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Reactions in a Protein-Polymeric Carrier System Used for the Immobilisation of Enzymes

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The review is devoted to methods for the formation of a covalent bond between a polymeric carrier and enzymes whereby the latter are immobilised.
The bibliography includes 474 references.

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I. INTRODUCTION

Enzymes immobilised in various ways in order to make them water insoluble have been attracting increasing attention recently. The preparations thus obtained (immobilised or stabilised enzymes) are used in chemical engineering, medicine, and analytical chemistry.

Numerous reviews and a series of monographs devoted to the immobilisation techniques and the characteristics of the employment of such artificial catalysts have been published in recent years in both foreign and Soviet literature[1-14]. A special review deals with kinetic-thermodynamic aspects of catalysis by immobilised enzymes[15]. Problems of the immobilisation of enzymes have been considered in a fundamental publication by a team of workers from Moscow University[16]. There is no doubt that the greatest number of studies on immobilisation methods have been devoted to methods involving the formation of a covalent bond between the enzyme and the carrier, particularly a polymeric carrier. The number of publications in this field is continuously increasing. Nevertheless, we believe that the extensive information about this important field of reactions between proteins and synthetic or artificial polymers has been inadequately dealti with in the review literature.

The immobilisation of enzymes by their attachment via a covalent bond to a polymeric carrier is based on the interaction of the reactive groups in the macromolecules of the polymeric carrier and the protein. In principle, the following functional groups forming part of protein aminoacids can react[4,16,17]: carboxy-groups (terminal groups and the groups in glutamic and aspartic acids), amino-groups (terminal groups and the groups of lysine and hydroxylysine), phenolic groups (in tyrosine), imidazole groups (in histidine), indole groups (into tryptophan), sulphydryl groups (in cysteine), disulphide groups (in cystine), hydroxy-groups (in serine, hydroxyproline, hydroxylysine, and hydroxyglutamic acid), guanidino-groups (in arginine), methylthio-groups (in methionine), phenyl groups (in tyrosine and phenylalanine), amido-groups (in asparagine and glutamine), and in certain cases peptide groups. In many cases it is difficult to determine with which protein group the carrier group reacts. For example, the acylating groups of the carrier can react both with amino- and with hydroxy- and mercapto-groups of the enzyme. The steric disposition of the reacting molecules plays an important role in such reactions.

A number of conditions must be observed in the immobilisation process. In particular, the reaction must proceed under conditions which rule out the denaturation of the protein. Furthermore, the functional groups of the carrier must not react with the groups of the protein responsible for enzyme activity[5].

Unfortunately the mechanisms of the reactions used to bind enzymes to polymeric carriers are obscure in many instances. This hinders significantly the classification of the known processes. However, among these one encounters both substitution and addition reactions involving aliphatic and aromatic carbon atoms as well as reactions at heteroatoms which proceed mainly via heterolytic mechanisms. Radical processes are used much more rarely.

II. ACYLATION REACTIONS

Amino-, hydroxy-, mercapto-, and guanidino-groups of enzymes can be involved in acylation reactions under the influence of the carrier. Although amino-groups are the most reactive, the mode of reaction depends significantly on many factors, particularly steric factors, the pH of the medium, etc.[4]

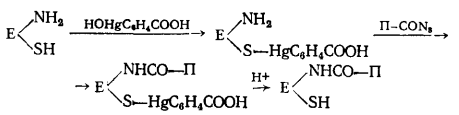
1. Acylation by Polymeric Azides

One of the first studies on the covalent binding of proteins to synthetic or artificial polymers was that of Micheel and Evers in 1949,[18] who used for this purpose carboxymethylcellulose activated by conversion into the azide[18]. In essence, this method is a modification of the familiar azide method for the synthesis of peptides proposed by Curtius in 1902.[19]

In this case the chemical reactions can be represented schematically as follows†:

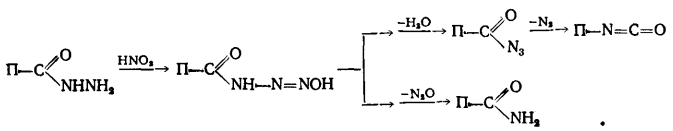
$$\Pi\text{—}OCH_2COOH \rightarrow \Pi\text{—}OCH_2COOC_2H_5 \rightarrow \Pi\text{—}OCH_2CONHNH_2 \rightarrow$$
$$\rightarrow \Pi\text{—}OCH_2CON_3 \xrightarrow{H_2N\text{—}E} \Pi\text{—}OCH_2CONH\text{—}E \quad .$$

The stage involving the interaction of the azide with the functional groups of the protein takes place at 0–5 °C in the course of 1–2 h in a weakly alkaline medium. Under these conditions, azides react preferentially with amino-groups; however, they can also react with hydroxy- and mercapto-groups. Since the latter are frequently involved in the active centre of the enzyme, an attempt is made to avoid the formation of thioesters in such cases. For this purpose, mercapto-groups, for example, are temporarily protected by the introduction of an organomercury residue by reaction with *p*-hydroxymercuribenzoic acid. After the reaction with the carrier has been carried out, the protecting group can be removed in a weakly acid medium (10^{-6} M HCl):[4,20,21]



The azide method has been used fairly widely[20-32,34-69]; it has been examined more or less in detail in a number of reviews[1-8,16,69].

In the general case the immobilisation process is significantly complicated in view of the possible occurrence of a number of side reactions involving the azide group and its conversion products. In particular, when HNO_2 and hydrazides react, inactive amide and active isocyanato-groups can be formed together with azido-groups:



The isocyanato-groups can react in their turn with various groups of the protein (preferentially amino-groups), but after hydrolysis to amino-groups they can also give rise to urea groups in the carrier itself. All these factors require a rigorous observation of optimum reaction conditions (specified temperature, pH, etc.) (see Berezin et al.[16], p.159). The low stability of the azido-groups, which precludes the retention of activity by the carriers for a long time, also creates certain disadvantages. In recent years the azide method has been used fairly rarely.

