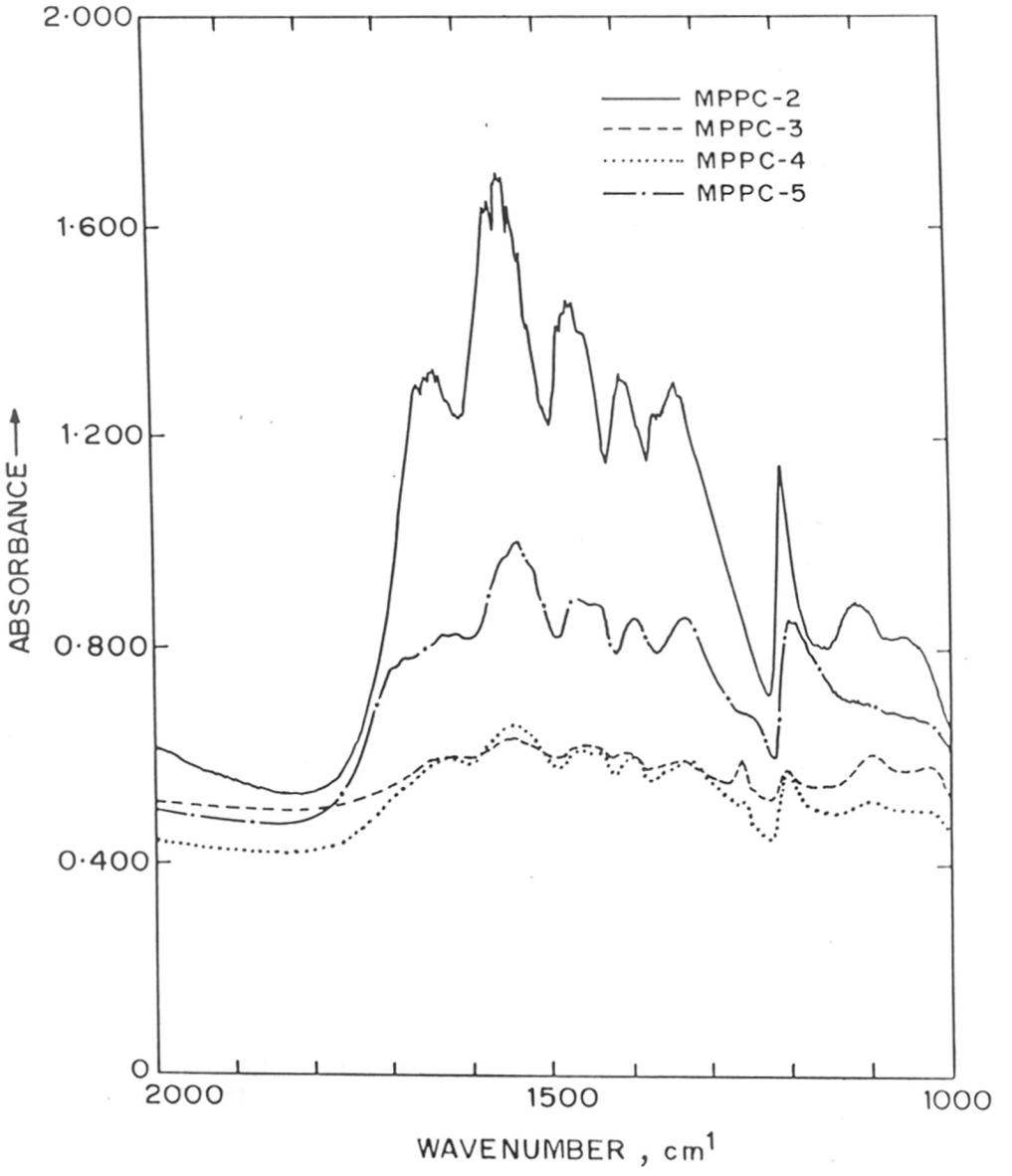


FIG.42: INFRA-RED SPECTRA OF POLY (METHACRYLIC ACID) BRANCHED POLY ETHYLENEIMINE COMPLEXES FORMED BY MIXING TWO AQUEOUS SOLUTIONS OF HOMOPOLYMERS

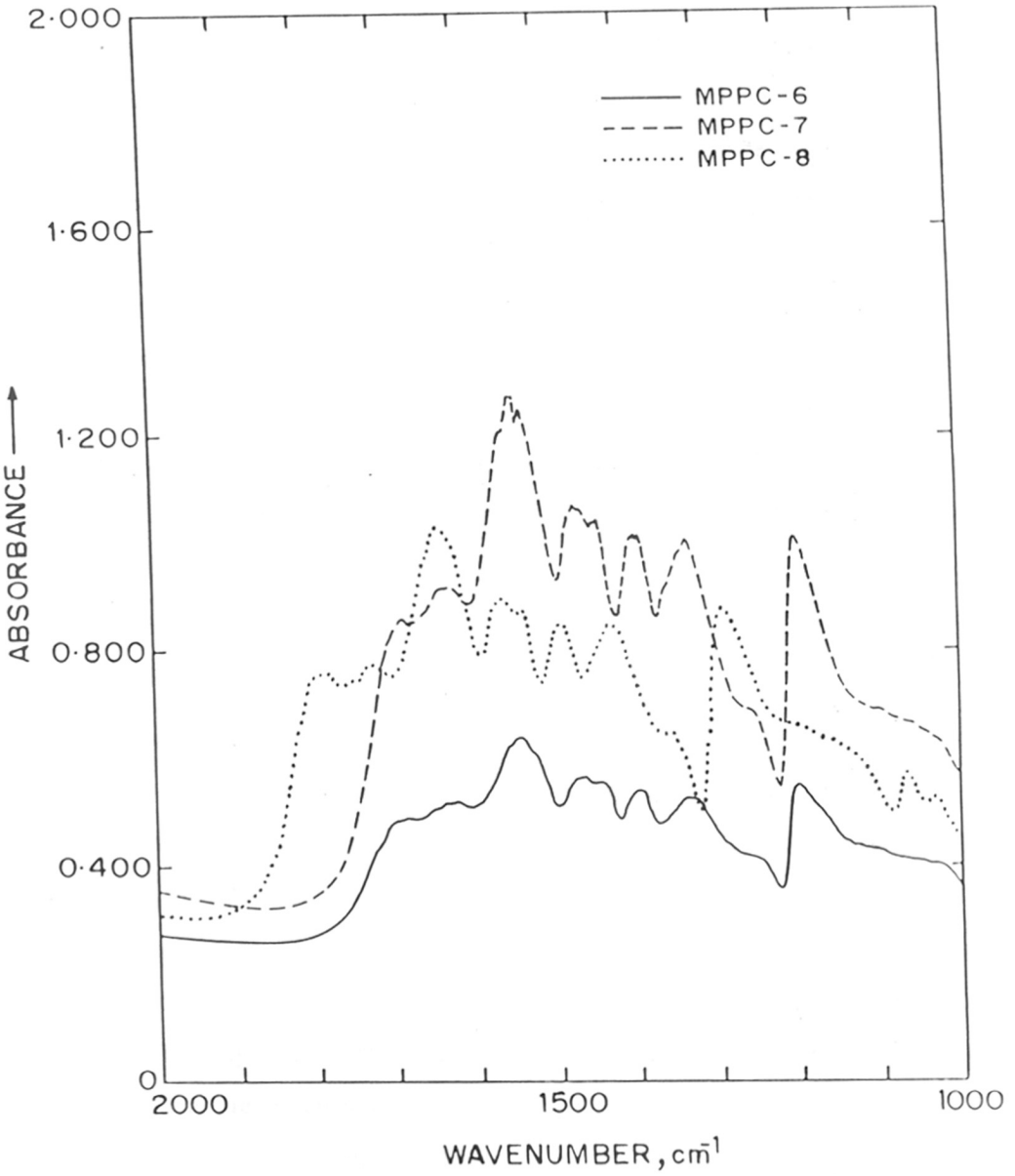


FIG. 43: INFRA-RED SPECTRA OF POLY (METHACRYLIC ACID) BRANCHED POLY ETHYLENEIMINE COMPLEXES FORMED BY MIXING TWO AQUEOUS SOLUTIONS OF HOMOPOLYMERS

