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OXIDATION OF AZAHETEROCYCLES BY FREE AND IMMOBILIZED XANTHINE OXIDASE AND XANTHINE DEHYDROGENASE

Proefschrift
ter verkrijging van de graad
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op gezag van de rector magnificus,
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1. INTRODUCTION

1.1. GENERAL

Nearly all chemical reactions occurring in living cells are catalyzed by enzymes. Enzymes have a unique reaction specificity, show in general a high catalytic activity and operate under very mild conditions in comparison with ordinary chemical catalysts. These properties make enzymes potentially very useful for numerous applications in laboratory and industry. However, the stability of enzymes isolated from their natural environment is usually low and their solubility in water makes repeated or continuous use difficult. These factors have limited their application for a long time. In the last two decades much research has been directed to find methods which make enzymes more suitable for application. Attachment of enzymes to solid supports, *i.e.*, immobilization, is a promising new technique and several immobilized enzymes have been applied in industry recently[1].

1.2. ENZYMES IN SYNTHETIC ORGANIC CHEMISTRY

The use of enzymes in synthetic organic chemistry has not received much attention yet, although in this area the advantages are obvious. First of all, enzymes may catalyze many reactions which are otherwise difficult or impossible to perform. Further, the use of enzymes has several practical advantages:

- As a result of the high reaction specificity very pure products are obtained; hence work-up of the reaction mixture becomes very easy.
- Enzymic reactions are very fast; acceleration rates of 10^{10} and much higher are common.
- The reactions can be easily executed since they are performed in aqueous media at atmospheric pressure and at moderate temperatures.

As such, enzymes are "environmental friends" and "energy savers" and thus potentially are of social relevance in future industrial development.

Despite these obvious advantages enzymes have been used very seldom in organic synthesis for various reasons. *i.* Most organic chemists are unfamiliar with working with enzymes. *ii.* Since there are only few enzymes commercially avai-

lable in sufficient quantity, isolation of enzymes from cells, tissues, plants, etc., is usually necessary or desired, and these isolations are often difficult and time-consuming. Purified enzymes are therefore scarce and expensive, and thus require efficient utilization. *iii.* The solubility of isolated enzymes in water prevents efficient use, since at the end of a reaction the enzyme is difficult to recover with retention of activity. *iv.* The instability of isolated enzymes limits their lifetimes.

1.3. IMMOBILIZED ENZYMES

Immobilization of enzymes is the key to the solution of most of the above-mentioned problems, since immobilized enzymes have a number of advantages in comparison with soluble enzymes:

- *Repeated use is possible.* At each desired moment the immobilized-enzyme preparation can be removed easily and rapidly, *e.g.* by filtration and centrifugation, and reused in the next batch or stored for later applications.
- *Continuous use is feasible.* The solid-supported enzymes can be utilized in columns and stirred tanks in a continuous fashion.
- *Greater stability.* Often immobilization improves the stability. Binding of the enzymes by more than one linkage (multipoint attachment) can fix the enzyme in its active conformation, so that it will better stand extremes in pH, temperature, and denaturing compounds like urea, guanidine-HCl and organic solvents. Furthermore, if the immobilization concerns proteases or if they are present as impurities, the fixation onto the support prevents autolysis or hydrolysis, simply because the enzymes cannot get together anymore.
- *Less-contaminated products are obtained.* Compounds not bound can easily be washed from the immobilized-enzyme system and thus cannot contaminate the product. At the end of the reaction the enzymes themselves are also not present in the product solution, since they are bound to the solid support.
- *Immobilized enzymes can be "tailor-made" for specific purposes.* Every application with immobilized enzymes has its specific demands, which can be taken into account when the support and coupling procedure are chosen. For example, if the experimenter wishes to use the immobilized enzyme in a packed bed, he requires a spherical rigid particle.
- *Less labour intensive.* Since immobilized enzymes can be used continuously and for a longer time than soluble enzymes, these processes require less labour and a minimal work-up.

For general acceptation as a routine catalyst in organic chemistry, an immobili-

zed enzyme should meet several requirements[2]:
- The enzyme must catalyze a reaction of general preparative interest.
- The enzyme should preferably be commercially available.
- The enzyme should be stable.
- Sufficient specificity data should be available to enable reliable predictions to be made.
- A convenient experimental procedure must be available.

A large number of enzymes have been documented[3] and the organic chemist is becoming increasingly aware of the great potential of immobilized enzymes. There are still a number of problems to be solved before immobilized enzymes can be applied routinely in the laboratory. Nevertheless, the use of immobilized enzymes as catalysts in organic synthesis is an area for which the long-term outlook is very favourable.

1.4. SCOPE AND INTENT

The study of "The application of immobilized enzymes in organic synthesis" was initiated in 1975 in the Departments of Organic Chemistry and Biochemistry of the Landbouwhogeschool in Wageningen. The objective was to show that immobilized enzymes can indeed be conveniently and profitably used in organic synthesis. To limit the number of potentially useful enzymes, the following restrictions were set:
- A close relation with the research already going on in these laboratories was desired.
- The enzyme to be chosen must have a broad substrate specificity and be available commercially.
- Research leading to new immobilization procedures was not intended, at least not in the initial stage.

These three restrictions appeared to be sufficient to allow the choice of the enzyme xanthine oxidase.

In the laboratory of Organic Chemistry there is strong interest in the chemistry of azaheterocyclic compounds like pyrimidines, purines, pteridines and related compounds[4,5]; while flavoproteins are a main subject of study in the laboratory of Biochemistry. The flavoprotein xanthine oxidase shows an interesting-wide substrate specificity as appears from the fact that it efficiently catalyses the oxidation of many azaheterocycles[7], which by other means are sometimes very reluctant to oxidation. Xanthine oxidase from bovine milk is available commercially and can easily be immobilized[8-10]. Immobilized xanthine oxidase was

therefore developed and evaluated for application in organic synthesis.

1.5. XANTHINE OXIDASE

Xanthine oxidase (EC 1.2.3.2) and xanthine dehydrogenase (EC 1.2.1.37) form a very closely related group of enzymes. Their wide specificities overlap to a large extend and all of them, from all sources so far as is known, contain FAD, together with molybdenum and iron-sulfur centers. The reactions they catalyze are generally of the form:

$$RH \xrightarrow[+OH^-]{-2e,\ -2H^+} ROH$$

where RH is the reducing substrate. The oxygen introduced into RH is derived from water. When molecular oxygen is preferentially used as terminal electron acceptor the enzyme is referred to as "oxidase", with other oxidizing substrates, *e.g.* NAD^+, "dehydrogenase".

Xanthine oxidase from cow's milk has been studied most extensively and several reviews exist, *e.g.* references 11-13 which were used as source for this section. The enzyme can easily be isolated from cow's milk, which usually contains around 50 mg of enzyme per litre of milk. "Pure" xanthine oxidase has a molecular weight of about 283000, with the following absorption ratios and extinction coefficients: (protein/FAD) A_{280}/A_{450} = 5.0; $A^{1\%}_{1cm}$ (280 nm) = 11.7; and $\varepsilon^{mM}_{450}/2$ FAD = 72. The isoelectric point in acetate buffer of ionic strength 0.2, was found to be 5.3 to 5.4. Bovine xanthine oxidase is able to oxidize a wide variety of compounds. A large number of purines containing hydroxyl, amino, methyl, mercapto, halogeno groups and purine N-oxides are oxidized, although at greatly differing rates. 2 or 8-azapurines are also effective substrates. Replacement of the imidazole ring of the purines by a pyrazolo ring gives a series of compounds which can be good substrates, although they are perhaps more remarkable with respect to the dramatic inhibitions they show in the oxidation of xanthine to uric acid by xanthine oxidase. A wide variety of pyrimidines, pteridines and other heterocyclic compounds is also oxidized, some of them quite rapidly. A comparison of the rates of oxidation is difficult, since the measurements of the activities towards reducing substrates were performed under widely different conditions using a variety of terminal electron acceptors. Furthermore, apart from the normal complications of two-substrate enzyme-catalyzed reactions, xanthine-oxidase reactions are frequently particularly sensitive to inhibition

by excess substrate. An overview was written by Massey[12]. Massey and co-workers[14] also have proposed a model of the catalysis, which is considered a fair approximation toward the true mechanism[13]. This model is discussed in more detail in Chapter 6.

Xanthine dehydrogenase from chicken liver is very similar to milk xanthine oxidase, except that it preferentially uses NAD^+ as terminal electron acceptor. It has a molecular weight of about 300000, A_{280}/A_{450} = 5.5 and $\varepsilon^{mM}_{460}/2$ FAD = 73. It also has a broad substrate specificity, but substrate inhibition has been observed only at very high concentrations of substrate.

Xanthine oxidase has also been isolated from Arthrobacter and studied only recently[15]. The properties of this bacterial enzyme seem to resemble more the chicken-liver enzyme than milk xanthine oxidase, except that it efficiently uses O_2 as terminal electron acceptor but not NAD^+. The molecular weight is about 146000. The absorption spectrum is remarkably similar to that of milk xanthine oxidase, suggesting that the prosthetic group content is very similar. The A_{280}/A_{450} ratio is about half that of the milk enzyme. According to Woolfolk and Downard[15] the bacterial enzyme is relatively specific, but only a few substrates have been investigated. Substrate activation instead of inhibition has been observed for xanthine, but the opposite for hypoxanthine. The specific activity is about 50 times that of the milk enzyme. Arthrobacter xanthine oxidase is thus also potentially useful for synthesis and the application was therefore studied. The high specificity of the subsequent enzym in the purine-oxidative-pathway sequence, uricase[16], and the development of several procedures for the immobilization of whole cells[17], induced us to immobilize the Arthrobacter cells without any prior enzyme isolation.

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2. THE SUPPORT

2.1. INTRODUCTION

The choice of the support is one of the most important factors to consider in developing an immobilized-enzyme system as it strongly influences the eventual characteristics of the immobilized enzyme. An ideal, preformed, solid support should possess the following properties:

- *Sufficient permeability and a large surface area for enzyme attachment.* In order to be able to immobilize significant amounts of enzyme, the support must have a large surface area (>10 $m^2.g^{-1}$). Since surface area is inversely related to the pore size, the ideal support will have a high porosity and small pores. Obviously, to be effective, the pores must have a minimum diameter to allow easy access of enzyme and substrate.
- *Presence of functional groups allowing attachment of the enzyme under mild conditions.* The support must possess a sufficient number of chemical groups which can be activated or modified such that they are able to bind enzymes under conditions that no denaturation occurs.
- *Hydrophilic character.* A hydrophilic character of the support is generally desirable because it permits relatively unhindered substrate diffusibility of the type normally met in aqueous systems[1]. Often, hydrophilic features enhance the stability of the attached enzyme. A hydrophobic support tends to decrease the stability and activity of immobilized enzymes by the occurrence of a mechanism similar to that of denaturation of enzymes by organic solvents.
- *Insolubility.* Insolubility of the support is essential, not only for prevention of losses of enzyme, but also to prevent contamination of the product by dissolved support and enzyme.
- *Chemical, mechanical and thermal stability.* The support must be chemically resistant under the conditions of its activation (if necessary), during the immobilization process of the enzyme and during catalysis. Those latter two must generally performed under mild conditions, since enzymes themselves have a limited stability and operate optimally under moderate circumstances. The mechanical stability should be sufficiently high to withstand treatments such as filtration, centrifugation and stirring, since the immobilization process

and the repeated or continuous use of the immobilized enzyme usually require these manipulations. The thermal stability of a support can also play an important role. The enzyme active site may be distorted or destroyed when a support has a large expansion coefficient, leading to contraction or expansion during temperature changes. Such changes can occur during a multistep immobilization at varying temperatures, or when the immobilized enzyme is brought from storage to operational temperature and *vice versa*. When bound at several places (multipoint attachment), these changes are particularly likely to "pull" the enzyme out of its active conformation.

- *High rigidity and a suitable form of particles*. The demand of rigid particles is connected with the problem of fluid flow. Rigid, spherical and uniformly sized particles (beads) are generally most suitable for continuous packed-bed reactors, since they provide for a small pressure drop and have good flow properties. A rigid pore structure also protects the enzyme against a turbulent external environment. In addition, once an enzyme has been immobilized in its active form on a rigid surface via multipoint attachment, the tertiary structure of the enzyme is stabilized by the lack of deformation of the support.
- *Resistance to microbial attack*. Another consideration in selecting a support is the inertness to microbial degradation. Obviously, if the support is attacked and metabolized by microbes, the enzyme is lost either by release into solution or by direct microbial consumption.
- *Regenerability*. Tightening budgets and increased public awareness with regard to pollution and finite resources have made regeneration and recycling of prime importance. The possibility of regeneration and reuse must be considered in the total economics of the immobilized-enzyme system, especially when considering a relatively expensive support for large-scale application.

At present an ideal and universal enzyme support does not exist and it is doubtful whether this will ever be found. It is necessary therefore to evaluate all aspects and determine which support meets best the demands set. Eaton[2] has presented a so-called "Support Study Decision Tree", enabling the researcher to make the right evaluation whether or not the pertinent support is suitable for the immobilization of the enzyme of interest. The keyrole of the support in the immobilization of enzymes appears from the extensive research efforts and the rapid progress in this area. Growing numbers of various types of supports are being developed and commercialyzed. In the following sections the support materials, used in our own experiments (agarose, cellulose, gelatin, acrylic copolymers, activated carbon and controlled-pore glass), are treated in some detail

taking into account the factors discussed above.

2.2. AGAROSE

Agarose is a purified linear-galactan hydrocolloïd isolated from agar, which is a mixture of polysaccharides extracted from certain red seaweeds (Rhodophyceae). In 1956 the formula for the repeating subunit, agarobiose, was presented by Araki[3], showing an alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose structure (Fig. 2.1).

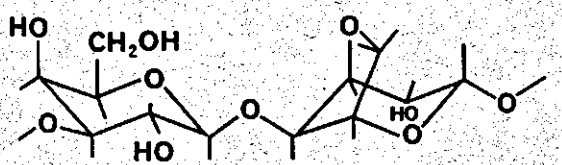


Figure 2.1 Structure of the basic repeating subunit of agarose, agarobiose.

Although not always present concurrently, sulfate, methoxyl, ketal pyruvate, and carboxyl groups, instead of hydroxyls, are also found to be present as substituents in the agar molecule in an almost infinite number of combinations. These substituents influence the properties of the various agar molecules considerably. The conditions used for the fractionation of agar determine in which fraction a specific molecule appears. Duckworth and Yaphe[4], as a result of their comprehensive chromatographic and enzymic studies, recommend as a practical definition of agarose: "... that mixture of agar molecules with the lowest charge content and therefore the greatest gelling ability, fractionated from a whole complex of molecules, called agar, all differing in the extent of masking with charged groups". Most agarose solutions gel at temperatures around 40°C. The precise gelling temperature, measured during cooling at a fixed rate, has been found to be related directly to the methoxyl content: the higher the degree of substitution the higher the temperature of gelling[5]. Once formed, a gel remains "stable" up to its melting point of about 90°C[6]. These and other properties have been explained by a model of parallel double helices[7]. The double helices are extensively aggregated and form cavities which extend along the length of the helix axis (Fig. 2.2). The relatively large voids are occupied with water molecules, which contribute to the stability of the structure through hydrogen bonding; no covalent crosslinks are present. Many procedures for the

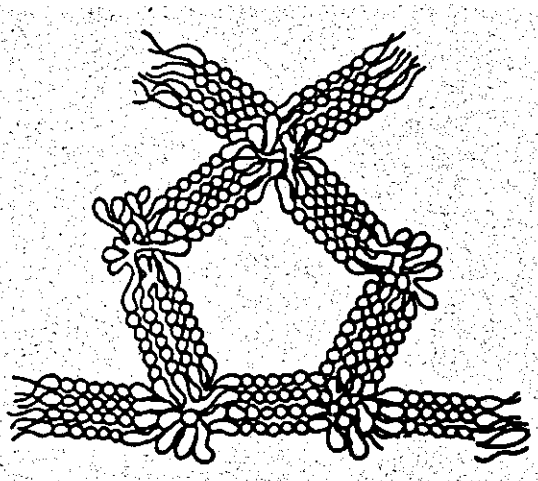


Figure 2.2 Schematic representation of an agarose gel.

isolation of agarose exist and have been developed into commercial processes for preparing beaded agarose[8]. The ready availability of beaded agarose and the fact that it meets many of the requirements of an ideal support have made agarose the support most-widely used.

The effective pore size of beaded agarose is inversely related to the weight concentration of agarose used during gelation. Agarose forms useful gels in the range of 2-6% (4% is commonly used; *e.g.* Sepharose 4B) and these gels allow free access to even the largest enzymes. Each agarobiose unit has 4 hydroxyl groups which in principal could be used for activation and coupling. However, the average number of hydroxyl groups available for reaction is somewhat lower due to the presence of the above-mentioned naturally occurring substituents. It has further been found that derivatization in aqueous solution never approaches the theoretical limit, at least not when the reactions are carried out in media of alkalinity lower than 2 M sodium or potassium hydroxide. Supports in which one of the four hydroxyl groups has been substituted, can occasionally be obtained[9]. This means that agarose gels have a moderately high capacity for substitution[10]. Several procedures exist for introducing reactive groups into agarose making the support more appropiate for immobilization of enzymes under mild conditions (Chapter 3). Since the hydroxyl groups in agarobiose are never fully substituted, the derivatized support remains very hydrophilic in nature. Agarose is chemically stable in aqueous media in the pH range 4-9 and at room temperature for a short time (2-3 h) in 0.1 M NaOH and 1 M HCl solutions. Agarose is also resistant to high salt concentrations, and denaturing agents such as 7 M urea and 6 M guanidine hydrochloride. For reaction with substances weakly soluble in water, 50% dimethylformamide in water or 50% ethylene glycol in water can be applied.

Temperatures below 0°C and above 40°C should be avoided. Lyophilization can be carried out only after the addition of protective substances such as dextran, glucose or serum albumin. The mechanical stability of agarose (4%) is adequate for most purposes, although magnetical stirring is not recommended. The rigidity of the beads is only moderate and high pressures cause compaction resulting in poor fluid flow. Agar polysaccharides are very resistant to microbial attack. This high stability is probably due to the unique molecular structure, which is not present in polysaccharides of terrestrial organisms[9]. Agar-degrading enzymes (agarases) are only found in certain microbes living in a marine environment. Regeneration of agarose supports is usually impossible.

Beaded agarose has thus many attractive features as a support, but there are a number of factors which limit its use, *i.e.*, *i.* lack of thermal and mechanical stability, *ii.* poor rigidity, *iii.* shrinkage or swelling due to changes in ionic strength or dielectric constant of the medium, *iv.* inability to be frozen or to be dried easily, *v.* ready solubility in the presence of denaturing or chaotropic ions, *vi.* drastical and irreversible changes in structure that occur in many organic solvents, and *vii.* the fairly high costs coupled with the impossibility of regeneration. However, the stability of agarose can be considerably increased in all respects by crosslinking with epichlorohydrin, 2,3-dibromopropanol or divinylsulfone[11]. The crosslinked-agarose beads are mechanically much stronger, more rigid, have better flow properties and can be employed with a far-wider range of buffers and solvents. As a consequence of the crosslinking the porosity is somewhat reduced and the hydroxyl groups being available for coupling decrease by about 50%. This can be compensated for by the addition of sorbitol or phloroglucinol during the crosslinking reaction[12].

It can be concluded that chemistry of agarose is developed sufficiently well to make agarose suitable as a starting support for most purposes. The relatively high costs of pure agarose limit large-scale applications, but in many such cases it may be that less pure and therefore cheaper agarose could be used.

2.3. CELLULOSE

Cellulose is a vegetable fiber and one of the most abundant natural organic compounds. It consists of linear polymers of varying length. Each molecule is composed of 1,4-linked β-D-glucose units (Fig. 2.3).

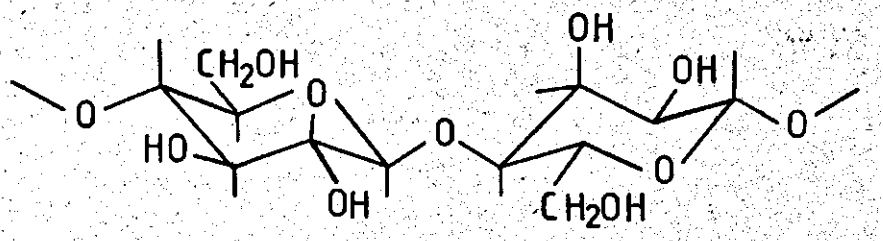


Figure 2.3 Structure of the repeating subunit of cellulose.

The polymers do strongly interact through hydrogen bonds and about 40 of these form cellulose strands with a diameter of about 3.5 nm, so-called elementary fibers. Elementary fibers contain highly ordered crystalline regions and more accessible amorphous regions of a low degree of order. Swelling of cellulose can occur when it is suspended in a solvent. Two types of swelling can be distinguished: inter and intracrystalline swelling. In the latter type the crystalline zones are also penetrated and a definite change in structure occurs. Cellulose goes into solution when treated with strong alkali. When native cellulose is regenerated from solution or passed through a highly swollen state following alkali treatment, the linear polymers shift to a different, less ordered and more accessible configuration and the cellulose is referred to as mercerized. The largest intercrystalline swelling is established in water[13].
The permeability, the surface area available for enzyme attachment, and the reactivity of cellulose largely depend on its crystallinity, the size of the compound to be bound and the swelling-inducing capacity of the reaction medium. In principle, cellulose can undergo all the usual reactions associated with polyhydric alcohols. Thus a wide range of modification and activation reactions is possible in theory[14]. Obviously, for reaction with the hydroxyl groups the reagent must have access to these groups. The first requirement is that cellulose is swellable in the reaction medium. Although a high degree of substitution of the three available hydroxyl groups in each subunit is possible, the ultimate capacity for enzyme binding is determined by the size of the enzyme itself. Although cellulose may be able to couple 500 times more small molecules than agarose, the capacity of cellulose to bind macromolecules (such as serum albumin) is actually lower than that of agarose[15]. If the hydroxyl groups are not substituted to a large extent, cellulose is very hydrophilic, easily wettable, but insoluble in aqueous medium. Substitution to a high degree with ionic func-

tions may lead to water solubility, *e.g.* when about one of every three hydroxyl groups is substituted with sodium-carboxymethyl groups, this cellulose derivative is water soluble[14]. Extremes in pH must be avoided: strong acids cause hydrolysis of the glucosidic bonds; strong alkali causes dissolution. The chemical, mechanical and thermal stability is sufficient to withstand most manipulations and conditions in the preparation and operation of enzyme/cellulose systems. Cellulose is commercially available in fibrous and granular form. In fibrous form it is easily clogged by particulate material and compressed by the application of even moderate pressure. The granular form is composed of microscopic cigar-shaped fairly rigid particles of uniform size (about 35 μm)[16], which can be packed into columns having reasonable flow properties. Cellulose in bead form with good mechanical strength and considerable porosity has also been described by various authors[12]. Cellulose is not completely inert to microbial attack, as it can be hydrolyzed by extracellular microbial cellulases. Regeneration of cellulose supports is in most instances impossible, but also not very relevant, since it is a relatively cheap material as result of its natural abundance.

