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Immobilised Enzymes

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Adsorption, covalent bonding, and occlusion into the structure of a gel can be used to attach enzymes to insoluble carriers in order to obtain heterogeneous enzymic catalysts. The kinetic characteristics of the enzymes are affected by immobilisation, but these changes—quite often more apparent than real—are due to changes not in the properties of the enzyme molecules themselves but in their microenvironment. Immobilised enzymes possess enhanced stability, and can be employed in technical processes, biochemical analyses, and medicine. A list of 506 references is included.

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I. INTRODUCTION

Artificial insoluble derivatives of enzymes are attracting increasing interest from chemists, biochemists, technologists, and medical men. These products—which, according to the recommendations of an international symposium[1], should be termed *immobilised enzymes*—have been recommended for use in technical processes, analytical chemistry, and medicine. Immobilised enzymes, especially in the form of membranes, may serve as models for subcellular structures. The prospects for the application of immobilised enzymes and certain convenient aspects for theoretical investigations have already been reflected in hundreds of papers and patents, which have been covered in dozens of reviews. Disregarding popular reviews and publicity, as well as relatively inaccessible publications, we can refer to several extremely serious and thorough surveys[2-20].

The immobilisation of enzymes forms part of the broader problem of the purposeful modification of biologically active substances. The present Review deals mainly with immobilised enzymes and insoluble complexes of enzymes with polymers. However, the immobilisation of enzyme inhibitors, antibodies, antigens, nucleic acids, hormones, and other biologically active substances is omitted, as well as the chemical modification of enzymes with simple substances. It must be emphasised here that the immobilisation of various biologically active substances capable of specific complex formation with biopolymers has become the foundation of an extremely effective technique for separating and purifying proteins and other biologically important substances by affinity chromatography (chromatography based on specific sorption). This method has developed especially rapidly during recent years, and has formed the subject of several detailed reviews[21-28].

The ability of enzymes to function when present in cell membranes has been known since the establishment of enzymology as a science. It may be supposed that most enzymes act in an insoluble form in cells, and that their total quantity contained in membrane structures exceeds the quantity present in dissolved form in the protoplasm. McLaren illustrates[11] the antiquity of heterogeneous enzymic catalysis by the use even in the nineteenth century of columns filled with earths to investigate nitrification. On the other hand, good yields of enzymic activity in adsorption chromatography have proved that the binding of enzymes with insoluble materials may not involve irreversible inactivation.

However, we can speak of the immobilisation of enzymes only after the specific catalytic action of intentionally prepared insolubilised enzymes with a substrate had been proved. The first results of this kind were published during the first half of the present century by Nelson[29,30] and by Langmuir[31,32]. The instability of enzymic adsorption complexes limited their application, and a broad investigation of immobilised enzymes began only in the 1950s, when the covalent bonding of enzymes and the occlusion of biological macromolecules in the three-dimensional structure of various gels came to be employed as well as adsorption. Methods for the retention of dissolved enzymes in microcapsules made of semipermeable membranes, in hollow fibres, and also in large-scale reaction vessels fitted with ultrafiltration membranes have now been developed together with molecular immobilisation (the immobilisation of each molecule).

Many immobilisation procedures are now known, hundreds of insoluble derivatives of enzymes have been obtained, and the possibility of their repeated and prolonged use has been shown. Technical processes, analytical techniques, and several therapeutic procedures have been based on the use of immobilised enzymes. The value of these products in industrial biochemistry is stressed in a special resolution of the Presidium of the USSR Academy of Sciences[33].

At the present time there are more than a thousand publications and two hundred patents on immobilised enzymes. It is clearly impossible to mention all these references in this Review, and the literature is therefore cited selectively. Since complete patent specifications are comparatively inaccessible and patented results are quite often published subsequently in the open press, a restriction was first imposed on the number of references to the patent literature. Nevertheless, the absence of an exhaustive bibliography does not prevent the reader from obtained a sufficiently general idea of the present position of the problem.

II. METHODS FOR THE IMMOBILISATION OF ENZYMES

1. Principles of Immobilisation

Methods of immobilising biologically active substances can be divided into two main groups: in molecular immobilisation each molecule is attached to the carrier individually; immobilisation occurs in solution when larger or smaller volumes of the enzyme solution are separated from the external medium by a semipermeable membrane. Molecular immobilisation can in turn be effected in three ways, according as the molecules are retained on the carrier by adsorption or covalent bonds or are held mechanically in the three-dimensional network of chains in the macromolecules of a polymeric carrier. Methods are also possible in which several of the above principles are combined.

2. Immobilisation of Enzymes by Adsorption

Ion exchangers and "neutral" adsorbents, which are not well defined exchangers, are used for the immobilisation of enzymes by adsorption.

Silicon dioxide as silica gel or glass is most often used as neutral carrier. Such materials have been applied to the immobilisation of glucose oxidase[34,35], cytochrome-c[36-38], malic dehydrogenase[39], succinic dehydrogenase[40-42], glucose 6-phosphate dehydrogenase[43], catalase[40-42], hexokinase[40-42,44,45], amyloglucosidase[34], trypsin[46,47], chymotrypsin[46], papain[35,48], alkaline protease[49], acid phosphatase[40-42,50], ribonuclease[46,51,52], aminoacylase[53], and phosphoglucomutase[40-42]. Interesting work has been done by Poltorak and his coworkers[54,55], who adsorbed enzymes on silica gel by means of an intermediate lipid layer. They thus achieved tenfold increases in activity of several enzymes, which is a very rare concomitant of immobilisation[40-42]. These results have been summarised and discussed in reviews by the same workers.

Various clays are suitable for obtaining active adsorbents. They have yielded immobilised glucose oxidase[56,57], catalase[56], glucoamylase[58,59], lysozyme[60], invertase[56], neuraminidase[56], trypsin[61], chymotrypsin[61-63], alkaline protease[49], bacterial protease[64], phosphatase[65,66], urease[67-69], and penicillinamidase[70]. Enzymes which have been immobilised on the mineral supports are nicotinamide—adenine dinucleotide pyrophosphorylase[71], invertase[30], proline iminopeptidase[72], alkaline protease[49], leucine aminopeptidase[73], aminoacylase[53,74], and penicillinamidase[75].

Activated carbon has been used as carrier for glucose oxidase[34], succinic dehydrogenase[40], invertase[29,30], glucoamylase[34,58,59,76], catalase[41 42 77], alkaline phosphatase[78], acid phosphatase[42,77], hexokinase[42,77], and phosphoglucomutase[42,77]. Peroxidase[79], cellulase[80], and deoxyribonuclease[81] have been adsorbed on cellulose; and glucose 6-phosphate dehydrogenase[82] and adenosine triphosphatase[83] have been immobilised on cellulose nitrate. Liver cells adsorbed on cellulose nitrate have been used to obtain material having ribonucleic-acid synthetase activity[84]. The efficiency of adsorption of amylase on Millipore filters can be enhanced by preliminary acylation of the enzyme with aliphatic acids[85,86]. Glycerol dehydrogenase has been adsorbed on a dextran gel—Sephadex[87].

Ion-exchange resins are used far more often than neutral carriers for immobilising enzymes. Insoluble derivatives of catalase[88], invertase[89], glucoamylase[90,91], aminoacylase[53,74,92-95], β-galactosidase[96], pepsin[96], kidney proteinase[96], asparaginase[97], adenosine triphosphate deaminase[98], glucose isomerase[99], and D-oxynitrilase[100] have been obtained on di- and tri-ethylaminoethylated celluloses. The enzyme column for the first industrial installation having an insoluble enzyme was obtained by adsorbing aminoacylase on diethylaminoethylated Sephadex[53,74,101,102]. Dextransucrase has been immobilised by the same method[103]. Catalase[104], glucose oxidase[34], glucoamylase[34,105], ribonuclease[51,52], aminoacylase[53], and penicillinamidase[106] have been attached to synthetic polymeric anion-exchange resins.