The formation of charge transfer complex is also confirmed from the decreased intensity of the carboxyl group of PMAA as determined from the band separation techniques. The carboxylic groups which were not involved in the charge transfer were estimated. When all the carboxylic groups are complexed with the tertiary amino groups which are present in equimolar quantities, a total disappearance of the band due to COOH (1700 - 1690 cm^{-1} , *Figure 42*) group was observed as expected. When the relative proportion of COOH group is higher than the tertiary amino group, both bands ascribable to COO^- (carboxylate ion) as well as COOH (carboxylic) groups were observed (*Figure 43*). When 1:1 proportion (MPPC 2, Table 29) of tertiary amino groups to PMAA was mixed, complete precipitation of polycomplex took place as would be expected from the amounts taken (0.17222 gm B-PEI and 0.0861 gm PMAA) and amount precipitated (0.2637 gm). In all other cases, where the ratio of tertiary amino to carboxylic groups is not 1:1, complete precipitation did not take place. This clearly shows that the interaction involved in the polycomplex formation is of a charge transfer type.

Polycomplexes precipitated during polymerisation (Polycomplex I) of methacrylic acid (MAA) in presence of B-PEI (molecular weight approx. 20,000), in the molar ratio 2:1 (MPEC 13 - MPEC 18) were subjected to infra-red characterisation. The infra-red spectra are presented in *Figure 45*. During polymerisation the time was varied from 2 to 12 minutes. Infra-red spectra in 2000 to 1000 cm^{-1} region were subjected to curve fitting (band separation) procedures. Curve fitting results are presented in *Figure 46 - 51*. The spectra were analysed for the relative intensities of carboxylate ion and carboxylic groups at 1542 cm^{-1} and 1699 cm^{-1} respectively. The area of resolved bands at these positions are presented in Table 30 It can be seen from the area estimations that there is a gradual increase in the intensities of both the bands. This can be explained on the basis of increased interaction of methacrylic acid with base with respect to time. The initial reaction mixture having large excess (approximately 8 times) of methacrylic acid (with respect to tertiary amino groups of B-PEI), would interact with all the tertiary amino groups which are available. However, due to the coiled nature and high molecular weight of B-PEI, many of the tertiary nitrogen

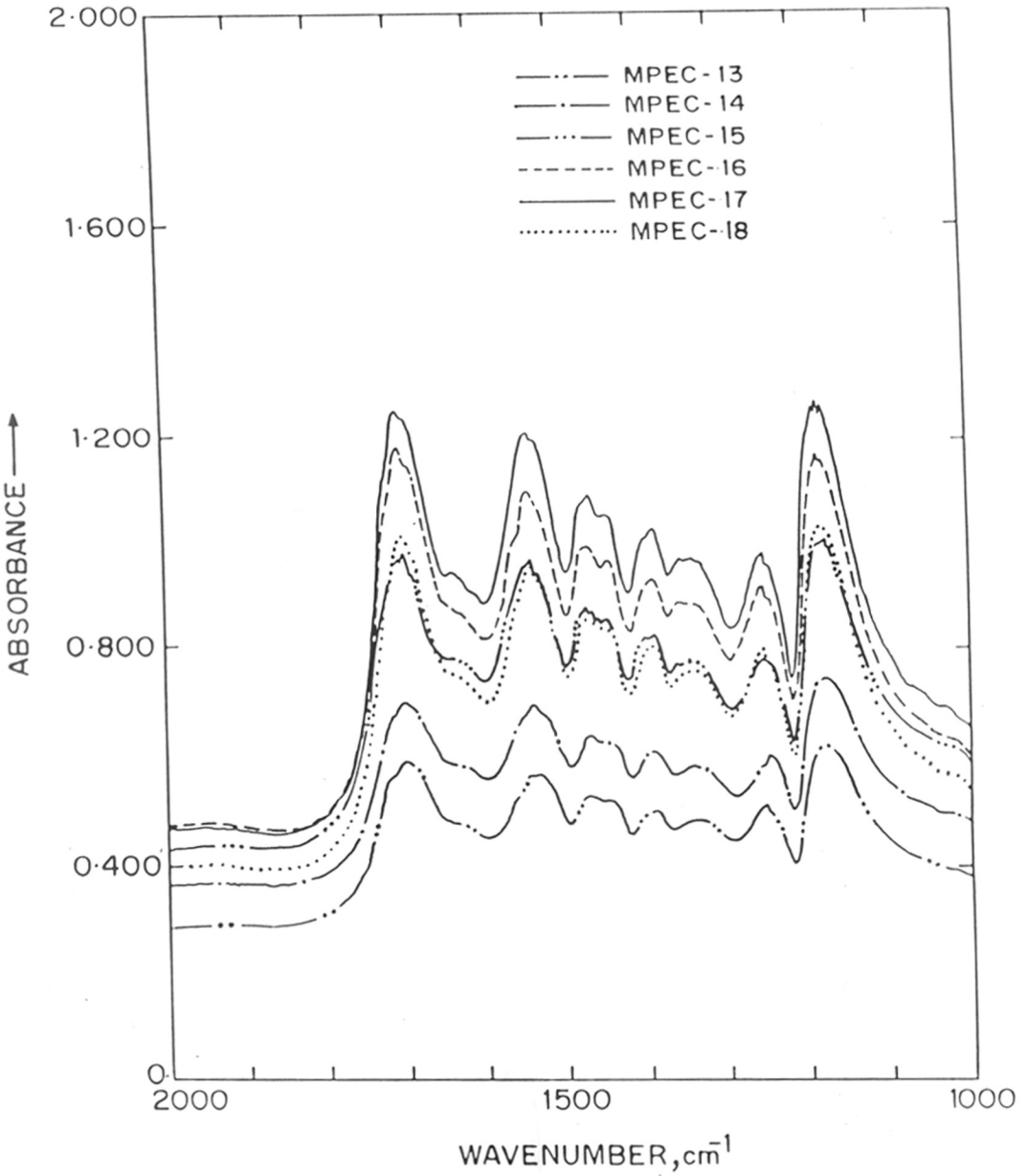


FIG. 45 : INFRA-RED SPECTRA OF A SERIES OF POLY (METHACRYLIC ACID) BRANCHED POLYETHYLENEIMINE POLYCOMPLEXES GENERATED AT DIFFERENT TIME INTERVALS DURING MATRIX POLYMERISATION (MONOMER : MATRIX RATIO 2 : 1 Molar)