Recapitulating, cellulose is an acceptable support provided that binding capacity homogeneous enzyme distribution and rapid high-pressure application are not essential. Cellulose can easily be derivatized in a variety of organic solvents, is hydrophilic, water insoluble, sufficiently stable and rigid for most applications, and commercially available in various inexpensive forms. It is therefore not surprising that cellulose and modified celluloses were some of the first supports to be used for enzyme immobilization and that over 40 different enzymes have now been immobilized succesfully on cellulose supports[14].

2.4. GELATIN

The protein gelatin has several proporties which make it attractive as a support for the immobilization of enzymes, also on a large scale. In the first place it is cheap and available in large quantities. It is easily obtained from collagen, the principal protein of skin and connective tissue of mammals, by boiling with water (Fig. 2.4). Glycine, proline and hydroxyproline are the main amino-acid building blocks of the collagen molecule. The regular repeating of proline and hydroxyproline residues force the peptide chain into a peculiar spiral and in native collagen three of these chains are intertwined to form a triple helix (Fig. 2.4), which gives collagen its strength.

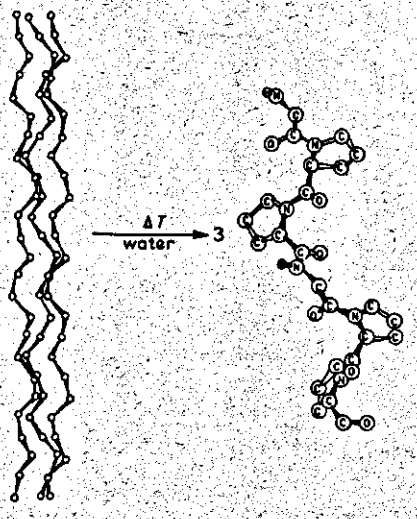


Figure 2.4 When boiled in water, the strands of the collagen "rope" come apart and gelatin is produced.

Crosslinking of gelatin is necessary in order to prepare a water-insoluble and mechanically stable enzyme support. This can be an advantage over the use of collagen, which has also been employed succesfully as a support for the immobilization of several enzymes[17]. Broun *et al.*[18] showed in their studies on immobilization procedures, that the most promising method consists of crosslinking the enzyme molecules together with inactive protein by means of a bifunctional reagent. This method does thus not use a preformed support. The crosslinking results in an insoluble support in which the enzyme is (covalently) entrapped and homogeneously distributed. The formation of a structure in which the enzyme is bound to other proteins does more closely resemble the *in vivo* conditions: Most enzymes *in vivo* function while embedded in membranes, adsorbed to interfaces or entrapped in other solid-state assemblages. When enzymes are isolated from their natural environment they usually are less stable. The presence of many surface charges and a large number of other potential binding sites for enzyme attachment and stabilization plea for the use of gelatin as a support for enzyme immobilization.

When evaluating the properties of gelatin using the criteria outlined above, we first note that only substrate permeability is relevant in this case, since the support is prepared from a homogeneous solution of enzyme and inactive protein. Substrates with a low molecular weight will generally have good access to the entrapped enzyme. For macromolecular substrates the access will largely depend on the degree of crosslinking of gelatin and on the dimension of the pertinent substrate molecules. The amount of enzyme immobilized in gelatin can be very

high (up to 20%)[19] and in the case of the immobilization of whole cells even much higher (dry weight cells : weight gelatin = 1:1)[20]. Gelatin has many functional groups allowing crosslinking to various degrees. Gelatin is also very hydrophilic in nature and when sufficiently crosslinked it becomes insoluble in water. The chemical, mechanical and thermal stability largely depend on the degree of crosslinking. Crosslinked gelatin is, however, more stable than enzymes, such that under the operational conditions the gelatin support is chemically inert and thermally stable. At least two immobilization procedures exist at present that produce mechanically stable gelatin preparations, which can withstand manipulations such as stirring, filtration and centrifugation[19,20]. The rigidity of the gelatin support again depends on the degree of crosslinking, but both immobilization techniques produce particles that cause, even in packed beds with large dimensions, low pressure drops. As a proteinaceous support, gelatin is subject to microbial attack. However, the pores of the crosslinked gelatin matrix do not allow entrance of microbes or proteases, such that microbial breakdown can only start at the outer surface and is thus likely to be slow. Van Velzen of Gist-Brocades found[19], when operating an invertase/gelatin reactor with a saccharose solution as substrate (sensitive to microbial infection), that during the 9th week of operation the conversion rate dropped as a result of microorganisms growing on the enzyme particles. However, the original conversion rate could be restored by thoroughly washing and sterilizing the column with a 2% glutaraldehyde solution. This most probably indicates that the gelatin support did not act as a substrate but merely as a support for growth of the microbes. Regeneration of gelatin is irrelevant as it is very cheap.
In conclusion, gelatin appears to be quite-generally applicable as a support for the immobilization of enzymes and whole cells, since the existing immobilization procedures using it are mild and yield an immobilized-enzyme preparation with favourable properties.

2.5. ACRYLIC COPOLYMERS

Solid supports derived from acrylic monomers, *e.g.* acrylamide, acrylic acid, methacrylic acid, N-acryloylmorpholine and hydroxyethyl methacrylate, comprise the largest class of wholly synthetic polymers used for the immobilization of enzymes[21]. In one respect the acrylic copolymers are outstanding: that is, in the possibility of preparing various polymers "tailor-made" for a specific application. Parameters that can easily be controlled are: *i.* the degree of porosity and *ii.* the chemical composition, which can be achieved by either

copolymerization of different monomers among the large number available or by chemical modification of preformed polymers. Acrylic copolymers are available from commercial sources and are also relatively easily prepared in the laboratory. By choosing the ratio of the participating monomers it is possible to prepare a polymer with the desired amount of a specific functional group. The structure of the gel (and thus the pore size and pore-size distribution within the gel) is dependent not only on polymerization kinetics but also on other factors, mainly on the total concentration of monomer, *e.g.* acrylamide, and on the relative concentration of crosslinking agent such as N,N'-methylene bisacrylamide (Bis). A minimal average pore size is obtained when Bis comprises about 5% by weight of the monomers. Above and below 5%, the pore size will be larger. Preparations above 5% turn more and more turbid, indicating microprecipitation, which leads to larger pores. However, gel structure can still be found in the microparticles of this coherent disperse system. These so-called macroreticular gels are characterized by having "interparticular" pores, which allow relatively unhindered diffusion into this type of gel, because they are large in comparison with normal gel pores. Another method of preparing bodies of varying porosity involves polymerization in different solvent systems. Regardless of how they are made, polyacrylic gels possess attractive features and are commercially available in beaded form, pregraded in sizes and porosities[22]. The permeability and surface area of acrylic copolymers depend on the monomeric mixture used and on modifications following the polymerization. For example, the commercially available polyacrylamide gel with the highest porosity is permeable for proteins with a molecular weight up to 300000, but during derivatization reactions the pores shrink so drastically, that the largest protein that can be bound has a molecular weight of only about 30000[15]. This problem can be eliminated by changing the compositions of the monomeric mixture. Spheron for instance, a copolymer of hydroxyethyl methacrylate and ethylene bismethacrylate, has large and stable pores with exclusion limits up to 5 million (Fig. 2.5). The principal advantage of polyacrylic gels is that they possess an abundant supply of easily modifiable groups which, together with a versatility in derivatization techniques, allow the covalent attachment of enzymes in many different ways. Reactive groups can even be introduced immediately by using monomers having active groups which do not participate in the polymerization reaction. Enzacryl was the first of these modified acrylamide supports to be marketed commercially and is available with various functional groups preattached to it (Fig. 2.6).

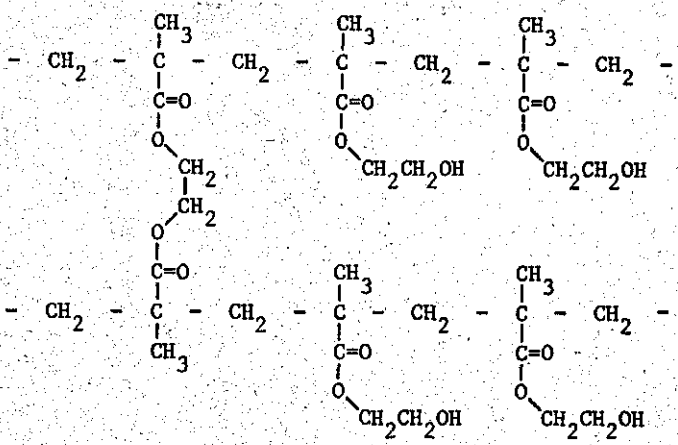


Figure 2.5 The chemical structure of Spheron.

Spheron is available with hydroxyl, carboxyl, sulfonyl and amino-aryl functions. Anhydride and oxirane-acrylic beads are also available commercially (Röhm). Polyacrylic supports are usually very hydrophilic in nature and insoluble in water. Their chemical stability is excellent, which is mainly the result of the polyethylene backbone, since carbon-carbon bonds are very stable. The mechanical stability of polyacrylamide is poor, but this property is greatly improved in the Enzacryl, Spheron and Röhm beads and manipulations as filtration, centrifugation and stirring are allowed. The thermal stability is sufficient to withstand all temperatures likely to occur in immobilized-enzyme systems (*e.g.* Spheron is stable up to 250°C). Enzacryl is commercially available as rough particles and Spheron and the Röhm products as beads. The rigidity of these supports is such that these materials produce colums with good flow properties. Polyacrylic copolymers are resistant to microbial breakdown. Regeneration of this type of supports is generally impossible.

Polyacrylic copolymers form an interesting group of solid supports for the immobilization of enzymes and the range of commercially available products is wide. The particular products are well characterized, such that a sensible choice can be made. Many-more variations are possible when the support is prepared in the laboratory. For a specific purpose "tailor-made" supports can then be made, although this demands a thorough knowledge of the polyacrylic chemistry and much technical skill and experience. Making one's own polyacrylic support also offers the possibility of immobilization by (covalent) entrapment. In this case, careful control of the temperature is desirable during the exothermic polymerization reaction in order to prevent thermal denaturation of the enzyme.

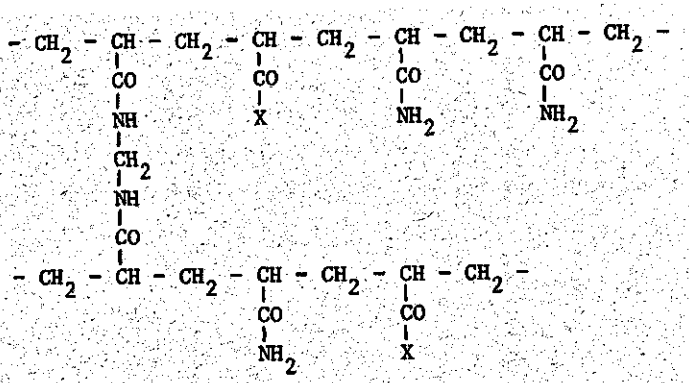


Figure 2.6A Enzacryl AA (X= $-NHC_6H_4NH_2$); Enzacryl AH (X= $-NHNH_2$); Enzacryl Polythiol (X= $-NHCH(COOH)CH_2SH$); Enzacryl Polythiolacton (X= $-NHCH(CH_2-S)-CO-$).

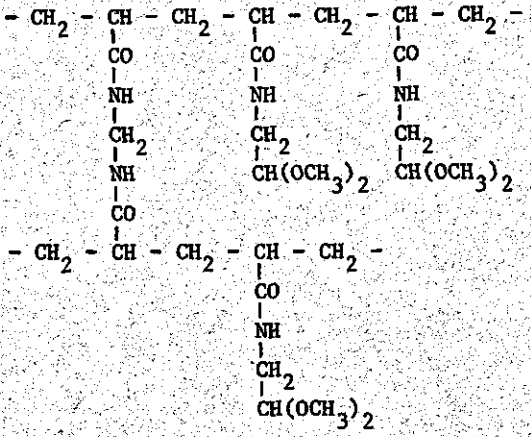


Figure 2.6B Enzacryl Polyacetal, which differs from the other polyamide supports in that all the amide groups are substituted.

2.6. ACTIVATED CARBON

Activated carbon is a highly porous carbonaceous material with a large internal pore surface[23]. It is prepared by dehydration and carbonization, followed by activation, of organic substances such as coconut shells, wood, coal and petroleum coke, bone, molasses, peat, and paper-mill waste (lignin)[24,25]. The source of carbon and the precise conditions of the various preparation steps determine the ultimate properties of the activated carbon. The permeability of activated carbon is largely determined by its pore structure. The permeability

of choice is determined by the size of the compound to be bound. The pore structure has been reported as "tridispersed", *i.e.*, containing micropores (0-0.2 nm radii), transitional pores (0.2-50 nm) and macropores (50-2000 nm)[26]. Activated carbon may be manufactured with high surface areas (600-1000 $m^2.g^{-1}$) and a significant fraction of its pore volume in the 30-100 nm range of pore diameter, suitable for enzyme immobilization. Thus, the morphology of activated carbon should be conducive to large loadings of immobilized enzyme[27]. The existence of vinyl, carboxylic, phenolic groups and other oxides on the carbon surface has been shown and many details on the surface chemistry of activated carbon are known[24]. The type of oxidation process used in the activation step of the carbon determines to a large extent the nature of these functional groups and the possible modifications of a specific activated-carbon preparation in order to make it suitable for covalent binding of enzymes. The manner in which the carbon is activated also influences the degree of hydrophilicity. When the carbon is activated at 1000°C either in pure CO_2 or under vacuum, followed by exposure to oxygen at room temperature, a hydrophobic surface results. In contrast, the oxidation of carbon by exposure to gaseous oxygen at temperatures between 200 and 400°C, or by adding it to an aqueous oxidizing solution, produces a hydrophilic surface. Activated carbon is insoluble and chemically very resistant, with the exception of the functional groups on the surface, which can be modified under mild conditions. Cho and Bailey[27] claimed that activated carbon possesses a mechanical strength comparable to porous-glass materials, although we found that a minimal amount of material easily abrases from the carbon, causing the reaction medium to become slightly turbid. The thermal stability is more than sufficient for all applications with immobilized enzymes. The rigidity strongly depends on the carbon source and manufacturing procedure. The granular form usually allows operation in large packed beds. Activated carbon is completely inert to microbial breakdown and finally, regeneration can easily be established in various ways[24].

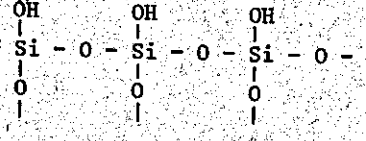
In summary, activated carbon can be produced in such a way that it has many advantageous properties for use as a support in the immobilization of enzymes. So far it has not been specifically designed for this purpose and not all the necessary specifications are given for the various preparations, making a sensible choice difficult. Nevertheless, several enzymes have been successfully immobilized on activated carbon. In fact, activated carbon was the first material to be used as enzyme support[26].

2.7. CONTROLLED-PORE GLASS (CPG)

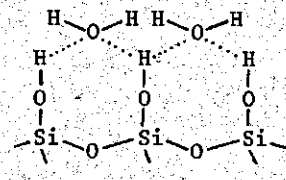
Many different kinds of inorganic materials have been used as a support for the immobilization of enzymes. Materials ranging from fabricated particles with specifically tailored properties to inexpensive minerals have been examined as inorganic supports. The variety in these inorganic substances is indeed wide, including aluminas, clays, sand, glass, charcoal, celite, kieselguhr, hornblende, nickel oxide, silicas, titanias and zirconias[18]. The distinct advantages of these supports are: high mechanical strength, resistance to solvent or microbial attack, reusability and easy handling. The scarcity of natural macroporous materials has made it desirable to prefabricate porous inorganic supports for the immobilization of enzymes.

The most important prefabricated inorganic solid support is undoubtedly controlled-pore glass (CPG). CPG is prepared by heating certain boro-silicate glasses to 500-700°C for prolonged periods of time, followed by various physical and chemical treatments[29]. Careful control of these treatments allows the production of glass beads of various diameters with narrow pore-size distributions in the range from 4.5 to 400 nm. Accurate determination of the pertinent nominal pore size is possible.

The commercially available CPG is well characterized with regard to mesh size, pore size and surface area. Consequently, if the dimensions of the enzyme and substrate to be used are known, an optimal choice with respect to enzyme or substrate permeability and binding capacity can be made beforehand. The surface of CPG is mainly composed of silanol functions:



These groups provide a mildly reactive surface for activation and enzyme binding. Several activation and coupling procedures have been developed (Chapter 3). The binding capacity is generally less than that of agarose[30], but the best properties of CPG and agarose can be combined by covalently covering of the CPG surface with a hydrophilic carbohydrate monolayer (Glycophase, Pierce). In addition to the SiOH functions, the surface of CPG contains tightly bonded water molecules, making the glass both hydrophilic in nature and well wettable. CPG



also has a wide solvent compatibility (no swelling or shrinkage in changing environments), a property which may be important for activation of the support. Glass is slightly soluble in alkali (pH>8), but because of the large surface area, CPG is appreciably more soluble, actually going into complete solution during prolonged operation under alkaline conditions. Since the enzyme is attached to the surface it goes into solution when the first silica surface layer dissolves and is thus rapidly lost. This solubility of CPG in alkali severely limits its application as enzyme support. Derivatization of CPG with a cross-linked organic coating, *e.g.* a polysaccharide, minimizes this problem of alkali solubility. Coating with metal oxides, less soluble in alkali, can also be employed. Zirconium-clad CPG, *i.e.*, CPG with a ZrO_2 surface, is available (Zirclad, Pierce) and can be derivatized in the same fashion as CPG, but the resulting support is more stable at alkaline pH. Except for the effect of alkali and hydrofluoric acid, the bulk of the CPG is chemically resistant and can withstand harsh conditions, so that various procedures for activation of the moderately reactive surface silanols can be applied (Chapter 3). The thermal stability is excellent and contraction or expansion is minimal upon temperature changes. The mechanical stability of CPG strongly depends on pore size: CPG with very large pores is very friable and easily breaks under pressure. Magnetical stirring also breaks CPG as a result of grinding of the particles. Furthermore, small glass particles, inhomogeneous in size, can easily become clogged with particulate matter. CPG is very rigid and can be prepared as almost spherical and uniformly sized particles. Therefore, CPG with moderate pore (<250 nm) and mesh size (40-80 mesh)[15] has very good flow properties even under high pressure operation. Other advantages of glass are its complete inertness with respect to microbial attack and the possiblity of regeneration of the CPG by pyrolysis of the organic matter. For laboratory purposes most CPG products are reasonably priced, yet they are too expensive for applications on larger scales. For these purposes porous ceramics, although less uniform in pore size, can be alternatives, since they can be fabricated more cheaply.

Like agarose, the advantages of using CPG often outweigh its disadvantages. The choice will eventually be determined by the specific purpose.

2.8. CONCLUSION

The support materials discussed above comprise the largest (>90%) and most successful part of applications with immobilized enzymes and are therefore representative for this field. The number of materials used one or more times as a support in immobilized-enzyme studies is, however, very large. Among these are gluten, starch, stainless steel, polystyrene copolymers, dacron, nylon, chitin, collagen and many more[31]. All have specific advantages, but no one is ideal in all respects and suits all applications best. Therefore, for a particular application a thorough evaluation of various supports, using the "Support Study Decision Tree", is advisable before a definite choice is made.

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3. IMMOBILIZATION TECHNIQUES

3.1. INTRODUCTION

The properties of immobilized enzymes, especially the specific activity, are influenced not only by the characteristics of the support to which the enzyme is bound, but also by the way in which an enzyme is coupled to the support. The most important factor is to avoid loss of enzymic activity during the immobilization process. The catalytic activity of enzymes resides in the active centre, the site where the substrate is bound and where it is converted into the product. The active centre usually consists of several amino-acid residues held in a specific spatial relationship to each other. The rest of the protein chain acts as "backbone" and keeps the amino-acid residues of the active site in the required spatial arrangement. This three-dimensional conformation, the tertiary structure, is essential for catalytic activity. Consequently, it is necessary that both during and after immobilization the amino-acid residues in the active centre are not altered and that the tertiary structure is maintained. The three-dimensional structure is based on the presence of relatively weak binding forces such as hydrogen, hydrophobic and ionic bonds, and sometimes a few disulfide bridges. Therefore it is essential to perform the coupling reaction under very mild and precise conditions. High temperatures, strong acid or alkali, must be avoided if the structural integrity of enzymes is to be preserved. Organic solvents or high salt concentrations may also cause denaturation and loss of activity.

It is thus logical that a large number of studies on immobilized enzymes have been devoted to immobilization techniques *per se* and the literature has now proliferated with a variety of methods for immobilizing enzymes. The various methods can be classified into three basically different approaches to immobilization:

1. *Support-binding method*: The binding of enzymes to solid supports.
2. *Crosslinking method*: Intermolecular crosslinking of enzymes by means of multifunctional reagents.
3. *Entrapping method*: Incorporating enzymes into the lattice of a semipermeable polymer gel or enclosing the enzymes in a semipermeable polymer membrane.

These three categories can be further subdivided as shown in Fig. 3.1.