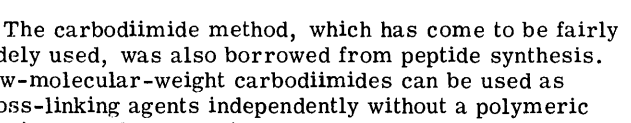
Carboxymethyl derivatives of polysaccharides, for example, cellulose and dextran, are usually employed as the carboxyl-containing polymers[33,50,60,63,69,165]. Using the azide method, it is possible to employ also polymers without carboxy-groups as carriers. Thus hydrazido-groups have been introduced[42] by treating a cross-linked polyacrylamide with hydrazine:

$$\Pi\text{—}CONH_2 \xrightarrow{H_2NNH_2} \Pi\text{—}CONHNH_2 \,.$$

A number of enzymes were immobilised on the resulting polyhydrazide (under the commercial name "Enzacryl AH") after its conversion into the azide[42,53-59]. The use of polyacrylic acid azide, which is soluble in borate buffer, for the binding of enzymes has also been described[44]. Among other carriers employed, one may mention the hydrazide derivative of agarose[66], the azide of polyglutamic acid[67], the copolymer of maleic acid azide with acrylamide and methylenebisacrylamide[68], and a polymeric azide obtained from the graft copolymer of cellulose and methyl acrylate[80].

2. Acylation in the Presence of Carbodiimides

The carbodiimide method, which has come to be fairly widely used, was also borrowed from peptide synthesis. Low-molecular-weight carbodiimides can be used as cross-linking agents independently without a polymeric carried; at the same time, numberous studies have been devoted to the activation of the carboxy-groups of the polymeric carrier by carbodiimides with subsequent attachment of the protein molecule[70-79,81-96]:



Water-soluble carbodiimides[78,87-90], for example, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[88,95], 1-cyclohexyl-3-(*p*-diethylaminocyclohexyl)carbodiimide hydrochloride, and methyl toluene-*p*-sulphonate–1-cyclohexyl-3-(4-ethyl-2-morpholino)carbodiimide[90] (see also Berezin et al.[16], p.170) are sometimes used in this reaction instead of the most frequently employed dicyclohexylcarbodiimide.

Carboxymethylcellulose[63,70,72,79,82,83] and the acrylamide–acrylic acid copolymer[77,78,83,88,90-92] are most widely used as carboxyl-containing polymers. However, other polymers, for example, carboxy-methyl-Sephadex[82,83], Sepharose derivatives[87], polyamides[62], and the 2-hydroxyethyl acrylate–methacrylic acid copolymer[86] can also be used. α-Amylase has been attached to a carboxyl-containing carrier with the aid of carbodiimides[96]. A polypropylene fabric to which polyacrylic acid had been grafted and partially hydrolysed poly(ethylene terephthalate film) were used as carriers.

Other carriers were unsubstituted cellulose[4], copolymers of *N*-vinylpyrrolidinone and unsaturated acids[93], CH-Sepharose 4B, and AH-Sepharose 4B, containing the $-OCONH(CH_2)_6COOH$ and $-OCONH(CH_2)_6NH_2$ groups (Berezin et al.[16], p.172).

Valentova[89] used particles of hydroxyalkyl methacrylate gels (under the commercial name "Spheron") for the immobilisation of pepsin by this procedure. The gel particles were activated with bromide and then hexamethylenediamine or aminocaproic acid were grafted to it, which results in polymers containing carboxy- and amino-groups. The immobilisation was carried out in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Not only the carboxy-groups of the carriers but also, in principle, those of the enzyme can be activated by carbodiimide:

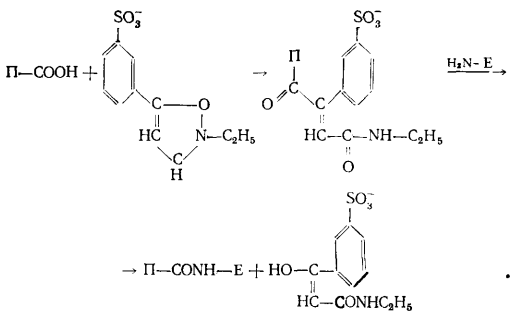
$$E\text{—}COOH + H_2N\text{—}\Pi \xrightarrow{R\text{—}N{=}C{=}N\text{—}R} E\text{—}CONH\text{—}\Pi + R\text{—}NH\text{—}CO\text{—}NH\text{—}R \quad .$$

In this case the activated enzyme adds to the amino-polymer, for example, aminoethylcellulose[6]. Carboxyl-containing enzymes have been bound with the aid of dicyclohexylcarbodiimide to the product of the interaction of the ethylene–maleic anhydride copolymer with hexamethylenediamine[94].

† Here and henceforth π is the residue of the polymer chain of the carrier and E is the residue of the protein chain of the enzyme.

3. Acylation in the Presence of Woodward's Reagent

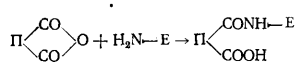
In 1961 Woodward et al.[97] described the use in peptide synthesis of activated esters obtained on the basis of *N*-ethyl-5-phenylisoxazolium 3′-sulphonate. Later this reaction was used[98,99] to obtained insoluble preparations with enzymatic activity; this method was also used in a number of other studies[41,100,101]:



Carboxy-containing polymers [carboxymethylcellulose[98] and the copolymer of L-alanine and L-glutamic acid (berezin et al.[16], p.173)] have also been activated by Woodward's reagent. The advantage of this process is that it proceeds under fairly mild conditions[4]. Furthermore, in this case it is easy to regulate the number of active groups introduced into the carrier[5]. This method can supplement the carbodiimide method just discussed, since in the former the reaction proceeds in an alkaline medium and in the latter in an acid medium[4].