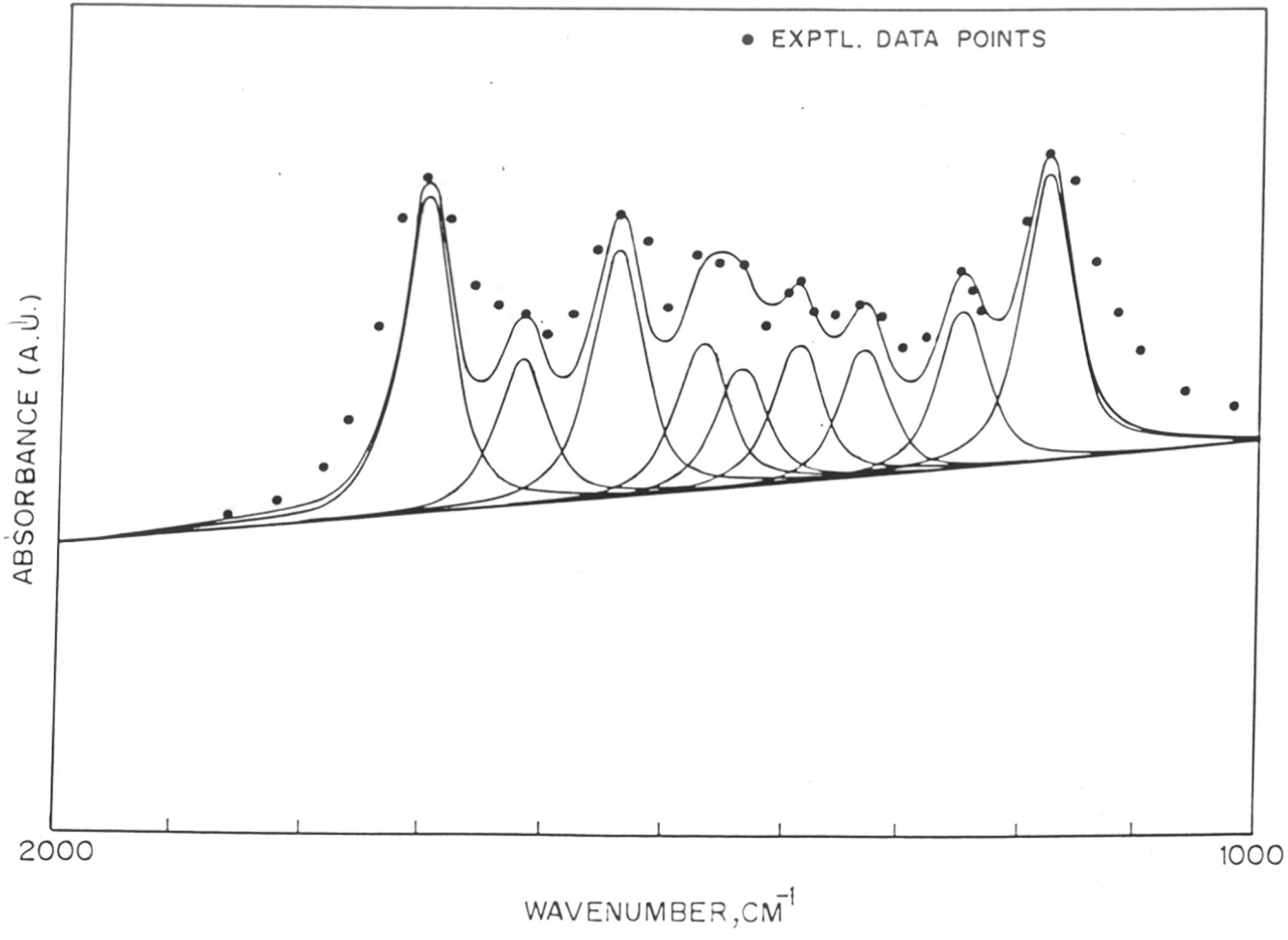


FIG. 46 : CURVE FITTING RESULTS FOR MPEC-13

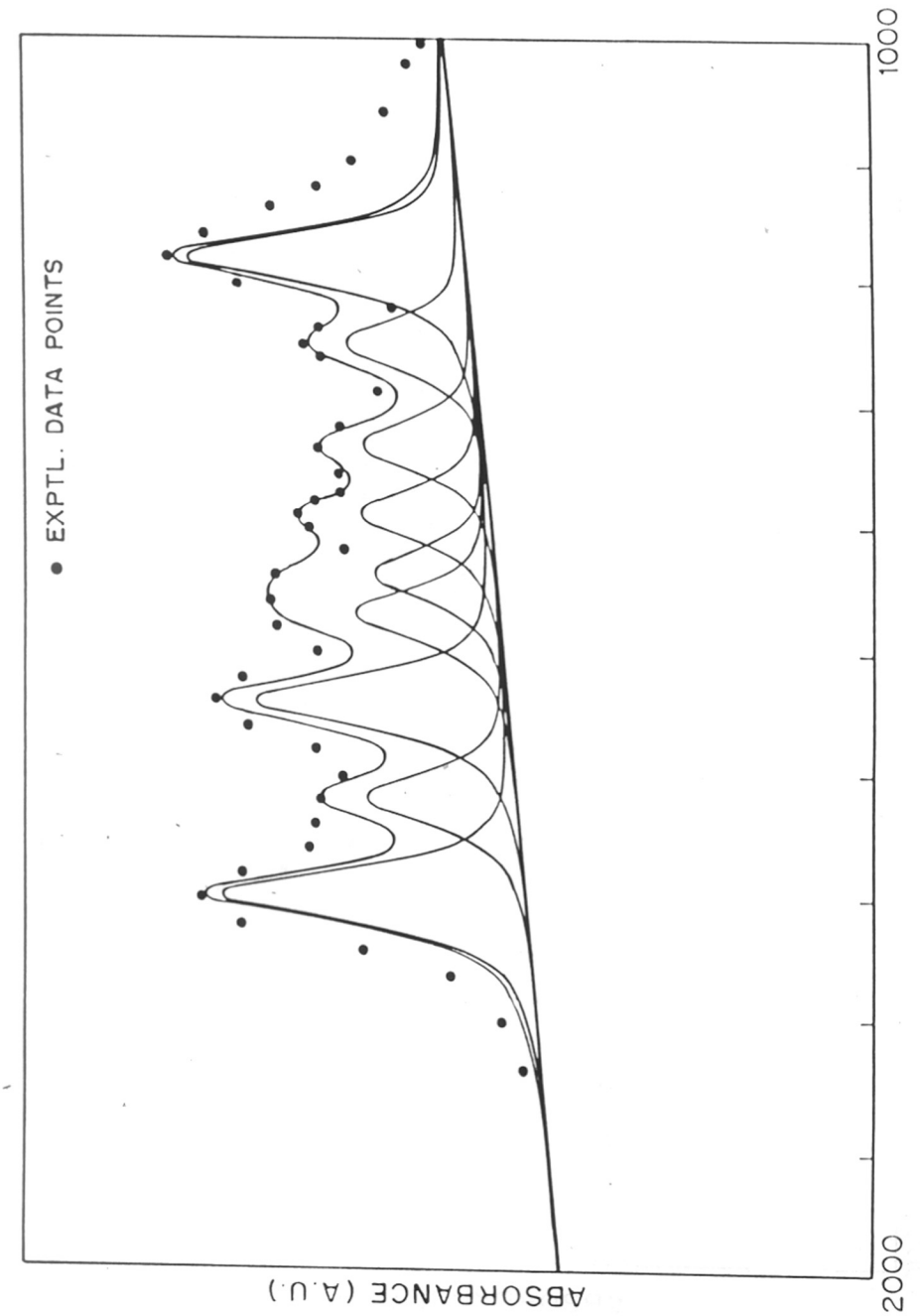


FIG. 47 : CURVE FITTING RESULTS FOR MPEC-14

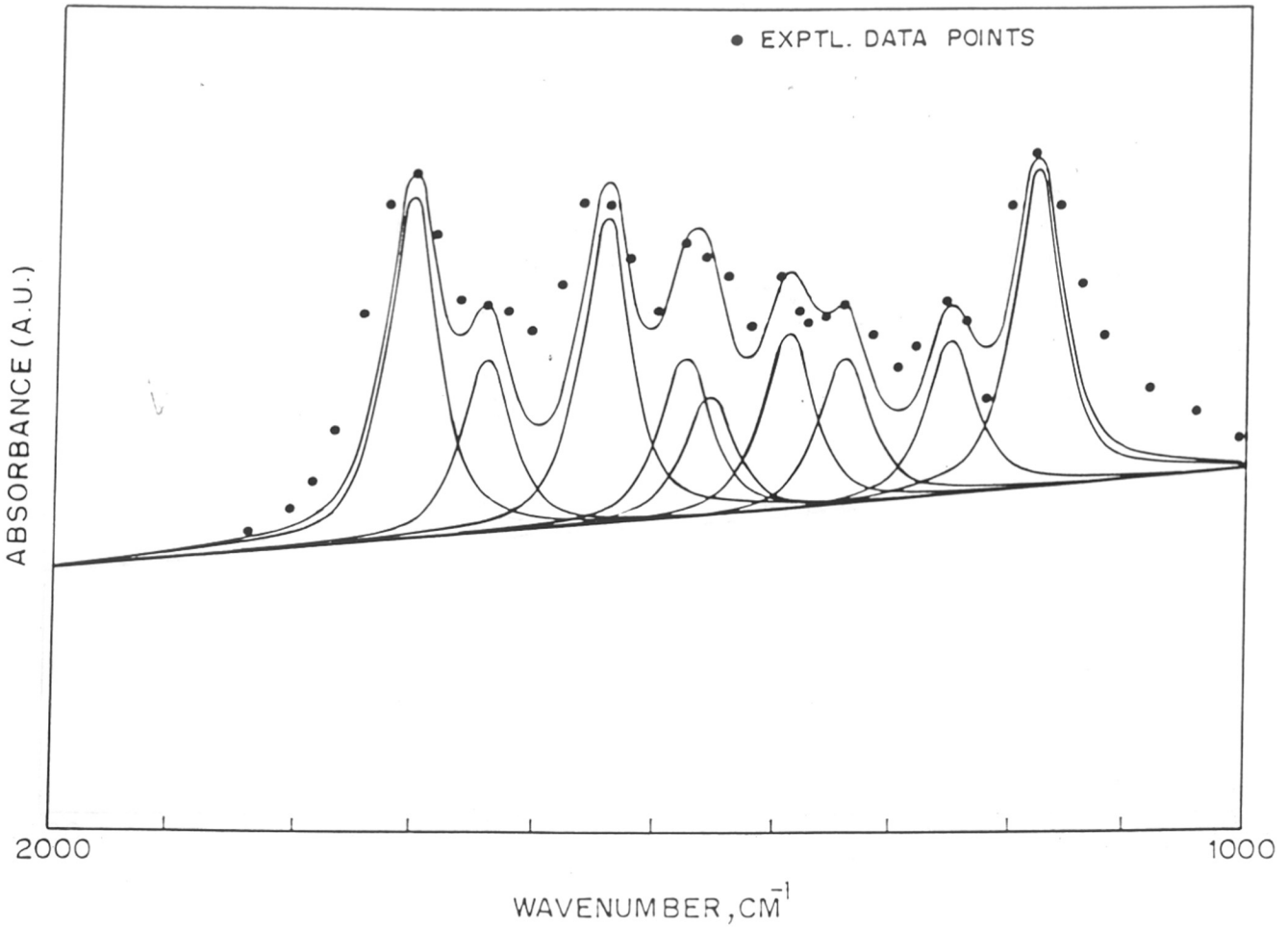


FIG. 48 : CURVE FITTING RESULTS FOR MPEC-15

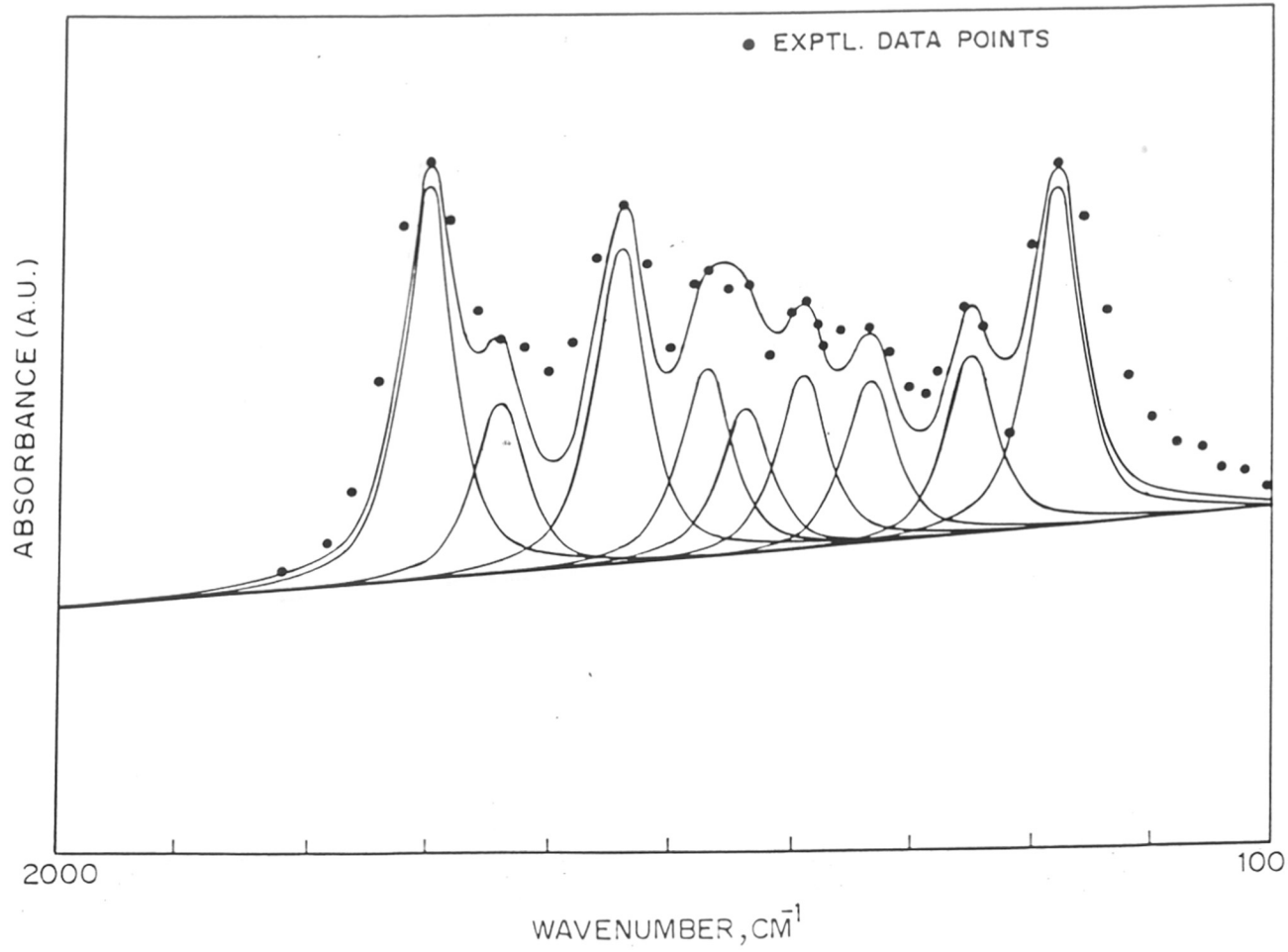


FIG. 49 : CURVE FITTING RESULTS FOR MPPC - 16

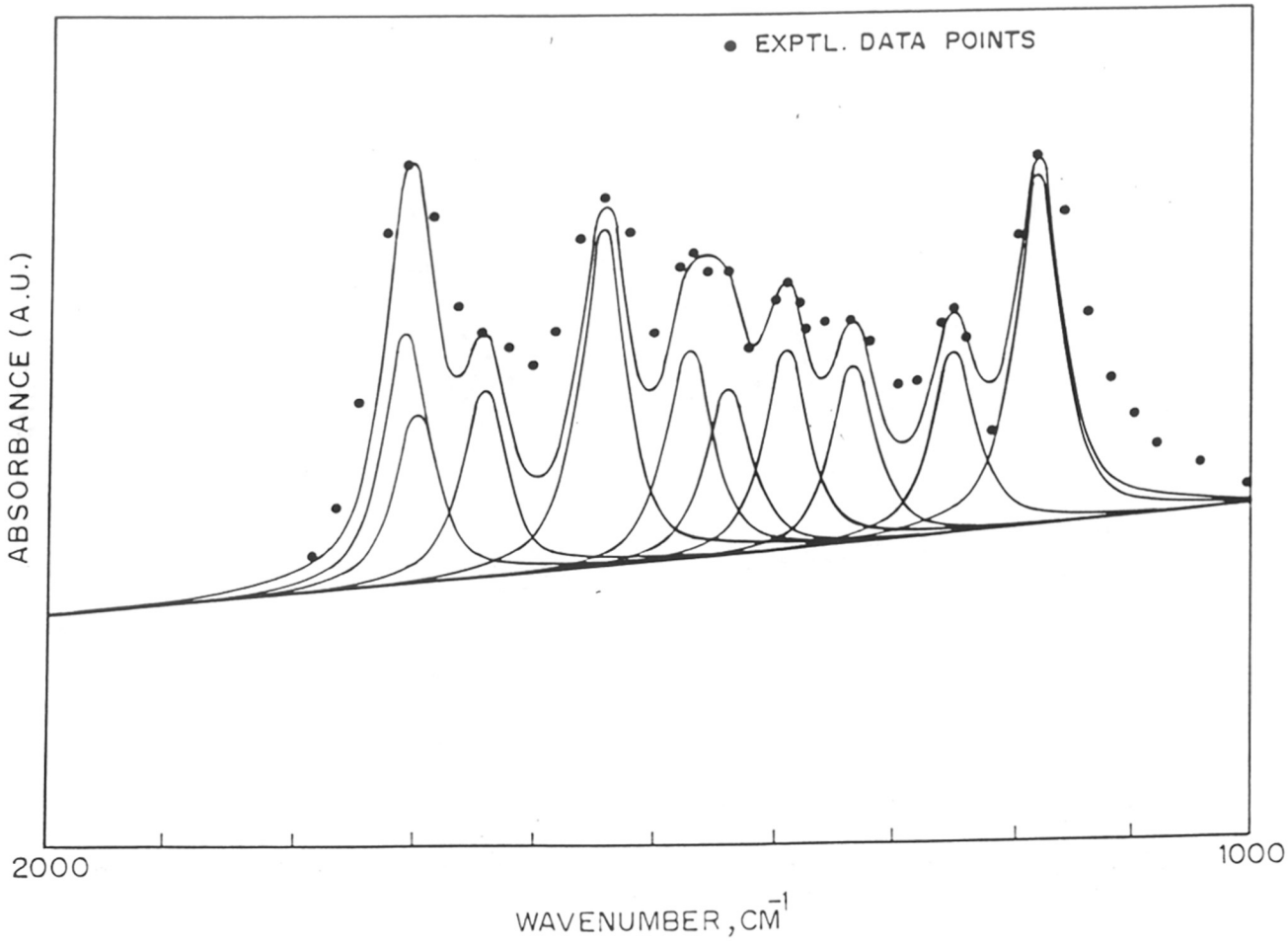


FIG. 50 : CURVE FITTING RESULTS FOR MPEC-17

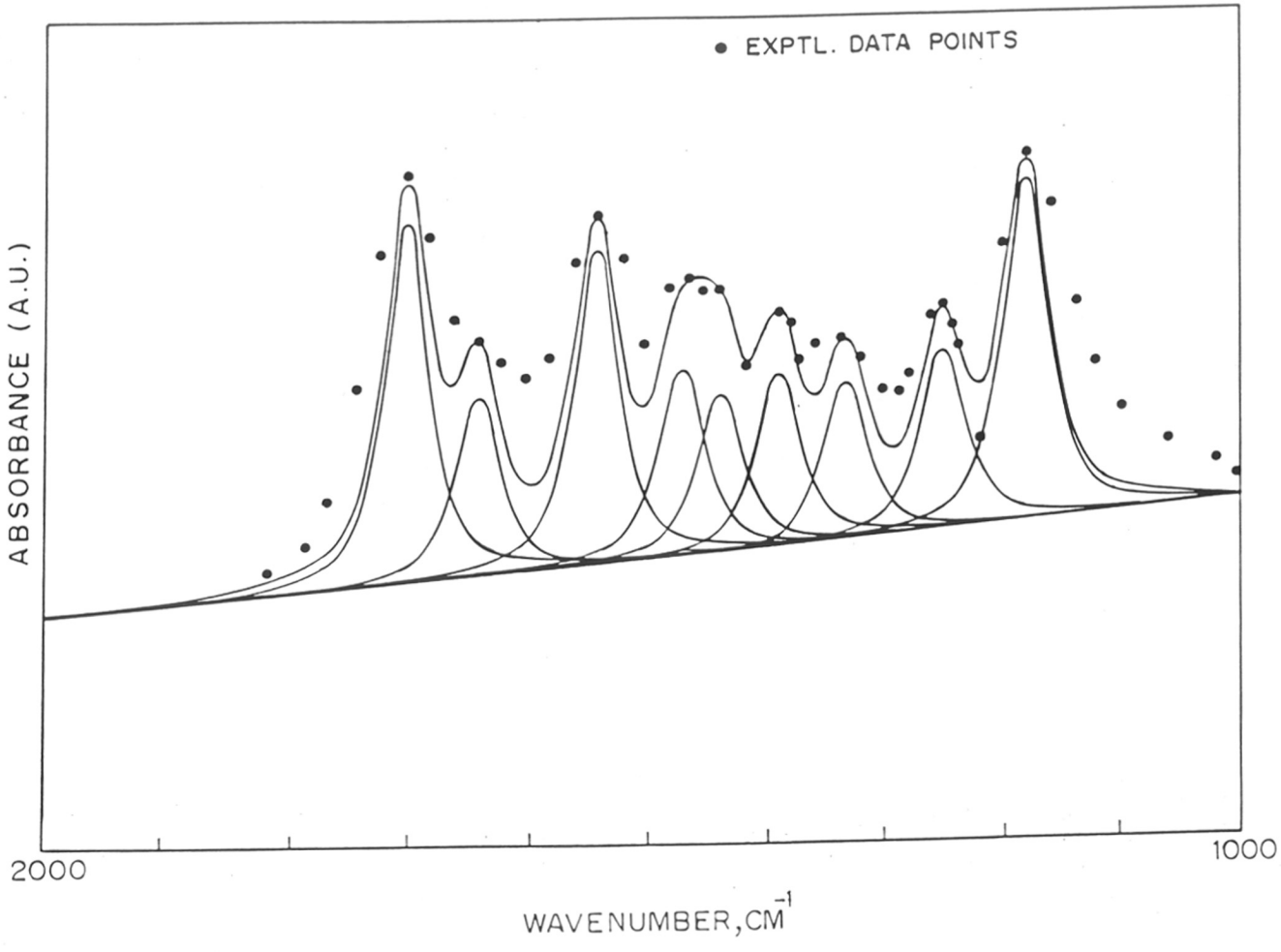


FIG. 51 : CURVE FITTING RESULTS FOR MPEC-18

Table - 30

Data of area estimations of resolved COOH and COO^- peaks.

MAA:B-PEI ratio is 2.0:1.0 molar

Sample code	Reaction time, min.	COOH Area CM^{-1}	COO^- Area CM^{-1}
MPEC 13	2	41.24	32.19
MPEC 14	4	46.03	38.38
MPEC 15	6	71.24	63.51
MPEC 16	8	95.00	76.59
MPEC 17	10	59.16	79.34