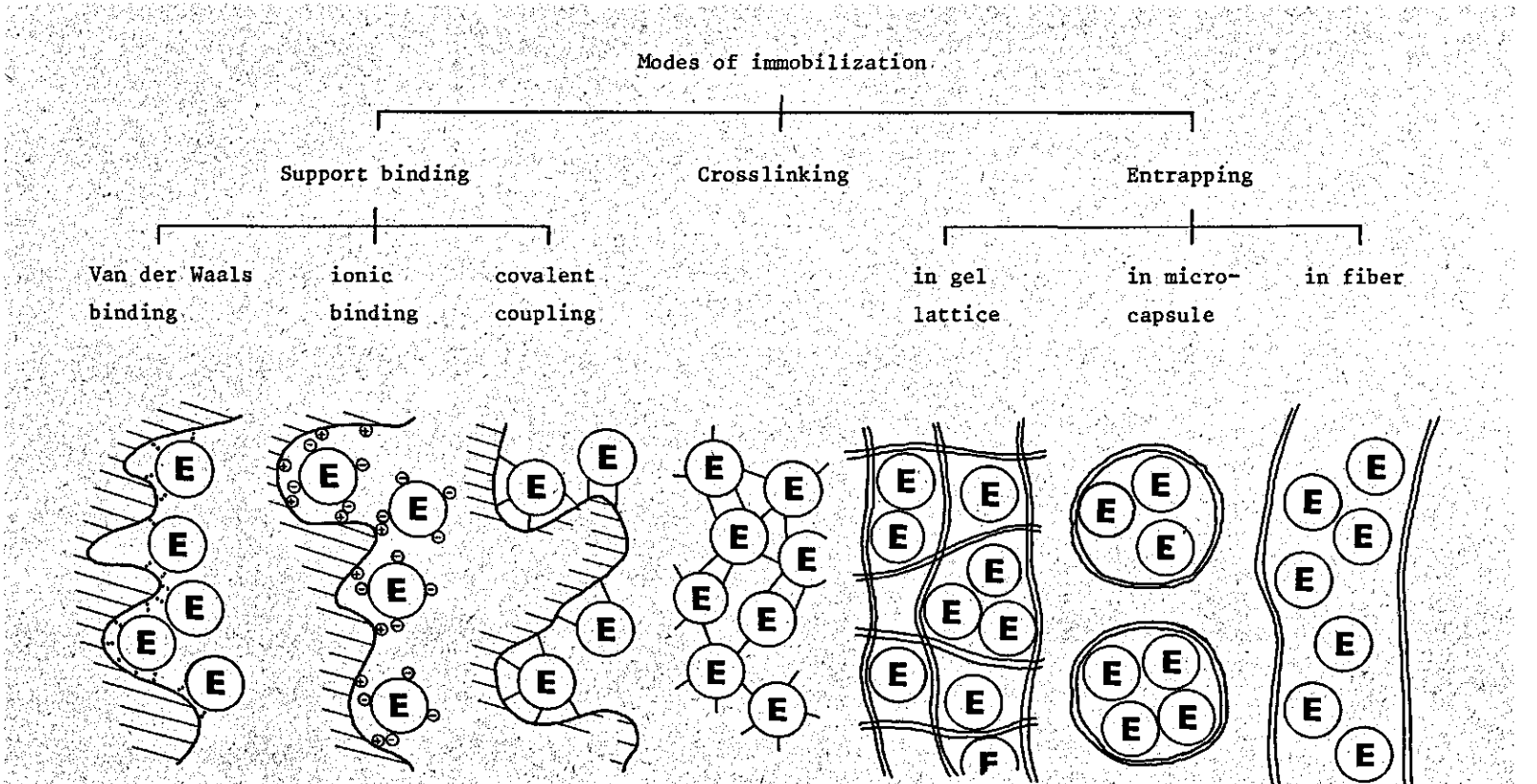


Figure 3.1 Division and schematic representation of immobilized enzymes.

None of the applied immobilization procedures depends on a single mechanism. In fact, each one is probably a combination of two or more of the binding modes. For example, in case of covalent coupling to supports, it is likely that part of the enzyme molecules is adsorbed to the support surface. In the description given below, it is merely implied that the proposed attachment is predominantly of the type discussed. The methods used in our own experiments are mainly given as examples and treated in more detail. Excellent reviews[1-7] discuss the various techniques extensively and are the main literature source for this chapter.

3.2. THE SUPPORT-BINDING METHOD

The support-binding method is the oldest immobilization technique and hundreds of papers have been published on this type of immobilization. The support-binding method can be further divided into three categories according to the binding mode of enzyme, that is, Van der Waals binding, ionic binding and covalent binding (Fig. 3.1).

3.2.1. Van der Waals binding

Adsorption based on Van der Waals attractive forces of enzymes and the surface of solid supports is undoubtedly one of the easiest immobilization procedures. Adsorption is established by simply contacting the enzyme solution with the surface of the support material and washing the resulting immobilized-enzyme preparation to remove any nonadsorbed enzyme. The binding forces between enzyme and support are generally weak, so that adsorbed enzymes usually are liable to desorption during the utilization. To minimize this problem it is advisable to wash the adsorbed-enzyme preparation thoroughly with the substrate solution under the conditions to be applied. The adsorption process is nonspecific and sometimes results in partial or total inactivation of the enzyme. Only supports which have a high affinity for the enzyme and also cause minimal denaturation are suitable. The adsorption of an enzyme is dependent on such experimental variables as pH, the nature of solvent, ionic strength, concentration of enzyme and adsorbent, and temperature. It is important to recognize these factors and to control them for optimal adsorption and retainment of activity. The more commonly employed adsorbents are alumina, activated carbon, clays, collagen, glass, diatomaceous earth and hydroxylapatite.

3.2.2. Ionic binding

Just as Van der Waals binding, ionic binding is also an old and simple way of preparing immobilized enzymes. This method is based on the ionic binding of enzymes to water-insoluble materials containing ionexchange residues. The binding of enzymes to this type of support is easily performed and the conditions are mild in comparison with those necessary in most covalent-bond-forming methods. The ionic-binding method usually causes little or no changes in the active site of the enzyme and in the tertiary structure, and often yields immobilized enzymes with high activity. The binding forces are relatively weak and leakage of enzyme from the support may occur in solutions of high ionic strength or upon variations in pH. Thoroughly washing with the substrate solution under the operational conditions may minimize leakage. Increasing the binding strength can be achieved by increasing the charge of the enzyme by chemical modification. For example, the slightly anionic enzyme, glucoamylase, was modified to a polyanionic derivative by covalent binding with a water-soluble copolymer of ethylene and maleic anhydride[8]. This polyanionic enzyme derivative was strongly adsorbed to cationic supports such as DEAE-cellulose and DEAE-Sephadex. Cellulose derivatives are the ionexchange materials most-widely used as enzyme support.

In practice, both ionic binding and Van der Waals binding usually occur concurrently. This is illustrated by the method of immobilization we have used most extensively in our own studies in which xanthine oxidase is adsorbed by both hydrophobic and ionic forces to an agarose derivative. Agarose is activated with cyanogen bromide, yielding mainly a reactive imidocarbonate[9] (Fig. 3.2). This activated

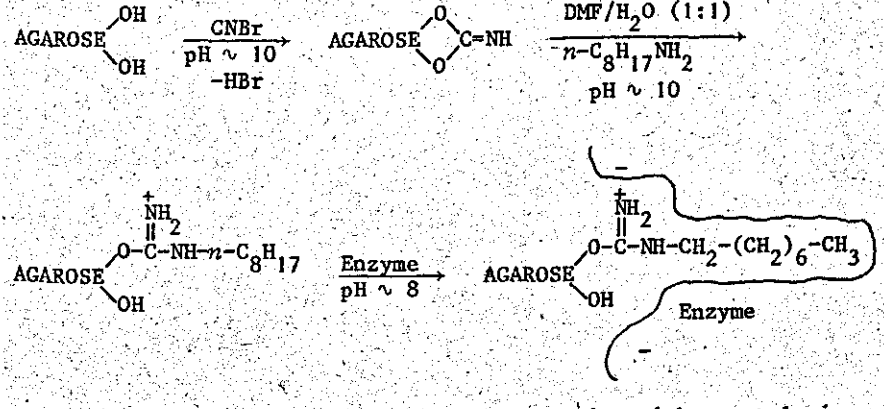


Figure 3.2 CNBr-activation of agarose, reaction with *n*-octylamine and immobilization of an enzyme.

agarose is reacted with *n*-octylamine and the support thus formed, strongly adsorbs, in weak-alkaline solutions, enzymes having an isoelectric point in the acid region[10]. The adsorption is based on both ionic and hydrophobic attractive forces. That is, in weak-alkaline solution (pH ~ 8) the isourea derivative (pK_a = 10.4) is protonated and the enzyme has an overall negative charge resulting in an ionic attraction between them. At the same time the freely mobile *n*-alkyl tail can reach the hydrophobic areas of the enzyme and thus hydrophobic attraction occurs in addition to the ionic interactions (Fig. 3.2). This dual binding character has the advantage that in solutions of high ionic strength the hydrophobic forces are largest, while at low ionic strength the ionic binding is strongest. An enzyme immobilized in this fashion is thus less liable to leakage from the support in a wider range of aqueous media than enzymes bound only by ionic forces.

3.2.3. *Covalent binding*

Covalent binding of an enzyme to a solid support is achieved by a chemical reaction between groups on the support surface and one or more functional groups of the enzyme. Usually ε-amino groups of lysine residues, but also other nitrogen containing groups such as the N-terminal aminogroup, the imidazole side chain of histidine and the guanidine group of arginine residues, can take part in the binding. The carboxyl group of glutamate and aspartate, the C-terminal carboxyl group, the hydroxyl group of serine and threonine, the hydroxyphenyl group of tyrosine and the sulfhydryl group in cysteine residues can also serve as coupling sites. It is important that minimal reaction occurs with the amino-acid residues in the active centre. The probability that reaction in the active centre occurs is small, since the pertinent amino acids are practically always more than once present in a nonessential form in the protein chain. By random chemical reaction it is thus likely that only part of the enzyme molecules will be inactivated, unless a large excess of reagent is present. It is nevertheless useful, before trying a specific covalent-binding method, to have knowledge of the effect of several reagents on enzyme activity and to know whether the enzyme can withstand the coupling conditions of that particular procedure, since covalent couplings cannot always be performed under mild conditions. The advantage of immobilization by covalent binding is that the binding is usually irreversible, so that the probability of enzyme loss by leakage is very small.

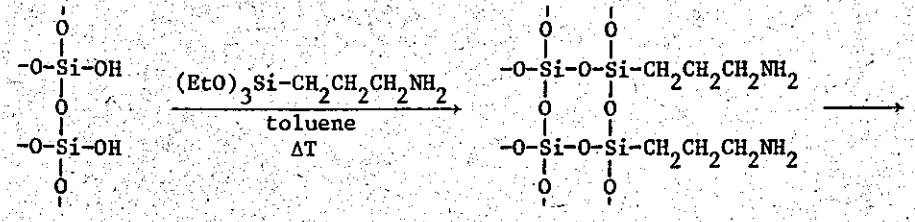
According to the mode of linkage, the method of covalent binding can be classified into diazo, peptide and alkylation methods, coupling by means of multifunctional reagents, and some miscellaneous procedures.

3.2.3.1. The diazo method

This coupling procedure is often used for immobilization of enzymes. The method is based on the reaction of the enzyme with diazonium derivatives of solid supports, that is supports containing aromatic amino groups, diazotized with nitrous acid, according to the overall reaction:

$$R\text{-}NH_2 \xrightarrow[HCl]{NaNO_2} (R\text{-}\overset{+}{N}\equiv N)Cl^- \xrightarrow{Enzyme} R\text{-}N{=}N\text{-}Enzyme$$

Functional groups of proteins likely to participate in diazo coupling include free amino groups, the imidazole side chain of histidine, and, especially, the phenolic group of tyrosine residues. As supports for this method aromatic amino derivatives of polysaccharides, of copolymers of amino acids, of polyacrylamide, of polystyrene, of copolymers of ethylene and maleic acid, and of porous glass, are commonly employed. See as an example the scheme in Fig. 3.3, showing the preparation of the diazo derivative of CPG and its coupling to the enzyme.



$$\xrightarrow[CHCl_3,\ \Delta T]{p\text{-nitrobenzoylchloride}} CPG\text{-}C_3H_6\text{-}NHCO\text{-}phenyl\text{-}p\text{-}NO_2 \xrightarrow[H_2O]{dithionite}$$

$$CPG\text{-}C_3H_6\text{-}NHCO\text{-}phenyl\text{-}p\text{-}NH_2 \xrightarrow[HCl]{NaNO_2} CPG\text{-}C_3H_6\text{-}NHCO\text{-}phenyl\text{-}p\text{-}\overset{+}{N}\equiv N\ Cl^- \longrightarrow$$

$$\xrightarrow{Enzyme} CPG\text{-}C_3H_6\text{-}NHCO\text{-}phenyl\text{-}p\text{-}N{=}N\text{-}Enzyme$$

Figure 3.3 Diazo coupling of enzymes to controlled-pore glass (CPG)

3.2.3.2. Peptide-binding method

This method is an application of the peptide-synthesis technique and is based on the formation of peptide bonds between enzyme and solid support. It can be cate-

gorized as follows:

1. Supports containing carboxyl groups can be converted to reactive derivatives such as acyl azide, acid chloride, or isocyanate. These derivatives form with free amino groups of the enzyme peptide bonds.
2. Peptide bonds are formed between free carboxyl or amino groups of the enzyme and carboxyl or amino groups, respectively, of the support using condensing agents such as carbodiimides and Woodward's reagent K.

An example of the first category is the acyl azide derivative of carboxymethylcellulose which preparation is shown in Fig. 3.4.

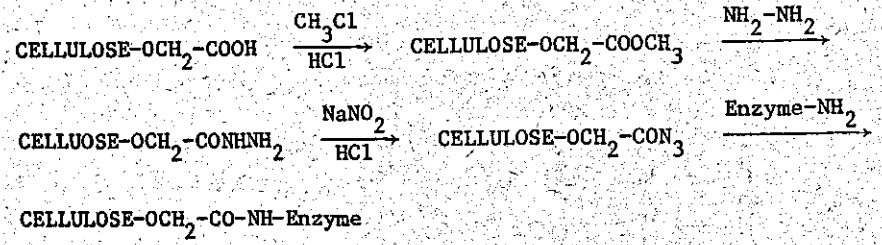


Figure 3.4 Azide coupling of an enzyme to carboxymethylcellulose.

Activation of polysaccharides with CNBr (Fig. 3.2) also belongs to the first category. Instead of reacting the reactive imidocarbonate with *n*-alkylamine, the reaction can immediately be performed with the enzyme, yielding mainly the isourea derivative. Of the second category activated carbon and controlled-pore glass are given as examples (Fig. 3.5).

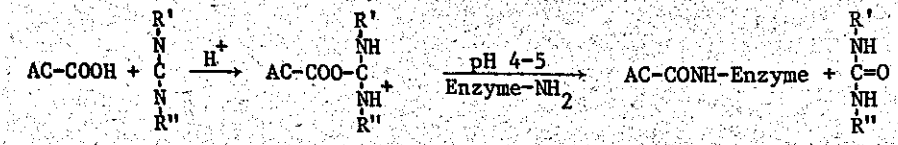


Figure 3.5.A Covalent immobilization of an enzyme onto activated carbon (AC) using a carbodiimide derivative

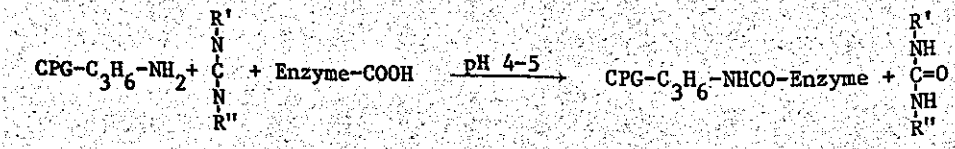


Figure 3.5.B Covalent immobilization of an enzyme onto the γ-aminopropyl-derivative of controlled-pore glass (CPG) using a carbodiimide derivative.

Amines form amide linkages with carbodiimide-activated carboxyl groups. The reaction can be carried out with both water-soluble and insoluble carbodiimides. They are performed under mildly acidic conditions, unlike most other amide-forming reactions.

3.2.3.3. Alkylation method

The alkylation method involves the alkylation of amino, phenolic or sulfhydryl groups of an enzyme with a reactive group of the support such as a halide. The alkylation method we have used to illustrate this principle, involves a reaction with s-trichlorotriazine, followed by an alkylation of a primary amine in the protein chain, primarily the ε-amino group of a lysine residue. The reaction is relatively simple and straightforward and appears to be a potential coupling method of choice (Fig. 3.6). For large-scale applications cost may be a limiting factor.

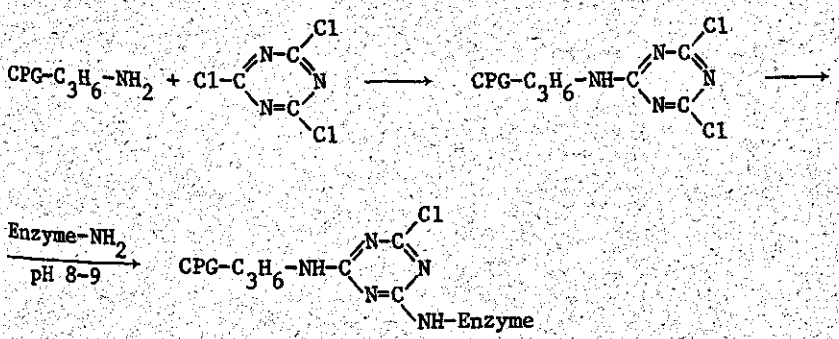


Figure 3.6 Covalent immobilization of an enzyme onto the γ-aminopropylderivative of controlled-pore glass (CPG) by means of s-trichlorotriazine.

Another example of the alkylation method is the binding via an epoxy group as shown in Fig. 3.7.

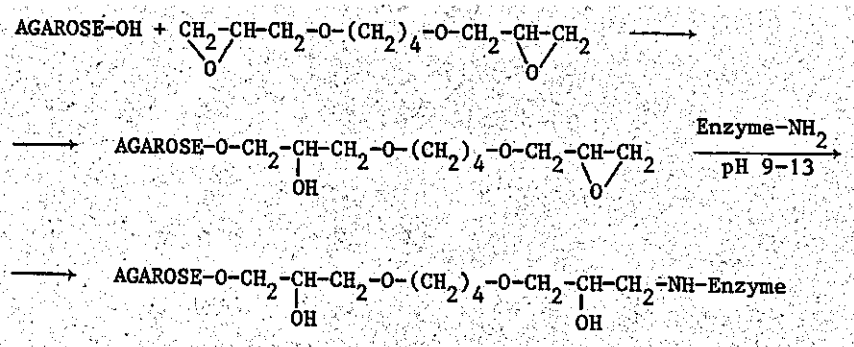


Figure 3.7 Covalent immobilization of an enzyme onto epoxy-activated agarose.

Derivatives of cellulose, agarose, porous glass, bentonite and acrylic copolymers have mainly been used as support.

3.2.3.4. Support binding with multifunctional reagents

This method is based on the formation of crosslinks between functional groups of support and enzyme by means of multifunctional reagents. Glutaraldehyde is by far the bifunctional reagent most-commonly employed, although its mechanism of binding is the least well understood. The reaction is usually presented as a Schiff's base formation, but according to Richard and Knowles[11] the actual reaction is much more complicated. They found that commercial glutaraldehyde solutions are invariably not pure and can be contaminated with acids, significant amounts of α,β-unsaturated aldehydes, and polymeric materials originating from glutaraldehyde itself. As a mechanism of the reaction of this bifunctional reagent with proteins they propose a Michael-type addition of amino groups of the protein and support to the α,β-unsaturated aldehydic polymer as shown in Fig. 3.8.
Both controlled-pore glass and activated carbon have been activated with glutaraldehyde for immobilization of xanthine oxidase in our own work. The reaction is gentle and can be carried out at a pH of about 7. By performing the reaction sequentially with an intermediate washing step, intermolecular enzyme crosslinking can be prevented. The reaction is very simple, extremely gentle and relatively inexpensive to scale up.

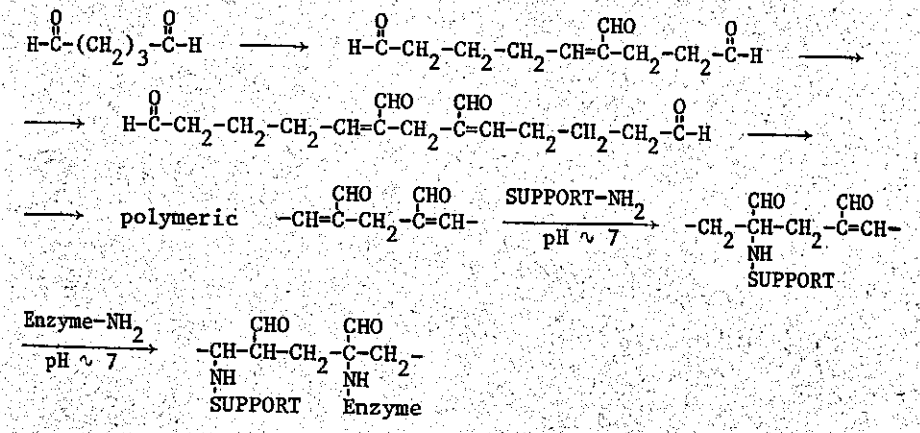


Figure 3.8 Proposed mechanism of enzyme immobilization with glutaraldehyde.

3.2.3.5. Miscellaneous covalent-binding methods

1. *The Ugi reaction.* This reaction is unusual because it involves four different functional groups, *i.e.*, carbonyl, amino, isocyano and carboxyl groups leading to the formation of an N-substituted amide according to the scheme as presented in Fig. 3.9.

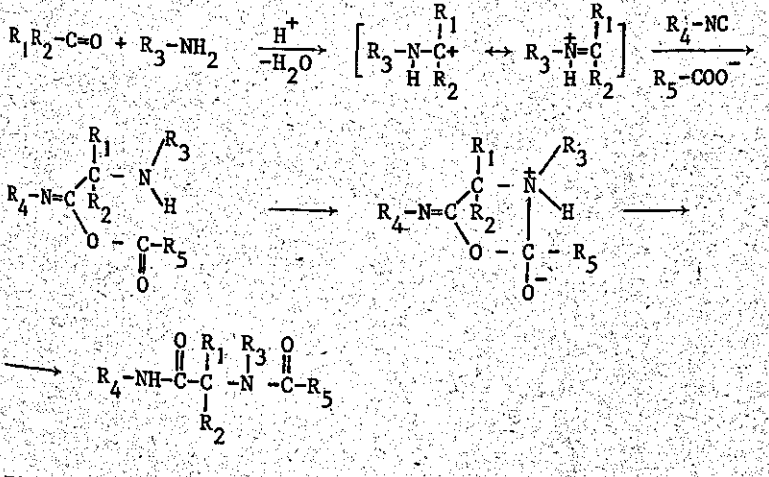


Figure 3.9 The Ugi reaction.

If one of the R_1-R_5 groups is a solid support, immobilization of enzymes is possible in principle. The enzymes can be coupled either through their amino or carboxyl groups. The reaction also works with a hydroxyl group replacing the carbo-

xyl group. It has been successfully applied with polysaccharide, polyacrylic and nylon supports mainly using α-chymotrypsin as the enzyme to be immobilized.

2. *Reversible immobilization by thiol-disulfide interchange.* A relatively new immobilization technique is based on thiol-disulfide interchange between thiol groups of enzymes and mixed disulfide residues of supports. The preparation of a mixed disulfide derivative of a support and its use for enzyme immobilization are pictured in Fig. 3.10. The special advantage of this technique is that the

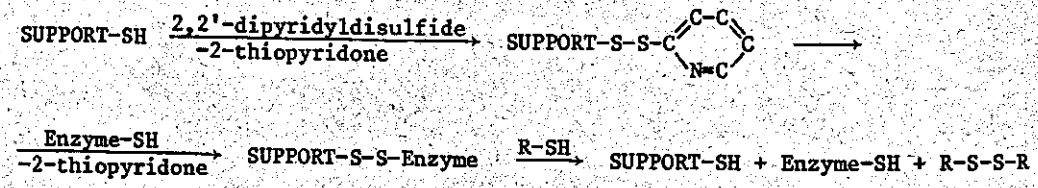


Figure 3.10 Reversible immobilization of enzymes by thiol-disulfide interchange.

enzyme can be removed with for instance dithiothreitol or 2-mercaptoethanol and the support subsequently reactivated. Thiolation of enzymes not containing thiol groups and subsequent immobilization via disulfide interchange has been reported[12]. Thiolation can be performed with methyl-3-mercaptopropioimidate as shown in Fig. 3.11. As activated thiol support agarose-glutathione-2-pyridyldisulfide is commercially available.