Among cation exchangers, carboxylated carriers are most often employed for immobilising enzymes. Carboxymethylcellulose has served for the immobilisation of catalase[88], peroxidase[107], asparaginase[108], and phosphomonoesterase[109]; and carboxymethylated Sephadex for glucose oxidase and glucoamylase[34], as well as aminoacylase[74]. Glucose oxidase[34], glucoamylase[34], ribonuclease[51,52,110], and trypsin[111] have been immobilised on carboxylated polymeric cation-exchange resins. Among special carboxylated carriers mention may be made of a copolymer of alanine and glutamic acid, as well as polygalacturonic acid for the immobilisation of acetylcholine esterase[112] and succinylated glass for binding lactate dehydrogenase[113]. Aminoacylase[74] and ribonuclease[114] have been immobilised on sulphonated cation-exchange resins.

Soluble polyelectrolytes are used, as well as insoluble exchangers, for the modification of enzymes. The resulting soluble polymer—enzyme complexes also possess enhanced stability[115-118].

3. Occlusion of Enzymes into Gel Structures

When enzyme and substrate differ considerably in size, a gel can be chosen whose pores are sufficiently large for diffusion of the substrate but completely exclude that of the large enzyme molecules. Such a situation ensures irreversible bonding of the enzyme with retention of its activity. A limited number of gel-forming substances have hitherto been used in this method of immobilisation. Trypsin[119] and acetylcholine esterase[120] have been occluded into gelatinous silicic acid. A process for obtaining choline esterase, alkaline phosphatase, trypsin, lipoxidase, urease, catalase, and prostaglandin synthetase occluded in silica gel has also been patented[121,122]. Aminoacylase has been trapped in hydroxymethylcellulose[53], acylase[123] and choline esterase[124] in agar-agar, choline esterase[124-126] and urease[127] in starch, trypsin[128], chymotrypsin[128], acetylcholine esterase[129], and a complex of glucose oxidase with peroxidase[129] in silastic silicone, and penicillinamidase[53] in polyurethane. An unusual method for obtaining complexes of this type is the electrodeposition of collagen fibres in a solution of the enzyme, which has been used to immobilise urease[127,130], α-amylase[131], amyloglucosidase[132], and invertase[132].

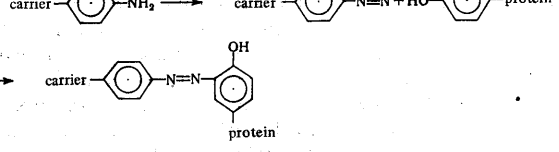
The most widely used carrier for obtaining enzymes enclosed in a gel is cross-linked polyacrylamide. Acrylamide and methylene-*NN*-bisacrylamide in various proportions are readily polymerised by a free-radical mechanism. The free radicals required are most often provided by a photochemical or a redox reaction, but ionising radiation is sometimes used[133,134]. Capture in a polyacrylamide gel has served for the immobilisation of glucose oxidase[135-139], L-amino-acid oxidase[140-142], D-amino-acid oxidase[143-147], xanthin oxidase[143], alcohol

dehydrogenase[143,144], lactic dehydrogenase[137,143,144], catalase[137,144], glucose 6-phosphate dehydrogenase[145,146], glyceraldehyde 3-phosphate dehydrogenase[143], peroxidase[148], hexokinase[146,149], phosphofructokinase[149], α-amylase[150], β-amylase[150], glucoamylase[151,152], invertase[153-155], β-glucosidase[156,157], β-galactosidase[158], trypsin[133,134,150,158,159], chymotrypsin[150], papain[150], urease[126,142,160,161], asparaginase[147], choline esterase[126,162], aminoacylase[53,163], phosphatases[164,165], adenosine trisphosphate diphosphohydrolase[166], ribonuclease[150], aldolase[149,150,167,168], enolase[167-169], decarboxylase[143,159], aspartase[170], phosphoglycerate mutase[167,168,171], glucoisomerase[172], phospho-gluco-isomerase[149], citrate synthetase[173], thiocyanase[174], and injectase[174]. Other polyacrylic gels have been used, e.g. hydroxyethyl methacrylate for the fixation of glucose oxidase[175], but considerably more rarely than polyacrylamide. The extremely long list of products obtained indicates the universality of this method. Polyacrylamide gel has been used to immobilise not only individual enzymes but also enzymic complexes[145,146,149,176-178] and entire cells of micro-organisms[159,179,180].

4. Covalent Bonding of Enzymes

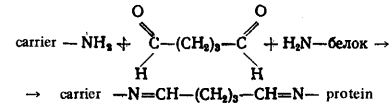
Covalent immobilisation is often considered separately for cases in which the new covalent bonds are formed between activated groups of the carrier and reactive groups in side-chains of the protein and for those in which the protein is cross-linked by a bifunctional reagent into an insoluble "superpolymer" in the pores or on the surface of an unreactive carrier or else in the complete absence of such a carrier. The distinction between these processes disappears when the carrier contains the same reactive groups as the protein, while immobilisation is effected by means of a bifunctional reagent. In such cases covalent bonding of the protein molecule with the carrier and immobilisation by formation of a superpolymer may take place simultaneously. In the present Review all these cases are regarded as the single method of covalent immobilisation. "Superpolymerisation" of an enzyme involves immobilisation in the absence of a carrier, although the resulting insoluble, intermolecularly cross-linked enzyme can then be regarded as a carrier.

One of the first processes suggested was the covalent bonding of proteins by azo-coupling, primarily with tyrosine residues of the protein molecule:



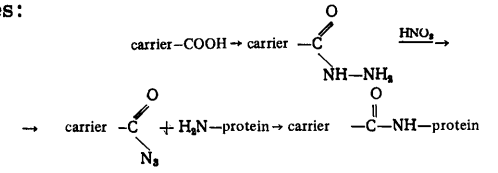
Bifunctional reagents, primarily bisdiazotised benzidine-2,2′-disulphonic acid, have also been used in this process. Immobilisation has thus been applied to papain in the absence of a carrier[181], glucose oxidase[182,183], trypsin[182], chymotrypsin[182], papain[184,185], apyrase[83], adenosine triphosphatase[83], and carbonic anhydrase[182,183] on cellulosic carriers, and papain on collagen[181]. Enzymes are more often bonded to an already diazotised carrier. Thus glass treated with 3-triethoxysilylpropylamine and *p*-nitrobenzoyl chloride, and then subjected to reduction and diazotisation, has been used for the fixation of glucose oxidase[186], peroxidase[186], L-amino-acid oxidase[187], invertase[188], trypsin[186,189], papain[189], ficin[186], pronase[190], alkaline phosphatase[186,191,192], urease[186,193], acetylcholine esterase[194], aminoacylase[52], aryl sulphatase[195], steroid esterase[196], and glucoisomerase[197]. Immobilisation of the coenzyme, nicotinamide–adenine dinucleotide, has been accomplished in the same way[198,199]. Carriers which already contain benzene rings are merely nitrated and reduced. Polystyrene has been used in this way for the immobilisation of glucose oxidase[200], catalase[201,202], lipase[201], α-amylase[203,204], pepsin[204], ribonuclease[204], carboxypeptidase[204], and aminoacylase[205]; and copolymers of *p*-amino-DL-phenylalanine with aliphatic amino-acids for that of trypsin[206,207], chymotrypsin[207], papain[181,207], thrombin[208], prothrombin[206,208], pronase[107,209], urease[207,210], and plasminogen[211]. Polytyrosyl derivatives of the enzymes—trypsin[212-214] and papain[215]—have been fixed by the same procedure. Polytyrosyl derivatives containing a larger number of phenolic groups attackable by diazo-compounds are obtained by treating the enzyme with *N*-carboxy-L-tyrosine anhydride. Diazotised *p*-aminobenzylcellulose has been attached to invertase[188], various amylases[216,217], pullulanase[218], dextranase[218], β-glucosidase[216], chymotrypsin[219], trypsin[216], thrombin[220], ficin[219], papain[218], carboxypeptidase[218], aminoacylase[53,205], and ribonuclease[219]. Aromatic amino-groups are present in the polyacrylic resin Enzacryl AA, which is marketed especially for the immobilisation of enzymes[221]. The diazotised resin has been used to bind amylase[222,223], cathepsin[224], aminoacylase[53], and adenosine deaminase[225,226]. Mention must be made also of the immobilisation of enzymes on diazotised polysaccharide anthranilates[227,228].