MPEC 18	12	69.77	60.75

sites may not be available for interaction. Moreover, as time goes on many new tertiary nitrogen sites may become accessible for interaction with the methacrylic acid. This explains the marginal increase in the relative intensity of COO^- band with time. The relative drop in the intensity of COO^- band in case of MPEC 18 (later stage of polymerisation) may be due to the precipitative nature of polymerisation. This makes tertiary nitrogen sites less accessible for interaction with the carboxylic groups of poly (methacrylic acid). The increase in the intensity of COOH band (1700 - 1699 cm^{-1}) is indicative of the polymerisation that is taking place within the charge transfer complex. The methacrylic acid units are being increasingly attached as the polymerisation proceeds.

Similar type of results were obtained with matrix polymerisation system having molar ratio 1.5:1.0 of MAA:B-PEI. The infra-red spectra of polycomplexes (MPEC 7 to MPEC 11) formed in this series are presented in *Figure 52*. Area estimations (of COOH and COO^- bands) are presented in Table 31.

In case of 1:1 ratio of MAA:B-PEI the extent of polymerisation is not increasing as rapidly as in 1.5:1.0 and 2.0:1.0 ratios. The infra-red spectra of polycomplexes (MPEC 1 to MPEC 6), generated in this series are presented in *Figure 53*. Area estimations (of COOH and COO^- bands) are presented in Table 32 The above results can be understood partly from the relatively lower concentration of MAA available for charge transfer interaction and polymerisation.