4. Acylation by Polymeric Anhydrides

The ability of polymeric anhydrides to interact in the cold with the protein molecules of enzymes (primarily with formation of amide groups) was first used probably in 1964[102,103] Subsequently this method gained wide currency[21,45,100,102-104,106-145]:



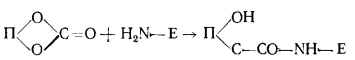
Mainly copolymers of maleic anhydride (see, for example, Refs. 46, 102, 103, 132, 134, and 139) were used. However, studies have been made on the use of copolymers of methacrylic acid anhydride[125,130] and also the anhydrides of itaconic and citraconic acids[133] and the homopolymer of the anhydride of methacrylic acid[208]. Ethylene[102,103,139], the divinyl ether of butanediol[45,131], ethyl vinyl ether and divinyl ether[133,134], methyl vinyl ether[136,144], and alkylene diacrylates[138] have also been used as comonomers with maleic anhydride.

Krömer and coworkers obtained and used for the immobilisation of enzymes a copolymer containing 32 mole % of methacrylic acid anhydride, 67 mole % of acrylamide, and 0.5–1.0 mole % of methylenebisacrylamide[125,130,143]. A homopolymer of maleic anhydride[142] and a number of other anhydride-containing polymers[145] have also been employed for immobilisation. In particular the anhydride groups intended for reaction with the protein molecule were obtained by heating to 180°C a copolymer of maleic acid, acrylamide, and methylenebisacrylamide[68].

The immobilisation on amino-carriers of the soluble products of the interaction of enzymes with a copolymer of ethylene and maleic anhydride has been described[137,140]. The influence of the number of anhydride groups in the carrier molecules on the activity of the enzyme has been investigated[141]. The copolymers of the anhydrides of unsaturated carboxylic acids can be used both in the form of beads and as films or coatings[132,136].

5. Acylation with Cyclic Carbonates of Polymeric Alcohols

This method was proposed by Kennedy and coworkers[146-154] and consists in the interaction of enzymes with polymers (mainly polysaccharides) containing *trans*-3,4-cyclic carbonate groups, which are introduced into the polymer by the reaction of the polymeric alcohol with ethyl chloroformate. The reaction with the protein proceeds via the following mechanism[154] at ~4°C in a citrate buffer solution at pH 5.4:

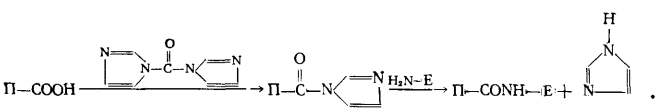


Later studies were carried out in which other polymeric alcohols[155], particularly polyallyl alcohol[153], were used for reactions of this kind.

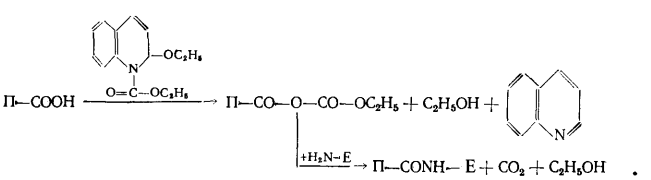
6. Other Acylation Methods

A number of other methods for immobilisation using acylation reactions, mainly reactions involving the formation of amide groups from the amino-groups of the protein molecule, have been developed in recent years.

Thus the copolymer of styrene and 4-vinylbenzoic acid has been used as a carrier[156-158], the reaction with the enzymes being carried out in the presence of carbonyldiimidazole in dimethylformamide:



The same workers suggested that the reaction of the carrier (the copolymer of 4-methacryloyloxybenzoic acid and divinylbenzene) with the amino-groups of enzymes be carried out in the presence of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline introduced into the reaction as an activator[159,160]. The reaction evidently proceeds via a stage involving the formation of a mixed anhydride:

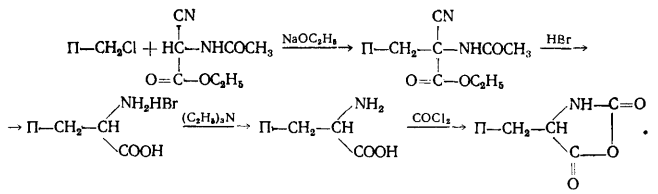


It has also been used by Sundaram[161], who used carboxymethyl-cellulose and carboxymethyldextran (Sephadex) as carriers.

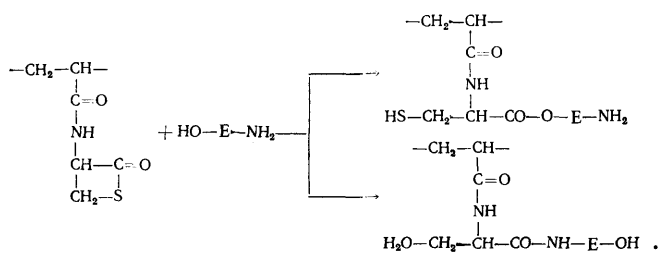
Polymeric acylating carriers containing acyl chloride groups have been described[162,164-166].

A polymeric cyclic *N*-carboxyanhydride, containing the 2,5-dioxa-4-oxaquinazolinedione group, has been used to bind a number of enzymes at 0°C in the course of 20 to 72 h.[167] This polymer was obtained from a chloromethylated polystyrene copolymer via the following

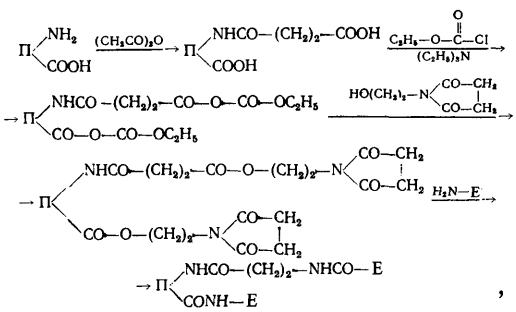
mechanism:



Derivatives of polyacrylamide or its copolymers containing thiolactone groups, manufactured under the commercial name "Enzacryl polythiolactone" can serve as acylating polymeric compounds [168,169]. This polymer can react with both amino- and hydroxy-groups of enzymes:



The use of polymeric carriers containing activated ester groups, for example, the copolymer of acrylamide and *p*-nitrophenyl acrylate [170], substituted cellulose [Cel–$OCH_2CH_2NHCO(CH_2)_2COOC_6H_4SO_2CH_3$] (see Berezin et al.[16], p. 167), polystyrene containing the $-CO(CH_2)_2-CO-O-C_6H_4NO_2$ group [171], and a partially hydrolysed polyamide in which the activated ester group with the alcoholic *N*-hydroxyethylsuccinimide residue was introduced by the procedure [172]

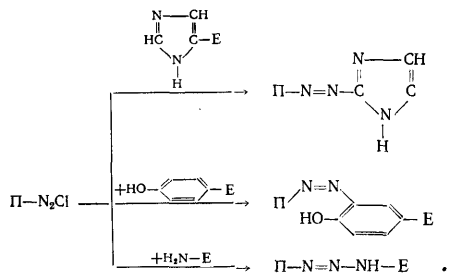


for the immobilisation of enzymes has been described.

III. THE AZO-COUPLING REACTION

The azo-coupling reaction has long attracted the attention of investigators in the field of the immobilisation of enzymes in view of its mild conditions and the ease with which the diazotisable aromatic amino-groups can be introduced into the carrier [173]. Later this reaction was employed in many investigations [26,34,38,42,70,102,104,109,112,163,169,174–202,204–207,209,211–243].

For example the following functional groups of the protein can be involved in the azo-coupling reaction:

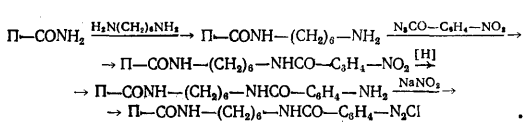


In addition, guanidino- and mercapto-groups can react with diazo-compounds. Under these conditions, two-diazo-groups can react with a single protein group [4].

The most frequently employed carriers are *p*-aminobenzylcellulose (see, for example, Refs. 26, 173, 174, 187, 188, and 235), *p*-aminostyrene (for example, Refs. 174–176, 199, 214, and 218), and copolypeptides based on *p*-aminophenylalanine (for example, Refs. 107, 177, 178, 184, 201, and 223). It has also been suggested that the product of the interaction of dialdehyde-starch and 4,4′-diaminodiphenylmethane be used as a diazotisable carrier [139,192]. On reacting with the aldehyde groups of dialdehyde-starch, the diamine forms a three-dimensional network. Among other carriers, we may mention the copolymer of glutaraldehyde and *m*-phenylenediamine [233], the reduced graft copolymers of *p*-nitrophenyl acrylate with poly(vinyl chloride), polypropylene, and polyethylene, and the styrene–propene copolymer [236], *p*-aminobenzamidohexyl Sepharose [237–240], polysaccharide anthranilates [221,226,227], aromatic amino-derivatives of polyacrylamide [169,209,210,219,220,225], the products of the interaction of a partially hydrolysed polyamide, aromatic diamines, diisonitriles, and aldehydes [234], aromatic amino-derivatives of wool, silk, chitin, and phenolic resins [241], and polyvinyl-(3-amino)benzylidene [242].

In order to obtain diazotisable carriers, the epoxy-resin based on di(hydroxyphenyl)propane and epichlorohydrin was treated with *p*-nitrophenylsulphonyl isocyanate with subsequent reduction of the nitro-group [243].

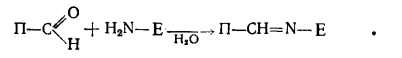
A multistage method has been proposed for the synthesis of diazo-derivatives of acrylamide [42]:



Diazotised aromatic diamines (benzidine-2,2′-sulphonic acid and *m*-phenylenediamine) have been used for the cross-linking of enzymes and polymeric carriers [107,197,205,211,224,228,232]. In order to increase the number of groups capable of undergoing the coupling reaction in the protein, the latter was treated with L-tyrosine *N*-carboxyanhydride [183]. The azo-coupling reaction has been used to bind not only enzymes but also other types of proteins, for example, antibodies, to the surface of the carrier [244].

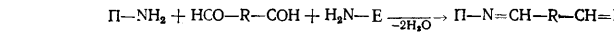
IV. THE FORMATION OF AZOMETHINE DERIVATIVES

Aldehydes react with the amino-groups of proteins under mild conditions with formation of Schiff bases:

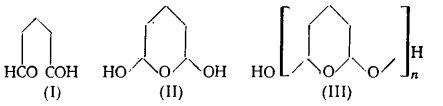


The characteristics of this reaction have been examined in detail in a review[105]. In recent years the reaction has served as the basis for one of the most vigorously developed procedures for the immobilisation of enzymes[21,88,94,171,245,247-253,255-298].

It is possible to isolate two trends in studies in this field—the use of polymers containing aldehyde groups (as shown in the above mechanism) and the use of low molecular weight aldehydes mainly glutaraldehyde) in order to bind enzymes to amino-carriers:



The glutaraldehyde used in the immobilisation reaction is in fact a fairly complex mixture of compounds. In particular, its aqueous solutions contain, apart from the aldehyde proper (I), also a low-molecular-weight cyclic acetal (II) and its oligomers (III). It has been stated (see Berezin[16], p. 208) that the mixture contains also derivatives of glutaraldehyde in which the α-position relative to the aldehyde group contains a double bond capable of involvement in an addition reaction with the amino-group[105]:



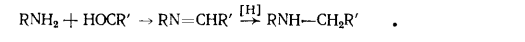
Among polymeric amino-carriers, aminoethylcellulose is most frequently used in combination with glutaraldehyde[21,245-247,250,257,277,287,290]. Among other amino-polymers, mention may be made of aminopolystyrene[21,171], partially hydrolysed polyamides[248,259-262,288,289], styrene–divinylbenzene copolymers modified by diamines[297], the maleic anhydride–ethylene copolymer treated with hexamethylenediamine[94], the products of the interaction of poly(methyl methacrylate) and 3-aminopropyltriethoxysilane[292], and polymers containing in the side chain lysine residues attached to the inner surface of a nylon tube activated with dimethyl sulphate[293]. Phenol-formaldehyde resins[241,265-267,275], gelatin[291], polyacrylamide[88,253], and certain other polymers[271,278] are also used for immobilisation in conjunction with glutaraldehyde.

