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Immobilised enzymes as drugs

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Contents

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Summary

The main objective of the work is to present the state of the art in a relatively new field of biotechnology – the use of immobilised enzymes in applied medicine. Enzyme therapy seems to be promising in the treatment of cardiovascular, oncological, intestinal, viral and hereditary diseases. At the same time there exist many limitations for the everyday clinical use of native enzymes. Enzymes are unstable, have a short lifetime in the circulation, cause toxic and immune reactions, and are costly, etc. These drawbacks can be at least partially eliminated by enzyme immobilisation onto different carriers. Such immobilisation can make enzymes more stable, less antigenic and impart a longer circulation lifetime in the organism. This present contribution reviews the data on natural and synthetic carriers used for enzyme immobilisation, chemistry of enzyme binding, in vitro and in vivo properties of immobilised enzymes as well as their clinical use. Immobilised enzymes can be used as drugs for either local or systemic application (including soluble and insoluble immobilised enzymes for thrombolytic therapy, and for the treatment of both malignant diseases and some in-born enzyme deficiences). Immobilised enzymes can be also used for preparation of artificial cells (based on semipermeable microcapsules, liposomes, cells and cell ghosts). Special attention is paid to the problem of using the immobilisation principles for the construction of drug targeting systems (vector protein and antibody immobilisation, enzyme-polymer-vector conjugates, etc.).

I. Introduction

IA. General considerations

During the last several years the evaluation of the molecular mechanisms underlying many diseases has lead to the increasing use of enzymes as therapeutic agents. This is based on the fact that, since enzymes are the natural regulators of biochemical processes, many pathologies occur when their normal functioning is somehow affected [1,2]. Furthermore, an increasing number of diseases has been found to be due to an inherent deficiency of some lysosomal enzymes (the so-called genetic storage diseases), which can only be treated in general by the administration of the deficient enzyme [3–5].

There exist several approaches in enzyme therapy, which for enzyme drugs can be grouped as: (1) preparations for enzyme replacement therapy (mainly for when surgery on digestive organs leads to a decrease in digestive activity); (2) antitumor enzymes possessing specific degrading ability towards some aminoacids which are the vital components for tumor development; (3) many different enzymes used for the treatment of genetic storage diseases; (4) enzymes for thrombolytic therapy which can act either directly (by degrading thrombi), or indirectly (via the activation of the thrombolytic system of the blood); (5) enzymes which possess activity against some bacterial or viral diseases; (6) different hydrolytic enzymes which act on pathological tissues and enzymes with anti-inflammatory activity.

In all these cases enzymes are extremely effective due to their high activity and specificity (i.e., the ability to affect only one metabolic process among many similar processes). Nevertheless, an analysis of the contemporary pharmaceutical market shows that despite the availability of some enzymatic drugs (produced mainly for replacement, anticancer or thrombolytic therapy), enzymes are used much more rarely than they could be expected to be. This leads to the question: what are the factors limiting the everyday clinical use of enzymes? These factors are of two types. The first includes economical difficulties – the availability of pure and homogeneous enzymes (especially from microbial sources) is relatively low, and they are still rather expensive. Hopefully, contemporary biotechnological methods connected with the cloning of appropriate genes into easily cultivated microorganisms capable of excreting the required enzymes into the cultural media can solve this problem. The second group of factors includes difficulties caused by the nature of enzymes as complex biological macromolecules, which are often heterologous for the treated patient. This can lead to pronounced immunological reactions accompanying the parenteral administration of therapeutic enzymes. In addition, other factors include the frequent nonspecific toxicity of enzymes; their fast degradation and inactivation in the body by endogenous proteases or inhibitors; the relatively fast clearance of therapeutic enzymes from the circulation; and the very low stability of foreign enzymes at physiological pH and temperature resulting in the conformational 'unfolding' of the active structure. Besides these problems, there exist others connected with the low storage stability of enzymatic drugs, which requires their preservation at low temperatures and in the darkness, even for relatively short times. The instability of enzyme preparations requires fast utilization of therapeutic enzyme solutions and leads to a frequent preparation of new drug samples during prolonged therapy. There also exists the impossibility of obtaining high local concentrations of enzymes in the desired region (as a rule, therapeutic enzymes have no specific affinity toward the target zone), without increasing the total body concentration – which is highly undesirable because of side-reactions and loss of expensive drugs. Attempts to solve some of these problems have already been made mainly by the modification of administration methods; for example, the development of catheterization enzymes. It is evident, nevertheless, that a general approach to solving these problems has to be made.

The recent advances evident in the field of enzyme engineering can be considered as a basis towards a general solution. The main goals of enzyme engineering are the study of fundamental problems of enzymes inactivation and stabilization, and the development of stable enzyme preparations for use in industry, medicine and analysis [6–8]. Such preparations, where an enzyme is usually bound with a protective and stabilizing matrix, are called 'immobilised enzymes'.

IB. Possibilities for the immobilisation of therapeutic enzymes

The study of immobilised enzymes for medical application is well documented [6,9,10]. The unique properties of enzymes have permitted their use in areas of medicine other than as parenteral drugs. Thus, enzymes can be used for the sur-

face modification of different prosthetic devices or extracorporeal apparatus (e.g., artificial heart, artificial lung, artificial kidney, equipment for haemodialysis and specific blood purification) in order to increase the biocompatibility of these de-

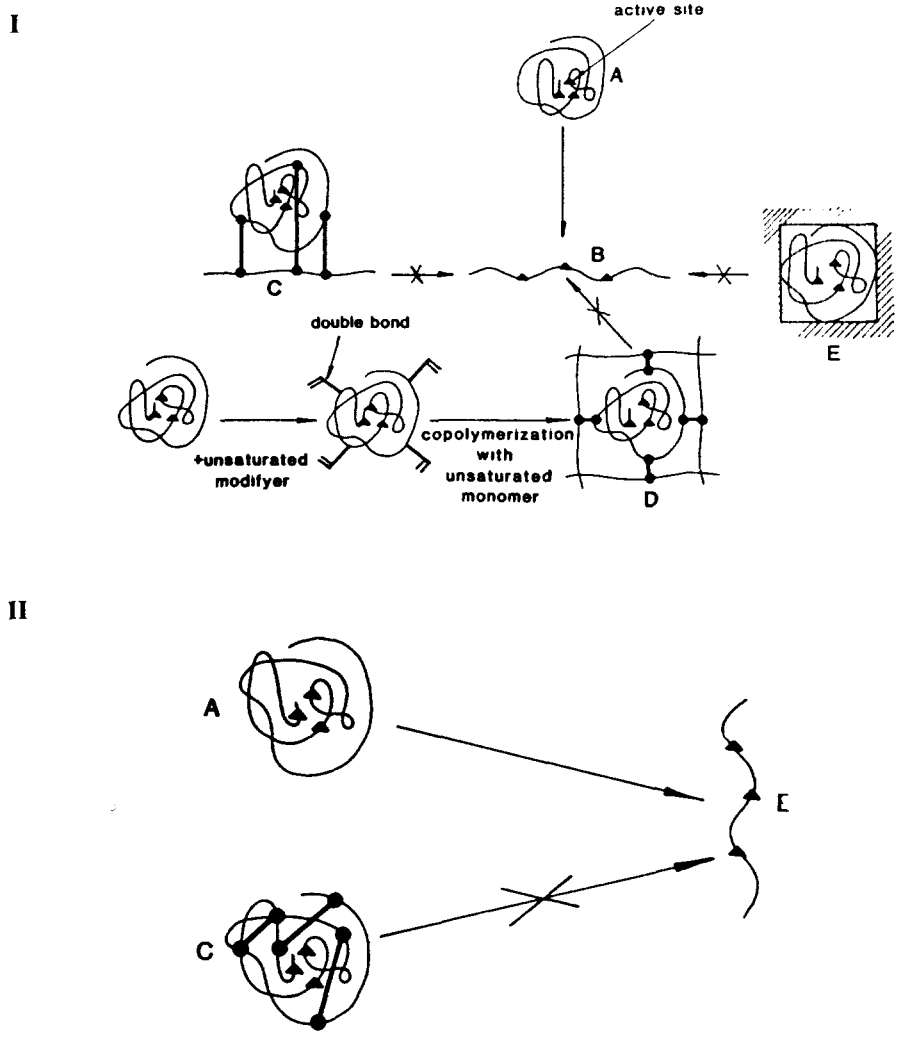

Fig. 1. Schematic representation of possible methods for enzyme stabilization. (I) Stabilization with the use of a carrier. A, native enzyme; B, denaturated form of the native enzyme; C, enzyme immobilization on soluble polymer; D, the copolymerization of a modified enzyme with unsaturated monomer into a three-dimensional gel; E, mechanical entrapment of an enzyme into a polymeric gel. (II) Stabilization of a single-chain enzyme by intramolecular cross-linking with bifunctional reagents. A, native enzyme; B, denaturated form of the native enzyme; C, intramolecularly cross-linked enzyme.

vices and to prevent blood clotting. Enzymes can be also immobilised on the surface of bandages and dressings to facilitate the purification and healing of infected wounds and burns. Enzymes immobilised in the structure of semipermeable polymeric membranes are key components of selective analytical systems and are already widely used in clinical analysis. Although there is a huge quantity of data available on these applications, the present review will deal exclusively with the use of immobilised enzymes as therapeutic drugs.

It is evident from general considerations that immobilised enzymes should possess those properties which are absent in native enzymes. Solutions of immobilised enzymes prepared for parenteral infusion should preserve their activity for at least several hours; once inside the body, immobilised enzymes should be relatively insensitive toward the action of endogenous degrading enzymes, antibodies and natural inhibitors; they should not provoke undesirable side effects in the host organism; upon single administration an immobilised enzyme should have a sufficiently long therapeutic activity; and, finally, but not the least, in the ideal case the carrier matrix should not only stabilize the bound enzyme, but somehow facilitate its accumulation in the affected region.

The most important approaches for stabilizing enzymes are shown in Fig. 1. All of these – enzyme immobilisation on soluble polymers, copolymerisation with vinylic monomers, gel entrapment, intramolecular cross-linking with bifunctional reagents – permit multipoint enzyme-carrier interactions (an intramolecularly cross-linking enzyme molecule can be considered as forming a carrier for itself), and improve upon enzyme stabilization by drastically increasing the activation barrier for the process of enzyme unfolding into inactive structural forms. All the above ap-

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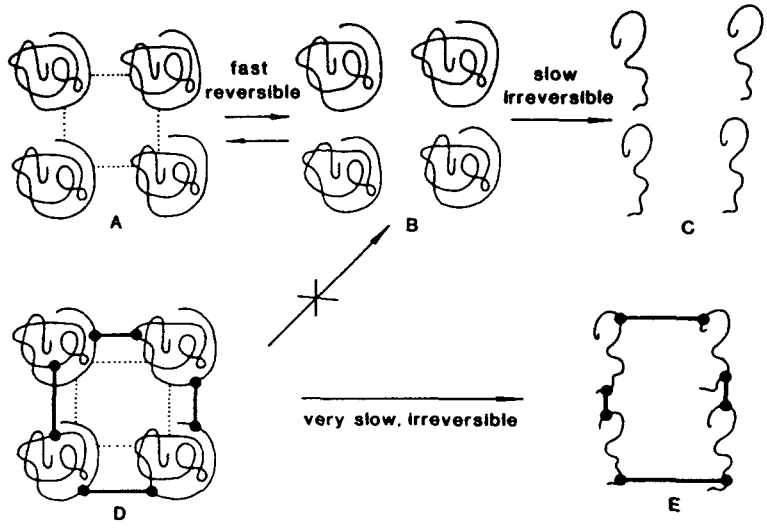

(III) Stabilization of a subunit enzyme by intramolecular intersubunit cross-linking with bifunctional reagents. A, native subunit enzyme; B, reversibly dissociated subunits; C, irreversibly denaturated subunits; D, intramolecularly cross-linked enzyme; E, irreversibly denaturated cross-linked enzyme.