Analysis of infra-red spectra of polycomplexes generated in systems having relatively lower MAA to B-PEI ratios (0.8:1.0, 0.6:1.0 and 0.4:1.0), was also attempted. The major obstacle with these polycomplexes was inability to obtain a good infra-red spectrum which hampered further investigation. This could be due to the highly amorphous nature of polycomplexes formed in these systems. In case of MAA:B-PEI ratio of 0.6:1.0, the polycomplexes were somewhat elastic in nature and KBr pellets could not be casted out.

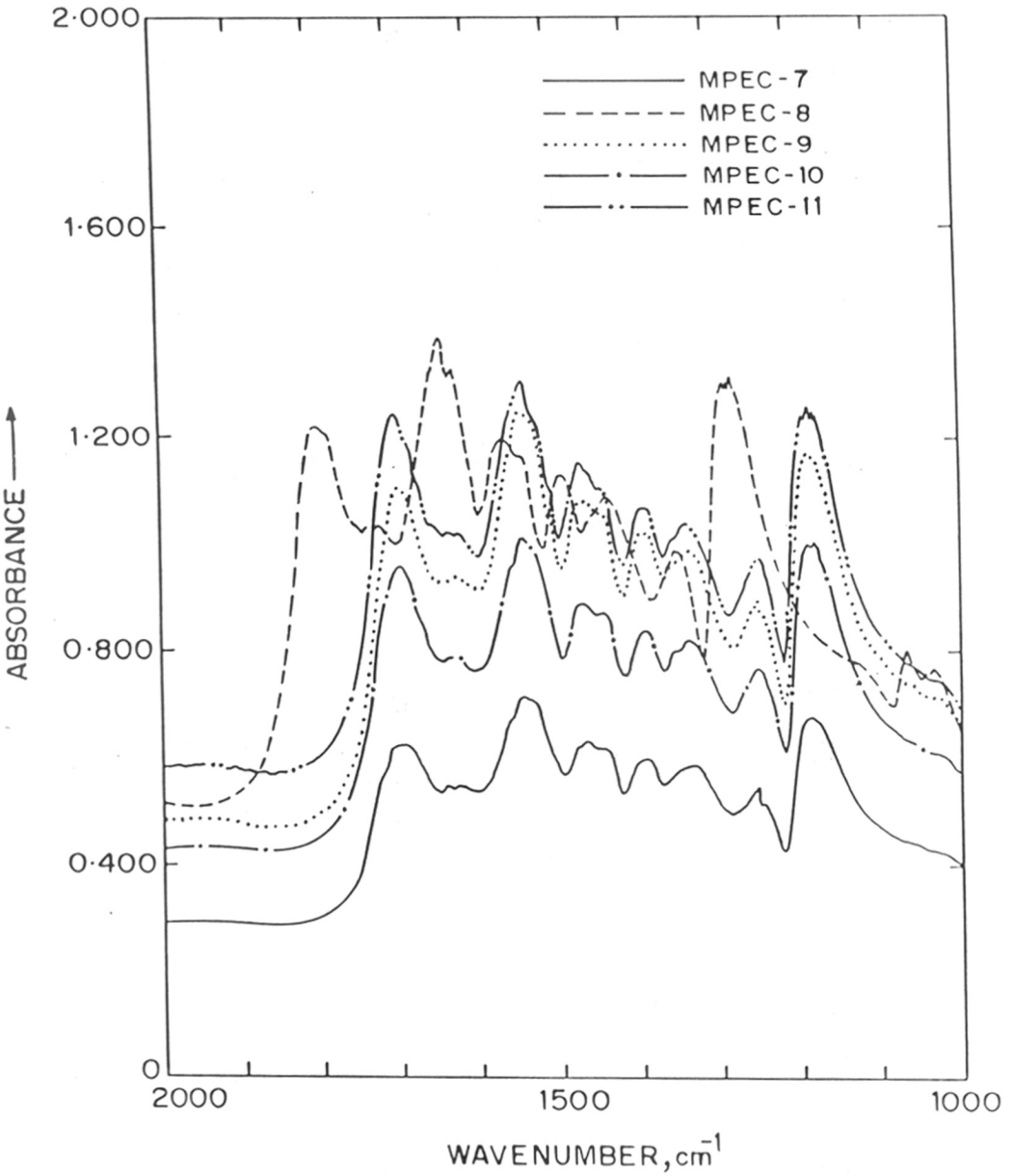


FIG.52: INFRA-RED SPECTRA OF A SERIES OF POLY(METHACRYLIC ACID) BRANCHED POLYETHYLENEIMINE POLYCOMPLEXES GENERATED AT DIFFERENT TIME INTERVALS DURING MATRIX POLYMERISATION (MONOMER:MATRIX RATIO 1·5:1 Molar)

Table - 31

Data of area estimations of resolved COOH and COO^- peaks.