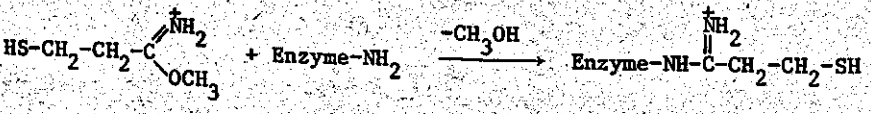


Figure 3.11 Thiolation of enzymes with methyl-3-mercaptopriopioimidate.

Several other covalent-binding methods have been used for the immobilization of enzymes. These methods have mostly found limited use and are therefore not treated here. They can be found in the more extensive reviews[1-7].

3.3. THE CROSSLINKING METHOD

A water-insoluble enzyme preparation can be produced by intermolecular crosslinking of the enzyme molecules in the absence of a solid support. If possible, a crosslinking agent is chosen which specifically binds functional groups on the

enzyme not involved in the catalysis at a concentration suitable for complete insolubilization with retention of activity. Although the procedure is simple to perform the establishment of the suitable conditions to achieve this goal is difficult and must be determined empirically. Only a few thorough studies have been reported, but these show that insolubilization of enzymes with maximum retention of activity depends on a delicate balance of factors such as concentration of enzyme and crosslinking reagent, pH, ionic strength, temperature and reaction time. The flow properties of the resulting preparations are generally poor as they are gelatinous in nature. This method is therefore usually combined with another immobilization technique. Adsorption to a solid support followed by crosslinking of the adsorbed enzyme molecules has worked for a variety of enzymes. Glutaraldehyde has been most extensively applied as a crosslinking agent, but a long list of multifunctional reagents have been used[4].

3.4. THE ENTRAPPING METHOD

The entrapping method is based on confining enzymes to the lattice of a polymer matrix or enclosing them in semipermeable membranes. This method is subdivided into entrapping in a lattice, microcapsule or fiber (Fig. 3.1).

3.4.1. Lattice type

This type involves entrapping enzymes within the interstitial spaces of a crosslinked water-insoluble polymer. The entrapment usually occurs by polymerization of an aqueous solution of the monomers and the enzyme in the presence of a crosslinking agent. Advantages of this method are the general applicability and the homogeneous distribution of the enzyme in the support. Several procedures exist, *e.g.* immobilization in gelatin crosslinked with glutaraldehyde, which can be performed under very mild conditions. The technique of this type most-widely used, *i.e.*, entrapment in a polyacrylamide gel, is also carried out under mild circumstances, but when polymerized in bulk the heat of reaction may denature the enzyme. Disadvantages of this method are that *i.* the polymerization must be performed in the presence of the enzyme, which may lead to chemical modification with concomitant inactivation, *ii.* a small amount of enzyme usually leaks from the support, and *iii.* the diffusion limitation of substrate and product in the gel pores is generally high, especially for high molecular weight compounds, leading to retardation in reaction velocity. Even complete inactivity has been

observed for amylases as a result of the inability of the substrate (starch) to penetrate into the gel.

3.4.2. Microcapsule type

Enzymes can be immobilized within microcapsules prepared from organic polymers. Microencapsulation is usually achieved by dispersing an aqueous enzyme solution containing 1,6-diaminohexane into a solution of adipoylchloride in an organic solvent immiscible with water (*e.g.* chloroform, tetra, toluene). They polymerize upon contact at the water/organic-solvent interface, forming a thin polyamide (nylon-6,6) membrane around aqueous droplets of enzyme solution:

$$n\ NH_2(CH_2)_6NH_2 + n\ ClCO(CH_2)_6COCl \xrightarrow{-HCl} -(NH(CH_2)_6NHCO(CH_2)_6CO)_n-$$

A solution of cellulose acetate in an organic solvent gives a precipitate and forms a membrane when contacted with water. This is also a common method used for the microencapsulation of enzymes.

3.4.3. Fiber type

A method strongly related to microencapsulation is fiberentrapment, a procedure developed by Dinelli in Italy[13]. In this process an emulsion is formed of a synthetic polymer in an organic solvent, *e.g.* cellulose acetate or polyvinylchloride in methylene chloride, with an aqueous enzyme solution. The emulsion is extruded through a spinneret into a precipitate, droplets of enzyme solution being trapped in the fiber.

The applicability of both microencapsulation and fiberentrapment is limited by the necessity of using water-immiscible solvents, which may in some cases cause inactivation of the enzyme. Both methods also result in immobilized-enzyme systems exhibiting diffusion-limited kinetics and hence are best suited for enzyme systems that involve substrates and products with a low molecular weight.

3.5. CONCLUSION

None of the above discussed immobilization procedures (the examples only covering part of the large number described in the literature) has pronounced advantages for all enzymes. Hence, the immobilization of a particular enzyme aimed at a specific application still requires an empirical, essentially trial and error ap-

proach. Of course, the choice of methods to be investigated should be made on the basis of need and on the available knowledge.

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4. IMMOBILIZED XANTHINE OXIDASE: KINETICS, (IN)STABILITY, AND STABILIZATION BY COIMMOBILIZATION WITH SUPEROXIDE DISMUTASE AND CATALASE

J. Tramper, F. Müller and H.C. van der Plas

4.1. SUMMARY

Milk xanthine oxidase was immobilized by covalent attachment to CNBr-activated Sepharose 4B and by adsorption to *n*-octylamine-substituted Sepharose 4B. The amounts of activity immobilized were 30% and 90%, respectively, for the two preparations. The pH optima for free and adsorbed xanthine oxidase were at 8.6 and 8.2, respectively. Both free and immobilized xanthine oxidase showed substrate inhibition. The apparent inhibition constant (K_i') found for adsorbed xanthine oxidase with xanthine as substrate was higher than the K_i for the free enzyme, which was shown to be due to substrate-diffusion limitation in the pores of the support beads (internal-diffusion limitation). Higher substrate concentrations as desirable for practical application in organic synthesis, can therefore be used with the immobilized enzyme without decreasing the rate. As a result of the internal-diffusion limitation the apparent Michaelis constant (K_m') for adsorbed xanthine oxidase was also higher than the K_m for the free enzyme.

Immobilized xanthine oxidase was more stable than the free enzyme during storage at 4° and 30°C. Both forms rapidly lost activity during catalysis. The loss was proportional to the amount of substrate converted. Coimmobilization of xanthine oxidase with superoxide dismutase and catalase improved the operational stability, suggesting that O_2^- and H_2O_2, side products of the enzymic reaction, were involved in the inactivation. Coimmobilization with albumin also had some stabilizing effect. Complete surrounding of xanthine oxidase by protein, however, by means of entrapment in glutaraldehyde-crosslinked gelatin, considerably enhanced the operational half life. This system was less efficient than the Sepharose preparations either because much activity was lost during the immobilization procedure and/or because it had poor flow properties.

Xanthine (15 mg) was converted by adsorbed xanthine oxidase and product (uric acid) was isolated in high yield (84%).

4.2. INTRODUCTION

Many enzymes have been immobilized in recent years and their potential applications in industry, medicine, and analytical chemistry have been recognized[1]. A largely unexplored area as yet is the use of immobilized enzymes in organic synthesis. The high reaction specificity and efficiency of enzymes, and the very simple work-up procedures that become possible when immobilized enzymes are used in a batch or column process, are very attractive features. The long-term outlook for this area is, therefore, very favorable[2a].

In one of our laboratories (Organic Chemistry) there is strong interest in the chemistry of aromatic heterocyclic compounds. Nucleophilic substitution, covalent amination, and ring transformations of azines, diazines, triazines, pteridines, and purines are subjects of continuous study[3]. Oxidation of azaheterocycles, which are electron-deficient compounds, is usually difficult. Suckling and Suckling[4], however, indicated that xanthine oxidase can potentially oxidize many electron-deficient heteroarenes not closely related to xanthine. Therefore, a study was initiated to investigate the suitability of immobilized xanthine oxidase for application in organic synthesis.

In order to make an immobilized enzyme acceptable for routine organic-chemistry practice, several criteria need to be met [2a]. The enzyme must catalyze a reaction of general preparative interest to chemists, possess broad specificity and narrow stereospecificity, be commercially available at an economical price, and be stable. Further, sufficient specificity data should be available to enable reliable predictions to be made for any previously unevaluated potential substrates.

Bovine-milk xanthine oxidase is commercially available and catalyzes the oxidation of many useful purines, pteridines and their analogs in methabolic pathways[5]. Xanthine oxidase can withstand a limited amount of organic solvents[6], a property which can be important in overcoming the general problem in organic chemistry of solubility. Although xanthine oxidase has been immobilized for different purposes[7-11] by convenient procedures, no data on pertinent parameters for efficient synthetic use (pH optimum, substrate optimum, stability under turnover conditions) were reported. We have therefore undertaken a detailed study on immobilized xanthine oxidase. It was found that xanthine oxidase is rather unstable in the presence of substrate, but coimmobilization with superoxide dismutase and catalase yielded a preparation sufficiently stable to use for conversion of xanthine on a preparative scale.

4.3. MATERIALS AND METHODS

4.3.1. Materials

Xanthine oxidase from cow's milk (EC 1.2.3.2) and catalase from beef liver (EC 1.11.1.6) were purchased from Boehringer, Mannheim. Superoxide dismutase (EC 1.15.1.1) from bovine erythrocytes was the gift of Dr.S.G.Mayhew. Bovine serum albumin was from Sigma. Freeze-dried CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Xanthine, puriss (Fluka A.G., Buchs S.G., Switzerland) and uric acid (Merck, Darmstadt) were at least 98% pure, based on specifications given and checked independently by elemental analysis and mass spectra. All other materials were at least reagent grade.

4.3.2. Covalent coupling of xanthine oxidase, superoxide dismutase, catalase, and albumin to CNBr-activated Sepharose 4B

Xanthine oxidase was immobilized by the general coupling procedure for CNBr-activated Sepharose[12]. Thirty ml of a dialyzed enzyme solution (0.2 mg protein.ml^{-1}; 0.03 M borate buffer, pH 8.2; 0.5 M NaCl) were used per gram of freeze-dried support. Xanthine oxidase, superoxide dismutase and catalase were coimmobilized by reacting the washed and swollen CNBr-activated Sepharose 4B (1 g of freeze-dried product) first with 50 ml of superoxide dismutase solution (8 µg protein.ml^{-1}) for 1 h at room temperature, before 5 ml of the above xanthine-oxidase solution and 5 ml catalase solution (0.2 mg protein.ml^{-1}) were added. Coimmobilization of xanthine oxidase and albumin was performed by the reaction of 15 ml of the above xanthine-oxidase solution and 15 ml of an albumin solution (0.2 mg.ml^{-1}) with CNBr-activated Sepharose 4B (1 g of freeze-dried product).

4.3.3. Adsorption of xanthine oxidase, superoxide dismutase, and catalase to Sepharose 4B substituted with n-octylamine

This immobilization procedure was analogous to the one described by Hofstee and Otillio[8]. Thirty ml of xanthine-oxidase solution (0.2 mg protein.ml^{-1}; 0.03 M borate buffer, pH 8.2) were used per gram of freeze-dried CNBr-activated Sepharose 4B. To accomplish coimmobilization with superoxide dismutase and/or catalase, solutions of these enzymes in concentrations as specified in Fig. 4.4 were applied to 300 mg of the filtered xanthine-oxidase/Sepharose preparation. Coimmobilization

was also accomplished by reaction of *n*-octylamine-substituted Sepharose 4B with a mixture of the 3 enzymes, or by first reacting with superoxide dismutase alone and then with catalase and xanthine oxidase.

4.3.4. *Immobilization of xantine oxidase, superoxide dismutase, and catalase in glutaraldehyde-crosslinked gelatin*

Ordinary cooking gelatine (0.5 g) was dissolved in 5.5 ml of glass-distilled water by heating. After cooling to room temperature the solution was thoroughly mixed with 0.2 ml of xanthine-oxidase solution (10 mg protein.ml^{-1}) and poured onto a polyethylene cover in such a way that a thin film formed. The stiff gel formed at 4°C was cut into small strips, which were immersed in a solution of 25 ml of 0.25% glutaraldehyde (0.1 M in citrate buffer, pH 6) at 4°C for 15 h. The strips were then cut into small cubes and treated again with a glutaraldehyde solution as mentioned above. The cubes were finally washed with glass-distilled water. Coimmobilization of a mixture of xanthine oxidase (0.2 ml; 10 mg protein.ml^{-1}), catalase (0.2 ml; 10 mg protein.ml^{-1}), and superoxide dismutase (1 ml; 0.3 mg protein.ml^{-1}) was performed by the same procedure, except that the film had to be immersed first in the glutaraldehyde solution for 4 h before it was strong enough to be cut into strips. To accomplish interenzyme crosslinking, a similar enzyme mixture was incubated with 1 ml of the above glutaraldehyde solution at 4°C for 16 h and then entrapped in the gelatin by the same procedure. All the above immobilized-enzyme preparations were stored in Tris-HCl buffer (pH 8.2; I=0.01; 0.1 mM EDTA) at 4°C until use.

4.3.5. *Enzyme-activity assays*

The enzyme activity was determined from the initial slope of the absorbance *vs* reaction-time curve, representing the rate of product (uric acid) formation. All assays were done on an Aminco-Chance dual-wavelength/split-beam recording spectrophotometer, equipped with a magnetical device to stir directly within the cuvet. The temperature of the cuvet holder was always kept at 30°C. Xanthine was used as substrate in all experiments, having the spectrophotometer in the dual-wavelength mode, the sample wavelength at 295 nm and the reference wavelength at 278 nm (isosbestic point of xanthine and uric acid). All the solutions were buffered with Tris-HCl of constant ionic strength (I= 0.01) containing 0.1 mM EDTA. The xanthine-oxidase/Sepharose preparations were suspended in the appropriate buffer

(250 ml per gram of freeze-dried Sepharose) and 0.5 ml aliquots of the homogeneous suspension rapidly pipetted into a cuvet (analogous to the procedure developed by Mort *et al.*[13] for aldolase bound to Sepharose). Two ml of substrate (30°C) was then added and the change in absorbance recorded, while stirring so fast that a homogeneous suspension was obtained. The activity of soluble xanthine oxidase was determined by adding 2 ml of substrate (30°C) to 0.1 ml of dialyzed-enzyme solution (0.2 mg protein.ml^{-1}) and recording the change in absorbance. pH profiles were determined by varying the pH by the addition of Tris. In this way the ionic strength was kept constant[14]. The molar differential absorption coefficients were determined from mixtures of substrate and product in the concentration range and pH applied. The activity of superoxide dismutase was determined by the method of McCord and Fridovich[15]. The catalase-activity assay was essentially that described by Beers and Sizer[16]. The protein content of the samples was determined by the modified Lowry microprocedure[17].

4.3.6. Enzyme stability

The stability of the soluble and immobilized enzyme was determined by incubation of a series of enzyme samples in the presence or absence of substrate at 4° and 30°C, and assaying the activity of the samples after different incubation times. Aliquots of the immobilized enzyme were also packed into columns (1 cm high x 0.5 cm i.d.). Enzyme stability under continuous operation was determined by pumping substrate at constant flow rate (46 ml.h^{-1}) and temperature (25°C) through the column and measuring the absorbance of the eluate at 290 nm as a function of time. Conversions were computed using the molar extinction coefficient of xanthine which is one third of the molar extinction coefficient of uric acid at 290 nm[18]. The aliquots were chosen such that complete conversion of substrate was obtained when the reaction was started.

4.3.7. Preparative-scale conversion of xanthine

Freeze-dried CNBr-activated Sepharose 4B (1.2 g) was substituted with *n*-octylamine as described above and reacted with 18 ml of xanthine oxidase (0.2 mg protein.ml^{-1}) and 18 ml of catalase (0.2 mg protein.ml^{-1}) solution. The washed enzyme preparation was suspended in 50 ml buffer, pH 8.2, and 5 ml of the suspension was packed into a column and the half life determined. The rest of the suspension was rotated in a round-bottomed flask and continuously fed with concen-

trated xanthine solution (2 mM) at such a speed that no substrate accumulated (based on the half life). This was regularly checked by taking small samples and measuring the extinction at 290 nm.
After 8 h of operation the addition was stopped and the reaction continued for another hour. The immobilized enzyme was filtered off, the filtrate (100 ml) acidified and concentrated to 10 ml. The precipitate formed upon cooling was filtered, washed with ethanol and ether, dried, weighed and analyzed.

4.4. RESULTS AND DISCUSSION

4.4.1. Activity of immobilized enzyme

Active immobilized xanthine oxidase was obtained through covalent attachment to CNBr-activated Sepharose 4B. Under the conditions used no remaining activity or protein was found in the supernatant after the coupling, suggesting that all the enzyme was covalently bound and/or adsorbed to the gel. When the immobilized enzyme was assayed before washing, about 80% of the original activity was found. However, when the gel was washed alternately with slightly acidic and basic buffers of high ionic strength, the activity rapidly dropped to 30%. This remaining activity decreased only slightly on further washing and it was therefore assumed that this activity was due to covalently bound enzyme. Similarly, up to 90% of the activity was retained upon adsorption of xanthine oxidase onto Sepharose 4B substituted with *n*-octylamine. Again, the supernatant did not contain residual enzyme activity. Since the retention of activity obtained by the latter procedure was much better, we chose the adsorbed-enzyme preparation for kinetic characterization.

4.4.2. Kinetic characterization

pH optima at about 8.3 have been reported in the literature[6] for the free enzyme and we therefore investigated the activity of the adsorbed enzyme as a function of the pH in the range 7.7-8.9. A pH optimum at 8.2 was found for the adsorbed enzyme and at 8.6 for the free enzyme (Fig. 4.1). This acid shift upon immobilization could be the result of the slight positive charge which the support takes on at these pH's (the isourea derivative mainly formed in the reaction of Sepharose with *n*-octylamine has a pK_a of about 10.5), thus creating a microenvironment around the enzyme, which contains in the applied buffer of low ionic strength,

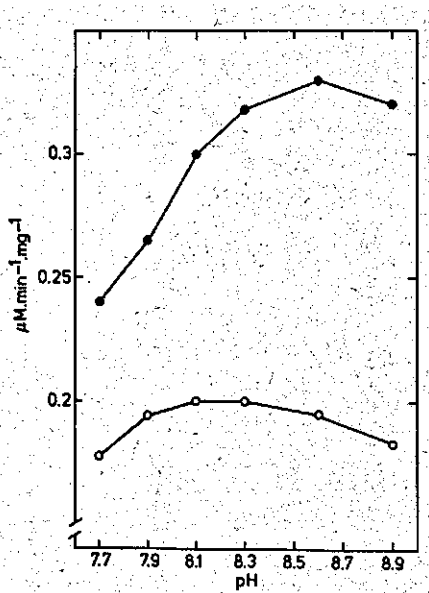

Figure 4.1 Effect of pH on the activity of (●-●) free and (O-O) adsorbed xanthine oxidase. Substrate: 20 μM xanthine in Tris-HCl buffer (I = 0.01; 0.1 mM EDTA; 30°C).

more hydroxyl ions than in the bulk of the solution. The pH in the direct environment of the immobilized enzyme is thus higher than the pH of the bulk, resulting in the apparent acid shift of the optimum[2b].

The K_m for the free enzyme with xanthine as substrate was found to be smaller than the apparent K_m (K_m') for the adsorbed enzyme, *i.e.*, 5 and 21 μM, respectively (Fig. 4.2a). Such changes in K_m to larger values are usually taken as an indicatio that the reaction could be limited by diffusion. Increasing the stirring speed in the cuvet had no effect on the rate of the reaction suggesting that diffusion of substrate from the bulk into the gel (external-diffusion limitation) was not limit ing. It was found that the K_m' depends on the amount of enzyme bound per unit volume of gel; in an experiment where the enzyme load of the support was one third that of the above preparation, the K_m' decreased from 21 to 11 μM (Fig. 4.2a). This indicates that the rate of reaction is limited by diffusion through the gel pores (internal-diffusion limitation).

Both free and immobilized xanthine oxidase show substrate inhibition (Fig. 4.2a). The substrate inhibition constant K_i can be calculated from a 1/v *vs* S plot[19,20]. Replotting the data of Fig. 4.2a in a 1/v *vs* S plot (Fig. 4.2b) shows that indeed fairly good linearity exists between 1/v and S at the highest substrate concentrations. From the slope and the y-intercept the inhibition constant K_i and the apparent K_i (K_i') for the free and immobilized enzyme were determined to be 94 and

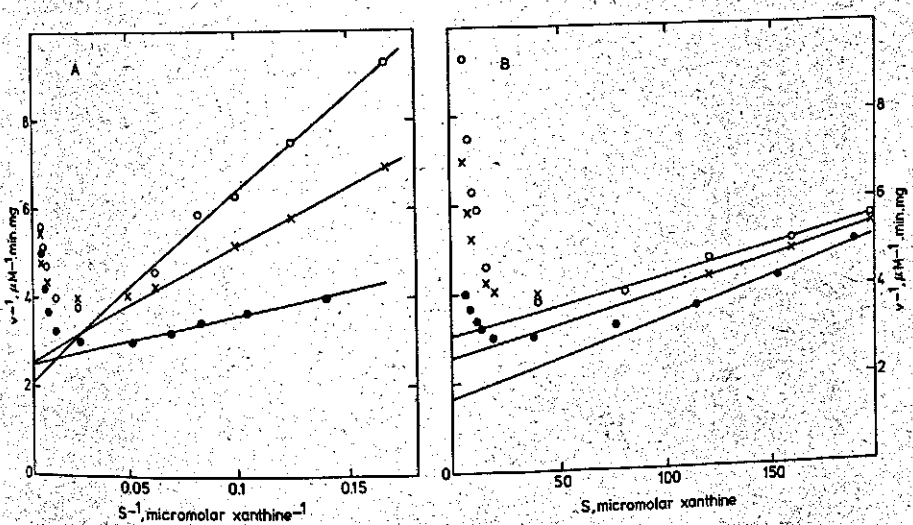

Figure 4.2 (a) Lineweaver-Burk plot of free and immobilized xanthine oxidase. (●-●) Free enzyme in Tris-HCl, pH 8.6 (I = 0.01; 0.1 mM EDTA; 30°C. Immobilized enzyme in Tris-HCl buffer, pH 8.2 (I = 0.01; 0.1 mM EDTA; 30°C): (O-O) 6 mg protein applied/g freeze-dried Sepharose 4B; (X-X) 2 mg protein applied/g freeze-dried Sepharose 4B. (b) Determination of substrate-inhibition constants (K_i, K_i') for (●-●) free and (O-O; X-X) immobilized xanthine oxidase, respectively. Experimental data taken from Fig. 4.2(a).