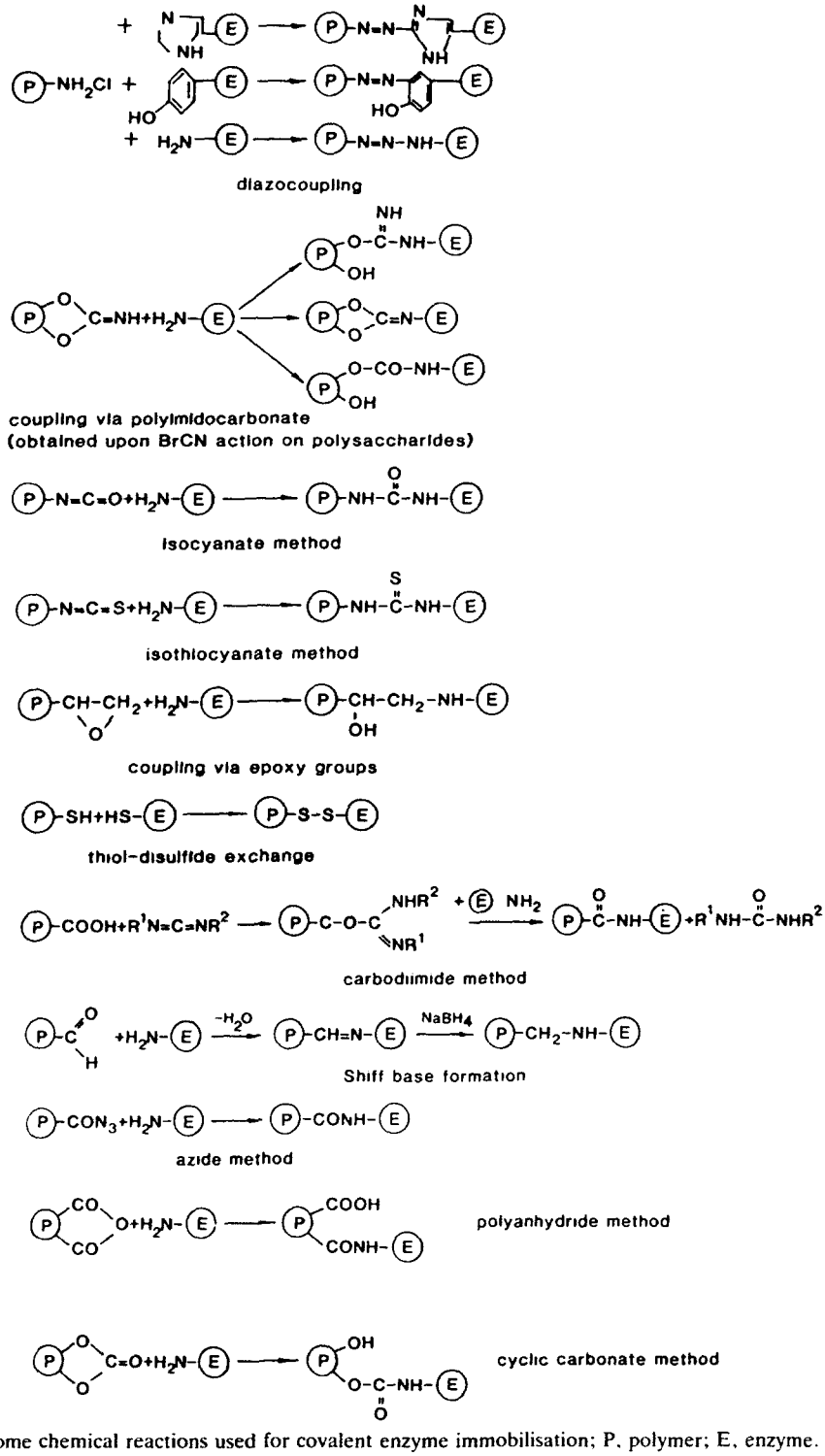

Fig. 2. Some chemical reactions used for covalent enzyme immobilisation; P, polymer; E, enzyme.

proaches have already been used to stabilize enzymes for medical purposes. The most popular are the chemical methods of enzyme-carrier binding. The problems of enzyme covalent immobilisation have been considered in detail [6–8]. Fig. 2 gives which reactive groups are usually introduced into a polymeric carrier, and how the interaction of the carrier with the protein molecule proceeds (presumably via ϵ-amino groups of lysine residues of polypeptide chain). During covalent binding other protein reactive groups can be involved, including: terminal aminogroups; terminal glutamic or asparaginic carboxy-groups; the imidazole of the histidine residue; phenolic groups of tyrosine residues; cysteine and cystine SH or S–S groups; hydroxypyrolines; hydroxyglutamines; hydroxylysines; hydroxy groups of serine residues; guanidines of arginine residues; and some other functional groups of both amino-acid residues and even of the main polypeptide chain.

Possible methods of immobilised enzymes administration may be divided into two principal groups (which can overlap); (1) immobilised enzymes intended for prolonged circulation in the blood, and enzymes which must be necessarily present in different tissues and organs of the body; and (2) immobilised enzymes intended for local deposition during the treatment of discrete lesions (e.g., thrombi, tumors, atherosclerotic injuries) or of individual organs.

In the first case it appears reasonable to create water-soluble stabilised enzymes by binding enzyme with soluble polymer in order to create constructs having a higher stability against different denaturating actions and a prolonged blood circulation life-time. It is desirable that the immunogenicity, toxicity and other undesirable side effects of such preparations be low. This group can also include the so-called 'artificial cells', which may be obtained by enzyme entrapment into microcapsules, liposomes, cells and cell ghosts.

In the second case, where an enzyme should be used locally and its presence in other organs and tissues is undesired, one should consider the synthesis of biocompatible and biodegradable enzyme derivatives (slow release systems), which can be localized in the affected zone and degraded there over the required period of time, continuously releasing an active agent into the local surroundings. The immobilised enzyme in this case either can leave the matrix via diffusion of the native form or, being covalently bound with the degradable matrix, it can be solubilized together with the matrix fragment. Either will result in an additional stabilisation of the enzyme (see schematic representation in Fig. 3).

The logical development of the enzyme localisation method is the concept of the targeted immobilised enzyme [11–13]. The principal idea of this concept is the immobilisation of an enzyme (or any other drug) on a carrier, which is capable of specific recognition and binding to components of a target organ or tissue, and which leads to its concentration in an affected zone.

A very important aspect of such carriers is the effect of immobilisation on the antigenic properties of enzymes. Optimally (especially when dealing with therapeutic enzymes of microbial origin), a decrease in protein allergic properties and immunogenicity can be caused by partial or complete blockage of protein antigenic determinants with a carrier macromolecule. Clearly, the carrier itself must be devoid of allergic properties or immunogenicity, and new antigenic determi-

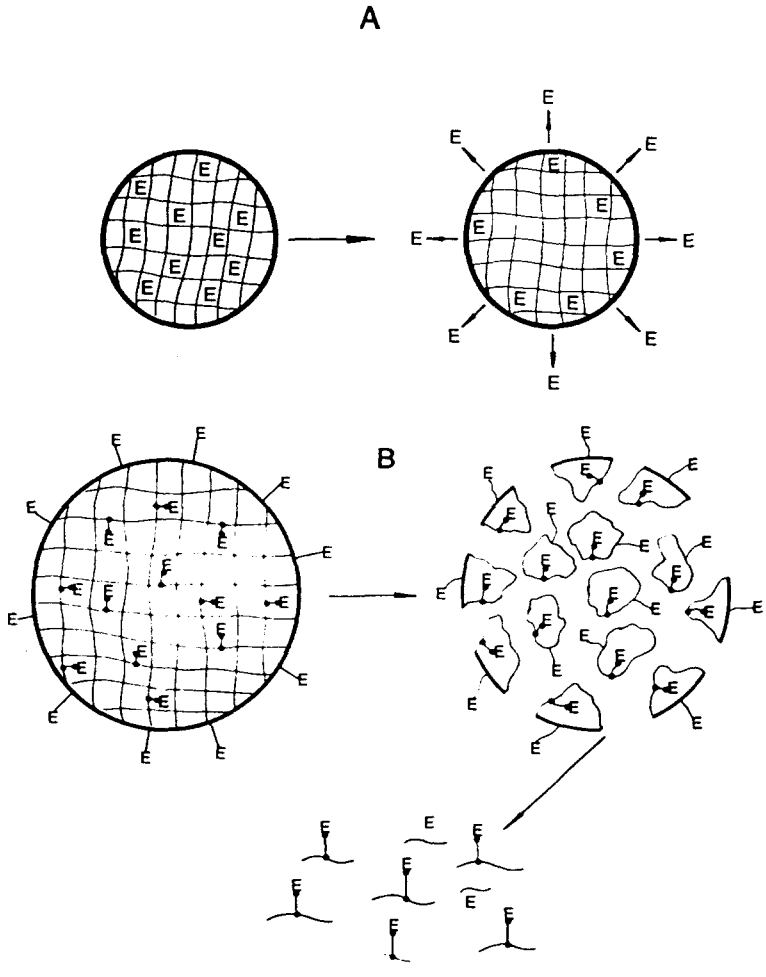

Fig. 3. Enzyme incorporation into polymeric microparticles (E, enzyme). A, non-degradable microparticles; enzyme releases via diffusional mechanism in the native form; B, degradable microparticles; enzyme releases simultaneously with microparticle destruction in the native form or being covalently coupled with a solubilised fragment of a carrier.

nants should not appear upon enzyme-carrier interaction. For the extracorporeal use of immobilised enzymes these problems do not seem so important, because the contact between an antigen and immunocompetent cell can be easily prevented.

The principal groups of therapeutic enzymes and possible methods for their immobilisation are presented in Table I.

Before a careful consideration of the problem is made two important points should be taken into account. First, so many data on immobilised therapeutic enzymes exist in the literature that it is just impossible to discuss all of them, and hence only some principal results will be given (especially those from the patent

TABLE I

THE PRINCIPAL TYPES OF IMMOBILISED THERAPEUTIC ENZYMES

Preparation, possible methods of application (1)	Typical examples (2)	Type of action (3)	Possible methods of immobilisation (4)
Enzymes for replacement therapy in the treatment of digestive organ diseases (implantation, parenteral application)	pepsin chymotrypsin trypsin amylase lipase or their mixtures	local	microcapsulation entrapment into polymeric gels and particles
Antitumour enzymes (implantation; intravascular, parenteral or extracorporeal application)	asparaginase arginase nuclease desoxyribonuclease	local and/or systemic	microencapsulation entrapment into liposomes entrapment into red blood cells ghosts entrapment into gel particles immobilisation on soluble polymers
Enzymes for therapy of inherited lysosomal enzymes insufficiency (intravascular application)	glucosidase glucuronidase galactosidase	local	entrapment into liposomes (with facultative preliminary stabilization by intermolecular cross-linking or immobilisation on soluble polymers)
Enzymes for thrombolytic therapy (intravascular application)	plasmin urokinase streptokinase tissue plasminogen activator	local and/or systemic	immobilisation on soluble polymers entrapment into red blood cells ghosts immobilisation on biodegradable microparticles
Antibacterial, antiviral, antiallergic enzymes (intramuscular or intravascular application)	penicillinase lysozyme nucleases	systemic	immobilisation on soluble polymers entrapment into microcapsules, red blood cells ghosts, liposomes
Hydrolytic enzymes for the action on pathological tissues and anti-inflammatory enzymes (local application)	trypsin chymotrypsin collagenase papain ribonuclease desoxyribonuclease peptidase lysozyme	local	immobilisation on material for dressing, drainages, tampons (films, fibres, etc.) entrapment into microcapsules immobilisation into powdered polymeric gels

sources). Second, the majority of experiments has only been performed either on cell cultures or in experimental animals. The clinical evaluation of immobilised therapeutic enzymes is at the beginning, with careful approaches by the appropriate regulatory authorities evident.