A considerable number of studies have been devoted in recent years to the synthesis of polymers containing aldehyde groups in side chains and to their use as carriers for the immobilisation of enzymes. For these purposes, a number of acrolein derivatives were employed: the adduct of polyacrolein and sodium bisulphite[269,279,283], the acrolein–styrene copolymer[263], and the acrolein–acrylic acid copolymer[274]. The products of the partial oxidation of polysaccharides, accompanied by ring opening, particularly dialdehyde-cellulose[258,264,270,276], dialdehyde-Epidex[210,429], dialdehyde-dextran[280], ethoxycarbonyl-cellulose[249], the hydrolysis products of polymeric acetals[255,256,282,294], oxidised phenol-formaldehyde resins[256,241], polymeric derivatives of pyridoxal phosphate[239,240,295], polystyrene formylated by 1,1-dichloromethyl ether[171], copolymers of unsaturated aldehydes and vinylpyrrolidinone[93], and a polyacroleine reaction product[254] have also been made to react with enzymes.

The polymer of methacryloylvanillin {poly[4-formyl-2-methoxyphenyl methacrylate]} and its copolymers with allyl alcohols (p moles of allyl alcohol per mole of the aldehyde), known under the commercial names of Vanacryl and Covanacryl-p,[272,273,276,298] merit attention. Enzyme preparations immobilised on such carriers show a decrease of activity on storage in an aqueous medium, which is probably associated with the dissolution of part of the enzyme as a result of the hydrolysis of the ester group in the methacrylic acid residues[272,284].

Mention should also be made of a study[286] in which a method was proposed for the immobilisation of glucose oxidase on polyaminostyrene after preliminary oxidation (to give a 60% content of carbohydrate residues) of the enzyme with formation of aldehyde groups.

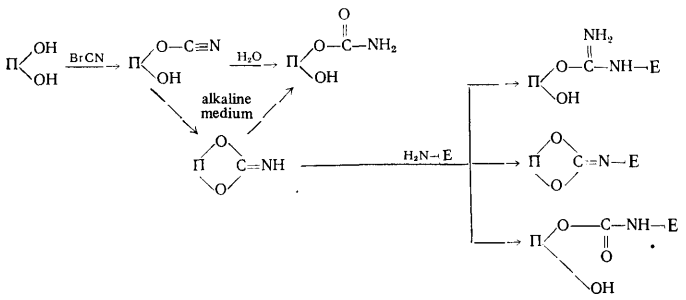
One should note that azomethine groups are insufficiently stable in acid media. In order to increase the stability of the polymer–protein system, it has been suggested that the azomethine groups be reduced with sodium tetrahydroborate[105,280,296]:



V. THE USE OF POLYIMIDOCARBONATES

The reaction of the amino-groups of proteins with polyimidocarbonates is widely used for the immobilisation of enzymes. These compounds are usually obtained by the reaction of polymeric alcohols with cyanogen halides (mainly with cyanogen bromide)[41,63,77,80,219,247,299-370]. This method has been proposed in two studies[299,300] and is attractive because of the high rate of reaction, the relatively high stability of the activated carrier, and its high reactivity in relation to the amino-groups of the enzyme.

The reaction proceeds via the following mechanism[5,335]:



The polyimidocarbonate, formed as the main product in the activation of a polyhydric alcohol, can give rise to an inactive unsubstituted carbamide in an alkaline medium. The carbamate can also be formed from the intermediate cyanate under the influence of water.

The reaction of imidocarbonates with the amino-groups of proteins can proceed via several pathways, which give rise to isoureas and N-substituted carbamates (urethanes). The formation of an interchain imidocarbonate, which can also interact with the amino-groups of the enzyme, is likewise very probable (see Berezin et al.[16], p. 176).

In the vast majority of studies polysaccharides are employed as the polyhydric alcohols: agarose (Sepharose)[77,88,238-240,290,294,296,307,308,316,317,321-323,330,339-343,347-353,354-367], dextran (Sephadex)[302,315-318,320-323,328,331,338,341,345,347,367-369], and (more rarely) starch[317] and cellulose[41,306,344,346]. Among other natural polymers, alginic acid has been used[370]; in this case the polymer–enzyme system is

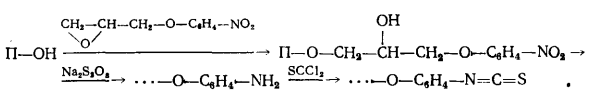
soluble at pH > 4, but is precipitated from solution at pH < 3. Synthetic polymers are used for these purposes much more rarely. In particular, the activation of the acrylamide–hydroxyethyl methacrylate copolymer with cyanogen bromide has been described [90]. Cyanogen chloride is much more rarely used for the activation of polymers in place of cyanogen bromide [371]. It has been stated that the imidocarbonate groups incorporated in the side chains of the polymer can be obtained by the interaction with polysaccharides with organic cyanates (see Berezin et al.[16], pp. 80 and 181).

Apart from enzymes, antibodies [372] and coenzymes [373] are also attached to carriers by the cyanogen bromide method.

VI. THE USE OF POLYMERIC CARRIERS CONTAINING ISOCYANATO- AND ISOTHIOCYANATO-GROUPS

Polymers containing these highly reactive groups have been used successfully for the immobilisation of enzymes despite their relatively high reactivity in hydrolysis [56,166,204,209,242,374–387]. Although, in principle, the isocyanato-groups can interact with many groups in the protein molecule (carboxy-groups—with formation of amides, hydroxy- and phenolic groups—with formation of urethanes, amide groups—with formation of acylureas, etc.), at the moderate temperatures used in the interaction with the protein, the amino-group is most reactive, giving rise to urea and thiourea groups on interaction with isocyanates and isothiocyanates.