240 μM, respectively. This means that the decrease in reaction rate as a result of substrate inhibition is less for the immobilized-enzyme system than for the free enzyme. The diffusional hindrance causes the actual local substrate concentration within the microenvironment of the immobilized enzyme deep in the pores to be much lower than in the bulk and consequently the overall reaction rate at high substrate concentrations is relatively higher than in a free-enzyme system without diffusion limitation. That this is indeed caused by diffusion limitation appears from the experiment with the preparation having a lower enzyme load for which K_i' decreased to 177 μM. Generally, diffusion limitation is undesirable since it reduces the effectiveness of the immobilized enzyme, but in this particular case of substrate inhibition it, in fact, enhances the rate of reaction compared with that of the free enzyme. A substrate concentration that would be more practical for organic synthesis can possibly be approached in an immobilized enzyme system having sufficiently high diffusion limitation and thus offers an extra processing advantage. It would then be unnecessary to recycle the product stream through a single reactor or pass it through a series of reactors with

additions of fresh substrate after each successive cycle/column, or running a continuous stirred tank reactor to obtain sufficiently high product concentrations.

4.4.3. Enzyme stability

If it is to be useful in organic synthesis, the immobilized enzyme should remain active during long periods of catalytic turnover and during storage. The immobilized xanthine oxidase (both covalently bound and adsorbed) was found to be much more stable than the soluble enzyme when incubated at 4° and 30°C in Tris-HCl or borate buffer at pH 8.2. The immobilized enzyme lost no activity during 6 months of storage at 4°C, while the activity of the soluble enzyme (0.2 mg protein.ml^{-1}) decreased by about 30% in 4 months. The half lifes ($t_{\frac{1}{2}}$) for the free and immobilized enzyme at 30°C were 55 h, and up to 300 h, respectively. The stability of the immobilized enzyme was not affected by EDTA or the enzyme product (0.2 mM uric acid).

The immobilized enzyme rapidly lost activity during continuous turnover, either in suspensions with substrate or in reactor columns in which a solution of substrate was continuously passed over the enzyme. The half lifes of the adsorbed enzyme (Table 4.1) and the covalently bound enzyme at 25°C during continuous turnover in a reactor column were about 1 h. The soluble enzyme (0.14 mg protein.ml^{-1}) lost all its activity in less than 3 h incubation with substrate (0.7 mM xanthine) at 25°C. The rapid inactivation was unexpected since Hofstee and Otillio[8] found, in an experiment where the strength of binding between matrix and enzyme was tested, at least 90% conversion of substrate during several days at 5°C. When we repeated Hofstee's experiment a similar high conversion was obtained during 1 week, but this sustained high conversion was due to the fact that the column initially contained much more enzyme than was needed for complete conversion and, at the constant pressure applied, the column became more tightly packed and consequently the flow rate continuously decreased. A constant flow rate was obtained by flowing the substrate from the bottom to the top at lower speed, or by using a pump. When, in addition, less enzyme was used, it was possible to determine the operational half life at 4°C. It was found to be 4 h. A similar value was obtained for the covalently bound enzyme under identical conditions.

Attempts were made to determine the cause of the rapid inactivation of soluble and immobilized enzyme that occurs in the presence of substrate. Incubation with product did not affect the stability or activity of either type of immobilized

Table 4.I Half lifes of various xanthine-oxidase preparations during catalysis (25°C)

Enzyme system	Half life (h)
Free xanthine oxidase	<1
Sepharose preparations:	
- covalently bound xanthine oxidase	1.3
- adsorbed xanthine oxidase	1.3
- xanthine oxidase and superoxide dismutase coadsorbed	2
- xanthine oxidase and catalase coadsorbed	3.3
- xanthine oxidase, superoxide dismutase and catalase coadsorbed	5.5
- covalently coimmobilized xanthine oxidase, superoxide dismutase and catalase	7.5
- covalently coimmobilized xanthine oxidase and albumin	2
Gelatin preparations:	
- xanthine oxidase entrapped in gelatin	26
- xanthine oxidase, superoxide dismutase and catalase entrapped in gelatin	26
- xanthine oxidase, superoxide dismutase and catalase crosslinked and then entrapped in gelatin	50

enzyme, indicating that the inactivation was not due to (ir)reversible product inhibition. The effluents from the columns did not contain activity or protein, and therefore no significant leaching of enzyme occurred as sometimes observed with other systems[21]. Accordingly, the preparations had the same protein content before and after continuous use. Incubation of partially inactive enzyme with FAD or Na_2S did not restore the activity, suggesting that loss of flavin[9,22] or labile persulphide[23] was not responsible for the inactivation. Avoiding normal levels of room light to prevent possible photoinactivation, did not influence the course of inactivation. Fig. 4.3 clearly shows that the rapid drop in activity is proportional to the absolute amount of substrate converted. When all the substrate is converted the decay is comparable with that in buffer alone. The rapid inactivation could be decreased, however, by adding superoxide dismutase and/or catalase to the incubation mixture. This observation suggested that O_2^- and H_2O_2 produced in the enzyme reaction, or $OH\cdot$ produced by the chemical reaction of O_2^- with H_2O_2[24], might be involved in the inactivation phenomenon. It has been observed that O_2^- has a damaging effect in biological systems[25] and that in dilute xanthine-oxidase solutions the enzyme is inactivated by relatively high H_2O_2 concentrations[26]. In the microenvironment of the immobilized enzyme, because of the slight positive

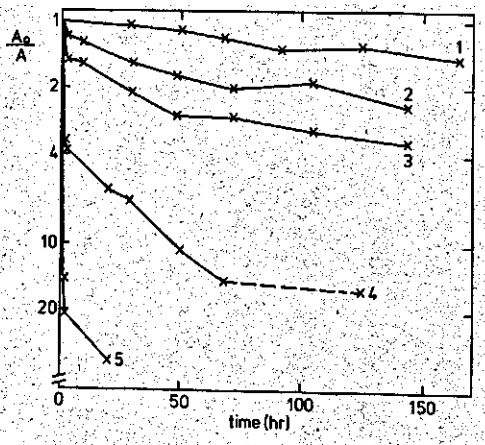

Figure 4.3 Dependence of the stability of adsorbed xanthine oxidase on the concentration of xanthine at 30°C. Assay consisted of aliquots containing 0.4 mg protein in 10 ml Tris-HCl buffer, pH 8.2 (I = 0.01; 0.1 mM EDTA) and various xanthine concentrations: 1, 0 µmol; 2, 0.6 µmol; 3, 1.2 µmol; 4, 4 µmol; and 5, 6 µmol. Ratio of the activity at t = 0 (A_o) and t(A) is plotted is a function of time.

charge of the matrix and as a result of the diffusion limitation, accumulation of O_2^- and H_2O_2 is likely to occur. Therefore, we expected that optimal protection of this system could be achieved by coimmobilization of superoxide dismutase and catalase with xanthine oxidase.

4.4.4. Coimmobilization of superoxide dismutase and/or catalase with xanthine oxidase

Superoxide dismutase and catalase have an isoelectric point in the acid region at pH 4.95[27] and 5.4[28], respectively, which makes them, like xanthine oxidase, suitable for immobilization by the procedure described by Hofstee and Otillio[8]. Both enzymes could indeed be coimmobilized with xanthine oxidase by adsorption on Sepharose 4B substituted with *n*-octylamine as described in Materials and Methods. The immobilized-enzyme material had activity of all three enzymes, although it should be noted that under the conditions used (Fig. 4.4) less than 30% of the initial soluble superoxide dismutase activity was immobilized, compared with much larger amounts of catalase (>90%). As the data of Fig. 4.4 show, either dismutase or catalase improved the stability of xanthine oxidase, and coimmobilization of both dismutase and catalase gave a further improvement. The protection given by catalase was constant during the time of operation, and, although a detectable amount of catalase activity was found in the effluent, the catalase activity of the immobilized-enzyme preparation was still very high after 8 h of operation. The protection afforded by superoxide dismutase activity decreased with time and the dismutase activity of the column material was zero after 8 h of operation. Low levels of superoxide-dismutase activity were detected in the first fractions of the ef-

fluent and the loss of protection can thus be explained by leakage of dismutase

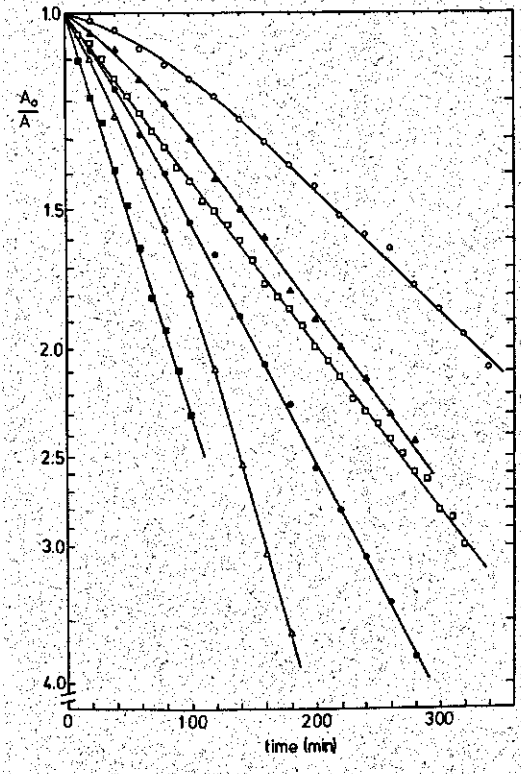

Figure 4.4 Stability of adsorbed xanthine oxidase (XO) under continuous operation at 25°C (constant flow rate: 46 ml. h^{-1}). Amount of XO was in all cases 0.4 mg (0.7 units.mg^{-1}) per column (1 cm high x 0.5 i.d.). Feed: 40 μM xanthine in Tris-HCl buffer (pH 8.2; I = 0.01; 0.1 mM EDTA. Ratio of the conversion at t = 0 (A_o) and t(A) is plotted. (■-■) Immobilized XO; (Δ-Δ) XO coimmobilized with SOD (0.4 mg); (●-●) XO coimmobilized with SOD (0.4 mg) and CAT (0.5 mg); (□-□) XO coimmobilized with CAT (1.0 mg); (▲-▲) XO coimmobilized with SOD (0.2 mg) and CAT (1.0 mg); (O-O) SOD = superoxide dismutase (3800 units. mg^{-1}); CAT = catalase (3100 units.mg^{-1}).

from the column. The coupling yield and strength of adsorption of superoxide dismutase could not be improved by reacting *n*-octylamine-substituted Sepharose first with only the dismutase.

To prevent the leakage of catalase and superoxide dismutase from the column material, the enzymes were reacted with CNBr-activated Sepharose 4B to covalently bind them. When all the three enzymes were reacted with Sepharose at the same time, xanthine oxidase and catalase were bound but no superoxide dismutase. Accordingly, this preparation showed a half life comparable with that of xanthine oxidase coadsorbed with only catalase. When superoxide dismutase was first reacted with Sepharose (xanthine oxidase and catalase were added after one h) about 10% of the activity applied was bound. The half life of xanthine oxidase in this system improved to 7½ h and no superoxide dismutase, catalase or xanthine oxidase activity was found in the effluent of the continuous reactor. Accordingly, the protein content of this preparation was the same before and after 24 h of operation.

In order to check if the protection afforded by superoxide dismutase and catalase was indeed due to the catalytic action of these enzymes on O_2^- and H_2O_2 rather than a stabilizing effect due to protein-protein interactions, xanthine oxidase was coimmobilized with albumin. The operational stability of this albumin/xanthine-oxidase preparation was slightly increased ($t_{\frac{1}{2}}$=2 h) as compared with the xanthine-oxidase preparation, but it was less than that of the three-enzyme system. To test further a possible effect of protein on the stability of xanthine oxidase, this enzyme was completely surrounded by protein by immobilization in glutaraldehyde crosslinked gelatin. This improved the operational stability considerably ($t_{\frac{1}{2}}$=26 h) No further improvement was achieved by coimmobilization of a mixture of xanthine oxidase, superoxide dismutase and catalase in gelatin. When, on the other hand, the three enzymes were first incubated with glutaraldehyde to accomplish interenzyme crosslinking the stability was further increased ($t_{\frac{1}{2}}$=50 h). This observation again suggests that O_2^- and H_2O_2 are involved in the inactivation, and that superoxide dismutase and catalase protect xanthine oxidase by catalyzing the destruction of these species. Operational half lifes of some preparations are collected in Table 4.I.

The gelatin system has, however, several disadvantages. To obtain a mechanically stable support the amount of glutaraldehyde needed, causes a severe inactivation of xanthine oxidase (retention of activity less than 10%). This observed low activity may also be the result of the poor flow properties of this material in a packed-bed reactor. Another disadvantage is the fact that a small amount of gelatin continuously leaks from the column. Thus, although their half lifes were shorter, the Sepharose preparations were superior.

In order to test the suitability for organic synthesis, a Sepharose preparation of xanthine oxidase and catalase ($t_{\frac{1}{2}}$=4 h) in a batch reactor was continuously fed with substrate at such a rate that no accummulation occurred. Fifty ml of 2 mM xanthine solution (*i.e.* 15 mg) were added in 8 h. The uric acid isolated after work-up was 14.2 mg which represented an 84% yield (UV and Mass spectra were identical with those of authentical material).

4.5. CONCLUSIONS

The kinetics, (in)stability, and stabilization of immobilized xanthine oxidase were studied. The diffusion limitation in the immobilized-enzyme system decreases the inhibitory effect of the substrate. Proper choice of support (high binding capacity, opposite charge as substrate) may further increase the diffusional

hindrance and, as a consequence, more practical substrate concentrations can be used, which further simplefies the isolation of the product. The operational stability of xanthine oxidase is poor, but can be improved by coimmobilization with superoxide dismutase, catalase or protein. In contrast to the operational stability, the storage stability of the immobilized enzyme at room temperature is good. Therefore, since the rapid decrease in activity during catalysis is proportional to the amount of substrate converted, we expect the operational half life to be much longer for poor substrates. This opens the possibility of also converting these compounds in relatively the same amounts as xanthine.

Note added to manuscript. It was pointed out to us by the editor that a paper by Johnson and Coughlan[29] entitled "Studies on the stability of immobilized xanthine oxidase and urate oxiase," was already in press while our paper was still under review. Some of the experiments in the two papers are similar; the results of these experiments are largely in agreement. A point of difference is that we observed stabilization of xanthine oxidase by catalase, whereas Johnson and Coughlan did not; consequently the approaches used to stabilize the enzyme in the two papers are different.

4.6. ACKNOWLEDGEMENTS

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5. KINETICS AND STABILITY OF IMMOBILIZED CHICKEN-LIVER XANTHINE DEHYDROGENASE

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5.1. SUMMARY

Xanthine dehydrogenase (EC 1.2.1.37) was isolated from chicken livers and immobilized by adsorption to a Sepharose derivative, prepared by reaction of *n*-octylamine with CNBr-activated Sepharose 4B. Using a crude preparation of enzyme for immobilization it was observed that relatively more activity was adsorbed than protein, but the yield of immobilized activity increased as a purer enzyme preparation was used. As more activity and protein were bound, relatively less immobilized activity was recovered. This effect was probably due to blocking of active xanthine dehydrogenase by protein impurities.

The kinetics of free and immobilized xanthine dehydrogenase were studied in the pH range 7.5 - 9.1. The K_m and V values estimated for free xanthine dehydrogenase increase as the pH increases; the K_m' and V' values for the immobilized enzyme go through a minimum at pH 8.1. By varying the amount of enzyme activity bound per unit volume of gel, it was shown that K_m' is larger than K_m as result of substrate diffusion limitation in the pores of the support material. Both free and immobilized xanthine dehydrogenase showed substrate activation at low concentrations (up to 2 μM xanthine).

Immobilized xanthine dehydrogenase was more stable than the free enzyme during storage in the temperature range of 4 - 50°C. The operational stability of immobilized xanthine dehydrogenase at 30°C was two orders of magnitude smaller than the storage stability, $t_{\frac{1}{2}}$ was 9 and 800 h, respectively. The operational stability was, however, better than that of immobilized milk xanthine oxidase ($t_{\frac{1}{2}}$ = 1 h). In addition, the amount of product formed per unit initial activity in one half life, was higher for immobilized xanthine dehydrogenase than for immobilized xanthine oxidase. Unless immobilized milk xanthine oxidase can be considerable stabilized, immobilized chicken-liver xanthine dehydrogenase is more promising for application in organic synthesis.

5.2. INTRODUCTION

The chemistry of purines and pteridines is an interesting and important area, because these compounds possess important biochemical (e.g., "one-carbon transfer", biological oxidations) and pharmacological (e.g., antineoplastic, antiprotozoal, diuretic and anti-arrhythmic) properties[1].

The oxidation of these azaheterocyclic compounds is usually difficult because the rings are π-deficient making electron donation to an oxidizing reagent difficult or impossible. The enzyme xanthine oxidase efficiently catalyzes the oxidation of many of these electron-deficient heteroarenes[2] and we are, therefore, studying the suitability of immobilized milk xanthine oxidase for application in organic synthesis[3]. With this technique we are able to conveniently oxidize substrates in milligram quantities, but until now two factors have prevented the efficient use of xanthine oxidase: 1) the low operational stability of the enzyme, even when its stability is improved by coimmobilization with superoxide dismutase and/or catalase and 2) the substrate inhibition. Immobilization can partially solve the second problem, because increasing the amount of enzyme bound per unit volume of support enhances the diffusional hindrance in the support material, and this results in a shift of inhibition to higher substrate concentrations[3,4]. This shift is of course limited by the binding capacity of the support.

Chicken-liver xanthine dehydrogenase is an enzyme closely related to milk xanthine oxidase[5] and also catalyzes the oxidation of many purines and pteridines. The distinguishing feature of the dehydrogenase is that it has very low activity with O_2 and preferentially uses NAD^+ as a terminal electron acceptor. It does not produce O_2^- or H_2O_2 when NAD^+ is used as electron acceptor and we expected, therefore, that it would be more stable than xanthine oxidase under turnover conditions. Another attractive feature of chicken-liver xanthine dehydrogenase is that the substrate concentration at which substrate inhibition actually lowers the reaction velocity, is much higher[6] (> 1 mM) than for milk xanthine oxidase[3] (> 0.1 mM). We have, therefore, initiated a detailed study of immobilized chicken-liver xanthine dehydrogenase. The isolation, immobilization, kinetics and stability of chicken-liver xanthine dehydrogenase are now reported and the relevant properties of the immobilized enzyme compared with those of immobilized milk xanthine oxidase. It is concluded that immobilized xanthine dehydrogenase is more promising for application in organic synthesis than immobilized xanthine oxidase, unless the latter enzyme can be considerably stabilized.

5.3. MATERIALS AND METHODS

5.3.1. Materials

Fresh chicken livers were obtained from Bekebrede BV (Barneveld, The Netherlands) and used immediately or stored at -20°C until required. Freeze-dried CNBr-activated Sepharose 4B and a suspension of Sepharose 4B were obtained from Pharmacia Fine Chemicals. Bovine-serum albumin and NADH (grade III) were from Sigma Chemical Co. Lactate dehydrogenase (EC 1.1.1.27) and NAD^+ (grade I) were purchased from Boehringer. 2,6-Di-chlorophenolindophenol (BDH), EDTA(BDH), xanthine (Fluka), *n*-butylamine (Aldrich), *n*-hexylamine (Aldrich), *n*-octylamine (Aldrich), uric acid (Merck), Folin-reagent (Merck), and all other materials were at least reagent grade.

5.3.2. Purification of xanthine dehydrogenase

Fresh or frozen chicken livers, (about 500 g) were cut in small pieces, suspended in 2 litre of distilled water, and homogenized. Xanthine dehydrogenase was then purified according to the procedure described by Rajagopalan and Handler[7]. Sometimes, however, only part of this procedure was carried out. The following steps were always performed (*basic purification procedure*): heating at 70°C for 2 min, centrifugation, ammonium-sulfate fractionation, acetone fractionation and finally a second ammonium-sulfate fractionation followed by dialysis. Where indicated, the enzyme was further purified on a DEAE-Sephadex column and/or on a Sephadex G-200 column. After each column the fractions containing the most activity were pooled, concentrated and desalted.
In addition to the specific activity, the ratio A_{280}/A_{450} is given as criterium for the purity of xanthine dehydrogenase. The lowest value reported[8] for highly purified xanthine dehydrogenase is 5.5.

5.3.3. Immobilization of xanthine dehydrogenase

Xanthine dehydrogenase was immobilized by adsorption to Sepharose 4B substituted with *n*-octylamine. The procedure was analogous to the one described for milk xanthine oxidase[3,9]. The amounts of enzyme used per gram of freeze-dried CNBr-activated Sepharose 4B are given in the figures and tables. The coupling reaction was terminated when the activity in the supernatant was zero or constant. Some-

times Sepharose 4B was used as starting material which was activated with CNBr according to March *et al*[10], and 3.5 ml of such a suspension was assumed to be equal to 1 g freeze-dried CNBr-activated Sepharose 4B[11]. The degree of substitution of Sepharose 4B with *n*-octylamine was determined by the method based on the irreversible binding of Ponceau S to this support material at low ionic strength[12]

5.3.4. Enzyme-activity assays

The standard activity assay for soluble xanthine dehydrogenase was performed as follows. Nine-tenths ml 2 mM xanthine solution and 0.5 ml 10 mM NAD^+ solution were brought to 10 ml with Tris-HCl buffer (pH 7.9; I = 0.01; 0.1 mM EDTA) and incubated at 30°C for 30 min. Enzyme solution (0.1 - 0.5 ml) was pipetted in a cuvet, brought to 0.5 ml with the Tris buffer (if necessary) and incubated at 30°C. When temperature equilibrium was reached, 2 ml substrate were added, the contents well mixed, and the increase in absorbance at 340 nm (NADH formation) recorded as a function of time on an Aminco-Chance dual-wavelength/split-beam recording spectrophotometer, equipped with a magnetical device to stir directly in the cuvet. The temperature of the cuvet holder was always kept at 30°C. End concentrations of substrates in the cuvet amounted to 144 and 400 µM for xanthine and NAD^+, respectively. The activity was determined from the initial slope of the absorbance *vs* reaction-time curve.

The standard assay for the immobilized enzyme was performed in the same way. In this case the sample was always 0.5 ml and taken from a suitably diluted homogeneous suspension with an Eppendorf pipet (analogous to the procedure developed by Mort *et al*[13] for aldolase bound to Sepharose). The mixture in the cuvet was stirred rapidly during the assays so that a homogeneous suspension was obtained and external-diffusion limitation (diffusion limitation of substrate from the bulk into the gel) was *excluded*.

Apparent Michaelis constants and V's of free and immobilized xanthine dehydrogenase for xanthine were determined as a function of pH in the range 7.5 - 9.1. The ionic strength of the Tris-HCl buffers used was kept constant at 0.01[14]. The xanthine concentration varied from 9 - 600 µM, the NAD^+ concentration was always 400 µM. No significant changes in A^{340}_{NADH} were found in the pH range used. The kinetic constants were estimated from Lineweaver-Burk plots.

Kinetic constants of free and immobilized xanthine dehydrogenase for both xanthine and NAD^+ were determined at pH 7.9. The xanthine concentration varied from 12 to 144 µM, the NAD^+ concentration from 10 to 400 µM. For the calculation of the con-

stants a "ping-pong" mechanism was assumed[15].