MAA:B-PEI ratio is 1.5:1.0 molar

Sample code	Reaction time, min.	COOH Area CM^{-1}	COO^- Area CM^{-1}
MPEC 7	2	38.91	46.52
MPEC 8	4	76.06	66.84
MPEC 9	6	80.29	92.64
MPEC 10	8	62.88	63.26
MPEC 11	10	75.49	78.29

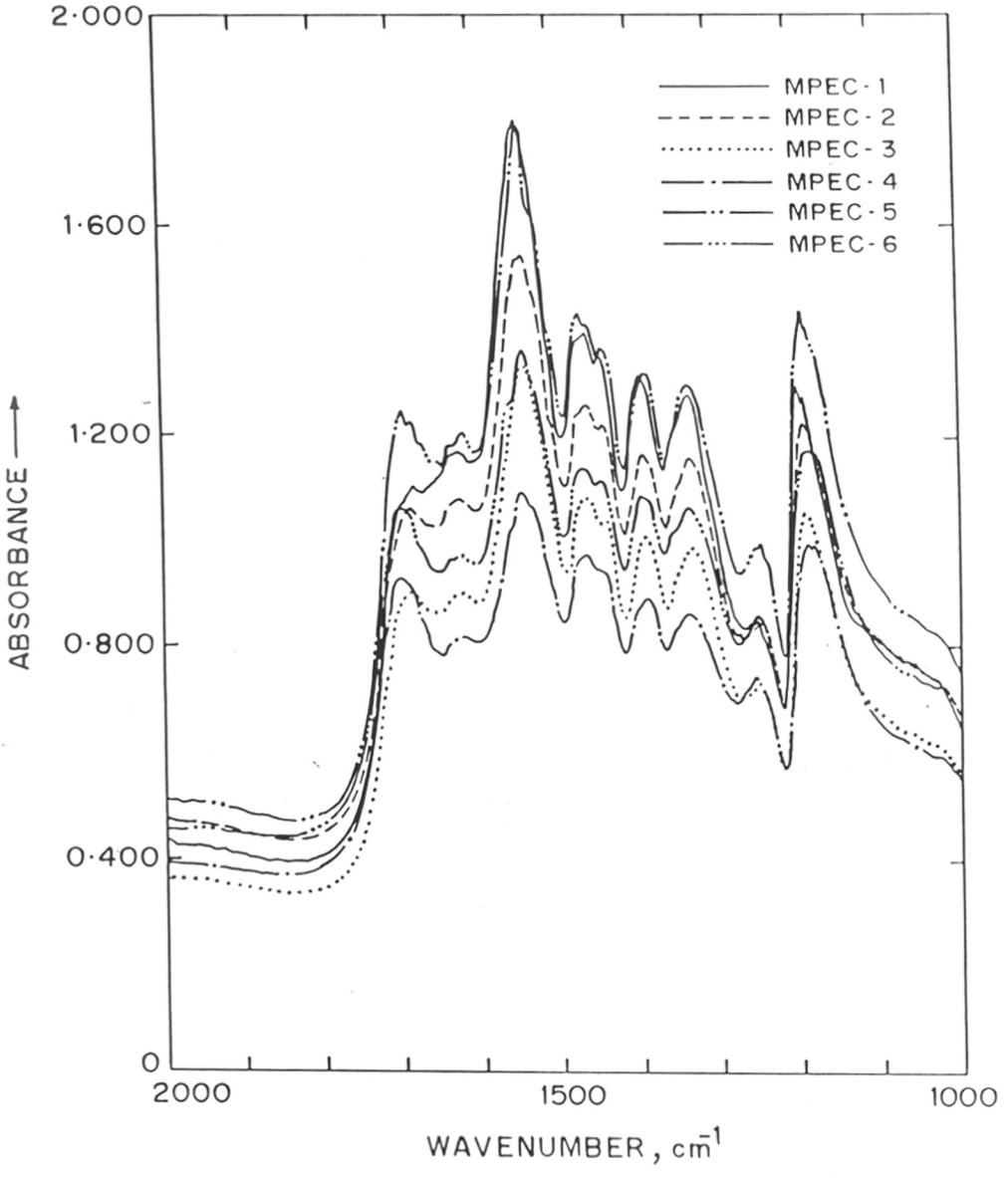


FIG.53: INFRA-RED SPECTRA OF A SERIES OF POLY (METHACRYLIC ACID) BRANCHED POLYETHYLENEIMINE POLYCOMPLEXES GENERATED AT DIFFERENT TIME INTERVALS DURING MATRIX POLYMERISATION (MONOMER : MATRIX RATIO 1:1 Molar)

Table - 32

Data of area estimations of resolved COOH and COO^- peaks.

MAA:B-PEI ratio is 1.0:1.0 molar

Sample code	Reaction time, min.	COOH Area CM^{-1}	COO^- Area CM^{-1}
MPEC 1	2	63.69	158.55
MPEC 2	4	75.43	133.46
MPEC 3	6	62.68	109.19
MPEC 4	8	69.15	83.77
MPEC 5	10	96.40	161.75
MPEC 6	12	76.06	110.60

SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

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SUMMARY

MATRIX POLYMERISATION

Free radical polymerisation of methacrylic and acrylic acid was studied in presence of branched polyethyleneimine (B-PEI), in aqueous medium at 30 ± 0.1°C. The polymerisations were investigated in the following relative monomer-matrix ratios: 0.4:1, 0.6:1, 0.8:1, 1:1, 1.5:1 and 2:1. Within each composition, samples were run for varied reaction times. The formed polycomplexes were quantitatively precipitated and isolated to estimate the rate of polymerisation. Polymerisation of methacrylic acid was also studied under identical conditions in absence of polyethyleneimine. The rate values of matrix and solution (in absence of matrix) polymerisations of methacrylic acid were estimated. The comparison the rate value reveals that branched polyethyleneimine accelerate the free radical polymerisation of methacrylic acid. The rate is 2-10 times faster than the normal redox polymerisation. The matrix effect of branched polyethyleneimine operates primarily due to hydrogen bond formation between the monomer and matrix. The polymerisation rate continues to increase even beyond the stoichiometric monomer: matrix ratio. Branched polyethyleneimine not only exert matrix effect on polymerisation of methacrylic and acrylic acid but also acts as a redox pair with potassium persulphate. In rate expression the value of monomer and initiator exponents were found to be 1.00 and 0.02 respectively. The lower value for initiator exponent may be due to the primary radical termination and heterogeneous nature of the reaction.