Both artificial and synthetic polymeric carriers have been used for immobilisation. In particular, the following scheme has been proposed for the preparation of polysaccharides containing isothiocyanato-groups [246,378]:



Among the synthetic polymers used for immobilisation, mentioned may be made of the copolymers of methacrylic acid, *p*-aminostyrene, and 3,5-diisocyanatostyrene [380], the copolymers of methacrylic and acrylic acids and 3-isocyanatostyrene [379], and graft copolymers of polypropylene and *p*-nitrostyrene, whose amino-groups, generated by reduction, were treated with thiophosgene [204,385].

Synthetic polymers containing terminal isocyanato-groups in the main or side chains, such as polyureas based on hexamethylene diisocyanate and tolylene diisocyanate or hexamethylenediamine and triethylenediamine [166], polyethers with terminal isocyanato-groups [381], polyurethanes based on poly(propylene glycol) and tolylene diisocyanate [386,387], and products obtained by treating poly(allyl alcohol) with 4-isothiocyanatophenyl isocyanate [242] have also been used for immobilisation.

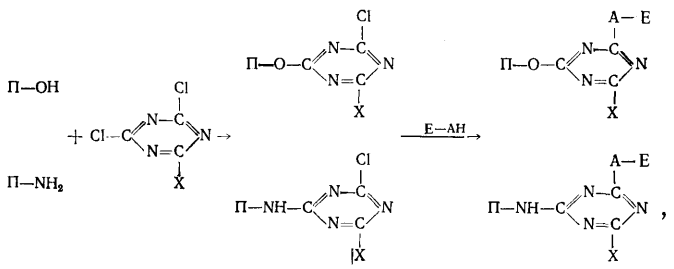
It has been suggested that the enzyme (trypsin) be treated initially with tolylene diisocyanate and that the resulting polyisocyanate be then cross-linked with poly(propylene glycol) [387].

VII. THE USE OF POLYMERS WITH AN ACTIVE HALOGEN ATOM

1. Polymers Containing Chlorotriazine Groups

In connection with the widespread use of carriers containing aldehyde and imidocarbonate groups, there has been some decrease in interest in recent years in polymers containing an active chlorine atom incorporated in a triazine group. However, studies in which polymers containing these groups are used for the immobilisation of enzymes are still being continued [63,163,195,206,388–412].

In this method the activated carrier is formed by the reaction of a polymer containing a group containing an active hydrogen atom (a hydroxy- or amino-group) with cyanuric chloride or a monosubstituted dichlorotriazine. The remaining unsubstituted chlorine atoms (atom) form hydrogen chloride by reaction with the protein molecule:



where X = Cl or R, A is the residue of a hydroxy-, amino-, guanidino-, phenolic, or SH group of the enzyme molecule.

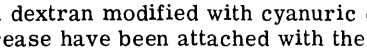
Among the polymers containing active atoms, polysaccharides (for example, Refs. 392, 398, and 410) or their aminoethyl derivatives (for example, Refs. 290, 308, and 399) have been most widely used. However, synthetic polymers, such as phenol-formaldehyde resins [409] and aminated polystyrene [171] have also been employed. Apart from cyanuric chloride, 2-amino-4,6-dichloro-*s*-triazine [63,290], 2-carboxymethylamino-4,6-dichloro-*s*-triazine [63], and Procion dyes [308] have been used to active these polymers.

Water-soluble derivatives of proteases have been obtained using a dextran modified with cyanuric chloride [93]. Arginase and urease have been attached with the aid of cyanuric chloride to different sides of a cellulose membrane [412]. The modification of enzymes by low-molecular-weight dichlorotriazine dyes followed by interaction with aminoethylcellulose has been described [407].

2. Polymers Containing An Active Halogen Atom Attached to Aliphatic and Aromatic Carbon Atoms

Publications about such carriers have appeared in the last 5–6 years. Thus it has been suggested that 4-iodo-n-butyl methacrylate polymers be employed; the exchange of a halogen in the reactions of monomeric and polymeric 4-chloro-n-butyl methacrylates with sodium iodide has been used to prepare such polymers [413–419].

The interaction of carriers of this kind with enzymes proceeds preferentially via the following mechanism:



Evidently other groups alkylated under these conditions can also react.

The copolymers of methacrylic acid and methacrylic acid 3-fluoro-4,6-dinitroanilide as well as the copolymers of these monomers with divinylbenzene [374-376,420-424] and the polymers and copolymers of fluorostyrenes and their derivatives [376,423] react similarly with enzymes.

Among other polymers containing an active halogen atom attached to aliphatic or aromatic carbon atoms used for the immobilisation of enzymes, we may mention bromo- and iodo-acetylcellulose [43,425,426], bromoacetamidohexyl-Sepharose [238-240,296], the products of the acylation of poly-(vinyl alcohol) with bromoacetyl bromide or bromoacetic anhydride [427], polyacrylamide derivatives containing iodoacetic acid residues [428], and 3-chloro-2-hydroxy-n-propyl-Epidex [429].

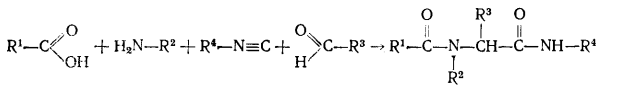
3. Polymers Containing Activated Halogen Atoms Forming Part of Inorganic Groups

Polymeric carriers containing activated halogen atoms in inorganic groups, mainly in inorganic acid residues, including acids derived from transition metals, are sometimes used. In particular, it has been suggested that synthetic polymers and polysaccharides be activated with thionyl chloride or sulphuryl chloride before interaction with enzymes [371,430,431]. A number of transition metal halides ($TiCl_4$, $SnCl_4$, VCl_3, $FeCl_2$, $FeCl_3$, and $ZrCl_4$), have also been used as activating agents. Both synthetic (polyamides) [432] and natural (cellulose [432-434] and chitin [435]) polymers have been treated with these compounds.