II. Immobilised enzymes for prolonged circulation in blood

As stated above, therapeutic immobilised enzymes capable of a prolonged functioning in the body without the loss of specific activity (due either to the degradation, inhibition or capture by cells of the reticuloendothelial system) can be formed either by enzyme immobilisation on soluble polymeric carriers or by their entrapment into 'artificial cells'. Both approaches are well developed.

IIA. Soluble polymeric derivatives of therapeutic enzymes

There exists a large number of water-soluble reactive polymers (including commercially available ones) which can be considered for enzyme immobilisation. However, some strict requirements for use reduce this number considerably. From the medical point of view a carrier polymer for the preparation of parenteral drugs should possess the following properties: high purity and homogenicity in respect of both composition and molecular-weight distribution; complete biocompatibility, i.e., the absence of any undesirable reactions in the site of application and in the whole organism; the ability to undergo biodegradation with a reasonable rate, and the absence of any biological activity in the products of biodegradation; and a good clearance from the body after the function of a polymer-bound drug has been fulfilled. The polymers which have been already tried as possible carriers for the immobilisation of therapeutic enzyme belong mainly to the following groups: soluble polysaccharides; polyaminoacids; synthetic polymers and copolymers obtained by polymerization or polycondensation; and proteins.

A great deal of attention has been paid to obtaining synthetic polymer carriers, as there exist considerable possibilities here for regulating the properties both of a carrier itself and of a bound enzyme. A detailed review on synthetic carriers intended for enzyme immobilisation is given in Refs. 14–18. Typical examples of inert biodegradable polymers used for obtaining soluble immobilised enzymes are shown in Fig. 4.

The behaviour of a (bio)polymer after its administration may depend on many factors. These include the molecular weight of both polymer and polymer-enzyme construct. This is of great importance as it defines to a considerable extent the clearance rate of the appropriate preparation from the circulation and its distribution in the body. An inert chemical polymer may cause undesirable side effects in the organism if its molecular weight exceeds some critical value. Thus, biologically stable polymers, or fragments of biodegradable polymers, which should presumably be released from the body via the kidneys, may accumulate there, decreasing the efficacy of the kidney filtration if a polymer molecular weight exceeds 60–80 kDa. This phenomenon may be termed 'physical toxicity'. Naturally, for biodegradable polymers the situation becomes seemingly more simplified, and this explains why methods for synthesising biodegradable polymers have been actively pursued.

There exists a number of approaches for the synthesis of polymers capable of splitting under physiological conditions [14,17]. As enzymatic degradation is the

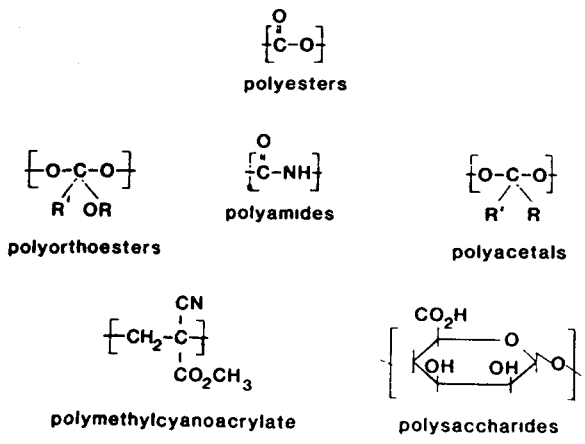

Fig. 4. Some examples of biodegradable polymers (as repeated units).

main route of biodegradation, the introduction of groups which resemble natural substrates of lytic enzymes (peptide, saccharide or nucleotide bonds) is the prime approach to obtaining biologically degradable polymers.

What are the main effects achieved upon the multipoint binding of enzymes (and other proteins) with water-soluble polymers? Firstly, there is an increase in both the conformational stability and the stability towards the action of endogenous proteases, due to the presence of a carrier molecule and the chemical modification of lysine residues sensitive to trypsin-like proteases by moieties which create steric hindrances for hydrolysis [19]; secondly, there is an increase in the circulation lifetime of the active form, due not only to an increase in enzyme stability, but also to a decrease in the rate of its filtration by the kidneys, as well as by the prevention of any interaction with cell receptors, which could result in removal of the proteins from the circulation; thirdly, protein modification by polymers enables a direct regulation of any host immune response.

In one of the initial studies it was shown that α-amylase and catalase bound with BrCN-activated dextran have a slow clearance from the blood in rats [20], such that 2 h after intravenous administration 75% of immobilised α-amylase (but only 16% of the native enzyme) remained in the circulation. After 2 h the blood activity of immobilised catalase was higher than 50% from the initial, whereas the native enzyme disappeared from the blood within 1 h. In the same work it was shown that upon intraperitoneal administration of immobilised enzymes, their activity in the blood was found over a more prolonged period of time: i.e., the native catalase was not found 4.5 h after administration, whereas the immobilised enzyme exhibited more than 30% of its initial activity 22 h after administration. Also, the intraperitoneally administered immobilised catalase was found to provoke a 3-fold higher increase in catalase activity in blood than did the same dose of the native enzyme. These authors explained these effects by a slow clearance of a high-molecular-weight enzyme/polymer conjugate, and a protective effect of the dextran matrix. It has been also shown that use of an immobilised amylase decreases a host immune response [21].

Analogous results have been obtained upon the immobilisation of enzymes used as antitumour agents. Thus, the covalent binding of carboxypeptidase G and arginase with soluble BrCN-treated dextran – with a molecular weight of 40000 – considerably prolongs the enzyme half-life in the blood of both healthy mice and mice with inoculated tumours [22]. The half-life for carboxypeptidase clearance, 3.5 h for normal mice and 7 h for mice with tumours, is increased up to 17–18 h for dextran-bound enzymes. The half-life for arginase clearance is also increased, from 1.4–2.5 to 12–17 h.

The methods for obtaining soluble derivatives of urokinase modified with BrCN-treated or partially oxidized dextran with a molecular weight of 20000 and possessing increased stability and lifetime in the circulation, are described in Refs. 23 and 24. In those studies the authors succeeded in binding between 100 and 10000 units of an active enzyme per g carrier, the binding being carried out both directly and via spacer groups containing 1–3 carbon atoms.

The protection afforded to enzymes by linking them to biocompatible synthethetic polymers and copolymers can be seen from the studies by Abuchowski et al. [25], who showed in mice that catalase immobilised on different polyethyleneglycols (via 2,4,4-trichlorotriazine) retains 90% of its initial activity and is removed from the circulation several orders of magnitude slower than the native enzyme. In the same way, the covalent binding of urease and trypsin with polyethyleneglycol noticeably increases the circulation time of enzymes in the blood of experimental animals [26]. Also, this group has shown that modification of glutamyl-L-asparaginase, phenylalanine ammonia lyase and uricase of animal and microbial origin with polyethyleneglycol increases the activity of the enzymes used in the treatment of mice bearing inoculated lymphosarcoma 6C3HE and other tumors [27], and it was demonstrated that the animals' survival time (20–24 days) was more than twice that for animals treated with native enzymes. These modified forms of proteolytic enzyme, the natural substrates of which are different high-molecular-weight substances, are of great potential importance clinically. A number of examples by the Abuchowski and Davis group demonstrates that protease conjugates with water-soluble polymers more often preserve the same activity towards low- and high-molecular-weight substrates than do the corresponding insoluble derivatives. This is rather important, as one of the main requirements of using a therapeutic enzyme in an immobilised form is its ability to interact with the natural, and usually high-molecular-weight substrates. Moreover, it has been demonstrated that the binding of protease enzyme with soluble polysaccharides and vinylic polymers leads to the formation of the derivatives which possess an increased heat stability as well as a decreased affinity toward natural enzyme inhibitors or suppressors. Hence, Lindenbaum et al. [28] have shown a correlation between a decrease in acute toxicity of dextran-bound enzymes and a decrease in immobilised enzyme affinity for natural macromolecular inhibitors. As a result of this effect the therapeutic potential of the immobilised proteolytic enzymes considerably increases. Among other examples there is kallikrein, which is covalently bound with activated polyvinylpyrrolidone, being capable of decreasing canine blood pressure for a considerably longer time than the native enzyme, with a simultaneous sharp

decrease in side effects [29]; and there are α-amylase and lysozyme bound with activated soluble dextran; or there is lysosomal hydrolase β-D-*N*-acetylhexoseaminidase – this last enzyme is of great importance, as it is used for the treatment of hereditary Tay–Sachs disease – bound with activated polyvinylpyrrolidone, possessing an increased stability towards exogenous proteases and having a noticeably increased clearance time from the circulation in experimental animals [30].

In cancer chemotherapy nucleodepolymerases (nucleases) of microbial origin stabilized by covalant binding with soluble aminobenzoyloxymethyldextran possess a higher activity than native enzymes [31]. Interestingly, these derivatives pentrate into the intact cells of Ehrlich's ascite carcinoma in the same manner as a native enzyme; this is found using dextrans having molecular weights between 20000 and 40000, and it suggests that such conjugates are able to penetrate the vascular barrier, since in mice it was shown that such immobilised enzymes, where immobilisation was performed via diazocoupling reaction, possess a 3–4-fold higher activity in the treatment of carcinoma than the native preparations. Further, intramuscular injection of such preparations in mice with inoculated sarcoma 37 also demonstrated an increased therapeutic activity.

Other examples of using immobilised enzymes include mannanase, collagenase and glucuronidase immobilised onto dextran and exhibiting an increased therapeutic activity upon their parenteral administration in rats with experimental arthritis [32].

It has been mentioned above that the possibility for regulation of immune response to foreign proteins is an important result of the immobilisation of therapeutic enzymes. The chemical nature of a carrier rather often defines the character of a polymer effect on the immunogenicity of conjugated protein antigen. It has been shown more than once that the use of polysaccharides and polyethyleneglycol [20,25,26,33] leads to a decrease in immunological and allergic reactions towards proteins upon the administration of immobilised proteins (e.g., for catalase, streptokinase, bovine serum albumin, etc.). This phenomenon is regarded as due to the decreased ability of modified enzymes to stimulate the formation of antibodies and bind the circulating ones. One of the reasons for this can be postulated as due to steric hindrances for normal protein–receptor or protein–antibody interaction, created by the presence of a polymeric carrier matrix. One can hope that for the use of heterologous therapeutic enzymes, enzyme-polymer conjugates can serve as an effective means of reducing allergic reactions upon the repeated administration of exogenous enzymes bound to synthetic polymers. For example immune responses to trypsin and urease are decreased upon their immobilisation onto synthetic polymers [26]. In a number of cases enzyme/carrier conjugates are capable of actively affecting immunogenesis. Thus, upon the immobilisation of proteins (enzymes) onto copolymers of D-glutamic acid and D-lysine conjugates are formed which possess the ability to suppress specific immune responses, leading to the prevention of the immunoglobulins E formation responsible for local and systemic allergic reactions [34]. However, conversely, it has been found that some synthetic polyelectrolyte carriers are capable of actively increasing immunogenesis, provoking an increased formation of antibodies in response to the administra-

tion of proteins bound with such carriers in an electrostatic complex which is capable of being destroyed under physiological conditions [35]. Polyelectrolytes used here include polyacrylic acid, poly-4-vinylpyridine, poly-2-methyl-5-vinylpyridine, copolymers of 4-vinyl-*N*-ethylpyridinium bromide, 4-vinyl-*N*-ethylpyridinium bromide, 4-vinyl-*N*-cetylpyridine and 4-vinyl-*N*-acetylpyridine. The principle of obtaining highly antigenic conjugates from binding weakly antigenic proteins to stimulating carriers can be used for preparing synthetic vaccines of a new type, which may be used to produce an immune response to all antigens of any individual including those who are poorly reactive.