To determine the effect of immobilization on substrate activation, reaction rates at low xanthine concentrations (< 12 μM) were measured with O_2, NAD^+ or dichlorophenolindophenol (DCP) as electron acceptors. When O_2 was used as oxidizing substrate (NAD^+ absent) the reaction was followed at 295 nm (uric acid production). Where DCP instead of NAD^+ was used as electron acceptor, the change in absorbance was recorded at 600 nm.

Activity assays for the free enzyme were always performed in duplicate, for the immobilized enzyme in triplicate. Average values are given in the tables and figures.

5.3.5. Protein determination

Protein was determined with the Biuret method during the enzyme purification. This method is not suitable for immobilized protein and, therefore, all other protein determinations were performed with the modified Lowry microprocedure[16], using bovine-serum albumin as standard. The samples were incubated with the Lowry reagents at room temperature in the dark and the mixtures shaken occasionally. After 24-27 h the mixtures were filtered and the absorbance at 700 nm measured. All the assays were performed in duplicate.

5.3.6. Enzyme stability

The storage stability of free and immobilized xanthine dehydrogenase was determined by incubation of a series of enzyme samples in 0.05 M potassium-phosphate buffer (pH 7.8; 0.1 mM EDTA; 0.02 % NaN_3 (w/v)) at several temperatures and assaying the activity of the samples as a function of time.

The operational stability at pH 7.9 and 30°C was determined by pumping substrate at a constant flow rate (43 ml h^{-1}) through a column packed with immobilized enzyme (specifications given in Table 5.VII) and measuring the absorbance of the eluate at 340 nm (NADH formation) as a function of time.

5.4. RESULTS AND DISCUSSION

5.4.1. Purification of xanthine dehydrogenase

The procedure that we used for the enzyme purification has been described by

Rajagopalan and Handler[7], but without details of the purification obtained in the intermediate steps. The data of Table 5.I show that considerable purification was obtained with the basic procedure and with up to 70% yield of activity. Some further purification was obtained after subsequent treatment on Sephadex G-200 and/or DEAE-Sephadex, but large losses in activity occurred during these two steps. The two steps are also time consuming because, in contrast to the basic procedure, which required only 2 days, the complete purification needed 7-8 days. For these reasons we used the less pure xanthine dehydrogenase prepared by the basic procedure in most of the experiments. The immobilization experiments showed (Table 5.II) that use of a purer enzyme preparation for immobilization yielded relatively more immobilized activity, but use of such purer preparation is an advantage only if the yields of activity in the final steps can be drastically improved. Some alternative purification procedures were therefore investigated. Several NAD^+-dependent dehydrogenases can be efficiently purified by Cibacron blue affinity chromatography[17]. We have found that xanthine dehydrogenase (preparation II) is not selectively bound to Sepharose 4B/Blue Dextran (prepared

Table 5.I Dependence of degree of purification of chicken-liver xanthine dehydrogenase on the procedure used.

Preparation	Purification procedure[a]	Specific activity (units/mg)	Purification (fold)	Yield (%)	A_{280}/A_{450}[b]
I	complete	2.4	41.7	2.4	5.6
II	basis + Sephadex G-200	2.8	45.0	21.9	8.0
III	basis	1.4	23.3	53.5	17.1
IV	basis	1.5	24.2	73.0	16.4
IV^a	IV + Sephadex G-200	-	-	31.2	11.4
IV^b_c	IV^a_b + DEAE-Sephadex	2.7	43.6	6.3	7.6
V	basis	1.0	17.4	52.7	18.5

[a] See Materials and Methods section.
[b] Purity criterium. The lowest value reported[8] is 5.5.

according to Ref. 18) and the enzyme is easily eluted from this material with 0.01 M potassium-phosphate buffer pH 7.8 (20 μM EDTA) without purification. Some purification was achieved by adsorption of xanthine dehydrogenase to *n*-octylamine-substituted Sepharose 4B (see below), but this method was not suitable for normal purification purposes because the adsorption could not be completely re-

Table 5.II Characterization of xanthine dehydrogenase immobilized by adsorption to *n*-octylamine-substituted Sepharose 4B

Immobilized-enzyme preparation[a]	Protein (mg/g Sepharose)[b]		Activity (units/g Sepharose)[b]		Yield of active immobilized enzyme (%)
	offered	immobilized (%)	offered	immobilized (%)	
III'_a	13.1	-	18.3	18.2(99)	9.6(52.6)
III'_b	12.5	-	17.5	17.4(99)	9.6(55.1)
III'_c	13.3	11.8(89)	18.6	18.5(99)	9.9(53.5)
IV'_a	14.8	11.7(79)	22.2	22.0(99)	15.5(70.4)
IV'_c	7.9	7.6(96)	21.3	20.9(98)	15.3(73.2)
V'_a	4.6	2.9(63)	4.6	4.2(92)	2.7(64.2)
V'_b	9.2	5.4(59)	9.2	7.6(83)	4.4(57.9)
V'_c	18.3	11.0(60)	18.3	14.8(81)	7.5(50.6)
V'_d	27.6	13.3(48)	27.6	19.3(70)	8.4(43.5)

[a] Number refers to the enzyme preparation as given in Table 5.I.

[b] For the preparations III'_a-IV'_c, 1 g freeze-dried CNBr-activated Sepharose 4B was washed, reacted with *n*-octylamine, and the thoroughly washed preparation reacted with the indicated amount of protein and activity at 4°C for 3 hr. For the preparation of V'_a-V'_d, 3.5 ml Sepharose 4B were activated with CNBr and further treated as described for the freeze-dried material; adsorption at 4°C for 18.5 hr.

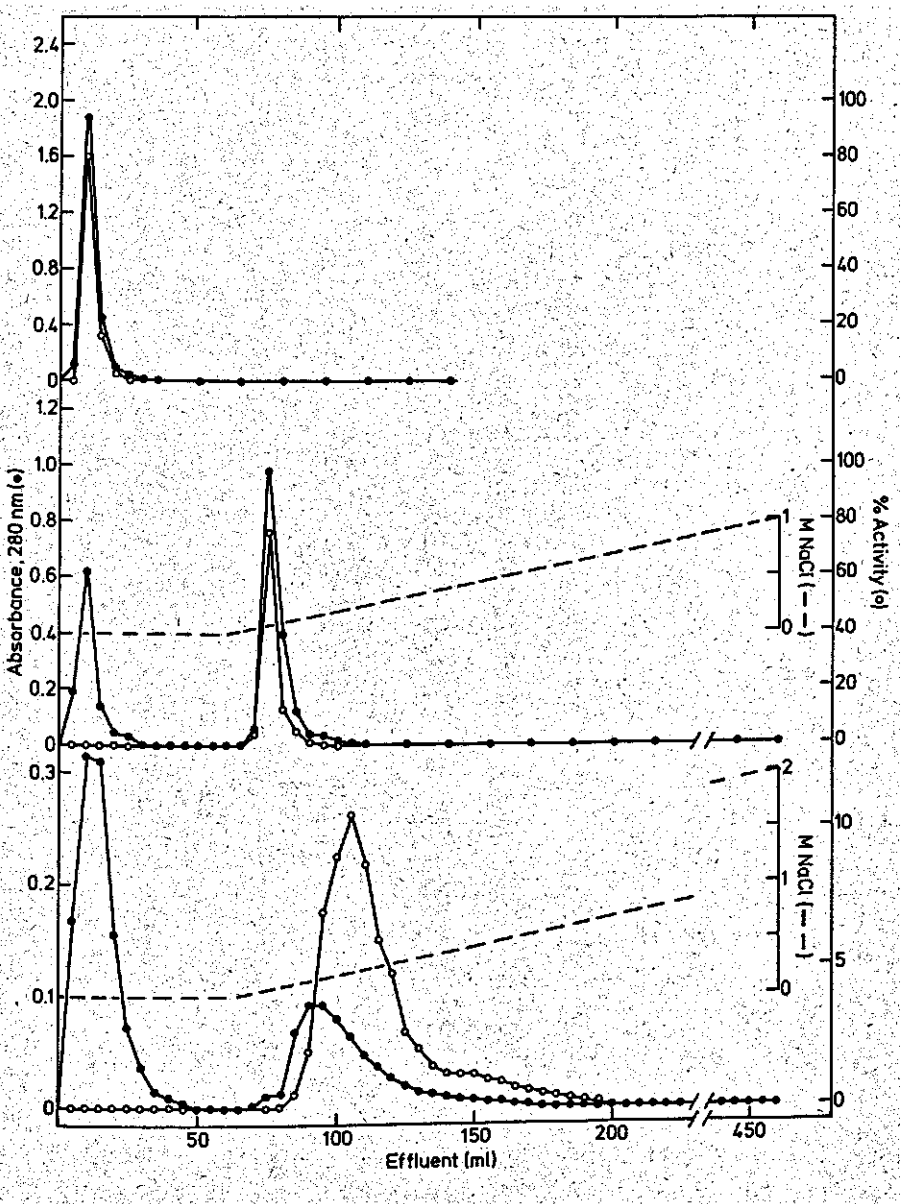

Figure 5.1 Purification of chicken-liver xanthine dehydrogenase on Sepharose 4B (top), *n*-butyl-substituted Sepharose 4B (middle), and *n*-hexyl-substituted Sepharose 4B (bottom). Preparation of the substituted gels was the same as for the *n*-octylamine derivative (see Materials and Methods section). 1 ml preparation V was applied to 3 ml packed gel.

versed; so that, *e.g.*, Tris-HCl buffer pH 7.9 (1 M NaCl/ethylene glycol (1 : 1 by volume)) only partially eluted the enzyme. According to Hofstee[9] the strength of adsorption of other proteins decreases when the number of methylene groups in *n*-alkylamine-substituted Sepharose 4B decreases. We found that xanthine dehydrogenase behaves similarly, and that considerable purification can be achieved with columns of *n*-hexyl- and *n*-butyl-Sepharose 4B (Fig. 5.1). The recovery of activity and protein from the *n*-butyl-substituted gel was 100%; the recovery from the *n*-hexyl-substituted material was lower (~70%) and a diffuse brown colour remained on the column. The purification obtained with the hexyl-Sepharose column was, however, higher than that with the butyl-Sepharose column: the A_{280}/A_{450} values of the fractions with the highest activity were about 6 and 10, respectively. At present this phenomenon is under active investigation in order to determine its suitability for large-scale purification.

5.4.2. *Immobilization of xanthine dehydrogenase*

Xanthine dehydrogenase can be efficiently immobilized by adsorption to *n*-octylamine-substituted Sepharose 4B. The data presented in Table 5.II and Fig. 5.2 were obtained from protein and activity assays of the enzyme solutions used for immobilization, the supernatants at the end of the immobilization reaction, the wash fluids and the immobilized-enzyme preparations. Also the A_{280}/A_{450} values of the various fluids were determined and were very high for the supernatants and wash fluids. All these data indicate that relatively more active xanthine dehydroge-

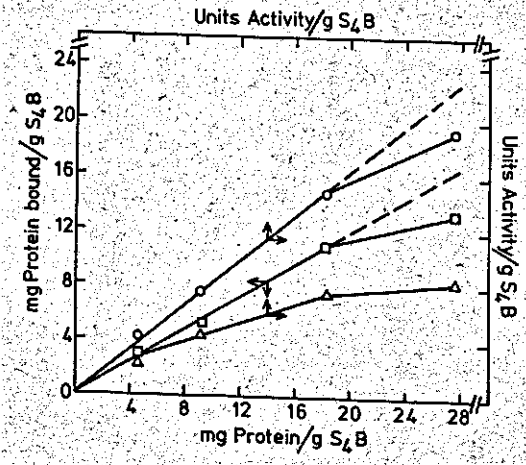

Figure 5.2 (Δ) Overall yield of active, immobilized chicken-liver xanthine dehydrogenase as function of the units of activity offered per g Sepharose 4B(S4B), and adsorption isotherms of (○) xanthine dehydrogenase activity and (□) protein.

nase was bound than protein and that thus a purification occurred as result of immobilization. This observation prompted us to set up the preliminary purification experiments (Fig. 5.1), which indeed show that part of the inert protein is not bound at all (probably a positively charged protein which can be removed on DEAE-Sepharose). Inspection of Table 5.II also shows that as more activity and protein were bound, relatively less immobilized activity was recovered. It is not very likely that this effect results from an increasing substrate-diffusion limitation, because in all cases the activity assay was performed under substrate-saturation conditions (see next section); it might be due to a complete blocking of active xanthine-dehydrogenase molecules by other protein molecules. There is also the trend that the yield of immobilized activity increased as a purer enzyme preparation was used for immobilization. This suggests that a blocking of active xanthine dehydrogenase occurred by protein impurity. This extra loss in activity as result of using a less pure enzyme preparation for immobilization is, however, more than cancelled by the loss which occurs when the enzyme is further purified (Table 5.I).

It was further observed that the adsorption of activity is affected by the manner in which the support material is prepared. The rate of adsorption of xanthine dehydrogenase activity was very rapid in the immobilization step of the preparations III'_a - IV'_c. Ninety-five and 98% of the activity disappeared from the supernatant in 5 and 10 min, respectively, an adsorption rate similar to that of β-amylase to agarose to which nonpolar side chains were introduced via ether bridges[19]. The rate of adsorption of activity, in the coupling step of preparations V'_{a-c} was much slower, however, and relatively less protein and activity were eventually adsorbed (Table 5.II). After 3 h of coupling, the activity still continued to decrease considerably in the supernatant, while in the preparation of III'_a - IV'_c in less than $2\frac{1}{2}$ h of coupling the activity was almost zero in the supernatant. A possible explanation for this observation is that the degree of substitution of Sepharose with *n*-octylamine varied. The degree of substitution was twice as high for the commercial CNBr-activated Sepharose 4B used in the preparation of III'_a - IV'_c as for the CNBr-activated material that was freshly prepared for V'_{a-d}, *i.e.*, 4.44 and 2.41 μM Ponceau S per g of gel (sucked dry in a Büchner funnel until the surface cracked), respectively[12]. The driving forces for the adsorption[9], *i.e.*, the number of positive charges and the degree of hydrophobicity of the support, are, therefore, much smaller in the latter material.

5.4.3. Kinetics of free and immobilized xanthine dehydrogenase

The pH dependencies of the kinetic constants of the free and immobilized enzyme have been investigated in the range of 7.5 - 9.1. The Michaelis constant and V of free xanthine dehydrogenase for xanthine as a variable substrate at a constant NAD^+ concentration increase as the pH increases, as previously observed for other soluble xanthine dehydrogenases[20]; the corresponding values for the immobilized enzyme go through a minimum at pH 8.1 (Table 5.III). The apparent Michaelis constant K_m' of immobilized xanthine dehydrogenase is larger than the K_m of the free enzyme throughout the pH range tested; this is usually taken as an indication that the reaction in the immobilized-enzyme system could be limited by diffusion of substrate. The Lineweaver-Burk plots from which the kinetic constants were calculated show a slight upward bend at the highest concentrations of substrate in the case of the immobilized enzyme; in soluble-enzyme systems such curvature indicates that substrate inhibition could be involved. In immobilized-enzyme systems, however, diffusion limitation shifts the inhibition to higher substrate concentrations[3,4] and a more likely explanation of the upward curvature in the present work is, therefore, that the diffusion of NAD^+ becomes rate limiting at the highest xanthine concentrations (see below). An upward deflection in Lineweaver-Burk plots of other immobilized-enzyme systems, where the reaction rate was controlled by diffusion, has indeed been observed at high substrate concentrations[21].

Table 5.III. Michaelis constants and V's of free and immobilized xanthine dehydrogenase for xanthine ($[NAD^+]$ = 400 µM as a function of pH.

pH	Free enzyme[a]		Immobilized enzyme[b]	
	K_m (µM)	V (units/mg)	K_m' (µM)	V' (units/mg)
7.5	23.0	1.37	78.7	1.28
7.7	23.5	1.59	59.5	1.17
7.9	24.2	1.72	50.0	1.03
8.1	29.4	2.00	41.8	1.00
8.3	31.3	2.00	61.3	1.10
8.5	33.3	2.08	87.0	1.41
8.7	53.2	2.17	94.3	1.41
8.9	57.5	2.18	111.1	1.41
9.1	97.1	2.86	250.0	2.68

a Preparation III (diluted 50 times).
b Preparation III' (material corresponding to 1 g freeze-dried CNBr-activated Sepharose 4B was suspended in 120 ml buffer).

The kinetics of free and immobilized xanthine dehydrogenase with xanthine and NAD^+ as substrates were studied in more detail at pH 7.9 and the data are presented in the form of double-reciprocal plots (Fig. 5.3 and 5.4). A pH of 7.9 was chosen, because both NAD^+ and NADH are relatively stable at this pH[22]. Over a limited concentration range, both free and immobilized xanthine dehydrogenase show a pattern of fairly parallel lines, suggesting that the mechanism involves two separable half reactions and two distinct forms of enzyme (ping-pong mechanism[15]). As other workers have previously assumed for soluble xanthine dehydrogenases[7,23-25] we assumed this mechanism for the calculation of (limiting) K_m and K_m' values (Table 5.IV) from plots of the intercepts of the reciprocal-velocity axis *vs* the reciprocal of the pertinent substrate concentration (inserts of Fig. 5.3 and 5.4). Again, the K_m' values of immobilized xanthine dehydrogenase are larger than the corresponding K_m values of the free enzyme. The relatively larger increase for NAD^+ probably results from some repulsion with the support material, which possesses a slight positive charge at pH 7.9 (the isourea derivative formed mainly in the reaction of CNBr-activated Sepharose with an alkylamine has a pK_a of about 10.4[26]). The diffusion limitation will, therefore, be larger for NAD^+ than for xanthine, resulting in a larger increase in Michaelis constant. This could also explain the upward deflection in the Lineweaver-Burk plots (see above) since diffusion of NAD^+ probably becomes the rate-limiting factor at the highest xanthine concentrations. That the rate of reaction in the immobilized-enzyme system is indeed limited by substrate diffusion was proved in an experiment in which the enzyme load of the support was varied. Table 5.V and Figure 5.5 clearly show that K_m' increases when the amount of enzyme activity bound per unit weight of gel increases. If the conversion of substrate in the support is faster than the supply by diffusion, a substrate-concentration gradient will develop in the support. This concentration gradient will be steeper the more enzyme present in the support, so that the effective substrate concentration in the microenvironment of the immobilized enzyme becomes lower and consequently K_m' increases[21]. Table 5.V and Fig. 5.5 also show that the increase in K_m' is coupled with a decrease in V'. Furthermore, the results in the v^{-1} *vs* S^{-1} plot (Fig. 5.6) indicate that a mechanism comparable to mixed-type inhibition is operative. The decrease in V' is in agreement with our conclusion above that a blocking of xanthine dehydrogenase occurs by other protein molecules bound in the pores of the support material. As more protein impurity is bound, more active xanthine dehydrogenase can be blocked and consequently V' decreases.

At low xanthine concentrations (<2 μM) substrate activation has been reported for chicken[7] and turkey[23]-liver xanthine dehydrogenase with O_2 as terminal electron

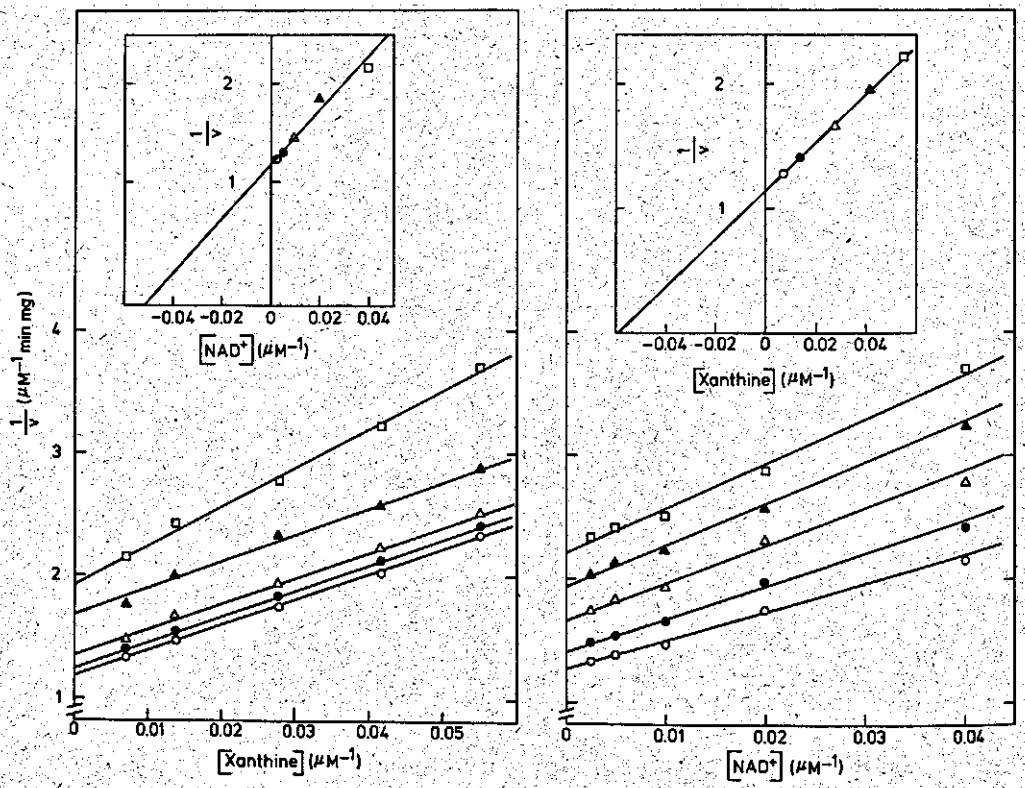

Figure 5.3 Kinetics of xanthine: NAD^+ oxidoreductase activity of soluble chicken-liver xanthine dehydrogenase. Reactions were carried out at 30°C in Tris-HCl buffer, pH 7.9, containing 0.1 mM EDTA. (a) Concentration of NAD^+ (μM) used were; (□) 25; (▲) 50; (Δ) 100; (●) 200; (o) 400. (b) Concentrations of xanthine (μM) used were: (□) 18; (▲) 24; (Δ) 36; (●) 72; (o) 144. Plot of the intercept vs. the reciprocal pertinent substrate concentration is shown in the insert.

Table 5.IV Michaelis constants of free and immobilized xanthine dehydrogenase for the substrates xanthine and NAD^+ at pH 7.9.

Substrate	Free enzyme[a] K_m (μM)	Immobilized enzyme[b] K'_m (μM)
Xanthine	17	35
NAD^+	19	54

[a,b] See corresponding footnotes in Table 5.III.