Most procedures for the covalent immobilisation of enzymes are based on the reactions of nucleophilic groups in the proteins—in particular their primary amino-groups—with electrophilic groups of an activated carrier on a bifunctional reagent. We shall firstly consider the reaction of amino-groups with aldehydes



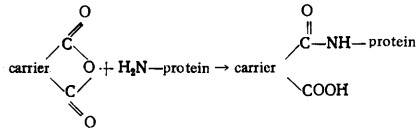
Dialdehydes, mainly glutaraldehyde, are mostly used in this reaction. This bifunctional reagent has been employed to immobilise, in the absence of a carrier, catalase[229-232], glucose oxidase[232], peroxidase[232], trypsin[232-234], chymotrypsin[233,235,236], papain[237-241], carboxypeptidase[242-244], lysozyme[127,232,236], subtilysine[245], urease[230], asparaginase[230], ribonuclease[232], aminoacylase[53], and certain zymogens[128,234,236]. The use of glutaraldehyde to cross-link a protein adsorbed on a neutral carrier has served to immobilise glucose oxidase, urate oxidase, trypsin, chymotrypsin, urease, carboanhydrase[246], and adenylate cyclase[247] on cellulose, as well as trypsin on silica gel[248]. Enzymes are more often cross-linked by glutaraldehyde with a carrier containing amino-groups: trypsin[234,249,250], pepsin[251], rennin[252], and aldolase[253] have been attached to aminoethylcellulose; alcohol dehydrogenase[217], lactate dehydrogenase[217], glucose oxidase[254], uric oxidase[255], urease[254,255], and L-asparaginase[256,257] to a partly hydrolysed polyamide; peroxidase[258], glucose oxidase[259], cytochrome[258,259], trypsin[258,259], chymotrypsin[259], acid phosphatase[259], and ribonuclease[250] to polyacylamide; and adenylate cyclase to aminopolystyrene[247]. Aldehyde-containing carriers are used considerably more rarely: e.g. poly(acrylamidoacetaldehyde dimethylacetal) has been employed for binding trypsin[260], papain[260], α-amylase[260,261], and dextranase[260,261], and an acrylaldehyde–styrene copolymer to bind invertase[262].

The reaction of acid azides with the amino-groups of a protein finds widespread use for the immobilisation of enzymes:



Diazides are seldom employed as bifunctional reagents because of their sparing solubility in water. It is preferred to convert carboxy-groups of a hydrophilic carrier, generally carboxymethylcellulose, into azides. Insoluble derivatives have been obtained by this method from catalase[263], chymotrypsin[219,264-269], trypsin[269], ficin[219,270], bromelain[56], pronase[271], ribonuclease[219,272], adenosine triphosphatase[83], alkaline phosphatase[273], apyrase[83,274], aminoacylase[53], penicillinamidase[275], asparaginase[276], streptokinase[277], adenylate cyclase[247], and various amylases[203,278,279]. Another widely used carrier of this type is polyacrylohydrazide, known also under the proprietary name Enzacryl AH,[221] on which have been fixed glucose oxidase[280], luciferase[281], α- and β-amylases[272,282], trypsin[283-286], chymotrypsin[286], aminoacylase[285,286], and ribonuclease[287]. Other carriers are comparatively rarely used in the azide method of immobilisation: carboxymethyldextran has given insoluble acetylcholine esterase[112], asparaginase[288], and streptokinase[277]; pectin has been used for trypsin and chymotrypsin[289]; and nylon for trypsin[290].

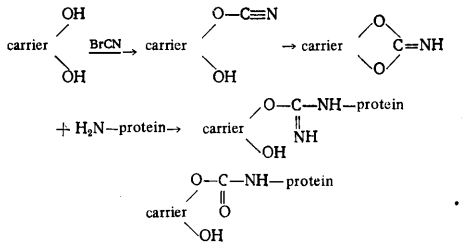
Reaction with anhydrides of dibasic acids is also extremely popular:



Since this involves the formation not only of a covalent bond but also of an ionisable carboxy-group, electrostatic interaction may be observed between protein and carrier molecules. Carriers of this type are usually obtained by the copolymerisation of maleic anhydride and ethylene, and are simply termed EMA polymers. This method of immobilisation has been applied predominantly to proteolytic enzymes, mainly trypsin[2,144,181,212,213,292-298], and a special review has been devoted to derivatives of this type[291]. Other proteinases are represented by chymotrypsin[2,144,297,298], pepsin[299], plasmin[2], kallikrein[144], papain[297,299,300], bromelain[300], ficin[300], pronase and neutral proteinase[299], and Novo and Carlsberg subtilisines[297].

Proteinases inhibited by mercury compounds have in certain cases been immobilised in order to protect the active centres of the enzymes from acylation: attachment of the enzymes to the carrier was followed by reactivation[300]. Immobilisation has been applied not only to proteolytic enzymes but also to catalase[298], D-amino-acid oxidase[298], xanthine oxidase[298], amylase[299,301], dextranase[301], cellulase[298,301], β-glucosidase[298], naringinase[302], ribonuclease[303], acetylcholinesterase[304], lipase[299,301], penicillinamidase[106,302], asparaginase[298,301], urease[301], alkaline phosphatase[273], pyrophosphatase[298], hexokinase[298], adenylate cyclase[247], and oxynitrilase[301]. Soluble EMA polymers have served for the preparation of soluble polymer—enzyme complexes[301]. Among other polymeric anhydrides only that of polymethacrylic acid has been used, to immobilise trypsin, chymotrypsin, and papain[362,386].

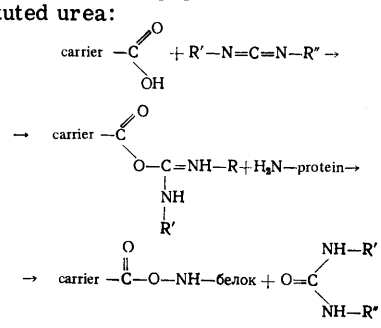
The activation of hydroxylated carriers with cyanogen bromide, proposed by Porath et al.[305], has gained very great popularity during recent years. This reagent acts on the carrier surface to form iminocarbonate groups capable of bonding with the amino-groups of proteins:



This method is mostly used to activate agarose (Sepharose), which, because of its ability to swell strongly in water (dry content 2%, 4%, or 6%), has an extremely large-pored structure. This ensures that large enzyme molecules are trapped in the pores of the carrier, and that diffusion coefficients shall be high even for substrates of high molecular weight. Enzymes which have been immobilised in this way are glucose oxidase[306], lactoperoxidase[307], lipoxygenase[308], isocitrate dehydrogenase[309,310], pyridine nucleotide transhydrogenase[311], protocatechuate 3,4-dioxygenase[312], glucose 6-phosphate dehydrogenase[146], glucoamylase[306], trypsin[313-317], chymotrypsin[252,305,316,318,319], pepsin[320,321], rennin[252,322], cathepsin[224], elastase[323], leucine aminopeptidase[324,325], amino- and carboxy-peptidases[326], papain[316], bacterial proteinase[327], various nucleases[328-334], choline esterases[335], aminoacylase[53], penicillinamidase[336], aldolase[337], glucogen phosphorylase[338], and transfer ribonucleic acid histidine[339] and isoleucine[340] synthetases.

Although Sepharose activated with cyanogen bromide enables extremely high activities to be attained with many immobilised enzymes, the poor mechanical and thermal stability of this material must not be overlooked. In order to obtain insoluble enzymes more suitable for technical application, other polysaccharide materials have been activated with cyanogen bromide. Thus cellulose has served for the attachment of glucoamylase[341], pronase[271], acetylcholinesterase[112], penicillinamidase[336], asparaginase[276], polynucleotide phosphorylase[342], and streptokinase[277]. The dextran gel Sephadex has been used to immobilise glucose oxidase[343], catalase[343], lipoxygenase[344], glucose 6-phosphate dehydrogenase[345], isocitrate dehydrogenase[309], glucoamylase[343], β-galactosidase[345], trypsin[314,346-348], chymotrypsin[319], leucine aminopeptidase[336], ribonuclease[331], hexokinase[345,349], and streptokinase[277].