VIII. OTHER METHODS

1. The Use of Ugi's Method

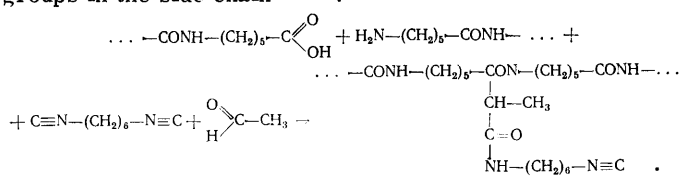
Ugi [436] showed that aldehydes (ketones), isonitriles, and carboxy- and amino-groups can interact:



This reaction has been used to bind a number of enzymes to polymeric carriers [437-443]. For example, Sepharose has been oxidised with formation of a polymer containing aldehyde groups. In the presence of 3-dimethylamino-propylisonitrile this polymer binds chymotrypsin at pH 6.5 in the course of 6 h. [438,439]

Carboxymethyl-Sephadex, aminoethylated polyacryl-amide [438], and a polyamine obtained by the interaction of diaminodiphenylmethane with Sepharose activated by cyanogen bromide [437] can be involved analogously in this reaction. The interaction of the last polymer with pepsin was carried out in the presence of phenylisonitrile and acetaldehyde.

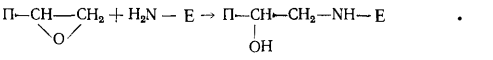
Another pathway leading to immobilisation involves the initial preparation of a carrier containing a sufficient number of carboxy- and amino-groups. A partly hydrol-ysed polycarproamide has been used for this purpose. The reaction of this polymer with hexamethylenediisonitrile and acetaldehyde yields a polyamide containing isonitrile groups in the side chain [442,443]:



Finally, the reaction of this carrier with trypsin in the presence of acetaldehyde results in the fixation of the enzyme.

2. The Reaction of Enzymes with Polymers Containing Epoxy-groups

Publications describing the immobilisation of enzymes and polymeric carriers containing epoxy-groups have begun to appear recently [444-449]:

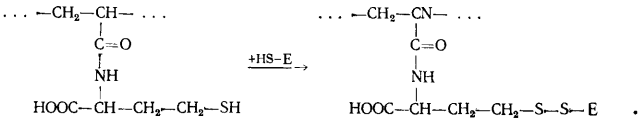


Thus the copolymers of epoxy-monomers (glycidyl meth-acrylate, glycidyl acrylate, and unsaturated glycidyl ethers) with other monomers, for example acrylonitrile and methacrylonitrile, have been used for this purpose [445,446]. The activity of the immobilised enzyme was higher when the carrier had been subjected to preliminary activation with an amine [444].

Urease was immobilised on a graft copolymer of carboxymethylcellulose and glycidyl methacrylate [447-449]. A method for the immobilisation of peroxidase, consisting in the copolymerisation with acrylamide of the enzyme subjected to a preliminary treatment with glycidyl meth-acrylate, is based on the ability of the amino-groups of proteins to react with epoxy-compounds [450].

3. The Thiol–Disulphide Exchange Reaction

The "Enzacryl polythiolactone" mentioned above can be attached to the mercapto-groups of enzymes when it is introduced into the reaction in the form of a mercapto-acid [169]:

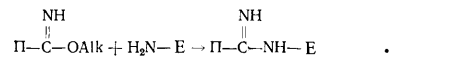


In order to increase the content of mercapto-groups in the initial enzyme, the latter can be subjected to a pre-liminary treatment with *N*-halogenoacetylcysteine thio-lactone [451] (see also Berezin et al. [16], p. 200).

Carlsson et al. [452] proposed another method for the immobilisation of enzymes (amylase and chymotrypsin) with the aid of the thiol–disulphide exchange reaction. The method includes two stages. In the first stage the NH_2 groups of the enzymes were modified with the aid of methyl 3-mercaptopropioimidate, which resulted in the introduction of a mercapto-group into the protein. In the second stage the enzyme with the mercapto-group was bound to disulphide derivatives of agarose obtained by treating the mercaptopropyl ether of the latter with 2,2′-dipyridyl disulphide.

4. Reactions with Polymeric Imidoesters

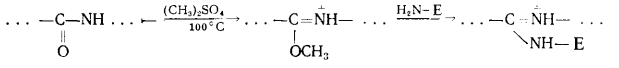
Polymers containing imidoester groups can interact with the amino-groups of proteins:



Thus Zaborsky introduced imidoester groups into a polyacrylonitrile powder by treating the latter with hydrogen chloride in methanol; this polymer was used as a carrier[453,454] and was also employed in a later study[456].

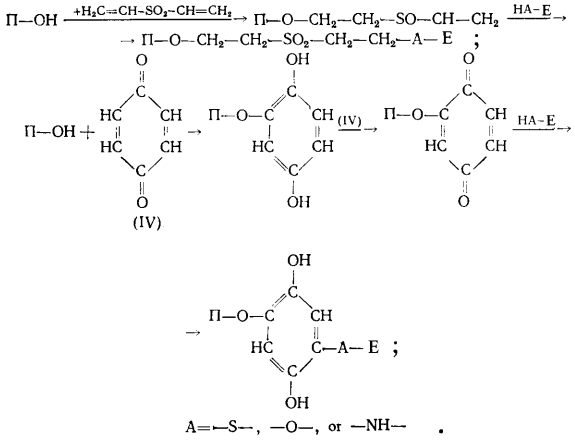
Imidoesters have been used also as cross-linking agents. In particular, a number of studies have been devoted to the interaction of enzymes and amino-polymers (aminoethylcellulose[455] and the products of the reaction of polyamides, activated by dimethyl sulphate, with lysine[293]) in the presence of diethyl adipimidate.