Characterisation:

Two first order transitions noted in the DSC thermogram, were thought to be originating from the crystalline nature of poly (methacrylic acid) - polyethyleneimine complex. Further investigation of polycomplex with X-ray and C-13 NMR spectroscopy, revealed that the poly (methacrylic acid) - polyethyleneimine complex generated during matrix polymerisation display amorphous nature and is not different from the polycomplex formed by mixing aqueous solution of poly (methacrylic acid) and polyethyleneimine. Infra-red spectrum of poly (methacrylic acid)- branched polyethyleneimine complex formed during matrix polymerisation and by mixing the aqueous solutions of homopolymers, indicates appearance of a new band at 1542 cm^{-1} and shift of COOH band position of PMAA to 1699 cm^{-1}. The 1542 cm^{-1} band was ascribed to carboxylate ion (COO^-) formed as a result of interaction with tertiary basic sites of B-PEI. It was therefore concluded that the nature of polycomplex is of charge transfer type. Curve resolution of infra-red spectra of a series of polycomplexes generated by matrix polymerisation at different time intervals, showed a gradual increase in the area of both the bands. This is due to the increased interaction of methacrylic acid with polyethyleneimine with time.

ENZYME IMMOBILISATION:

Immobilisation of penicillin G acylase was studied on

(i) Porous alumina with varying amounts of MgO content and porous alumina coated with polyethyleneimine and derivatised with glutaraldehyde.

(ii) Macroporous beaded hydroxy ethyl methacrylate - divinyl benzene copolymers coated with polyethyleneimine and derivatised with glutaraldehyde.

(iii) Macroporous beaded glycidyl terpolymers of varying degree of hydrophilicity and hydrophobicity

Adsorption and expression of penicillin G acylase was found to vary with the hydrophobic/hydrophilic nature of support. The pH at the surface of the porous alumina was varied by incorporating differing amounts of magnesium oxide. The pH increases with increase in magnesium oxide content. Since penicillin G acylase has optimum pH of 7.8, the adsorption of penicillin G acylase was found to increase with increase in magnesium oxide content. Polyethyleneimine treatment of porous alumina sample imparted hydrophilic character to the support. This treatment decreased the adsorption of penicillin G acylase by 40 percent and increased the percent expression of bound penicillin G acylase by 63 percent. This indicates a crucial role of hydrophobicity of alumina and pH in the microenvironment of the support, in adsorption and expression of penicillin G acylase.

Adsorption of penicillin G acylase was also investigated on a series of macroporous hydroxyethyl methacrylate-divinyl benzene copolymers. Adsorption was found to increase with DVB content of upto 0.7610 mole fraction (copolymer HD 6). This was due to the increased hydrophobic character of the beads. This copolymer was further investigated for covalent binding of penicillin G acylase, by treatment with polyethyleneimine and glutaraldehyde. Studies with penicillin G acylase, thus immobilised ,reveals that there is substantial change in the kinetic and thermal properties of enzyme. Immobilised penicillin G acylase display lower K_m (12.8 $\mu mole$) as compared to native enzyme (31 $\mu mole$. This indicates less steric hindrance and increased affinity of the penicillin G molecule for the enzyme. Thermal stability of penicillin G acylase was enhanced after immobilisation. The shift in optimum pH was marginal. The comparison of pH-activity profile between native and immobilised penicillin G acylase indicates that latter is stable over a wider pH range. Optimum temperature shifted from 40°C (native enzyme) to 57°C (immobilised enzyme). Broadening of temperature-activity profile in case of immobilised penicillin G acylase, near optimum activity was noted. This indicates that immobilised penicillin G acylase is stable over a wider temperature. The immobilised penicillin G acylase permitted repeated use upto 32 work cycles, in a batch mode, without any noticeable change in its activity.

Three series of beaded terpolymers containing oxirane groups namely, GMA-HEMA-EGDM, GMA-HEMA-DVB and GMA-MMA-EGDM were investigated for immobilisation of penicillin G acylase. The microenvironment in the vicinity of oxirane was varied. Increase in the hydrophilic character in the microenvironment of the support by incorporating hydroxyethyl methacrylate as well as by modification with polyethyleneimine, decreased the binding efficiency and expression of the bound penicillin G acylase. Modification of oxirane groups to a lowest extent (3.13 percent) with polyethyleneimine causes significant drop in binding efficiency. The hydrophilic microenvironment generated by modification with polyethyleneimine, hinders interaction between penicillin G acylase and support. Interactions between bound penicillin G acylase and penicillin G molecules are also hindered during catalysis. This leads to a remarkable drop in the expression of bound penicillin G acylase. The binding efficiency was found to decrease less dramatically with divinyl benzene containing terpolymers. This is due to the more hydrophobic nature of these polymers. However, expression of the bound enzyme was drastically reduced.

RECOMMENDATIONS

A number of systems have been investigated hitherto by various research groups all over the world. An unambiguous solution to many aspects of matrix polymerisation are not available. These revolve around

* Kinetics and mechanistic aspects
* Structural features of daughter polymer. This includes molecular weight and molecular weight distribution and microstructure of daughter polymer.

The research should be directed at an understanding of the elementary processes underlying the matrix polymerisations. A differential scanning calorimetric (DSC) investigation will be of immense importance. Methods ought to be developed to quantitatively separate daughter polymer from matrix (parent polymer) without any decomposition of the former. Since a lot of information about the

matrix polymerisation can be obtained from the mode and degree of preassociation of monomer with the matrix, the adsorption constant of monomer, K_m should be quantified. This would give (particularly in type I systems where K_m is very large) more or less exact information regarding the local monomer concentration and rate enhancements due to monomer ordering can be accurately estimated.

Information about the structural features (i.e. molecular weight and molecular weight distribution and topochemistry) of the daughter polymer play a vital role in ascribing the matrix effect. Methodology should be developed to permit characterisation of the daughter polymer either directly or indirectly.

The exact concentration of free radical species should be estimated by using electron spin resonance (ESR) spectroscopy to obtain quantitative estimates of the mechanistic course of matrix polymerisation initiated by free radicals. This would allow the estimation of propagation rate constant (k_p) accurately. In cases where the matrix effect is a function of chain length of parent polymer, it will be beneficial to employ matrices having narrow molecular weight distribution.

It is highly intriguing to investigate the matrix polymerisation of concentrated matrix systems by using photoinitiation. Lastly it will be worth while to use copolymers (instead of homopolymers) as well as crosslinked polymers as matrices and to study their matrix effect.

ENZYME IMMOBILISATION:

There is tremendous scope in future for immobilised enzymes. Many techniques for enzyme immobilisation are becoming well developed and are now generally available to researchers. However, future research trend should be directed at a more effective utilisation of existing techniques. Immobilised enzyme should be applied for performing the task of a variety chemical transformations including complex sequential chemical reactions for organic synthesis. Production of specialised chemicals of pharmaceutical importance, using immobilised enzymes will continue to represent an important aspect in future. A number of enzymes require coenzyme, such as NAD^+

or $NADP^+$, ATP, FAD or coenzyme A, for their catalytic activity, are very expensive. Techniques should be developed to immobilise enzymes together with their coenzymes and to generate them after the reaction is over.

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PATENTS

1. An Improved Process for the Production of Immobilized Penicillin G Acylase Using Crosslinked Spherical Macroporous Hydroxyethyl Methacrylate Terpolymers Useful for the Preparation of 6-Amino Penicillanic Acid.
   Indian Patent No. 186/90.

2. A Process for the Preparation of Crosslinked Spherical Macroporous Hydroxyethyl Methacrylate Terpolymers Beads of Controlled Pore Size Distribution for Enzyme Immobilization.
   Indian Patent No. 185/90.