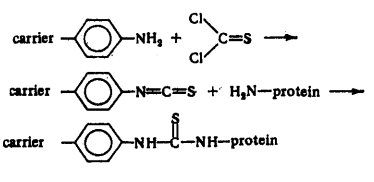
The carbodi-imides employed in peptide synthesis are good condensing agents. The reaction takes place in two stages, addition of the agent to a carboxy-group being followed by formation of a peptide link with elimination of a disubstituted urea:



This reaction can be used to form intra- and inter-molecular amide bonds[360], and enables an immobilised enzyme to be obtained in the absence of a carrier (e.g. the immobilisation of aminoacylase[53]). In most cases carbodi-imides are used to condense carboxy-groups of the carrier with amino-groups of the enzyme. In this way glutamate dehydrogenase[351], peroxidase[352,353], chymotrypsin[351], pronase[354], asparaginase[276], and acetylcholinesterase[112] have been immobilised on carboxymethylcellulose; glutamate dehydrogenase[351], chymotrypsin[351], and pronase[355] on dextran; glucose 6-phosphate dehydrogenase[146], glutamate dehydrogenase[351], β-galactosidase[158], pullulanase[356], trypsin[158], chymotrypsin[351], and citrate synthetase[159] on acrylic polymers; and trypsin[290] on nylon.

Several compounds having mobile halogen atoms—in particular bromo- and iodo-acetyl derivatives, acid chlorides, fluoronitrophenyl compounds, and cyanuryl halides (triazinyl trichlorides, Procion dyes)—have been used for immobilisation. They are able to react with amines and mercapto, hydroxy, and imidazole groups. Fluorophenyl-containing polymers have served for the immobilisation of alcohol dehydrogenase[357,358], invertase[358,359], amylases[357,358,360-362], pepsin[357,358], papain[360-362], alkaline phosphatase[273], and aminoacylase[205]. Triazinyl halides have been used for the immobilisation of enzymes mainly on aminoethylated celluloses, as well as on other polysaccharides. Insoluble preparations have been obtained in this way from glucose oxidase[363], lactate dehydrogenase[364], glucoamylase[363,365,366], dextranase[363], α-galactosidase[363], β-galactosidase[367,368], invertase[369], hyaluronidase[370], trypsin[363,371], chymotrypsin[189,372,373], papain[263], penicillinamidase[363,374,375], acetylcholinesterase[112,376], alkaline phosphatase[363], ribonuclease[363], pyruvate kinase[363,368,377], and creatine phosphokinase[363]. Derivatives of iodo- and bromo-acetic acids and also iodoalkyl esters have effected the immobilisation of chymotrypsin[378,379], trypsin[380], thrombin[381], pronase[355], glucoamylase[341,382], aminoacylase[53,383], penicillinamidase[275], urease[380], aldolase[384], and streptokinase[277]. The action of ethyl chloroformate has immobilised pepsin[385], trypsin[289], chymotrypsin[289], and ficin[289]. Penicillinamidase[106,275] and alkaline proteinase[386] have been attached to acid chlorides of carboxylated cation-exchange resins. Among inorganic compounds sulphuryl and thionyl chlorides have been used[387,388] to immobilise lactate dehydrogenase, invertase, pepsin, alkaline phosphatase, and threonine deaminase. New bonding agents of this type are transition-metal chlorides (in particular titanium tetrachloride[389,390]), by means of which glucose oxidase, uricase, glucoamylase, α-amylase, invertase, trypsin, urease, and glucose isomerase have been attached to various carriers, including yeast cells.

Various cyanates are quite often used to establish covalent bonds between protein and carrier. Among them we must first mention isothiocyanates, obtained by the action of thiocarbonyl chloride on amines:



Similarly, treatment with carbonyl chloride leads to the formation of isocyanate groups. Insoluble derivatives of glucose oxidase[391], catalase[201], various amylases[222,282,361,392,393], proteinases[358,361,393,394], and lipase[201] have been obtained by these procedures. Isocyanides[319,395] and cyanates[396] are employed considerably more rarely for immobilisation.

Among the less widely used methods for the covalent fixation of enzymes is the application of 2-ethyl-5-phenylisoxazole-3-sulphonate[276,397-401] to the immobilisation of trypsin and chymotrypsin, as well as asparaginase[276], apyrase[399], deoxyribonuclease[399], and choline esterase[399]. β-D-Glucosidase, α-amylase, glucoamylase, and trypsin have been immobilised on cellulose carbonate[402]. Reaction with compounds containing epoxy-groups has been used to immobilise certain proteinases[403]. Several enzymes have been immobilised on Enzacryls containing thiolactam and thiol groups[221,284]. Commercial preparations of insoluble enzymes have been produced by the above methods[404-410].

5. Isolation of Enzymes by means of Membranes

Repeated application of enzymes is achieved also by isolating the enzyme solution by means of membranes whose pores are impermeable to protein macromolecules but permeable to the substrate and the reaction products. This is accomplished by microencapsulation, in which polymeric envelopes are formed around the enzyme solution, by producing artifical fibres containing minute droplets of the enzyme solution, and by enclosing the enzyme solution between membranes on an electrode surface. The application of enzymes in a reaction vessel with ultrafiltration of the reaction mixture through a membrane which retains protein macromolecules might have been included in this type of isolation. In the last case a solution of the substrate is introduced into the reaction vessel, omitting the membrane, and the enzyme can act on macromolecular substrates provided that the products are of low molecular weight.

Since in these systems the enzymes retain a certain mobility in solution, isolation of this type is difficult to relate completely to our problem of immobilisation. In view of the similarity between the methods of practical application of microencapsulated enzymes—in particular those enclosed in fibres—and of molecularly immobilised enzymes, we shall mention some of the relevant work and give a list of products obtained in this way (Table 1).

Table 1. List of immobilised enzymes.

No.	Enzyme	Ref.
1	Alcohol dehydrogenase	143, 204, 214, 357, 406
2	Acetaldehyde dehydrogenase	421
3	Glycerophosphate dehydrogenase	406
4	Lactate dehydrogenase	113, 137, 143, 144, 217, 388, 406, 411
5	Glycerate dehydrogenase	39, 40—42
6	Malate dehydrogenase	38, 217, 406
7	Isocitrate dehydrogenase	309, 310
8	Glucose 6-phosphate dehydrogenase	43, 82, 145, 146, 345, 406
9	Steroid dehydrogenase	179
10	Glucose oxidase	34, 35, 56, 57, 129, 135, 136, 137, 139, 175, 182, 183, 186, 200, 232, 246, 254, 259, 280, 306, 343, 363, 390, 391, 406, 408, 409, 420
11	Glyceraldehyde phosphate dehydrogenase	406
12	Xanthin oxidase	377
13	Glutamate dehydrogenase	351
14	L-Amino-acid oxidase	140—142, 187
15	D-Amino-acid oxidase	143, 147, 298
16	NAD transhydrogenase	311
17	Nitrite reductase	421
18	Urate oxidase	246, 255, 390
19	Catalase	32, 41, 42, 56, 77, 88, 104, 121, 122, 144, 187, 188, 202, 229, 231, 263, 298, 343, 411, 418, 420

Table 1 (continued).