Most therapeutic enzymes immobilised onto soluble carriers have been studied only in animals. One of the few exceptions is streptokinase, which is an enzyme produced by a haemolytic streptococci and which activates plasminogen (the precursor of the fibrinolytic enzyme plasmin). Due to its low cost and high thrombolytic activity this enzyme is widely used in many countries for the treatment of myocardial infarct, and pulmonary arterial thromboembolism as well as other thromboses. At the same time, this enzyme clearly demonstrates the drawbacks of using therapeutic enzymes in the native form, especially in case of thrombolytic therapy (see Tables II and III). This had led to numerous attempts to improve streptokinase properties by its modification with soluble polymers. Thus, it has been shown that streptokinase bound with BrCN-activated polysaccharide possesses an increased stability as well as retaining its activity by lysing model thrombi [36,37]. (The use of toxic intermediate products in Ref. 37 for obtaining conjugates appears to mitigate against their use for practical applications.) In several studies streptokinase, or its binary complexes with plasminogen, have been modified by polyethyleneglycols of different molecular weights (2000, 4000, 5000) and by polyole F 38 [38–40]. The activation of polymer was carried out with cyanurochloride. As a result it is possible to obtain streptokinase derivatives with increased stability against both heat denaturation and the action of proteolytic enzymes. At the same time the antigenicity of modified streptokinase or streptokinase-plasminogen complex was considerably decreased as compared with the native protein. Modi-

TABLE II

NATURAL SHORTCOMINGS OF NATIVE THROMBOLYTIC ENZYMES

Antigenicity
Non-specific toxicity
Low stability in physiological conditions
Depression of haemostasis coagulative chain

TABLE III

CLINICAL LIMITATIONS IN THE USE OF NATIVE THROMBOLYTIC ENZYMES

Frequent allergic reactions
The necessity for prolonged infusion
Hemorrhagic and rethrombotic complications
The difficulty of control and combined therapy with heparin

fied streptokinase has also been obtained using a vinylpyrrolidone copolymer which contained reactive aldehyde groups [41]. Here, under optimal conditions 14–16 covalent bonds between the protein globule and the carrier molecule can be formed. It was found that the specific activity of the immobilised enzyme decreases with the increase in the molecular weight of the carrier, which is probably due to the development of a steric barrier for the interaction between streptokinase and plasminogen.

To date, the best results have been obtained using streptokinase immobilised onto soluble dextran activated by partial oxidation. The covalent binding of an enzyme takes place via the reaction of the Schiff base formation between the enzyme free amino groups and aldehyde groups of the activated carrier. To prevent the hydrolysis of Schiff bases under physiological conditions the bases can be reduced by sodium borohydride or cyanoborohydride, and simultaneously, the reduction of non-reacted aldehyde groups of the carrier into inert oxidative groups will take place. It has been shown that the enzyme immobilised onto dextrans with molecular weights of 35000–50000 became more stable in the circulation, and caused less pronounced toxic and allergic reactions; also its antigenicity decreased by approx. 30–fold [42–45]. Immobilised streptokinase, under the trade mark of 'Streptodekaza'®, has been produced in the U.S.S.R. on an industrial scale since 1980, and is approved for clinical use in the treatment of cardio-vascular and ophthalmological pathologies caused by thromboses. When using Streptodekaza an increase in fibrinolytic activity in the blood is observed up to 80 h after administration. The use of Streptodekaza so far has had practically no complications [46]. Using native streptokinase 72% of the cases give rise to hemorrhagic complications, 14% to rethromboses and 27% to allergic reactions; in the case of Streptodekaza these figures are 6%, 5.5% and 2%, respectively, for patients with an acute myocardial infarct. Such properties have permitted therapy to be changed from continuous intravenous infusion used in the case of the native enzyme, to a single injection of the whole dose in a small volume of the physiological solution. For the treatment of cardiovascular diseases the whole therapeutic dose of the preparation equals between 1.5 to 6 million units in 20–40 ml of physiological solution, and the time for dose administration does not exceed 5 min. When using Streptodekaza, in contrast to the native enzyme, heparin therapy can be started after 6–12 h. Upon the treatment of eye thrombotic diseases the whole dose (30–50 thousand units in 0.2–0.3 ml of the physiological solution) is administered as a single injection into the vitreous body. Blood lysis has been observed in the vitreous humour in 90% of the cases, with the visual functions increasing in 60% of the cases. The details of Streptodekaza action have been considered in Refs. 42–46.

Animal studies of plasmin and urokinase derivatives which possess analogous properties to streptokinase have been described [47,48]. Thus, preparations of plasmin immobilized onto partially oxidized dextran contain up to 190 mg of an enzyme per g of a carrier, with enzyme activity being 85% of the native activity (against *N*-benzoyl-L-arginine ethyl ester as a substrate), and having a specific fibrinolytic activity of 22 U/mg. Stabilized urokinase has also been obtained by immobilisation onto soluble copolymer of acrylamide and acrylic acid (molecular

weight: 70000, with the amount of acrylic acid units being 10%). In the latter case, the immobilisation was carried out using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide as a coupling agent. Optimization of the reaction conditions permitted up to 180 mg of an enzyme per g of a polymer to be bound with an immobilisation yield of up to 90% and a retention of the specific activity of up to 95% [49].

The modification by water-soluble polymers has been used for obtaining not only stabilised enzymes, but also some other therapeutic agents of protein origin. In particular, attempts to obtain haemoglobin polymeric derivatives in order to create semisynthetic blood substitutes are of great importance. Is has been shown that haemoglobin binding with bromo, amino or aldehyde derivatives of dextrans gives products which possess 3–20-fold higher circulation lifetime compared to the native haemoglobin, with haemoglobin functional properties being the same [50,51]. It is encouraging to observe that the experiments carried out on animals with hematocrit values of less than 2% showed that the transfusion of dextran-haemoglobin complex allowed animals to survive and to recover fully without additional enrichment of the air with oxygen.

The problem of using protein inhibitors of enzymes is very closely connected with the clinical application of enzymes. One of the inhibitory polypeptides which is widely used in clinical practice is a polyvalent inhibitor of proteolytic enzymes from bovine pancreas. The pancreatic inhibitor effectively inhibits kallikrein, trypsin, chymotrypsin, plasmin, plasminogen activators, blood clotting factors, tissue and leucocytic proteases. It is used in the treatment of allergic pathologies, various shocks, sepsis, acute pancreatitis, mechanical and thermal injuries, artrozoarthritis, primary hyperfibrinolytic bleeding, and also for the treatment of myocardial infarct when it demonstrates a pronounced anti-ischaemic effect. Unfortunately, the therapeutic doses of pancreatic inhibitor are very large because of its fast clearance from the circulation; the half-life of the preparation in the circulation depends on the animal species and is only between 10 and 70 min. To prolong an inhibitor action a number of derivatives modified by soluble polymers have been obtained. Thus, inhibitor preparations, possessing a prolonged lifetime (in rats this increased 10-fold!), and an unchanged activity have been described [52,53]. So have been the kinetic parameters of the association reaction with proteases. The application of soluble high-molecular-weight pancreatic inhibitor in dogs with experimental acute pancreatitis increases animal survival by 1.5-fold and by 2-fold, respectively. Most interestingly, introduction into the carrier of sugar moieties which have their receptors in the liver cells permitted direct accumulation of the modified inhibitor in the liver [54].

Apart from the pancreatic inhibitor of kininogenases, some other inhibitors of the kallikrein system have been immobilised onto water-soluble carriers [55], including the nonapeptide bradykinin, and a synthetic hexapeptide inhibitor of angiotensin-I-converting enzyme. It has been shown in vitro that this latter peptide, modified by soluble dextran, expresses 24% of inhibitory activity of the native peptide [55]. Bradykinin bound with dextran activated by cyanogen bromide or periodate has an unchanged immunological activity, and retains about 6% and 25%

of biological activity, respectively. Similarly, angiotensin-I-converting enzyme in vitro hydrolyses dextran-bound bradykinin considerably slower than the native form; at the same time, the macromolecular forms of kinin inhibitors inhibit the substrate hydrolysis by angiotensin-I-converting enzyme effectively enough. These results lead to optimism that dextran-modified bradykinin will be more active in vivo than its native form.

In a number of cases the comparison of biological activity of native enzymes and their macromolecular derivative counterparts allows elucidation of some of the important aspects of the functioning of physiologically active substances. Such studies have been usually carried out with low-molecular-weight drugs, hormones and neurotransmitters (see a detailed review in Ref. 56). Nevertheless, the technique has been fruitful in studying the biological activity of the hexapeptide hormone gastrin, the physiological role of which is to stimulate some of the main functions of the alimentary tract. With the help of gastrin conjugated to different proteins, autoantibodies against gastrin have been found in blood, which can be considered [57] as evidence for the existence of an autoimmune regulation of gastric gland action, as antibodies against gastrin lowered a normal gastric secretion. Macromolecular forms of gastrin, unlike the native hormone, affect only functioning gastric glands. Also, the post-effect of albumin/gastrin conjugates is found within several hours after the injection. From these results the authors suggested a hypothesis of gastrin action via firm binding with antibodies leading to their removal from the circulation as immune complexes.

It is expedient to include into this section on water-soluble preparations of immobilised enzymes for clinical application, mention of enzymes stabilized without the use of a carrier, i.e., by chemical modification of protein or by the introduction into a protein of intramolecular cross-links, which hamper the unfolding of an enzyme molecule (see review Ref. 58). The principle of stabilisation of single-chain and subunit enzymes by intramolecular cross-linking with the help of bifunctional reagents [59,60] is illustrated in Fig. 1. To achieve an optimal stabilisation effect, it is currently necessary in each case to find empirically cross-linking agents of such a size which maximally correspond to the distance between the groups to be linked in the protein molecule. The preparations obtained possess higher stability against unfolding and the action of proteases. The clearance rate and inhibition activity of these modified enzymes should be the same as for the native enzyme. Nevertheless, the approach can be applied to the enzymes which need to penetrate a cell or bind with receptors in the cellular membrane, where the presence of a polymeric carrier may hinder these processes.

A relatively new method of stabilizing therapeutic enzymes is their immobilisation onto the substances which are either characteristic of the organism itself (e.g. proteins), or even possess their own useful biological activity. A partial review of such examples is given in Ref. 61. Examples include the immobilisation of lysozyme and asparaginase onto collagen, the antigenic properties of which may be decreased by a preliminary limited proteolysis [62]. Also enzyme immobilisation onto fibrin, preliminarily treated by heating to reduce antigenicity, has been described in Ref. 63.

The immobilisation of α-galactosidase, uricase and β-asparaginase onto albumin ensures a considerable stabilisation of the enzymes against thermal and proteolytic inactivation in vitro [64,65]. However, trypsin modified with albumin is subject to autolysis, though the action of serum inhibitors is less than found with the native enzyme [66]. Poznansky [67] has shown in animal experiments that the circulation half-life of uricase/albumin conjugate in dog after intravenous injection totals 26 h as compared with 4 h for the native uricase. In the same work it was shown that for the native enzyme the first injection decreased the amount of uric acid in the blood plasma by 35% with a subsequent return to the initial level with the half-time of about 24 h; the second injection is less effective; and the third one causes an anaphylatic shock. This fact points out the quick formation of antibodies against uricase. In contrast, repeated injections of uricase immobilised onto albumin remain as effective as the first one, with no immunologic complications.

Taking into account the fact that thrombolytic therapy usually goes together with heparinisation, it has been suggested that thrombolytic enzymes be immobilised onto heparin – which is a rigid chain macromolecule suitable for the stabilisation of a protein bound to it [68]. The protein can easily be coupled with carboxy groups of the heparin molecule via activation with soluble carbodiimides [68] (as given in Fig. 2.) Using α-chymotrypsin and thrombolytic enzyme urokinase it was shown that the immobilisation onto heparin causes significant enzyme stabilization against heat inactivation without affecting either enzyme specific activity or heparin's ability to slow down ADP-induced platelet aggregation. Moreover, it was shown [68] that urokinase bound with heparin can be additionally modified by low-molecular-weight physiologically active substances, for example by sodium nitroprusside, which is a strong hypertensive agent [69,70]. As a result of such binding, nitroprusside circulation half-life in hypertensive rats increases (7–10)-fold, and its efficacy remains normal. Such experiments open the way for obtaining complex-immobilised enzymes, which possess simultaneously a number of useful physiological activities, that could lead to both a considerable simplification in the therapy, and to making it more effective.