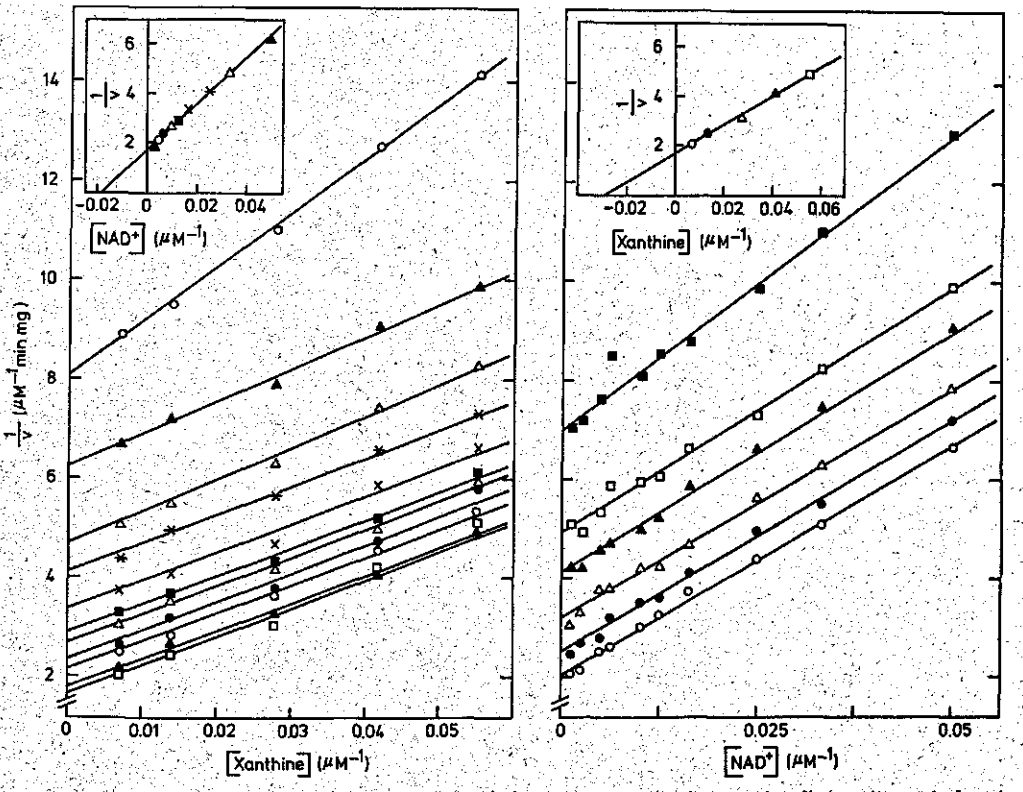

Figure 5.4 *Kinetics of xanthine: NAD^+ oxidoreductase activity of immobilized* chicken-liver xanthine dehydrogenase. Reactions were carried out at 30°C in Tris-HCl buffer, pH 7.9, containing 0.1 mM EDTA. (a) Concentrations of NAD^+ (μM) used were: (o) 10; (▲) 20; (Δ) 30; (*) 40; (x) 50; (■) 80; (Δ) 100; (●) 160; (o) 200; (▲) 400; (□) 800. (b) Concentrations of xanthine (μM) used were: (■) 12; (□) 18; (▲) 24; (Δ) 36; (●) 72; (o) 144. Plot of the intercept vs. the reciprocal pertinent substrate concentration is shown in the insert.

Table 5.V Apparent kinetic constants for immobilized xanthine dehydrogenase as a function of the amount of activity bound per unit weight of support.

Preparation	Units activity bound/g Sepharose 4B	K'_m (μM)	V' (units/mg)
V'_a	4.2	39.1	0.81
V'_b	7.6	45.5	0.74
V'_c	14.8	52.6	0.65
V'_d	19.3	62.5	0.56

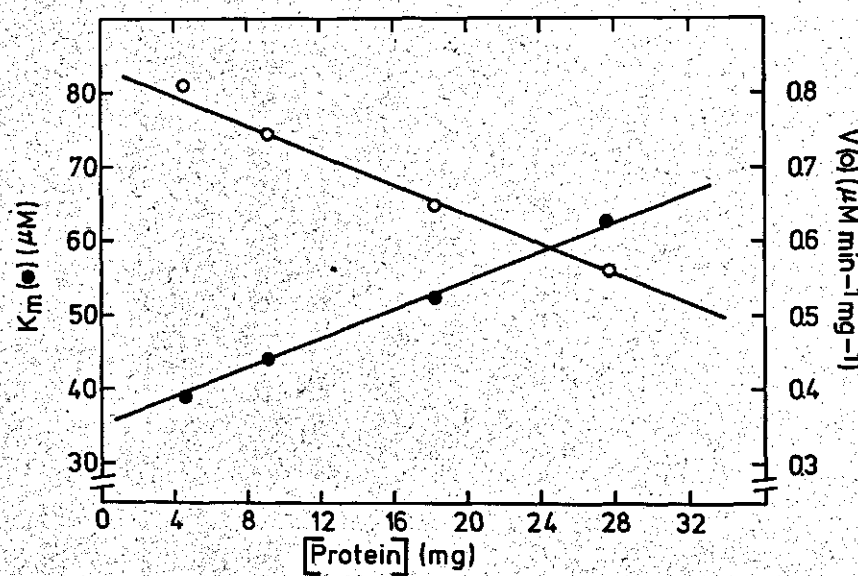

Figure 5.5 Apparent kinetic constants K_m' and V' of immobilized chicken-liver xanthine dehydrogenase (preparations V'_{a-d}) as a function of the amount of protein offered/g gel.

acceptor. We observed that immobilized xanthine dehydrogenase is also activated by substrate in the same low concentration range, not only with O_2, but also with NAD^+ or DCP as the electron acceptor. Activation of free xanthine dehydrogenase occurs with DCP, but not with NAD^+ as oxidizing substrate. DCP at increasing concentrations shows a marked inhibitory effect on the rate of oxidiation of xanthine, an effect also observed with bovine-milk xanthine oxidase[27]. The reaction with DCP was, therefore, not studied further because the electron acceptor must be used at high concentration if the reaction is to be useful for large-scale organic synthesis.

5.4.4. Stability of xanthine dehydrogenase

In its resting state, the immobilized enzyme is more stable than the free enzyme over a range of temperatures (Table 5.VI). The temperature at which the half life of the enzyme preparations drastically decreases, is also higher for the immobilized enzyme than for the free enzyme. This stabilization might be due to multipoint attachment of xanthine dehydrogenase to the support, creating a more rigid enzyme molecule. Transition to an inactive or less stable molecule will, therefore

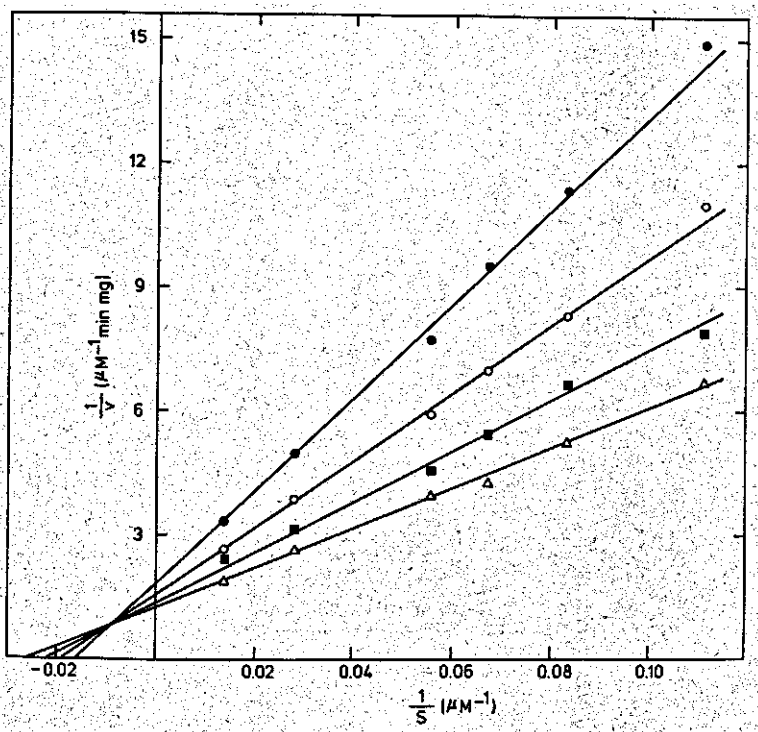

Figure 5.6 Lineweaver-Burk plots for immobilized chicken-liver xanthine dehydrogenase (preparations V'_{a-d}) with varying amounts of enzyme bound per unit weight of gel. (Δ) V'_a; (□) V'_b; (o) V'_c; (o) V'_d.

Table 5.VI Storage stability of free and immobilized xanthine dehydrogenase as a function of temperature at pH 7.8[a].

T (°C)	Free enzyme[b] $t_{\frac{1}{2}}$ (hr)	Immobilized enzyme[c] $t_{\frac{1}{2}}$ (hr)
4	423	2114
20	292	1167
30	71	810
40	21	33
50	11	15

[a] 0.05 *M* phosphate buffer; 0.1 m*M* EDTA; 0.02% $NaNO_3$ (w/v).
[b] Preparations III and IV (diluted 50 times).
[c] Preparations III_c' and IV_a'.

be retarded[28].

The activity as function of the temperature was measured in order to determine the optimum temperature for reaction (Fig. 5.7). From the slopes of the lines the activation energies for the free and immobilized enzyme were calculated to be 33 and 19 kJ $mole^{-1}$, respectively. That the activation energy for the immobilized enzyme is about half that for the free enzyme is another indication that the reaction is diffusion controlled[21]. When both the rate of reaction and the half life are taken into consideration, 30°C is the optimum temperature, and this temperature was therefore, choosen for the following experiments.

The ultimate goal of our work is to use immobilized xanthine dehydrogenase in organic synthesis. For efficient use the enzyme must be adequately stable during prolonged catalysis. The operational stability of immobilized xanthine dehydrogenase was therefore tested in small packed-bed reactors, operated under continuous

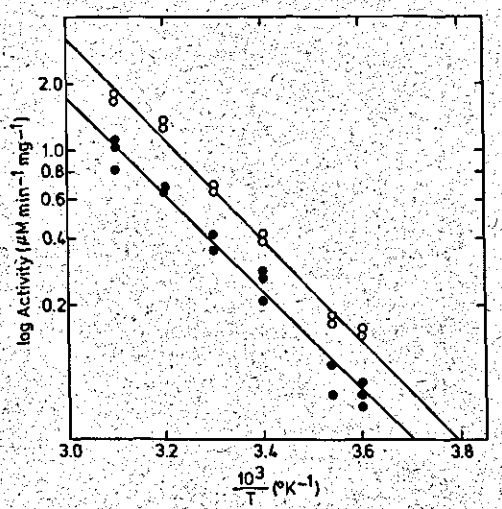

Figure 5.7 Activity of (o) free and (•) immobilized chicken-liver xanthine dehydrogenase as a function of temperature.

flow with various substrate concentrations. The half lifes and the amounts of product formed in one half time, are recorded in Table 5.VII. From the results it follows that the optimum product yield is found at about 500 μM xanthine. A problem not yet understood is the fact that the operational stability of this enzyme is drastically lower than the storage stability. It is our intention to search for probable causes of the instability and to stabilize the enzyme.

Table 5.VII Operational stability of immobilized xanthine dehydrogenase[a] and yield of product per mg protein in one-half time; 30°C.

Xanthine conc. (μM)	NAD^+ conc. (μM)	$t_{\frac{1}{2}}$ (hr)	Yield of product in $t_{\frac{1}{2}}$ (μmol/mg)
180	250	7.2	44.5
450	450	9.3	61.9
720	750	8.9	57.2
1000	1000	10.7	42.2

a Preparation IVa.
b Solution in Tris-HCl buffer (pH 7.9; I = 0.1 mM EDTA).

5.4.5. Comparison of immobilized chicken-liver xanthine dehydrogenase with immobilized milk xanthine oxidase

The study of immobilized chicken-liver xanthine dehydrogenase was started, because we found previously[3] that the low operational stability of immobilized milk xanthine oxidase results in part from inactivation by O_2^- and H_2O_2. These reaction products are not formed when xanthine is converted by xanthine dehydrogenase, since this enzyme preferentially uses NAD^+ as electron acceptor, and we expected, therefore, that xanthine dehydrogenase would be more stable. The operational stability of immobilized xanthine dehydrogenase is, however, about two orders of magnitude smaller than the storage stability, 9 and 800 h, respectively. A large(r) decrease was also observed for milk xanthine oxidase, 1 *vs* 300 h, so the main inactivation is here also probably not by O_2^- and H_2O_2. The operational stability of immobilized xanthine dehydrogenase is better, however, than that of immobilized milk xanthine oxidase, even when the latter enzyme is coimmobilized with superoxide dismuatase and catalase. Also the formation of product per initial unit of activity in one half time is higher. Moreover, the use of immobilized xanthine dehydrogenase is much more convenient because a continuous reactor fed with a high substrate concentration can be utilized. In the case of immobilized xanthine oxidase, the substrate inhibition makes it necessary to recycle the product stream through a single reactor or to pass it through a series of reactors with additions of fresh substrate after each successive cycle/column, or to run a continuously stirred tank reactor to obtain sufficiently high product concentrations.
The yield of immobilized activity is slightly less (~15%) for xanthine dehydrogenase than for xanthine oxidase. A disadvantage of xanthine dehydrogenase is also that the oxidizing substrate NAD^+ is rather expensive, and that the reduced by-

product, NADH, may interfere in the isolation of the hydroxylated substrate. Preliminary experiments, however, with xanthine dehydrogenase coimmobilized with lactate dehydrogenase or alcohol dehydrogenase show that *in situ* NAD^+ regeneration is feasible. At present xanthine dehydrogenase seems, therefore, more promising for application in organic synthesis than xanthine oxidase.

5.5. ACKNOWLEDGEMENTS

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6. THE OXIDATION OF 7-(p-X-PHENYL)PTERIDIN-4-ONES (X=OCH_3, H, Br, CN, NO_2) WITH FREE AND IMMOBILIZED XANTHINE OXIDASE

J. Tramper, A. Nagel, H.C. van der Plas and F. Müller

6.1. SUMMARY

Bovine-milk xanthine oxidase was used as a catalyst for the oxidation of various 7-(p-X-phenyl)pteridin-4-ones into 7-(p-X-phenyl)lumazines. On a preparative scale the derivatives with X = Br, CN and NO_2 were most conveniently oxidized with the free enzyme in a solution continuously fed with concentrated substrate. The lumazines obtained separated during the reaction and were easily isolated in good yield (> 90%) and high purity by filtration. The compounds with X = H and OCH_3, on the other hand, were more conveniently oxidized with the immobilized enzyme, *i.e.*, xanthine oxidase adsorbed to *n*-octyl-substituted Sepharose 4B. Immobilized xanthine oxidase was used either as a suspension in a stirred batch reactor which was continuously fed with concentrated substrate, or packed in a column through which substrate solution was recirculated. After completion of the reaction the filtrate of the suspension, or the concentrated recirculation solution, respectively, were acidified and the precipitate was collected in good yield (> 90%) and high purity by filtration.
The rate of oxidation was found to be influenced by the substituent X. The more electron attracting X, by either resonance or induction, the lower the reaction rate. Accordingly, a negative reaction constant ρ of about -0.5 was calculated from the data for the free and immobilized enzyme. The formation of the enzyme-substrate complex, as reflected in Michaelis constant K_m, is largely determined by the hydrophobicity of the phenyl group.

6.2. INTRODUCTION

Almost all chemical reactions which occur in biological systems are mediated by the action of enzymes. Enzymic reactions *in vivo* are generally rapid, highly specific and usually take place under mild conditions. This is in contrast to the

high temperatures and/or high pressures often required in many ordinary *in vitro* reactions. The advantages of reactions being catalyzed by enzymes have induced considerable interest in employing biocatalysts for industrial applications. However, the usually low stability of enzymes isolated from their natural environment and their high solubility in water makes repeated or continuous use of these often expensive biocatalysts difficult or nearly impossible.

These properties have confined the use of enzymes in industry for a long time. However, attachment of enzymes to a solid support can improve the stability considerably. This immobilization also makes a repeated or continuous use of enzymes possible and is therefore a very promising technique. Several immobilized enzymes have reached the production stage recently.

The use of immobilized enzymes in organic synthesis is largely unexplored. The advantages of applying this technique are obvious: The great potential of immobilized enzymes in organic synthesis lies in the fact that under mild conditions specific products may be obtained in a fast reaction which otherwise cannot be formed or can be obtained only by laborious synthetic procedures. Moreover, due to the two-phase system, the reaction product formed can be easily isolated and is usually very pure as result of the high specificity of the reaction. For many years we have been interested in the chemistry of azaaromatics. Recently much attention is paid to the purines and pteridines[2], a class of compounds with important biochemical and pharmacological implications. Specific oxidation of these electron-deficient azaheterocycles by chemical means is usually difficult. However, since it is known that the enzymes xanthine oxidase and xanthine dehydrogenase catalyze the oxidation of many heteroarenes, we initiated a study[3] on the suitability of these enzymes, in free and immobilized form, for obtaining specifically oxidized products on a preparative scale. Although the operational stability of xanthine oxidase is rather poor[3a], we found that this enzyme can be conveniently and profitably used for the preparation of oxo-azaheterocylces. In this paper we report the preparation of 7-(p-X-phenyl)pteridine-2,4-diones by oxidation of 7-(p-X-phenyl)pteridin-4-ones using free as well as immobilized xanthine oxidase.

6.3. THEORY

The kinetic and chemical scheme representing the oxidation of xanthine by xanthine oxidase (Fig. 6.1) as proposed by Olson *et al.*[4] is at present accepted as the best approximation of the true mechanism[5]. In the Michaelis-type complex (I) a nucleo-

Step 1. *Xanthine (S) + Xanthine Oxidase (E)* $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ *Complex* I

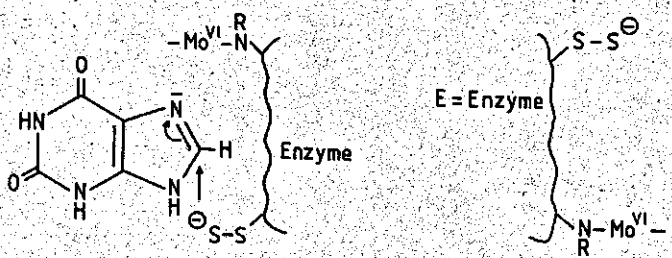
Step 2. *Complex* I $\xrightarrow{k_2}$ *Complex* II

Step 3. *Complex* II $\xrightarrow{k_3}$ *Complex* III

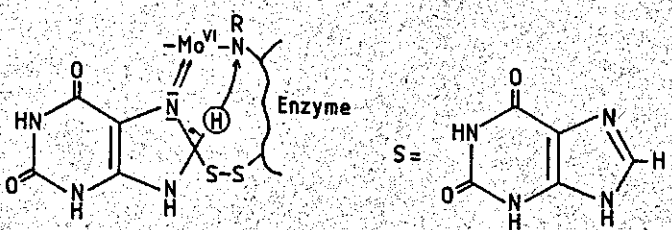
Step 4. *Complex* III + H_2O $\xrightarrow{k_4}$ *Uric Acid (UA) + Reduced Enzyme (E')*

Step 5. $E' + O_2 \xrightarrow{k_5} E + H_2O_2$.

Complex (I)



Complex (II)



Complex (III)

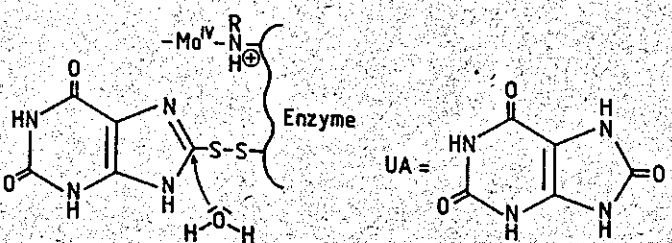
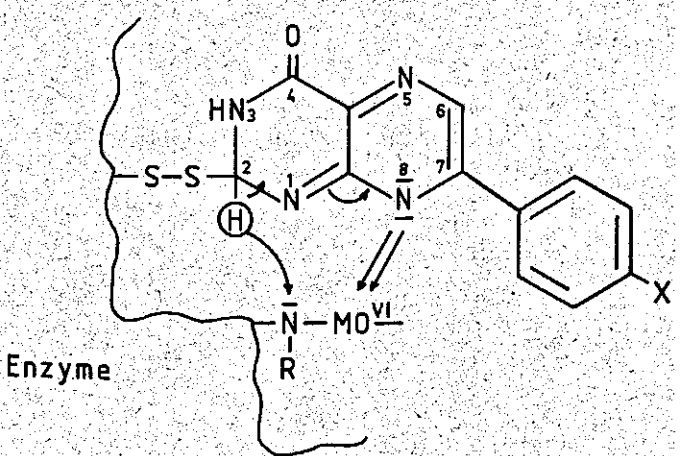


Figure 6.1 Representation of the oxidation of xanthine by xanthine oxidase as proposed by Olsen *et al.*[3]. Complex II is introduced as additional intermediate complex to make the model chemically more feasible.

philic attack by the disulfide occurs at C-8 of the purine ring. In complex (II) a two-electron donation to Mo^{VI} takes place leading to Mo^{IV} and a proton is transferred to the nitrogen ligand, yielding complex (III). In this complex the N=C-S-S moiety is highly susceptible to a nucleophilic attack. Reaction with water produces uric acid and a reduced enzyme, which by reaction with molecular oxygen is oxidized to E. It is assumed that water and not the hydroxide ion is involved in the formation of uric acid in view of the fact that the oxidation rate is independent of the pH over a wide range. It is further assumed that the formation of the Michaelis complex (I) (k_1) and the reoxidation of the enzyme (k_5) are relatively fast.

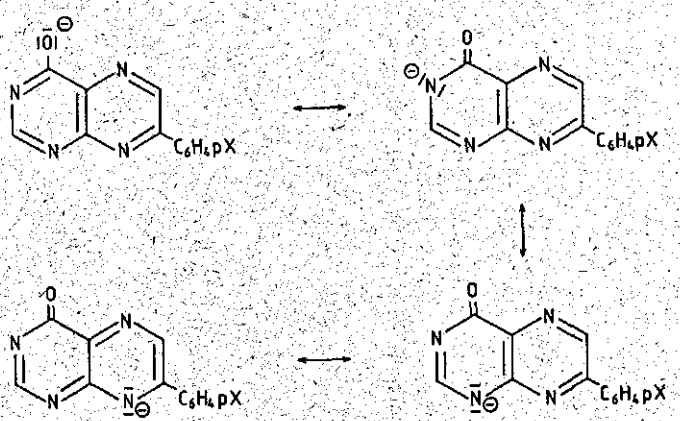
It seems reasonable to assume that the same mechanism is also involved in the oxidation of 7-(p-X-phenyl)pteridin-4-ones. The disulfide group is then attached to position 2 of the pteridine system and in that position the nucleophilic attack of water can take place. Analogous to the model for xanthine the two-electron donation to Mo^{VI} can take place from N-1 or N-3, but may in this case occur from N-8, which can also accommodate a negative charge (Scheme 1).



Scheme 1

In order to obtain more detailed insight into the mechanism of this enzymic oxidation we tried to establish the influence of substituent X on the maximum oxidation rate (V) and on the affinity of the enzyme for the substrate, as reflected in the K_m value. Detailed studies were carried out at pH 9.1. Since the pK_a values of these compounds vary between 8.0 and 8.2, they are largely present in the an-

ionic forms at this pH. The negative charge is delocalized over both rings, oxygen, N-3, N-1 and N-8 having the greatest electron density (Scheme 2).