No.	Enzyme	Ref.
20	Peroxidase	120, 129, 148, 186, 232, 258, 259, 352, 353, 405, 406, 409
21	Cytochrome-c	36, 37, 258, 405, 409, 121, 122, 308, 344
23	Protocatechuate 3,4-dioxygenase	312
24	Polyphenol oxidase	422
25	Luciferase	281
26	Lactoperoxidase	307
27	β-Tyrosinase	419
28	α-Glucan phosphorylase	338
29	Dextransucrase	103
30	Galactosyl transferase	423
31	Nucleoside-ribosyl transferase	350
32	Aminotransferase	420
33	Hexokinase	40, 41, 42, 44, 77, 146, 149, 298, 338, 345, 349, 377, 406, 419
34	Creatine kinase	363
35	Phosphoglycerate mutase	406
36	NAD pyrophosphorylase	71
37	Polynucleotide phosphorylase	342
38	Thiocyanase	423
39	Lipase	51, 201, 299, 301, 411
40	Acetylcholesterase	89, 112, 120, 129, 335, 376
41	Cholinesterase	121, 122, 124—126, 162, 335
42	Glycerol dehydratase	87
43	Acid phosphatase	40—42, 51, 77, 164, 259, 408
44	Alkaline phosphatase	40—42, 78, 122, 164, 165, 186, 191, 192, 273, 363, 388, 404, 408
45	Phosphatase	65, 66
46	Phosphomonoesterase	109
47	Phosphodiesterase	404
48	Deoxyribonuclease	81, 404
49	Nuclease	330, 332, 334
50	α-Amylase	85, 86, 131, 150, 203, 216, 217, 222, 223, 260, 261, 282, 389, 390, 393, 405, 406
51	β-Amylase	150, 217, 222, 358
52	Glucoamylase	34, 58, 59, 76, 90, 91, 105, 132, 151, 152, 200, 216, 261, 279, 306, 341, 363, 365, 366, 389, 390, 404
53	Maltase	416
54	Diastase	204, 301, 357, 361, 362
55	Cellulase	80, 298, 301
56	Dextralase	363
57	Lysozyme	60, 128, 232, 236
58	Neuraminidase	56, 406
59	β-Glucosidase	156, 157, 216, 298, 397
60	α-Galactosidase	363
61	β-Galactosidase	96, 158, 345, 367, 419, 420
62	Invertase	29, 30, 56, 89, 153—155, 262, 416, 419, 420
63	Naringinase	302
64	Pullulanase	218, 261, 301
65	Ribonuclease	46, 51, 52, 110, 114, 156, 204, 219, 232, 259, 272, 287, 328, 329, 331, 333, 334, 363, 404—409, 419, 420
66	Leucine aminopeptidase	73, 324, 325, 405
67	Aminopeptidase	326, 393
68	Carboxypeptidase	204, 218, 242, 243, 244, 326, 406, 408, 420
69	Iminopeptidase	72
70	Alkaline protease	49, 186, 228, 299, 386
71	Bacterial protease	198, 228
72	Neutral protease	299
73	Pepsin	31, 95, 204, 251, 299, 320, 321, 357, 358, 385, 387, 393
74	Kallikrein	144
75	Trypsin	46, 47, 61, 111, 119, 121, 128, 133, 144, 150, 158, 159, 182, 186, 289, 206, 207, 212, 213, 216, 228, 232, 234, 246, 248, 250, 258, 260, 269, 283, 286, 289, 290, 292, 293—298, 304, 313, 314, 316, 346—348, 358, 363, 371, 380, 389, 390, 393, 401, 404—410
76	Papain	48, 140, 148, 150, 155, 181, 184—186, 189, 207, 215, 227, 236, 238—241, 260, 278, 300, 304, 316, 346, 361, 363, 394, 397, 405—410, 420
77	Chymotrypsin	5, 41, 61—63, 112, 128, 144, 150, 182, 207, 219, 233, 236—238, 246, 252, 259, 264—267, 286, 297, 305, 315, 316, 319, 351, 358, 373, 378, 393, 395, 397, 398, 401, 405—410
78	Elastase	323
79	Cathepsin	224
80	Ficin	106, 186, 219, 270, 289, 300, 406, 407, 409
81	Thrombin	206, 208, 220 381,
82	Plasmin	47, 211
83	Rennin	252, 322
84	Bromelain	300, 407, 409
85	Pronase	107, 190, 209, 271, 355, 406
86	Subtilisin	64, 297, 405—407
87	Asparaginase	27, 108, 147, 230, 256—277, 288, 298, 301, 406
88	Aminoacylase	51—53, 74, 92—95, 101, 102, 163, 205, 285, 383
89	Urease	31, 32, 67, 68, 69, 122, 126, 129, 160, 161, 186, 193, 210, 230, 246, 254, 255, 286, 301, 380, 390, 405, 406, 411, 413, 414, 416, 420
90	Penicillinamidase	53, 70, 75, 106, 274, 336, 363, 374, 375, 419
91	Arginase	420
92	Adenosine deaminase	225, 226
93	Pyrophosphatase	298
94	Adenosine triphosphatase	83
95	Adenosine triphosphate deaminase	98
96	Adenosine triphosphate dihydrolase	166
97	Apyrase	83, 271
98	Orsellin decarboxylase	159
99	Arginine decarboxylase	143, 196
100	Cysteinesulphinate decarboxylase	143, 195
101	Oxynitrile lyase	145
102	Aldolase	149, 150, 167, 168, 253, 337, 384, 406, 419
103	Carbonic anhydrase	182, 183, 246, 317, 412, 417
104	Enolase	167—169
105	Hyaluronate lyase	370
106	Threonine deaminase	388
107	Aspartase	170
108	Glucose isomerase	99, 172, 416, 419
109	Glucose phosphate isomerase	149, 406
110	Triose 3-phosphate mutase	406
111	Phosphoglycerate phosphomutase	41, 42, 40, 77, 145, 167, 168, 171
112	Tryptophan synthetase	419
113	Isoleucine-tRNA synthetase	340
114	RNA synthetase	84
115	Histidine-tRNA synthetase	339
116	Citrate synthetase	159, 173
117	Prostaglandin synthetase	121, 122
118	Injectase	174

Inclusion in microcapsules has been applied mainly to urease[230,411-415], and also to lipase[411], maltase[416], glucose isomerase[416], carbonic anhydrase[317,412,417], catalase[411,418], and asparaginase[230]. By dispersing an aqueous solution of one or more enzymes in a solution of a linear polymer in an organic solvent, various enzymically active fibres and cloths have been obtained, which are able to retain such activity for a long time[419,420].

In order to facilitate the finding of references on the immobilisation of any individual enzyme, Table 1 gives a list of the immobilised enzymes known at the present time, their sequence corresponding to the generally accepted classification of enzymes.

III. EFFECT OF IMMOBILISATION ON THE PROPERTIES OF ENZYMES

1. General Description of Changes

It can be concluded from all the accumulated experimental results that immobilisation produces no fundamental changes in the catalytic properties of enzymes. Furthermore, even the quantitative parameters of their action—the catalytic constants, activation energies, optimum pH and temperature—quite often undergo no significant change. Henceforward, therefore, we shall consider only those cases in which the properties of the immobilised enzyme differ from its original properties.

In examining the experimental differences in properties between soluble and insoluble enzymes, we must bear in mind that the molecules of an immobilised enzyme function in a microenvironment which may differ considerably from the macroenvironment, i.e. that the medium may differ in composition inside and outside a particle of a heterogeneous enzymic catalyst. For this reason the International Commission has recommended that the experimental kinetic characteristics of immobilised enzymes should be qualified as "apparent".

We propose to discuss the kinetics of immobilised enzymes, and hence the production of the observed kinetic changes, at several levels (Table 2). Changes in enzymic kinetics at the molecular level (No. 5) are of greatest interest to theoretical enzymology, of course, but study of the kinetics of enzymes at this level requires that perturbations at levels 1—4 be taken into account.

In principle it is easiest to eliminate the effect of the apparatus, for which purpose we must operate at sufficiently low degrees of conversion of the substrate. The necessity must then be emphasised of verifying experimentally that the reaction velocity is independent of the degree of conversion. Our own experimental results have shown that, when the enzyme is inhibited by

reaction products, non-linearity arises even when only a few percent of the substrate has been converted. Development of the kinetics of apparatus with immobilised enzymes is of immense value for the introduction of heterogeneous enzymic catalysis into technical processes, and several theoretical and experimental papers have been devoted to this problem. Lack of space prevents discussion of all this work here, and the Reviewer prefers to refer only to a few of the most recent and interesting publications[424-429].

Table 2. Changes in kinetic properties of immobilised enzymes at various levels.

No.	Kinetic level	Causes of changes	Nature of effect	Conditions for maximum changes
1	Apparatus	conc. gradients along apparatus; variation in concn. during operation	dynamic: depends on activity of enzyme prep.	thorough conversion of substrate, especially when a product is an activator
2	Material	concn. gradients at interface of soln. with enzymically active material	as above	high moduli $\kappa = E/\rho\beta c^*$
3	Particle	concn. gradients through enzymically active material	as above	high moduli $\lambda = R^2E/D^*$
4	Matrix	effect of matrix on activity coeff. of solutes; partition of substrate and activator between soln. and matrix	static: independent of activity of gel	charged matrix; hydrophobic matrix when substrate hydrophobic
5	Molecule	direct action of matrix on conformation of protein macromolecule and on active centre	as above	not generalised

*E = activity of enzyme preparation; ρ = specific surface of material; β = mass-transfer coefficient; R = radius (half-thickness) of material; D = diffusion coefficient of substrate (product) in the grain.