One more example of using 'natural' carrier may be the immobilisation of asparaginase on γ-globulin as described in Ref. 71. The enzyme in such a conjugate is not only more stable than the native asparaginase, but is more slowly removed from the blood of experimental mice and rats.

Finally, soluble stabilised enzymes can be obtained by their intermolecular cross-linking using bifunctional reagent. Thus, the cross-linking of β-galactosidase (the absence in the body of which provokes Fabry disease) leads to a noticeable enzyme stabilization [72]. Other properties of intermolecular cross-linked enzymes include a slow down in the rate of their digestion within lysosomes [73]. Increase in circulation half-time for enzymes linked intermolecularly can be due to an increase in their stability against physiological conditions and natural mechanisms of inactivation, and simply because of the increase in the enzyme molecular weight upon intermolecular cross-linking. For example, the dimer of pancreatic ribonuclease has a half-time for clearance from the circulation of rats which is 12-fold higher than that found for the native enzyme [74].

One should bear in mind that the formation of protein conjugates, having a great number of groups capable of simultaneous interaction with cell surfaces, may lead to an increase in their capture by cells. High capture of ribonuclease dimers and polymers has been demonstrated in both cell cultures [75] and rat in in vivo experiments [76]. An increased capture of antibody aggregates in kidney capillaries has also been demonstrated [77]. Nevertheless, according to Ref. 78 soluble cross-linked enzyme polymers may be of great interest for enzyme therapy.

Thus, the studies given above show that the field of soluble, stabilised therapeutic enzymes is widespread and touch upon practically all groups of enzymes used as drugs. Notwithstanding the fact that the majority of studies have been in the laboratory, the results obtained permit hope for a wide application of immobilised enzymes, though it must be emphasised that from the practical point of view the synthesis of such preparations is facile and cost-effective.

IIB. Artificial cells

The so-called 'artificial cells' are one more form of immobilised therapeutic enzymes meant for prolonged circulation in the organism. This term, introduced by Chang [9,79], initially included impermeable and semi-permeable microcapsules, into the inner space of which an enzyme (or, in a more general form, any other drug) was encapsulated. More recently, the term included normal cells, cell ghosts and synthetic liposomes.

Microcapsulated enzymes possess a number of advantages for therapeutic purposes: (1) the microcapsule membrane, on one hand, protects an enzyme from contact with an aggressive physiological medium, and on the other hand, protects tissues and organs from undesirable effects of the immobilised drug; (2) considerable amounts of the enzyme may be encapsulated into microcapsules, which increases the enzyme/carrier ratio up to 1 000 000:1; (3) the surface area/volume ratio of microcapsules is maximal at a given volume, as a result there is a fast penetration of substrates and exiting products of enzymatic reaction via the membrane; and (4) an enzyme may be encapsulated into microcapsules as an additionally stabilised form; moreover, multienzyme complexes, enzyme mixtures or even intact cells may be encapsulated inside 'artificial cells'. Fig. 5 presents some peculiarities of enzyme encapsulation either in synthetic semi-permeable microcapsules or in red blood cell ghosts.

Disadvantages in the use of microcapsules include the problems connected with the use of synthetic materials (polyamides, polyurethanes, etc.) in the body, and the risk of vessel embolisation upon the accumulation of microcapsules in the circulation as a result of prolonged and repeated application. That is why during the last few years a great volume of work on obtaining biodegradable microcapsules (made from polyactic acid, alginate, etc.) has been carried out, in parallel with development of methods for the extracorporeal application of reactors with microcapsulated enzymes for detoxication of biological fluids.

Microencapsulated enzymes can be administered intravenously, intramuscularly, intraperitoneally [79,80–82], or applied perorally [83,84], or locally [85] as a

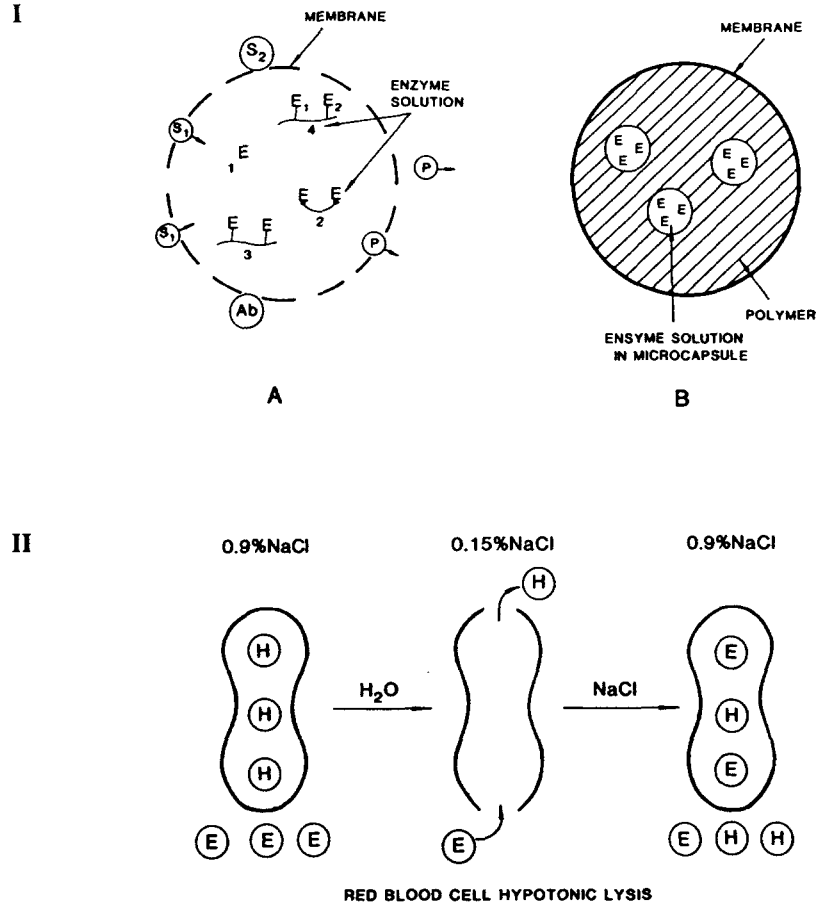

Fig. 5. Schematic representation of enzyme (E) entrapment into artificial cells. (I) Enzyme entrapment into microcapsules. A, semipermeable microcapsule. The enzyme can be immobilised in the native form (1), as intermolecularly cross-linked derivative (2), being immobilised on a soluble carrier (3), or as a component of an immobilised multienzyme complex (4). Low-molecular-weight substrates (S_1) and products (P) can easily penetrate the membrane; at the same time high-molecular-weight substrates (S_2) and antibodies (Ab) cannot enter the microcapsule; B, 'Double' entrapment of the enzyme into polymeric microcapsules. (II) Enzyme entrapment into red blood cell ghosts by hypotonic lysis; H, haemoglobin.

fine suspension. Urease is a good example of microcapsulated enzyme which serves as a detoxicant or component of artificial organs. For example, the administration of urease-containing microcapsules sharply decreased the urea content in the blood of experimental animals; the enzyme may be encapsulated either as an additionally stabilized form, or together with an ammonia adsorbent for the more effective removal of the latter [79,83,84].

Further examples include catalase (the deficiency of which causes acatalasemia), which can also be used in a microencapsulated form. Catalase activity in the blood and tissues of diseased animals rises 2% and 20% above normal, respectively, and it has been found that the injection of a 50% (w/v) aqueous suspension of microencapsulated catalase levels of 0.5 mmol per 1 g of the body weight, increased blood catalase activity in mice by 50% after the preliminary administration of substrate (perborate)in 20 min without any undesirable side effects [86]. Microencapsulated catalase has also been used for local wound healing. For example, in the oral cavity of patients suffering from acatalasemia, lesions are caused by hydrogen peroxide; local administration of microencapsulated catalase caused an effective degradation of hydrogen peroxide and wound healing, but, contrary to the native enzyme, provoked no allergic reactions [85].

A great deal of effort has been devoted to obtaining microencapsulated asparaginase, which has been suggested for the treatment of asparagine-dependent tumors. A number of studies (for example Refs. 87–90) has shown a clear possibility of effective suppression of tumour growth using a microencapsulated enzyme; e.g., asparaginase administered in polyamide microcapsules functions in the circulation much longer than does the native enzyme. Also, the use of a treatment which includes microencapsulated asparaginase and an artificial capillary system containing immobilised heparin to prevent thrombogenesis has permitted haemoperfusion to be carried out [80].

Interestingly, biodegradable microcapsules made from lactic acid have been used for vaccination and immunization of the population by single injections of microencapsulated vaccines and antigens [91–93]. Also, in the treatment of different metabolic disorders, microencapsulated multienzyme systems may be used therapeutically, and reported examples include the possibility of recyclisation of cofactors [94] and amino-acids synthesis from urea and ammonia [95].

Microencapsulation can also be used to protect enzymes from acid degradation after oral administration. For example, phenylalanine ammonia lyase has been used in the microencapsulated form for replacement therapy in phenylketonuria [96,97]. These authors demonstrated that oral administration of microencapsulated enzyme over a 2-day treatment course decreased the content of phenylalanine in the blood of mice with phenylketonuria by 35%, and over a 7-day course by 75%.

During the last decade a further method has been developed for the immobilization of physiologically active compounds, including enzymes, i.e., the encapsulation into various cells or cell 'ghosts' (which are cell membranes, either fully or partially cleared of their natural contents). The majority of such studies have been devoted to the immobilisation of enzymes in red blood cell ghosts (see Fig. 5). The approach is understandable, since cells and their ghosts possess all of the advantages of polymeric microcapsules as well as remaining fully biocompatible and biodegradable and, as a rule, having long life-times in the circulation. Enzymes can be introduced into red blood cells by different methods, including hypotonic lysis which gives cells that are filled with the enzyme solution [98]. Enzymes administered in red blood cell ghosts are stable toward the pH change of the outer medium and the action of endogenic proteases and inhibitors, and as given above

do not cause undesirable side reactions. Red blood cell ghosts also possess a large capacity for enzymes, and their membrane does not differ much from the one of normal cells, which ensures their prolonged circulation. Although the leakage of enzyme from such cells is a current problem, various approaches are being examined to solve this.

β-Glucosidase, β-galactosidase [99,100] and some other enzymes including asparaginase [101,102] have been incorporated into red blood cells. For entrapped asparaginase, in vivo and in vitro studies showed a considerably increased life-time of the enzyme in the circulation and a prolonged therapeutic activity of the immobilised enzymes towards inoculated tumours 6C3 HED (in mice), as well as leading to a decrease in the glutamine content in blood [102]. Analogous results have been obtained in mice and monkeys using entrapped β-glucuronidase [103, 104]. Additionally, red blood cell ghosts containing immobilised glucocerebrosidase have been used for the treatment of patients exhibiting a lysosomal storage disease – Gaucher's disease [105].

Attempts have been made to include proteins into other cells, in particular hepatocytes (for transplantation) and leucocytes. In the latter case the reported ability of such cells to penetrate the blood–brain barrier and then to deliver potentially drugs to different zones of the brain is of possible importance [106]. It is rather possible that enzymes can be also included in macrophages due to high endocytotic ability of the latter, and since macrophages can accumulate at zones of inflammation this may be regarded as a novel approach of drug delivery to such sites.

A number of studies have described methods for enzyme immobilization at the surface of cell membranes. Thus, glucosidase, arginase and peroxidase have been immobilized onto the surface of rabbit platelets and human red blood cells by simple adsorption [107]. This latter study has also suggested that therapeutic enzymes be modified in such a way that upon intravenous administration they can bind with red blood cell membranes and then be transported in the circulation in an immobilised state [108].

Evidence from the studies described above raises hope for a possible use of autologous red blood cells of a patient being used for fast enzyme drug loading and subsequent return to the circulation, with the aim of providing a long-term enzyme therapy resulting in a marked decrease in untoward reactions.