Yet another method for immobilisation (of glucose oxidase) using polymeric imidoesters has been proposed, namely on a polyamide treated with dimethyl sulphate[293]:



5. The Use of Carriers With an Activated Double Bond

Hydroxylated polymers can be activated by divinyl sulphone (Mosbach[460], p. 34) or quinone (Mosbach[460], p.35, and Tarasova et al.[461]). Such carriers can interact with the amino-, hydroxy-, or mercapto-groups of enzymes. The relevant processes can be represented schematically as follows:



A=—S—, —O—, or —NH— .

6. Radical Reactions

Radical procesees also play a definite role in the immobilisation of enzymes. This applies in the first place to the familiar processes involving the incorporation of an enzyme in a gel in the course of the polymerisation of unsaturated monomers (for example, acrylamide or its mixtures with methyl bisacrylamide, etc.), which is frequently accompanied by the formation of a covalent bond between the molecules of the enzyme and the gel-forming polymer as a result of a chain transfer reaction followed by grafting (see Berezin et al.[16], p.144). Cases of radical reactions in other systems are also known: for example, chymotrypsin is immobilised using the free radicals formed in aqueous solution as a result of ^{60}Co γ-irradiation[457]. Instances of the polymerisation of unsaturated derivatives of enzymes should also be classified as radical processes[458,459].

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This review shows that many processes have now been developed whereby enzymes can be linked by covalent bonds to polymeric carriers and that the possibility of introducing various functional groups into the latter is widely used. Naturally, in the course of the development of this field of enzyme immobilisation many methods have lost or are gradually losing their importance, giving way to more effective procedures (this can be seen from the analysis of the literature quoted). Polymeric carriers have been used in different forms—as grains, coatings, fibres, tubes, membranes, and gels.

Data, permitting the prediction to some extent of the efficiency of a particular carrier have now accumulated. Thus Porath formulated a series of properties, which the "ideal carrier" should possess: total insolubility in water; high chemical and biological stability; high mechanical stability; high reactivity with respect to the enzyme and ease of imparting such reactivity to the carrier; a form suitable for practical application; finally, highly hydrophilic properties of the carrier permitting its employment in aqueous media (see Berezin et al.[16], p.68). Some attention has been given in certain investigations to other properties of carriers, particularly their polarity (ionic and electrical effects[15,462]).

However, systematic studies have not so far been made of the influence of the nature of the carrier, particularly its chemical structure, geometrical structure, and molecular-weight distribution (for soluble forms) both on the characteristics of its binding to the protein and on the efficiency of the resulting biocatalysts. In the vast majority of studies quoted in this review very moderate attention has been devoted to these problems. According to the data quoted in many of the investigations, it is impossible to reach conclusions about the number of functional groups in the carrier responsible for the reaction with the protein, about the degree of their reaction in the course of binding to the enzyme, and about the influence of the conditions used in the preparation of the polymer–enzyme system on its catalytic activity.

As an example of a publication of this kind, one may quote the work of Baum[171], who compared the activities of immobilised amyloglucosidase and papain, obtained using polystyrenes containing different functional groups, and established that the carriers containing different groups (aldehyde, chlorotriazine, and activated ester groups) yield preparations with different activities. However, the author[171] does not state, for example, how many groups were present in each initial carrier nor is it stated what kind of carrier was used, particularly whether it was a linear homopolymer or a three-dimensional carrier obtained using a cross-linking agent. In our view, all these factors play a significant role in the preparation of effective biocatalysts. In this connection, one may refer to the known relation, with an extremum, between the content of functional groups in the initial carrier (ethylene–maleic anhydride copolymer) and the activity of the enzyme immobilised on this carrier[141].

Aspects of the influence of the chemical structure of the carrier on the properties of immobilised enzymes, which are already well known, such as the fact that the disposition of the active groups at the ends of side branches rather than in the main polymer chain is preferable[293], the negative influence of the enrichment of such branches

in aromatic groups (see Berezin et al.[16], p.188), the negative influence of the presence of hydrolytically labile groups at the carrier–enzyme binding sites[454,455], as well as the presence of groups promoting such hydrolysis (see Berezin et al.[16], p.166) have not been investigated in detail either.

All these findings show that detailed and systematic study of the influence of the properties of the carrier on those of the systems obtained can reveal considerable further possibilities for both an increase of the activity of the resulting preparations and for an increase of their efficiency. Studies in this field should probably be developed in the following directions: (1) investigation of the influence of the chemical structure and the physical properties of the carrier on the characteristics of the polymer–enzyme reaction; (2) investigation of the influence of the chemical structure and physical properties of the carrier on those of the immobilised enzyme obtained.

We believe that particular attention should be devoted to the following features of the chemical structure of the polymeric carrier: the content of functional groups and the degree of their utilisation in the reaction with the protein, the structure of the surrounding groups and sections of the polymer chain adjoining the carrier-enzyme binding site, and the structure and content of the groups responsible for the hydrophilic properties and polarity of the carrier.

In this connection mention should be made of the studies of Berezin and coworkers, who proposed a method using a carrier which is "complementary" to the surface of the enzyme globule and consists in the copolymerisation of a hydrophilic monomer (sodium methacrylate) and a cross-linking agent (methylene bismethacrylate) with an enzyme (chymotrypsin) modified by an analogue of the monomer (methacryloyl chloride). An increase of the thermal stability threshold of the enzyme by 40°C was achieved in this way relative to the native specimen[458,459].

It is quite possible that definite information about the characteristic features of the reaction between the carrier and the enzyme can be obtained from kinetic studies on the formation of the immobilised enzyme.

The study of the conformational changes in the enzyme occurring during its reaction with the carrier merits the closest attention. The importance of this factor was demonstrated by Martinek and coworkers, who showed that the activity of the enzyme (chymotrypsin) can change significantly as the extention of the nylon or viscose thread or the human hair to which it has been attached by covalent bonds is varied[285,288].

During the preparation of the review for the press, numerous publications in the field under consideration appeared in the literature, among which mention should be made of Refs. 463–474.

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