By solving numerically the appropriate equations following from the mass-transfer balance at the surface and within the particle we have shown[430] that the mass-transfer resistance at the interface does not affect the form of the Michaelis–Menten equation, but increases the apparent value of K_m. The main parameter determining the concentration drop in the mass-transfer layer, and responsible also for the increase in the apparent K_m, is the modulus

$$\varkappa = \frac{E}{\rho \cdot \beta \cdot c} \quad , \tag{4.1}$$

where E is the enzymic activity of the product, ρ the specific surface of the material, β the mass-transfer coefficient, and c the concentration of the substrate in the solution. When $\kappa \leqslant 0.1$, the mass-transfer resistance can be neglected. Values of $\kappa \geqslant 10$, on the other hand, correspond to processes which are completely controlled by external diffusion, i.e. to infinite K_m.

Rony[431] has described the kinetics in a multilayer material in the presence of mass-transfer resistance. The kinetics of mass transfer in an enzymically active membrane has been described also for the presence of an electrostatic potential[432], for inhibition of the reaction by a product[433], and for a membrane with two consecutive reactions[434].

Disregarding external diffusion, we can describe the kinetics of reactions in a particle (membrane) in a steady state by the general equation

$$\operatorname{div}(D \cdot \operatorname{grad} c) = g(c) \, , \tag{4.2}$$

where D is the diffusion coefficient of the product within the material, and $g(c)$ a function describing the dependence of reaction velocity on substrate concentration. The concrete form of this equation and hence the possibility of its analytical solution depend on the geometry, the material, and the function $g(c)$. It is of practical importance to solve Eqn. (4.2) for an infinite sheet (membrane), for a sphere, and in certain cases also for an infinite cylinder (fibre). In the case of a membrane, solution presents no difficulty for various forms of kinetic equation. Among the many papers giving solutions of this equation, mention must be made of that by Moo-Young and Kobayashi[435], who give a general solution for certain of the most often encountered forms of the function $g(c)$.

For a sphere Eqn. (4.2) becomes

$$\frac{d^2c}{dr^2} + \frac{2}{r} \cdot \frac{dc}{dr} = \frac{1}{D} g(c) \tag{4.3}$$

and can be solved analytically only for reactions of zero or the first kinetic order: only an approximate solution is available for real enzyme kinetics. In the case of Michaelis–Menten kinetics better approximate solutions of the same equation were obtained by Blum and Jenden[436], and subsequently developed by O'Sullivan[437]. Murray[438] solved the same equation by a single-perturbation method. The paper mentioned above[435] gives approximate solutions of Eqn. (4.3) for several forms of the function $g(c)$, and exact numerical solutions for Michaelis–Menten kinetics have been obtained by Keller[439] and by Kěstner[440,441].

From all the approximate and numerical solutions it may be concluded that the form of the fundamental kinetic equation is unaffected by either internal or external diffusion, but the apparent value of K_m increases. In this case the diffusion resistance may be measured by the dimensionless modulus

$$\lambda = R \sqrt{\frac{E}{K_m \cdot D}} \quad , \tag{4.3'}$$

where R is the radius of the grain, and K_m the Michaelis constant for the immobilised enzyme. To a first approximation the apparent K_m varies linearly with λ:

$$K_{m(\text{app.})} = K_m(1 + 0.018 \cdot \lambda) \, , \tag{4.4}$$

in which 0.018 is a coefficient determined from the numerical solutions. Thus diffusion resistance at the surface and inside the material itself leads to an increase in the apparent K_m. The smallest difference between observed and actual values of K_m is ensured by the lowest activity and minimum linear dimensions of the material. Increasing the diffusion coefficient by controlling the pore size and raising the mass-transfer coefficient by stirring assists the achievement of this aim.

Interaction of solutes with the matrix of an immobilised enzyme may lead to considerable changes in the concentration of the solutes within the carrier. A typical example is the variation in the effective hydrogen-ion concentration in an electrically charged matrix. The change in the effective pH is given by

$$\Delta\text{pH} = 0.43 \cdot \varepsilon\psi/kT, \tag{4.5}$$

where ϵ is the charge on the proton, ψ the electrostatic potential in the matrix, k Boltzmann's constant, and T the

absolute temperature. A similar equation describes the variation of the concentration of an electrically charged substrate in the matrix. The applicability of the above equation has been shown by Goldman, e.g. in his review[7]. Agreement between calculated and experimental shifts in pH has been found[119] for trypsin trapped in silica gel.

The crowding of a hydrophobic substrate into a comparatively hydrophobic matrix has also been confirmed experimentally[129,442], but a quantitative description of this phenomenon is lacking. Laurent[443] examined the effect of a polymer medium on the activity coefficients of enzyme, substrate, and enzyme–substrate complex, which may produce a change in the apparent value of K_m.

Thus the microenvironment of the molecules of an immobilised enzyme is formed under the influence of various factors, which are quite often difficult to assess quantitatively.

2. Change in Activity and Stability of Enzymes

The main purpose of obtaining immobilised enzymes is to increase the integral activity of the enzyme defined by the total quantity of product obtained. The effectiveness of immobilisation η_{imm} is given by the ratio of the integral activities of the insoluble derivatives and of the initial enzyme:

$$\eta_{imm} = \frac{P_{imm}}{P_{sol}} \quad , \tag{4.6}$$

where P_{imm} is the quantity of product formed by the insoluble enzyme under practical conditions, and P_{sol} the corresponding quantity for the soluble enzyme.

We shall suppose that P_{sol} is determined mainly by the quantity of enzyme employed A_{sol} and the duration of the reaction t_{sol}. The latter is governed by the practical requirements of completing the reaction within acceptable periods. On the assumption that the activity of the soluble enzyme does not diminish significantly under the operating conditions during the period t_{sol}, we obtain for the effectiveness the formula

$$\eta_{imm} = \frac{A_{imm}}{A_{sol}} \cdot \frac{1}{t_{sol}} \int_0^{t_{imm}} [1 - f(t)]\, dt \quad , \tag{4.7}$$

where A_{imm} is the initial activity of the immobilised enzyme, t_{imm} its time of operation determined by its stability, and $f(t)$ the rate of inactivation of the immobilised enzyme under the working conditions.

The ratio A_{imm}/A_{sol} determines the retention of activity on immobilisation (or the "activity yield" of immobilisation), and will henceforward be denoted by γ. The remaining part of Eqn. (4.7)—the integral coefficient of prolongation of the action of the enzyme—is denoted by I_{pr}:

$$I_{pr} = \frac{1}{t_{sol}} \int_0^{t_{imm}} [1 - f(t)]\, dt \quad . \tag{4.8}$$

Equation (4.7) thus simplifies to

$$\eta = \gamma \cdot I_{pr} \quad .$$

The quantity I_{pr} is evaluated from two fairly arbitrary values of t_{sol} and t_{imm}, and can hardly be determined accurately. Immobilisation is regarded as advisable in practice for values of I_{pr} exceeding unity.

The coefficients γ can be regarded as the product of the coefficients α and β of immobilisation of the enzyme and development of activity:

$$\gamma = \alpha \cdot \beta = \frac{B_{imm}}{B_{sol}} \cdot \frac{E_{imm}}{E_{sol}} \quad , \tag{4.9}$$

where B_{sol} is the initial quantity of enzymic protein, B_{imm} the quantity of protein in the preparation obtained, E_{sol} the specific activity of the soluble enzyme, and E_{imm} the observed specific activity of the immobilised enzyme. The enzymic protein remaining in the reaction mixture or in the wash-waters can hardly be utilised, so that there is no need to practice to separate the coefficient γ into two components. However, such an approach is quite often used in elucidating the nature of immobilisation processes.

Complete bonding of a protein, when $\alpha = 1$, is well known in ion-exchange sorption, but α is considerably less than unity in most of the methods described for obtaining immobilised enzymes. The actual values of α depend significantly on the conditions of immobilisation, and it is extremely difficult to make any generalisations. In some cases, especially with adsorption, an enzyme washes out for weeks, which prevents accurate determination of α.