During the past 15 years a novel method of immobilisation – encapsulation in liposomes – has been widely studied. Liposomes are artificial phospholipid vesicles, obtained by different methods from phospholipid water dispersions. Having been discovered about 20 years ago liposomes quickly became popular as convenient models to study certain properties of phospholipid components of cell membranes and as possible microcarriers for in vivo drug transport. To obtain bilayered spheric structures, neutral (lecithin, phosphatidylcholine dipalmitoyl, phosphatidylcholine dimyristoyl) and charged (phosphatidylethanolamine, phosphatidic acid) phospholipids have been used. To impart liposomes with a sufficient in vivo stability a certain amount of cholesterol is generally added to a phospholipid mixture. The size of liposomes depends on their composition and preparation method, and can vary from 25 nm to greater than 1 μm in diameter. Liposomes

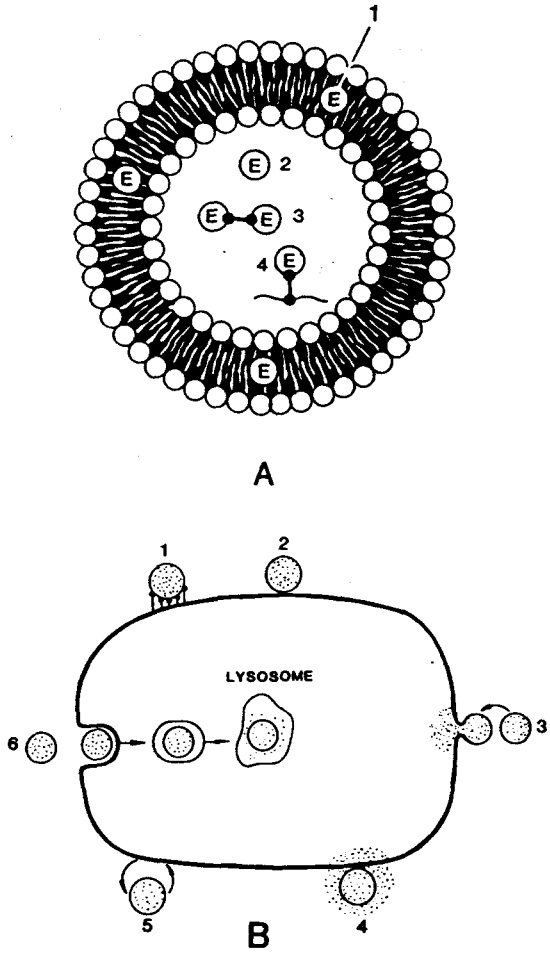

Fig. 6. Enzyme immobilisation into liposomes and liposome interaction with cells. A, enzyme (E) entrapment into liposomes; hydrophobic enzymes are included into the phospholipid membrane (1); hydrophilic enzymes can be immobilised in the native (2) or stabilized form (3: intermolecularly cross-linked; 4: bound with soluble carrier); B, a liposome can interact with a cell via specific adsorption (1), non-specific adsorption (2), fusion (3), release of an entrapped matter in the vicinity of a cell membrane and the penetration into the cell (4), lipid exchange with the cell membrane (5) and endocytosis (6).

are suitable for the immobilisation of various substances – water-soluble compounds are entrapped into the liposome inner-water space or/and water space between bilayers in case of multilammellar liposomes, and lipophilic compounds may be incorporated into the liposome membrane (Fig. 6). We will not consider here the details of liposome preparation, properties and interaction with cells. All these problems have been described elsewhere [109–113]. A detailed description of possible methods of liposome preparation is given in Ref. 114, though since then a number of original techniques has been suggested [115,116].

Evidently, enzyme encapsulation into liposomes should protect them from inactivating effects of the outer medium, i.e., the pH and the action of endogenous proteases and inhibitors. Also, prior to encapsulation in liposomes, enzymes can be additionally stabilized by immobilisation onto soluble carriers or by intramolecular or/and intermolecular cross-linking (Fig. 6). From the biomedical point of view, liposomes have many ideal characteristics of carriers for drugs – they appear to be completely biocompatible, causing no antigenic, pyrogenic, allergic and toxic reactions; they undergo rapid biodegradation; their components may be utilised as the material for the synthesis of cell membranes; and they do afford host protection from any undesirable effects of the encapsulated enzyme. Since liposomes are taken up by phagocytic cells, they do present a unique opportunity to deliver encapsulated enzymes into cells (Fig. 6). This opens new possibilities for the therapy of a number of inherited diseases connected with the deficiency of some lysosomal enzymes in liver phagocytic cells. Currently, these diseases may be treated only by the administering of an exogenous enzyme, which, if presented in the native form, can lead to the formation of anti-enzyme antibodies [117].

To date, numerous enzymes have been encapsulated in liposomes, including glucosidase [118], glucose-6-phosphate dehydrogenase, hexokinase and β-galactosidase [119,120], glucoronidase [121], glucocerebrosidase [122], α-mannosidase [123], amiloglucosidase [124], hexoseaminidase A [125], lysozyme [126], peroxidase [127], β-D-fructofuranosidase [128], neuraminidase [129], superoxydismutase and catalase [130]. The modern methods of liposome preparation, in particular detergent dialysis and reverse-phase evaporation [114], permit encapsulation in liposomes of more than 50% of the enzyme from water phase without any loss in its specific activity.

To predict the behaviour of liposome-encapsulated enzymes in the body it is necessary to know a number of the characteristics of the liposome, including size, charge, lipid composition, permeability of the liposomal membrane for the encapsulated substance, membrane stability, etc. Taking just one of these features for example, it is now known that the rate of leakage of low-molecular weight species from liposomes is highly dependent upon the cholesterol content of the liposomal membrane [131].

Many studies have shown that within the first 15–30 min after the intravenous administration of liposomes, between 50 and 80% of the dose is absorbed by the cells of the reticuloendothelial system, primarily, by the Kupffer cells of the liver [121, 124, 128, 132, 133]. Considerable amounts of liposomes are also found in the spleen and the lungs, and degradation products are either to be found in the bone marrow or the circulation. These phenomena point to a use of liposomes for the delivery of enzymes to the liver where many pathologies are localised. Two principal classes of diseases localised in the liver cells appear amenable to attack in this way; i.e., first, diseases connected with a certain enzyme deficiency leading to a harmful accumulation of certain metabolites in lysosomes (e.g., Tay-Sachs disease, Gaucher's disease, peroxidase insufficiency, mucopolysaccharasidoses); and second, disease caused by the disorder of normal functioning of non-membrane enzymes in the cytoplasm (e.g., Lesch–Nychan syndrome, adenosinedeaminase insufficiency).

In the field of liposome immobilised therapeutic enzymes, a transfer from experimental to clinical studies can already be noticed. The majority of clinical studies have been carried out on liver diseases. Particularly impressive results have been obtained upon the treatment of a patient suffering from Gaucher's disease (glucocerebrosidase insufficiency causing excessive glucocerebrosides accumulation in liver lysosomes), using liposomal glucocerebrosidase [122,134]. Here, therapy with the native enzyme gave no result because of its inability to penetrate cells. The reported therapy using enzyme encapsulated in liposomes over a period of 13 months produced a pronounced effect, in particular a decrease in liver size at a very low amount of the active enzyme used [122,134]. An attempt to treat a patient suffering from type II glycogenosis (caused by the deficiency of lysosomal amiloglucosidase and glycogen accumulation), is also very encouraging. Here, 3 mg amiloglucosidase encapsulated in liposomes (170 mg phospholipids) was injected over a period of 1 week to a sick child, which led to a measurable decrease in the liver size, though later the patient died [123].

Although in a number of studies liposomes have been shown to accumulate in other organs and tissues, particularly those exhibiting pathological lesions (see for example Refs. 135 and 136 on the accumulation of neutral and positively charged liposomes at zones of experimental myocardial infarction), the application of liposome-immobilised enzymes can hardly be considered yet as a universal approach. Approaches need to be developed to prolong liposome circulation life-time and to decrease their capture by the cells of the reticulo-endothelial system. For the former problem, methods of obtaining long circulating liposomes are being worked out. To prevent liposome capture by liver three principal approaches have been suggested: first, blockade or saturation of the reticulo-endothelial system using inert particulates such as empty liposomes [124,137]; second, coating of liposome surfaces by different substances which prevent liposome interaction with liver cells [138,139]; third, increasing liposome stability against the action of enzymes such as phospholipid acyl hydrolases and phospholipid acyl transferases [140,141].

Whilst these issues are still to be resolved, animal experiments have clearly demonstrated the suitability of liposomes for encapsulation of enzymes used for the therapy of disease not located in the liver. Thus, the increase in circulation life-time of liposomal asparaginase, the decrease in its antigenicity and the increase in the efficacy of treating experimental tumours in mice have been shown [142]. Similarly, urease entrapped in liposomes can function in the organism during a longer time than the native enzyme [143]; δ-aminolevulinate dehydralase, which is used for the replacement therapy of porphyriasis has also been immobilised in liposomes [144]; and liposomal β-galactosidase was proved to be (40–100)-times more effective in the treatment of mice leucodystrophia compared to the native enzyme [145]. It is interesting to note that a model enzyme, peroxidase, has been introduced into rat-brain tissues by means of liposomes [146]. It appears unclear whether this suggests that liposomes can be used to aid in penetrating the blood-brain barrier. Other studies show the possibility for using liposomal superoxide dismutase and catalase to eliminate the toxic effects of oxygen upon their intravenous injection in rats [130]. A number of other examples of therapeutic en-

TABLE IV

SOME EXAMPLES OF IMMOBILISED THERAPEUTIC ENZYMES

Enzymes, EC-number (1)	Intended use (2)	Carrier (3)	Comments (4)	References (5)
Acetylhexose-aminidase 3.2.1.52	Tay–Sachs disease	soluble activated polyvinyl pyrrolidone	animal experiments	30, 184
δ-Aminolevulinate dehydratase 4.2.1.24	porphyriasis	liposome		144
α-Amylase 3.2.1.1	burns abscesses	liposome	amoeba	185
	digestive insufficiency	soluble cyanogen bromide activated dextran	rat	20,21
		glutenin	(β-amilase)	186
		insoluble cross-linked polymers containing methacrylic acid residues		147
Amyloglucosidase 3.2.1.33	type II glycogenosis, storage diseases	liposome	man, rat	123, 187
Arginase 3.5.3.1	oncology	soluble cyanogen bromide activated dextran	mice	22
Asparaginase 3.5.1.1	oncology blood treatment	insulin-globulin	mice, rat	188
		albumin		71
				65
		insoluble polyacrylamide and polydextran microspheres	mice	149, 189
		collagen		62, 190, 191
		liposome	mice	142, 192
		microcapsules	rat, mice	80, 89, 90
		red blood cells ghosts		102
		modified polyacrylamide microgranules		162
Carboxypeptidase 3.4.22.12	oncology	soluble cyanogen bromide activated dextran	mice	22
Catalase 1.11.1.6	burns abscesses wound healing	soluble cyanogen bromide activated dextran	rat, mice	20
	acatasemia	polyethyleneglycol microcapsule		25
		liposome	rat	130
			fibroblasts	193
		microgranules made of polyvinylpyrrolidone, polyvinylalcohol, or their copolymers		
Cholesterol-oxidase 1.1.3.6	atherosclerotic deposits	soluble polymer	model-targeted systems	169, 175

Enzymes, EC-number (1)	Intended use (2)	Carrier (3)	Comments (4)	References (5)
Collagenase 3.4.24.3	rheumatism atherosclerotic deposits adjuvant arthritis	dextran	rat	32, 169, 175
Cytochrome oxidase 1.9.3.1	oncology	liposome		194
Desoxyribonuclease 3.1.22.-	puzulen inflammation	insoluble polymer		196
β-D-Fructofuranosidase 3.2.1.26	storage diseases	liposome	rat	128, 187
α-Galactosidase 3.2.1.22	Fabry disease	albumin		64
β-Galactosidase 3.2.1.23	Fabry disease	intermolecular cross-linking		72
		red blood cell ghosts		99
		liposome	fibroblasts	120
			mice	145
		microcapsule		196
α-Glucosidase 3.2.1.20	Pompe disease	soluble polymer; low-density liproprotein complex		197
		albumin		198, 199
β-Glucosidase 3.2.1.21	Gaucher disease Krabbe desease	liposome	man	200, 201
	gangliosidosis	red blood cell ghosts		99, 202
Glucose oxidase 1.1.3.4	oncology	liposome		203
		collagen		191
		adsorption on rat platelets; human red blood cells		107
β-Glucuronidase 3.2.1.31	rheumatism oncology	soluble polymer		32
	adjuvant arthritis	liposome	mice	121
		red blood cell ghosts	mice	103
			monkey	104
Glutaminase 3.5.1.2	liver diseases oncology	asialoorosomucoside		171
Glutaminase-L-asparaginase 3.5.1.38	liver diseases oncology	soluble polyethyleneglycol	mice lymphosarcoma	27
		concanavalin A-Sepharose		204
Hexoseaminidase A 3.5.4.8	G_{M2} gangliosidosis Tay–Sachs disease	liposome		125, 205
Kallikrein 3.4.21.8	inflammation	soluble activated polyvinylpyrrolidone	dog	29

Enzymes, EC-number (1)	Intended use (2)	Carrier (3)	Comments (4)	References (5)
Lactase 3.2.1.23	inherited lactase deficiency	microcapsule		196
	liver diseases	covalent binding to polymers		172
Lysosome 3.2.1.17	inflammation	collagen	animal experiments	62
	liver diseases	activated dextran		30
		asialofetuin		170
		liposome		126, 131
		microcapsules made of polyvinylpyrrolidone, polyvinyl alcohol or their copolymer		153, 155
Mannase 3.2.1.25	rheumatism, adjuvant arthritis	soluble dextran	rat	32
α-Mannosidase 3.2.1.24	glycoproteinosis	liposome		124
Neuraminidase 3.2.1.18	sialidosis	liposome	rat	129
Nitrate reductase 1.7.99.4	oncology	microcapsule	mice lymphosarcoma	31, 206
Peroxidase 1.11.1.7	peroxidase insufficiency	liposome	rat	127, 146
		adsorption on rat platelets and human red blood cells		107
Phenylalanine-ammonia lyase 4.3.1.5	oncology	soluble polymer	mice	27
		microcapsule from cellulose nitrate	mice	96, 97
Plasmin 3.4.21.7	thrombolytic therapy	soluble dextran	animal experiments	47, 48
		oxidized Sephadex	dog	158, 159, 160
Ribonuclease 3.1.-	inflammation	intermolecular cross-linking	rat	74, 76
	oncology	insoluble polymer		185, 195
Streptokinase 3.4.24.4	thrombolytic therapy	insoluble polymer		195
		oxidised Sephadex		158, 159
	ophthalmological pathology	soluble oxidised dextran	dog	42
			man	46
Superoxide dismutase 1.15.1.1	toxic shock	soluble polymer		208
		liposome	rat	130
Trypsin 3.4.21.4	burns	soluble polyethyleneglycol	animal experiments	26, 165, 166
	wound healing			
	blood kallikrein activation	insoluble polymer; dressing material		208, 209
Thrombin 3.4.21.5	cardiovascular diseases	insoluble polymer		208
Urate oxidase 1.7.3.3	detoxication	polyacrylamide, polyhydroxymethylmethacrylate, polyamide and polyisocyanate gels		150, 151

Enzymes, EC-number (1)	Intended use (2)	Carrier (3)	Comments (4)	References (5)
Urease 3.5.1.5	blood detoxication	soluble polymer	animal experiments	26
	diabetes	insulin		211
		microcapsule		83, 84
		liposome		143
		insoluble polymer		211
Uricase 1.7.3.3	oncology blood detoxication	soluble polymer	mice	67
		collagen		62
		microcapsule		212, 213
Urokinase 3.4.21.31	thrombolytic therapy	soluble polymer		69, 70
		soluble cyanogen bromide activated dextran	lysis of fibrin clot in vitro	23, 24
		oxidised Sephadex		158, 159

zymes immobilised in liposomes and their use in experimental therapy is shown in Table IV.

All of these results are encouraging and suggest that the use of liposomes as carriers of therapeutic enzymes may be considered a clinical reality, though many problems remain, including the development of the methods of liposome industrial production, sterilisation and storage, etc.

III. Insoluble preparations of immobilised enzymes

There is a clear opportunity with insoluble immobilised enzymes for their application as either a local system (with administration to discrete anatomical regions), or as an implanted system for controlled and sustained enzyme effects. Thus, such preparations may be indicated for the oral treatment of enzyme deficiencies or for the regulation of enzyme activity within the alimentary canal. For these cases it is necessary to obtain preparations which are stable towards the action of acid and proteolytic enzymes, and at the same time have a prolonged life-time in the stomach or intestine.

It has been found that ion-exchange resins are good carriers for the treatment of alimentary canal diseases. For example, binding of α-amylase to a cross-linked polymer which included methacrylic acid units led to a preparation stable to the action of gastric juice [147]. In a number of cases the use of ion-exchange resin allows preparations able to release bound protein at certain pH values of the surroundings, as has been demonstrated with insulin, which, upon an increase in pH, is released in an active form from its complex with an ion-exchange polyelectrolyte [148].

Numerous studies have shown that the enzymes which catalyse the conversion of low-molecular-weight substrates may generally be used in the form of of en-

zyme-containing polymeric gel particles. Such gels have two functions, on the one hand to protect an enzyme from the action of the aggressive biological medium, and on the other hand to permit diffusion of both substrate to the enzyme and product from the matrix. Again, with the much studied enzyme asparaginase it has been shown with experiments on mice with asparaginase-sensitive lymphoma that the administration of asparaginase immobilised on or in either polyacrylamide or polydextran microspheres gave a better therapeutic effect than did the use of the native enzyme [149]. These workers were able to show that the enzyme does not leave microparticles and preserves its activity over a long period of time. Similar effects have been found upon the encapsulation of urate oxidase in polyacrylamide gel, hydroxyethylmethacrylate gel or gel formed by polyamine and polyisocyanate [150,151].

Numerous studies have addressed the issue of obtaining immobilised enzymes

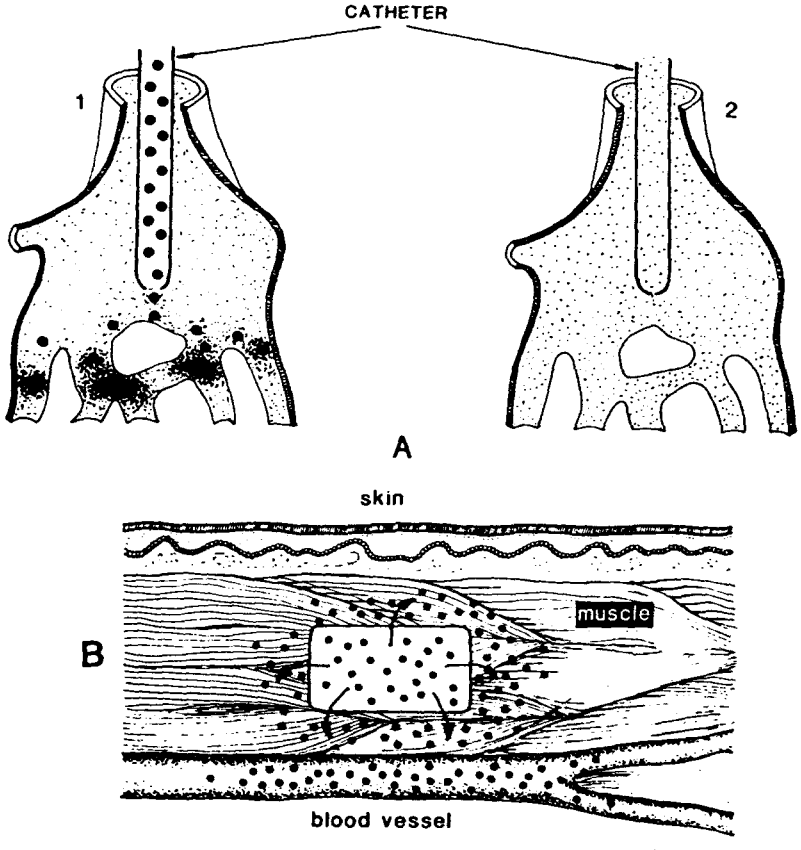

Fig. 7. Different methods of drug depot obtaining in the organism. A, intravascular administration of enzyme-containing microparticles via catheter (1, immobilized enzyme use creates high local concentration; 2, the native enzyme is distributed all over the circulation); B, intramuscular implantation of enzyme-containing polymeric material. The enzyme releases from the pellet and creates a high local concentration; it can also reach the circulation system.

capable of secreting an active matter into the surroundings at a prescribed rate and duration. Such slow release systems may be administered either via the vasculature or by implantation either subcutaneously or intramuscularly (Fig. 7). A limitation here is that in general immobilised enzymes do not pass through the endothelial barrier. The question of extravasation has been analysed in detail elsewhere [152].

There exist two principal approaches to obtain slow release systems. The first, which we shall mention briefly involves encapsulating biologically active macromolecules into polymeric tablets or granules during their moulding from various synthetic biocompatible polymers. A granule for implantation is prepared by the following method: a polymer is dissolved in water or in the mixture of water and a non-denaturing organic solvent, a certain amount of a protein to be immobilised is added, the solution obtained is put in the vessel and vacuum evaporation of the solvent is carried out. The carrier permeability for an enzyme is regulated by polymer concentration and the enzyme/polymer ratio. Enzymes encapsulated in this manner include lysozyme, catalase, and trypsin protein inhibitor [153–155].

The second approach is where an enzyme is immobilised onto a biodegradable carrier which is capable of being destroyed under physiological conditions and hence releases into the local surroundings an enzyme either in a free form or as a conjugate with a solubilised fragment of a carrier (Fig. 3B). The rate of the enzyme release will be determined by the rate of the carrier destruction. To obtain such systems it has been suggested that enzymes can be immobilised onto different insoluble polysaccharide derivatives like Sephadex [156,157]. Such carriers can be activated by partial oxidation by periodates which causes them to have a slow solubilisation in water at physiological pH, the rate of dissolution being regulated by the degree of modification. Different thrombolytic enzymes can be immobilised onto polysaccharide carriers, including plasmin, urokinase and streptokinase. Depending on the conditions, it is possible to bind up to 80 mg of an active enzyme per g of a carrier. Such preparations may be effective for local therapy of thromboses and thromboemboli of various vessels available for catheterization. For example, upon the experimental thrombosis of dog femoral artery, local delivery directly to the thrombus of fibrinolysin immobilised onto partially oxidised Sephadex resulted in a full restoration of the blood flow in the vessel within 1 h [158,159]. The total enzyme dose is about 1% of the native enzyme dose needed to achieve the same effect. Such a combination of intra-arterial administration, particle fixation at the point of the thrombus, and a slow release of enzyme results in a delivery modality that only acts at a well-defined prescribed position [160]. This latter study also showed that the use of such carriers allows a local depot of protein preparations to exist for more than 24 h in the hearts of dogs (after arterial infusion). These encouraging results are being continued with the use of partially oxidised cellulose and starch as carriers, where the rate of dissolution is much slower than that of dextran [161,162].

An alternative scheme for the use of polysaccharides to obtain biodegradable microsphere carriers has been suggested in Refs. 162–164. According to this approach, acrylic derivatives of corresponding polysaccharides are obtained, and by

using emulsion polymerization this leads to microcarriers which contain reactive carboxy groups. It is postulated that under physiological conditions a gradual dissolution of polysaccharide component, gel destruction and leaching out of immobilised enzyme will take place. Experiments in mice have shown that the half-life of polyacryldextran microparticles is between 8 and 10 weeks, and that asparaginase entrapped in such carriers possesses an increased stability and pronounced therapeutic activity towards asparagine-dependent tumours compared to the free enzyme [162].