The coefficient β is also almost always smaller than unity, but an increase in enzymic activity has been demonstrated quite reliably in several researches[39,42,69,72,78,95,391,444]. An increase is often observed when enzymes are adsorbed, the activity sometimes increasing dozens of times[42,444]. This is fully consistent with the considerable conformational changes in a native protein detected by various methods on adsorption, which in other cases leads to almost zero values of β. Nor is the nature of such a phenomenon as the enhanced activity of trypsin on freezing or coagulation of its solutions entirely clear. These processes for enhancing enzymic activity have been patented[445,446].

An interesting example of an increase in the total activity of a jointly immobilised three-enzyme system has been reported by Mattiasson and Mosbach[345]. The increase is attributed to facilitation of the transfer of the product of the first reaction to molecules of the enzymes which effect subsequent stages.

The diminution in specific activity of enzymes on fixation may be due to chemical modification of certain groups in the active centre, shielding of the enzyme molecule or of its active centre from access by the substrate, and change in conformation of the protein macromolecule. Undesirable modification of the active centre on covalent immobilisation can be diminished by preliminary formation of a complex of the enzyme with an inhibitor, which is subsequently rinsed out of the preparation. The presence of shielding is proved by a difference in the activity of the preparation towards substrates of low and of high molecular weight. A linear decrease in observed activity with increase in the logarithm of the molecular weight of the substrate has been shown for certain immobilised enzymes[107,109]. The existence of this "sieve effect" has been demonstrated also by the restoration of the original activity on solubilisation of the matrix[313]. To eliminate undesirable effects of the matrix on the enzyme molecule it is advisable to effect attachment through an "extension bridge" or "arm" of 6–10 methylene groups[113,178,258,447-449]. Carriers with ligands immobilised "on the arm" are most suitable for use as affinity sorbents.

The reasons for the well known increase in stability of enzymes on immobilisation have been summarised by Zaborsky[450]. They are indirect, such as shielding of the enzymes from the action of lytic micro-organisms and elimination of autolysis (or proteolysis by proteinase impurities), owing to separation of the enzyme molecules on the carrier. These factors may partly explain the prolonged retention of enzymic activity, but the enhancement of resistance to the short-lived action of denaturing factors indicates the operation of molecular mechanisms of stabilisation. It is most often reported that immobilised enzymes resist the action of high temperatures[87,120,152,166,173,198,217,219,226,256,270,281,285,350,376,383].

It is interesting that Surovtsev et al.[265] have shown the presence of two fractions differing in thermal stability in an immobilised enzyme. However, other workers have reported a worsening of the temperature stability of enzymes[185,225,281,433,451-453]. Nevertheless, on the whole an increase in lability of enzymes is rarely encountered on immobilisation.

Extremely long periods of operation or retention of activity during storage have been reported for immobilised enzymes. A column of a gel containing invertase, for example, functioned for between 18 months and 3 years[419,454,455]. Specimens of certain covalently bound dehydrogenases have been used for more than a thousand analytical determinations[217].

It has been quite often reported that immobilised enzymes are less liable to be inhibited[120,249,452,456]. It is astonishing that they retain their activity in 4–8 M solutions of urea and other reagents which rupture hydrogen bonds[101,203,314,347]. The protective action may depend on the nature of the matrix: the immobilisation of trypsin on cyanogen bromide-Sephadex, for example, protects the enzyme from the action of urea, but bonding to agarose by the same method has no protective effect[347]. According to several workers[101,314] urea may increase the vulnerability of a substrate to attack by an enzyme, and may thus even increase the effectiveness of the latter. If a denaturing agent leads to a loss of enzymic activity, the bonds between the protein molecule and the matrix may help to restore the native three-dimensional structure[113,249,309,328,457].

Deoxyribonuclease immobilised on glass is able to develop its activity in the absence of the bivalent cations required by the soluble enzymes[465,458].

Immobilisation protects enzymes also from oxidation and denaturation on exposure to γ-rays[202].

3. Effect of Immobilisation on Fundamental Characteristics of Enzymes

Differences in the substrate specificity of immobilised and soluble enzymes can be explained by differences in the accessibility of the enzyme molecule to substrates of different molecular weights. This is true, for example, for proteolytic enzymes[107,209,219,268,294]. Such differences enable other peptide fragments to be obtained by means of immobilised proteinases than by the action of the soluble enzymes, and hence yield additional information in establishing the primary structure of proteins[294]. Immobilised amylases do not act on amylopectin[152], but form more products of low molecular weight from starch[459]. Immobilised luciferase is more sensitive to variation in the molecular weight of synthetic substrates[281]. Immobilisation of dextransucrase eliminates the factor controlling the type of glucosidic bonds formed, and therefore leads to more strongly branched products[103].

The increase in the Michaelis constant predicted by theory is observed in many cases. Increases by factors of 2–10 are found with creatine phosphotransferase[460], apyrase[274], cholinesterase[376], lactase[461], phosphomonoesterase[109], and glucoamylase[279] covalently fixed on cellulose. The same effect is observed with lipoxygenase attached to Sephadex[308], with adsorbed invertase[452] and glucoamylase[59], and also with many enzymes enclosed in microcellular fibres[419]. Phosphatase in a collodion membrane shows increases in K_m by factors up to 350.[433] For unknown reasons lyophilisation increases the apparent K_m of immobilised lactase[461]. When immobilised enzymes are used in a column, increasing the rate of flow of the substrate diminishes K_m,[5,256,452,462] in good agreement with the hypothesis that the apparent K_m depends on the conditions of mass transfer. Decreases of 90% or more are observed with glucose oxidase[391], succinate dehydrogenase[39], trypsin[292], bromelain[463], cholinesterase[129], and penicillinamidase[374]. This phenomenon can in principle also be explained by concentration of the substrate into the matrix. In particular, a 90% decrease in K_m is observed with bromelain covalently bound to carboxymethylcellulose only at low ionic strengths, whereas at high ionic strengths, when interaction between the substrate and the carboxy-groups of the carrier is suppressed, insoluble and soluble enzymes have the same Michaelis constant.

It is interesting that an improvement in thermal stability on immobilisation is sometimes accompanied by a fall in the temperature optimum of the enzyme[90,188,383,452]. However, the optimum temperature usually rises[169]. The apparent activation energy of an enzymic reaction most often remains unchanged, but increases[391] or decreases[169,383] have been shown in some cases. A change in form of the Arrhenius plot may occur[169].

The change in the pH dependence of enzyme activity depends on the carrier used. The optimum pH has become more acid with protocatechate dioxygenase[312], trypsin[292], chymotrypsin[63], invertase[89], urease[193], and penicillinamidase[374]; and more alkaline with lipoxygenase[308,344], trypsin[119,292], chymotrypsin[267,313], elastase[323], and invertase[188,369]. The shifts in the optimum pH are usually 0.5–1.0, and seldom reach 2. They are explained by the difference between the local pH of the microenvironment of the active centre of the enzyme and the pH measured in the bulk of the solution. This produces a change in the measured apparent values of pK_a for ionisation of the functional groups in the enzyme. The local microenvironmental pH is influenced by the charge on the matrix, the change in the charge on the substrate on coversion into the products, diffusion of substrate and product, change in the total charge of the protein on immobilisation, etc. The variation in the optimum pH of a two-enzyme system can be explained by the cooperative action of the enzymes[306].

Furthermore, immobilisation quite often produces a change in the form of the pH dependence. Broadening of the bell-shaped curve is observed with adsorbed succinate dehydrogenase[39], covalently bound acetylcholinesterase[194], pronase[190], enolase[169], adenosine deaminase[226], and also with many enzymes enclosed in cellular fibres[419]. Narrowing of the bell is observed with adsorbed glucoamylase[90] and with covalently bound papain[300], invertase[464], asparaginase[256], and penicillinamidase[374]. A detailed analysis of the reasons for the variation in the pH dependence will be found in the review by Goldman and his coauthors[7].

IV. APPLICATION OF IMMOBILISED ENZYMES

1. Use in Technical Processes

Most papers on immobilised enzymes are concerned with the preparation of heterogeneous enzymic catalysts for use in industrial technology. General aspects of the introduction of enzymic catalysis, prospects for its development, and possible difficulties are discussed in several reviews and symposia[17,19,465-469]. In the present Review we shall disregard specific aspects of the technical application of immobilised enzymes, and give only the principles underlying their use.