Although not directly related to drug delivery it may be useful to the reader to be directed to the literature on the immobilisation of proteolytic enzymes onto different textiles used as wound dressings [165,166]. Enzymes immobilised onto such dressings include trypsin, which accelerate the cleaning and healing of infected wounds and reduces the amount of enzyme used as compared with the use of enzyme solutions.

Further areas of interest include different implanted polymeric prosthetic devices, the surface of which are modified by thrombolytic enzymes to produce non-thrombogenic material.

To conclude this section, it is clear that there is a great number of immobilised enzymes appropriate for local application, and whose efficacy has been shown in animal experiments. The next step in this field should be to translate these findings into clinical studies.

IV. Enzyme immobilisation and targeted drug transport

One of the major difficulties in using native enzymes in therapy is their general non-selectivity for the target site when this is extravascular. Hence, for obtaining high local concentration of the enzyme it is necessary to increase the systemic concentration, which often causes undesirable complications. Immobilisation of enzymes onto carriers opens up new possibilities for the solution of this problem. The importance of drug and enzyme targeting, and numerous ways of solving the problem, have been discussed in detail [11–13]. Targeted enzyme preparations obtained by co-immobilisation onto a common carrier of an enzyme and some vector molecule capable of recognizing the target and binding to it have been described in Refs. 167–169. Examples of such carriers, and/or vector moieties, include both antibodies against characteristic components of target organ or tissue, or other recognition species, for example asialoglycoproteins which are able to interact specifically with liver parenchymal cells. For example, using covalent binding of lysozyme with asialofetuin [170] and glutaminase with asialoorosomucoside [171] it is possible to target these proteins to the liver. It is interesting to note that in the latter study, notwithstanding fast inactivation of glutamine in the liver due to its digestion by lysosomal enzymes, a selective decrease in glutaminase content in the liver was found [171].

Wilson has demonstrated that similar effects can be obtained by introducing galactose residues into an enzyme molecule by binding activated lactose to protein amino groups [172]. He found that to ensure 50% capture by the liver 8–10 gal-

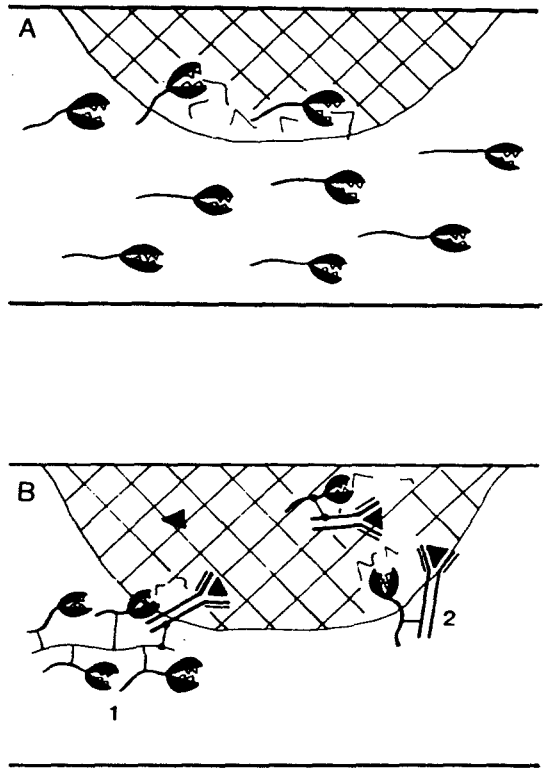

Fig. 8. The advantages of targeted enzyme therapy (thrombolytic therapy). A, the native enzyme does not concentrate on the substrate, and its quantity there corresponds to the average concentration in the blood; B, the enzyme bound with specific antibody via polymer (1) or directly (2) can be concentrated on the substrate.

actose residues per protein molecule should be present. The results obtained using reductive lactosamination of asparaginase [172] and ribonuclease dimer [173] show that the method suggested by Wilson ensures targeted drug transport to the liver even when the protein has a low molecular weight and is very quickly cleared from the organism via kidneys.

Detailed reviews on the use of other carriers which are suitable for enzyme targeting, given in Refs. 168 and 174, and clearly indicate that tissue/organ specific antigens need to be defined, and specific ligands (including antibodies) need to be produced to interact with them before successful targeting can occur.

There exists a number of original systems for targeting immobilised enzymes using antibodies as vectors. In Refs. 169 and 175 it has been suggested that soluble polymeric carriers, which carry protease, collagenase, fibrinolysin and cholesteroloxidase may be used together with antibodies against apoproteins of atherogenic lipoproteins for the targeted destruction of atherosclerotic deposits; similarly, carriers with bound protease and gialuronidase combined with antibodies against pro-

tein component of kidney and urinary bladder stones are suggested for the destruction of these stones.

Although we have shown earlier that insoluble immobilised enzymes can be used to treat thromboses, the creation of systems for local thrombolysis is still an important problem (Fig. 8). Recently, it has been suggested that conjugates of thrombolytic enzymes (in particular urokinase), with fibrinogen or antifibrinogen antibodies, which possess increased affinity towards thrombus material can be used for this purpose [176,177]. These studies have also shown that in vitro enzyme modification by specific reagents sharply increased the rate of fibrinolysis.

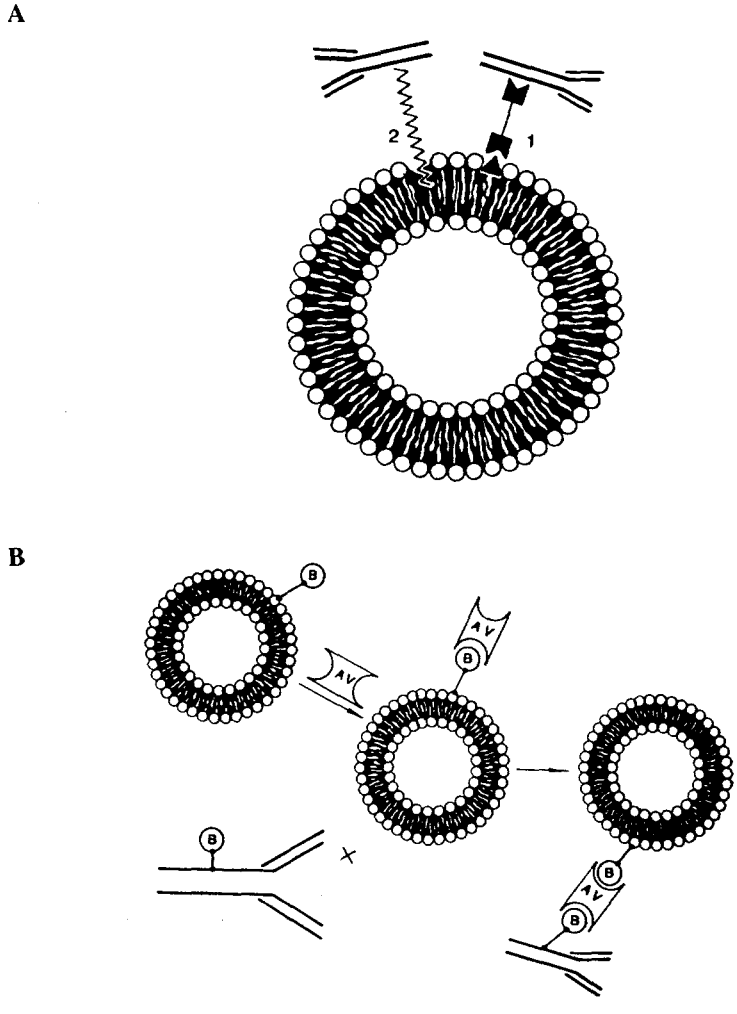

Fig. 9. Protein (antibody) immobilisation on liposome surface. A, covalent immobilisation via bifunctional reagent (1) and hydrophobic immobilisation via the residue of hydrophobic modifier (2); B, protein immobilisation via a biotin (B)-avidin (AV) system.

Different microcarriers (e.g., microcapsules, liposomes, cell ghosts), where the outer surface can be modified with specific antibodies, have been suggested for increasing the targeting potential of immobilised enzymes. However, it is clear that a major limitation in the use of these carriers is their poor extravasation. Notwithstanding this problem, much attention has been given to methods for immunoglobulin immobilisation onto liposomes [178,179] and red blood cells [180], and many simple and effective methods for such immobilisation have been developed (Fig. 9), and these are reviewed in Refs. 178 and 179. Although in vitro the use of immunoliposomes containing drugs and enzymes is successful in terms of targeting, in vivo results are disappointing, and this appears to be due, again, to the question of extravasation.

A relatively new approach in the targeting of immobilized drugs is their immobilization on carriers which possess ferromagnetic properties, and which after their administration into the circulation, can be concentrated in the appropriate region of the vascular bed under the action of the external magnetic field. One of the first examples of this kind is the work described in Ref. 181, where particles of Fe_3O_4 were coated with polysaccharide, then activated by cyanogen bromide and then used for protein immobilisation. In rabbit experiments the preparations described were concentrated by magnetic field in the desired place. Such studies are still being developed [182], though they may be considered to be a promising method for drug delivery to tumours and thromboses, and it is encouraging to note that recently in rabbit experiments the possibility of concentrating protein immobilised onto activated 'magnetic' Sephadex in the rabbit ear marginal vein has been demonstrated (see Ref. 183 and Fig. 10 of this article).

V. Conclusions

(1) At present there exists a great number for methods for obtaining immobilised therapeutic enzymes in different forms, i.e., in soluble, insoluble form or as artificial cells and systems for targeted transport.

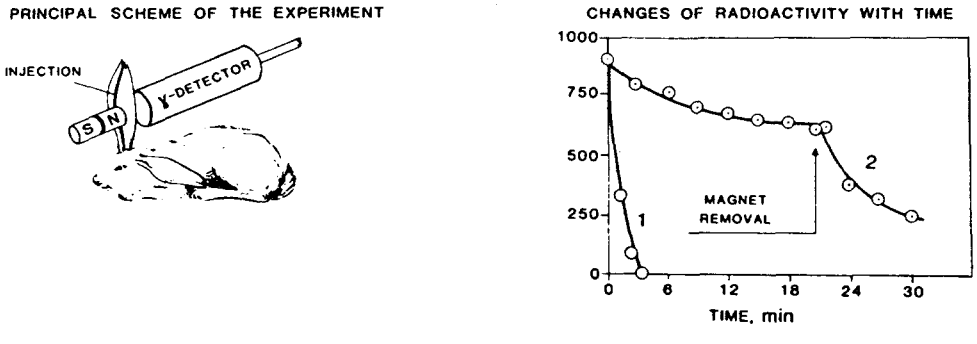

Fig. 10. The scheme of the experiment on targeted transport of ^{131}I-labeled albumin immobilised on Fe_3O_4-containing aldehyde-Sephadex G-25 in rabbit ear marginal vein. For detailed conditions, see Ref. 183. (1) Control injection of labelled sephadex suspension. (2) Injection 'under' the magnet.

(2) In the majority of cases high efficacy has been demonstrated in animal experiments.

(3) Immobilisation often eliminates undesirable side effects characteristic of native enzymes.

(4) For a number of preparations there already exist impressive clinical data.

VI. Future perspective

To facilitate and accelerate the everyday clinical use of immobilised therapeutic enzymes the following are still necessary.

(1) To have a set of commercially available biocompatible soluble and insoluble polymeric carriers with standardised properties specially designed for medical purposes.

(2) To further our theoretical and experimental appreciation of enzyme antigenicity changes occurring upon immobilisation, and to make these changes predictable.

(3) To choose an optimal form of immobilised enzyme for the treatment of any particular disease.

(4) To find for any particular preparation an optimal balance between the lifetime in the circulation and the clearance rate by the liver.

(5) To develop industrial methods for the production of microcarrier-entrapped enzymes possessing good storage stability (e.g., for liposomes and red blood cells).

(6) To prove the safety of all carriers.

(7) To study further and then to develop manufacture procedures for targeted enzymes, and, finally

(8) To develop reasonable regimens for the clinical use of immobilised enzymes.

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