Processes in which immobilised enzymes are employed can be classified according to their purpose—(*a*) preparation of a required product as a pure substance or as a component of a mixture, (*b*) removal of an undesirable impurity from production or from industrial wastes, (*c*) chemical conversion of one optical isomer to facilitate resolution of a racemic mixture, or (*d*) concentration or isolation of a required substance by specific sorption. Table 3 gives examples of specific proposals for the application of immobilised enzymes. It includes only those suggestions which have been subjected to at least preliminary testing on a laboratory scale: it omits those for which such testing is lacking.

According to the Reviewer's information, processes for the separation of racemic amino-acids and for the production of 6-aminopenicillanic acid have been introduced on an industrial scale. In view of the comparatively high cost of immobilised enzymes we may expect in the near future the introduction firstly of processes in which expensive products—medicinals and biochemical reagents—are manufactured. The following stage may be large-scale processes in the food and chemical industries.

2. Application in Laboratory Investigations and in Medicine

Immobilised enzymes are being increasingly widely used in fundamental biochemical research as models of subcellular structures and for the study of steric effects on enzyme kinetics. Working models of active transfer have been produced by means of enzyme membranes[486,487]. Brown et al.[487] have effected the active transfer of glucose in a membrane containing hexokinase and phosphatase, and termed the phenomenon "vector catalysis". Certain other researches[281,327] can be regarded as an approach to the production of model biological systems.

The application of immobilised enzymes in practical analysis is also being extended. Several reviews have appeared on this topic[3,21,488-490]. Analytical applications of enzymes can be divided into three groups—(*a*) affinity chromatography, (*b*) determination of substrates in flow-type systems with optical transducers or by means of enzyme electrodes, and (*c*) determination of enzyme inhibitors.

Very many individual papers have been devoted to affinity chromatography, several reviews have been written[21,26,491,492], and Cherkasov has recently published a review in this journal[28]. Here only a few references are given to work in which an immobilised enzyme has been used as affinity adsorbent[291,346,485,493,494].

The use of enzyme electrodes offers a convenient method for determining substrates. They are made from a specific electrochemical transducer, whose sensitive surface is covered with a membrane containing the enzyme[235]. Enzyme electrodes are divided into two groups according to the principle on which they act, i.e. to the nature of the substance determined: (*a*) the product is determined; (*b*) the decrease in the diffusion of the substrate through the enzyme membrane is determined. Data on enzyme electrodes are given in Table 4. In most cases, however, the stability of such electrodes is inadequate, and they are not manufactured by industry, probably for this reason.

Table 3. Proposals for the introduction of enzyme catalysis into industrial technology.

Enzyme	Initial substance or mixture	Product and effect	Reference
	preparation of required product		
Amylase	starch	glucose, glucose syrup	34, 85, 282, 365, 470-473
Invertase	sucrose	invert syrup	89, 369, 441, 454, 455, 474
Proteinase	milk	curdled milk for cheese product	252, 322, 475, 476
Penicillinamidase	benzylpenicillin	6-aminopenicillanic acid	8, 31, 70, 336, 363, 374, 477, 478
Glucose oxidase	glucose	gluconic acid	7
Glucose isomerase	glucose	invert syrup	172, 479
Steroid dehydrogenase	cortisol	prednisolone	179
Oxynitrilase	benzaldehyde	mandelonitrile	100
Polynucleotide phosphorylase	mononucleotides	oligonucleotides, polynucleotides	342, 428
Laevansucrase	sucrose	laevans	103
Adenylate kinase	acetyl phosphate; adenosine diphosphate	adenosine triphosphate	480
NAD pyrophosphorylase		nicotinamide-adenine dinucleotide	71
	removal of undesirable impurities		
β-Galactosidase	milk, whey	hydrolysis of difficultly soluble and assimilable lactose	481, 482, 483
Protease	beer	hydrolysis of proteins which cause cloudiness	225, 423
Protease	antibiotics	hydrolysis of immunogenic protein impurities	355
Naringinase	citrus joices	removal of bitter taste of naringin	302
	resolution of racemic mixtures		
Aminoacylase	acetylated DL-amino-acids	free L-amino-acids and acetyl-D-amino acids	94, 102, 383, 484
	concentration by specific sorption		
Proteases	treated stock	natural protease inhibitors	144, 291, 346, 485

Substrates can be determined also in a system comprising measuring devices, a column of an immobilised enzyme, and a flow-type colorimeter (or fluorometer). The column can be replaced by a tube with the enzyme attached to its inner surface[217,200]. Such systems have been assembled for determining glucose (by means of glucose oxidase[136,137,254]), urea (by means of urease[210,254,255]), uric acid (by means of urate oxidase[254]), and ethanol and lactic and malic acids (by means of the appropriate dehydrogenases[217]).

Fundamentally similar types of equipment are used for determining enzyme inhibitors. Especially high sensitivity is achieved when fluorogenic substrates are used. This method has been employed to determine organophosphorus compounds which inhibit immobilised cholinesterase[126,142,268,500]. Besides high sensitivity, it has the further substantial advantage that, in contrast to chemical

methods of analysis, in which substances are determined by means of their functional groups, enzymic analysis enables toxic substances to be determined from their biological action, which consists precisely in the inhibition of certain key enzymes.

Another application of immobilised enzymes is to controlled proteolysis in determining the primary structure of proteins[159,224,501]. The electrophoretograms of certain enzymes can be developed by applying to the test strip a polyacrylamide sheet in which have been immobilised the enzymes and substrates required for the development[145]. Thus a film containing glucose 6-phosphate dehydrogenase is used to develop phosphoglucomutase, and a film containing these two enzymes to develop pyrophosphorylase.

Table 4. Principles of the action of enzyme electrodes.

Compound determined	Enzyme in membrane	Species determined by transducers	References
Glucose	glucose oxidase	O_2	135, 280, 495, 496
Urea	urease	NH_4^+	142, 161, 497, 498
Glutamine	glutaminase	NH_4^+	466
Asparagine	asparaginase	NH_4^+	147
L-Amino-acids	L-amino-oxidase	NH_4^+	140–142, 499
D-Amino-acids	D-amino-oxidase	NH_4^+	147
Ethanol	alcohol oxidase	H_2O_2	491
Amygdalin	β-glucosidase	CN^-	156, 157

The therapeutic effect of the parenteral introduction of soluble enzymes has been known for a long time. Among the many applications of enzyme therapy the greatest attention has recently been attracted by the treatment of certain malignant growths with asparaginase. But the success of enzyme therapy is very often brought to naught by the rapid decomposition of the enzyme in the living organism and because of the serious immunological reactions caused by the added enzyme as a foreign protein. A way to overcome these obstacles is to replace native enzymes by insoluble or soluble derivatives[502]. Immobilised enzymes can be introduced into the bloodstream, of course, only when their particle size does not exceed a few micrometres. In particular, the use of microencapsulated enzymes is recommended for these purposes. The effectiveness of encapsulated asparaginase[242,276,503,504], catalase[418,505], and streptokinase[277] has already been demonstrated by animal experiments. The injected enzymes possessed negligible immunogenicity and greater stability. But it is not yet clear how the organism will react to the prolonged introduction of considerable quantities of a polymeric carrier of the enzyme. It may therefore be advisable to use biodegradable polysaccharide carriers.

A fundamentally different way of using immobilised enzymes for the controlled variation of blood composition consists in passing the blood through an enzyme column outside the organism. It has been recommended, for example, that the cumbersome and expensive dialysis equipment of the "artificial kidney" should be replaced by a small column and immobilised urease[129,414,458]. The column should contain also a sorbent to bind the ammonia formed.

Many polymeric materials on contact with blood cause haemolysis, which is harmful to the human organism, and extremely dangerous thrombosis. In order to obtain improved implantation materials it has been recommended that polymers should be produced with proteolytic enzymes or proteins attached to them[151]. Materials of this type have been used for the treatment of burns and wounds[446].

The above examples show the possibilities of the use of immobilised enzymes in medical practice. However, the realisation of these possibilities is evidently still more complicated than the modification of technical processes or the establishment of new analytical methods.

NOTE ADDED IN PROOF

While the Review was being prepared for press the number of papers and patents on immobilised enzymes almost doubled, but it was impossible to include this material in the Review. Several symposia have also been held on this topic, among which it is necessary to mention the First All-Union Symposium on the Preparation and Application of Immobilised Enzymes[506] (held in Tallinn in May 1974).

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