Scheme 2

If the nucleophilic attack of either $-S-S^-$ or H_2O (step 2 or step 4) is rate limiting, it can be expected that the influence of X on the oxidation rate is relatively small, as there is no important contribution of a mesomeric effect of X on C-2 and the influence of the inductive effect of X on the electron density of C-2 can be considered to be insignificant. On the other hand, if the electron transfer to the metal ion is rate limiting, one can expect a relatively large effect of X on the rate: If X has a +M effect it will promote the electron flow from one of the substrate nitrogens to Mo^{VI} and rate acceleration will be observed; when X is electron withdrawing the oxidation rate will be decreased.

According to Bergmann *et al.*[6] the groupings $\overset{1}{\text{N}}\text{-C-}\overset{8}{\text{N}}$ and $\text{O=C-C-}\overset{4}{\text{N}}$ are important in the association of the substrate with the enzyme. Baker *et al.*[7] showed that there must be a hydrophobic group in or near the active site of the enzyme. Therefore, in the 7-(p-X-phenyl)pteridin-4-ones the hydrophobicity of the phenyl group possibly determines the formation of the enzyme-substrate complex to a certain extent. An influence of X on K_m can be expected as X has both an effect on the groupings involved in the binding and on the hydrophobicity of the phenyl group.

6.4. RESULTS AND DISCUSSION

6.4.1. Synthesis of the 7-(p-X-phenyl)pteridin-4-ones

Pteridines are conveniently synthesized by condensation of 4,5-diaminopyrimidine with a 1,2-diketone. However, in the case of substituted 4,5-diaminopyrimidines and asymmetric 1,2-diketones, product mixtures are often obtained, consisting of the isomeric 6 and 7-substituted pteridines. The separation of these mixtures is usually laborious and the characterization of the isomeric pteridines by classical methods like UV and ^{1}H NMR[8,9] is not always straightforward. ^{13}C NMR was recently reported to be the method of choice for the structural elucidation of 6 or 7-phenylpteridines[10]. The formation of 6 and 7-(p-X-phenyl)pteridin-4-ones by the condensation of 4,5-diaminopyrimidin-6-one (1) and a substituted phenyl glyoxal (2), under various conditions (Scheme 3) leads to the isomeric pteridines 3 and 4 which were found to be extremely difficult to separate.

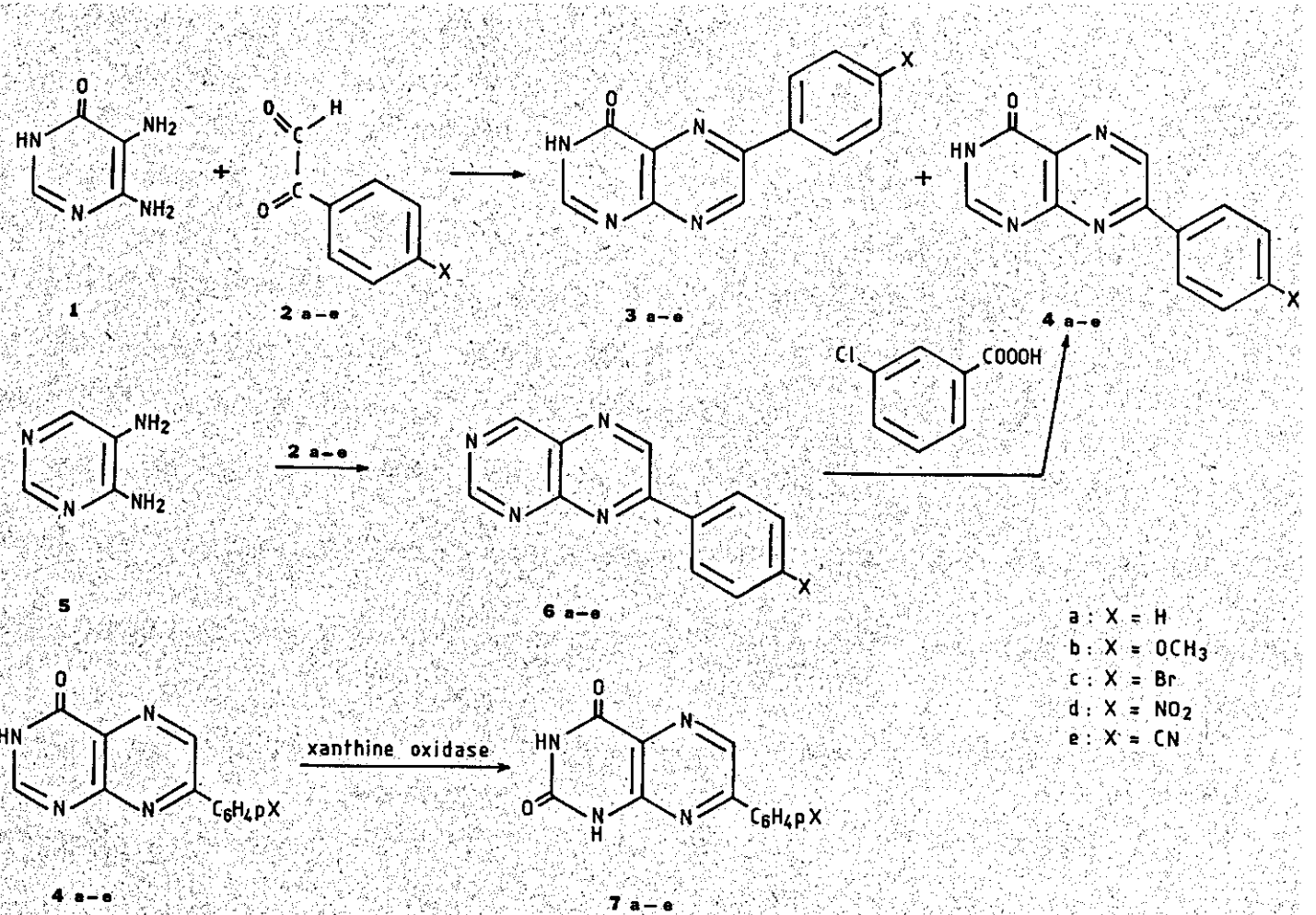
Since our studies required the utmost purity of the 7-(p-X-phenyl)pteridin-4-ones used as substrates for the enzyme, it was necessary to use a different synthetic approach (Scheme 3). We found that condensation of 4,5-diaminopyrimidine (5) with the appropriate phenyl glyoxal 2a-e does not yield a mixture of the 6 and 7-substituted pteridines but only the pure 7-isomers 6a-e, as determined by their melting points and ^{13}C NMR spectra. Oxidation of the 7-(p-X-phenyl)pteridines 6a-e by m-chloroperbenzoic acid in chloroform solution produced the pteridin-4-ones 4a-e in high yield.

6.4.2. Enzymic oxidation of the 7-(p-X-phenyl)pteridin-4-ones on preparative scale

The enzymic oxidation of the 7-(p-X-phenyl)pteridin-4-ones to the 7-(p-X-phenyl)-lumazines (Scheme 3) on preparative scale was performed at pH 9.1. At this pH these substrates are fairly soluble and the rate of oxidation is only somewhat less than the optimum rate (Fig. 6.2).

6.4.2.1. Oxidation of the derivatives 4c-e (X = Br, CN and NO_2) with soluble xanthine oxidase.

The solubility of the lumazines formed in case of X = Br, CN and NO_2 is so low, that precipitation occurred during the reaction, even if the oxidation is carried out at a very low concentration. The substrates 4c-e were, therefore, most con-


1
2 a–e
3 a–e
4 a–e
5
6 a–e
7 a–e
xanthine oxidase
a : X = H
b : X = OCH3
c : X = Br
d : X = NO2
e : X = CN

Scheme 3

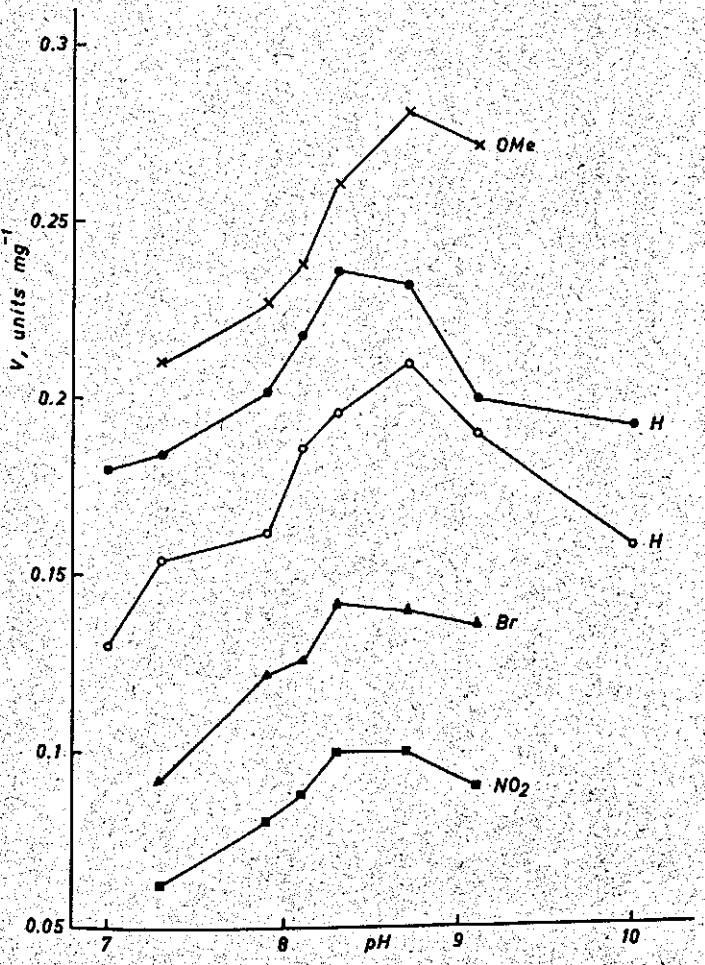


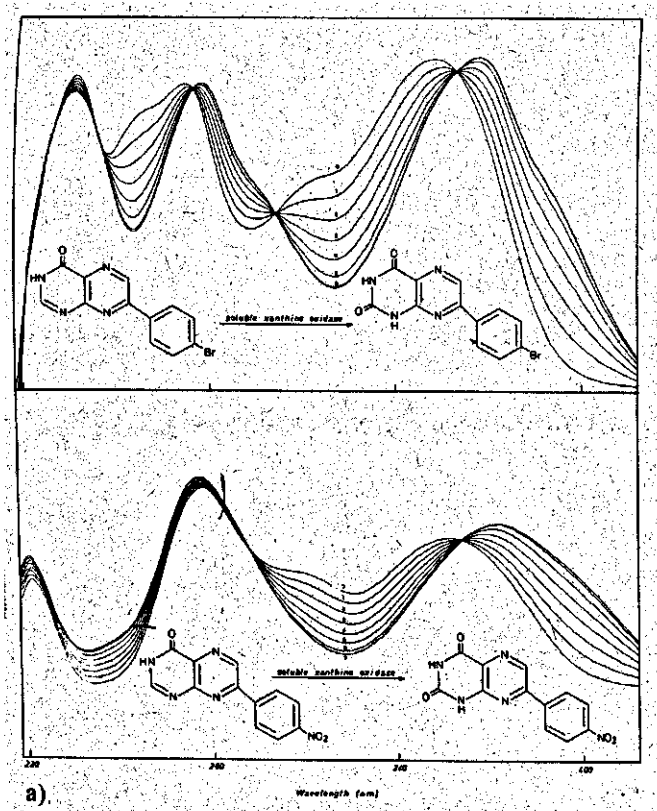
Figure 6.2 Maximal oxidation rates (V) of 7-(p-X-phenyl)pteridin-4-ones as a function of pH at 20°C, using xanthine oxidase as a catalyst. (x) X = OMe; (●) X = H; (o) X = H, immobilized xanthine oxidase; (▲) X = Br. (■) X = NO_2.

veniently oxidized with the free enzyme in buffer solution to which a concentrated solution of substrate was added slowly. The produced lumazine derivatives 7c-e have a high purity and were easily isolated in good yield (> 90%) by simple filtration of the reaction mixture at the end of the reaction.

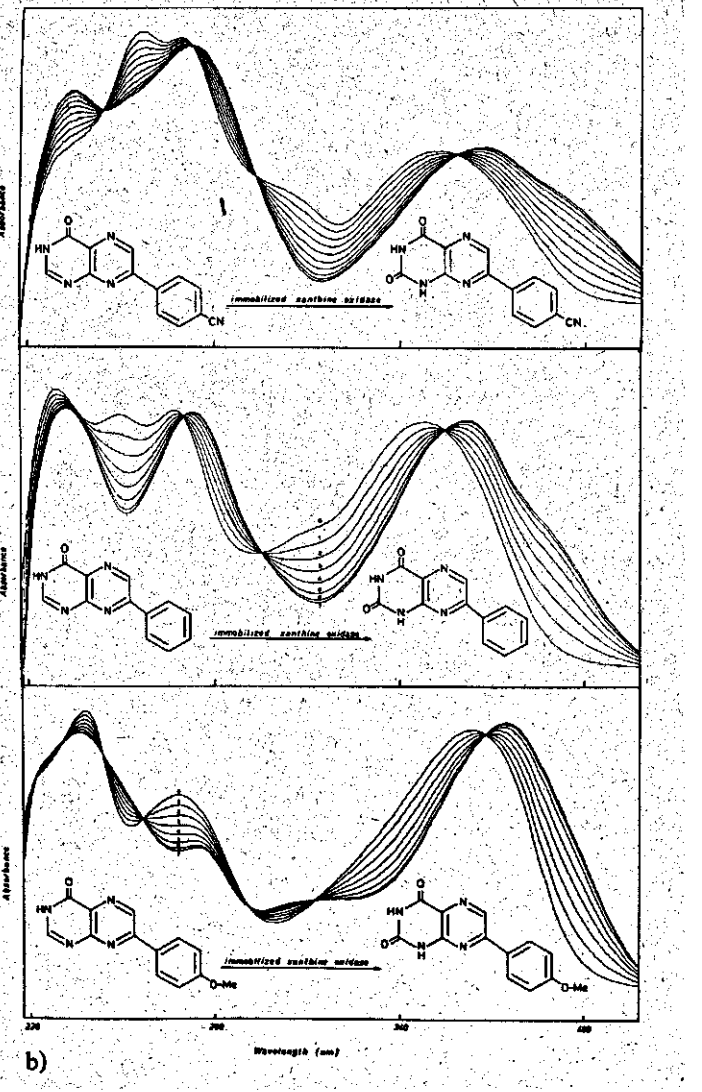
6.4.2.2. Oxidation of the derivatives 4a,b (X = H and OCH_3) with immobilized xanthine oxidase.

The compounds 4a and 4b (X = H and X = OCH_3), on the other hand, were more conveniently oxidized with the immobilized enzyme. The lumazines 7a and 7b are relatively soluble as the sodium salts and acidification of the reaction mixtures after removal of the immobilized enzyme by filtration quantitatively affords the required products. We observed that when the free enzyme was used, acidification resulted several times in coprecipitation of the enzyme. Oxidation is, therefore, most conveniently performed with the immobilized enzyme either by using it as a suspension in a stirred batch reactor which is continuously fed with concentrated substrate, or by packing the enzyme system in a column through which a solution of the substrate is recirculated. The former method has the advantage that, after the immobilized enzyme is removed by filtration, the reaction mixture can be immediately acidified without prior concentration. During the reaction the pH must be controlled. The recirculation reactor is easier to operate but the solution of the product must be concentrated in order to obtain maximum yield. In both procedures the yields (> 90%) are high.

The structures of the 7-(p-X-phenyl)lumazines were proved by UV, IR and MS-comparison with compounds prepared by an independent synthesis involving condensation of 5,6-diaminouracil hydrochloride and the appropriate phenyl glyoxal. For the parent substance, *i.e.*, 7-phenyllumazine, structural similarity was proved by comparing the ^{13}C-NMR spectra of both compounds in DMSO-d_6 solutions. When the enzymic oxidations were followed by measuring the UV-spectra of the reaction mixture at regular time intervals (Fig. 6.3) it can be seen that several sharp isosbestic points are present in the spectra. This strongly indicates the absence of significant amounts of by-products in the reaction mixture. Accordingly, the isolated lumazines are very pure. Our experiments thus show, that xanthine oxidase can be used with advantage in organic syntheses. The high yields obtained and the high purity of the formed compounds are attractive aspects of this method.



a)



b)

Figure 6.3 Oxidation of 7-(p-X-phenyl)pteridin-4-ones into 7-(p-X-phenyl)pteridine-4-diones by free (a) and immobilized xanthine oxidase (b). Spectra were continuously scanned during the reaction (cycle time 1 min.).

Table 6.I Kinetic constants of free and immobilized xanthine oxidase for various substrates in Tris-HCl buffer pH 9.1 (I = 0.01; 0.1 mM EDTA) at 20°C.

Substrate	pKa	λ (Δε) used in assay nm (mM^{-1} cm^{-1})	Free xanthine oxidase V(units mg^{-1})	K_m (μM)	Immobilized xanthine oxidase V'(units mg^{-1})	K'_m (μM)
4a	8.17 ± 0.05	315 (3.7)	0.20	1.3	0.19	4
4b	8.2 ± 0.1	335 (1.9)	0.27	1.3	0.30	7
4c	8.12 ± 0.05	320 (4.7)	0.14	0.7	0.14	3
4d	8.0 ± 0.1	320 (5.3)	0.09	3.6	0.09	6
4e	8.0 ± 0.1	320 (4.2)	0.09	3.3	0.09	6
8[a]		315 (3.7)	0.20	3.5	0.18	7
7-*n*-propylpteridin-4-one[b]	8.11 ± 0.03	269 (6.2)	0.9	3×10^2	0.8	3×10^2
xanthine[b]	7.5[c]	296 (8.9)	0.64	27	0.46	31

a. ^{2}H-2 content: 65%.
b. Unpublished results[16].
c. Bergmann and Levene[16].

6.4.3. The influence of substituent X on the rate of oxidation

The results of the kinetic experiments show that the rate of oxidation with free and immobilized xanthine oxidase are nearly the same (see Table 6.I). A plot of the logarithm of the ratio of the maximal oxidation rate of the 7-(p-X-phenyl)pteridin-4-ones and 7-phenylpteridin-4-one *versus* the substituent constant σ of X, is given in Fig. 6.4. The lines were obtained by linear regression; a reaction constant ρ of about -0.5 for free and immobilized enzyme is calculated from the slopes. This means that the rate-limiting step in both the free and immobilized-enzyme system is facilitated by a high electron density at the reaction site and moderately sensitive to substituent effects. This indicates that neither the nucleophilic attacks by the active-site disulfide and water or hydroxide ion (the more likely nucleophile in alkaline solution) nor the C-H bond breaking is the rate limiting step. That the C-H bond breaking is not rate limiting is confirmed by our observation that there is no significant difference in the maximum rate of oxidation of 2-D-7-phenylpteridin-4-one (8) and the parent compound 4a (Table 6.I). A similar observation has been made for xanthine and 1-methylxanthine[5]. Apparently the electron transfer from the substrate nitrogen to the Mo^{VI} in the active centre is rate-limiting. Accordingly, the rate of oxidation of the anion is higher than that of the neutral substrate (Fig. 6.2).

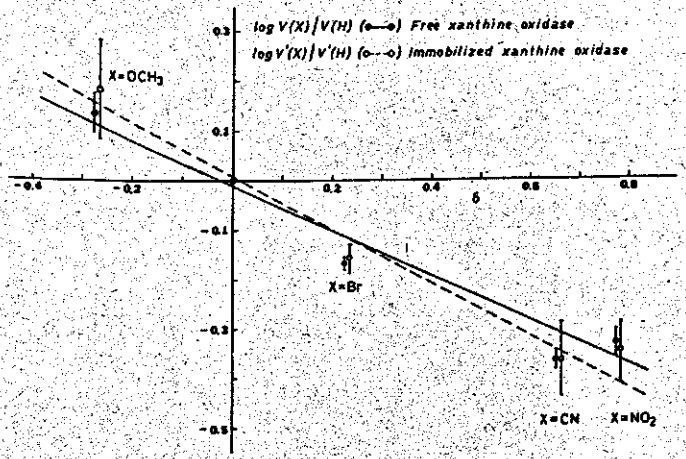


Figure 6.4 Plot of the logarithm of the ratio of the maximal oxidation rate of the 7-(p-X-phenyl)pteridin-4-ones and 7-phenylpteridin-4-one versus the substituent constant σ of X. Oxidation by free and immobilized xanthine oxidase at pH 9.1 and 20°C.

6.4.4. The effect of substituent X on K_m and K'_m.

The K_m and K'_m values for free and immobilized xanthine oxidase, respectively, are given in Table 6.I. As we showed previously for xanthine[3a], the K'_m values of the immobilized enzyme are larger than the K_m values of the free enzyme as result of diffusion limitation in the pores of the support material. All the K_m and K'_m values for the 7-(p-X-phenyl)pteridin-4-ones are much lower than the corresponding values found for xanthine and 7-*n*-propylpteridin-4-one[11] (Table 6.I). This increase in affinity is probably to a large extent due to the presence of the phenyl group at position 7. This is in agreement with the proposal of Baker *et al.*[7] that there is a hydrophobic group in the vicinity of the active centre which strongly increases the binding of inhibitors substituted with a phenyl group. Apparently the 7-(p-X-phenyl)pteridin-4-ones fit with the right orientation in the active centre of the enzyme, since they are good substrates. That a hydrophobic interaction is indeed responsible for the high affinity of the enzyme for the 7-(p-X-phenyl)pteridin-4-ones is supported by the data in Table 6.I, showing that if the polarity of X increases (NO_2, CN) K_m tends to increase and thus the affinity decreases. Accordingly the affinity also decreases upon anion formation (Fig. 6.5). As an interesting contrast, Bergmann *et al.*[12] reported that the corresponding 8-(p-X-phenyl)purin-6-ones (X = OCH_3, H, NO_2) are strongly bound, but clearly in the wrong orientation, as they act as very poor substrates but strong inhibitors. That the position of the phenyl groups indeed plays an essential role is underlined by our observation[11] that the isomeric 6-(p-X-phenyl)pteridin-4-ones (3a-e) are also poor substrates of xanthine oxidase and inhibit the oxidation of xanthine to uric acid too.

The observation that the K_m value of the deuterated substrate is higher than that for ordinary 7-phenylpteridin-4-one is in agreement with a similar observation for 1-methylxanthine[5].

6.4.5. Stability of xanthine oxidase

The operational stability of both free and immobilized xanthine oxidase with xanthine as substrate is rather low[3a]. Also with the 7-(p-X-phenyl)pteridin-4-ones the operational stability is in the same order of magnitude, the half lifes of the free and immobilized enzyme being about 1 and 2 h, respectively (Fig. 6.6). This means that relatively a large amount of enzyme is needed per mole of substrate oxidized (in the synthetic experiments 1 mole of enzyme converts 3000 moles of substrate, assuming that all the protein applied is xanthine oxidase). This is

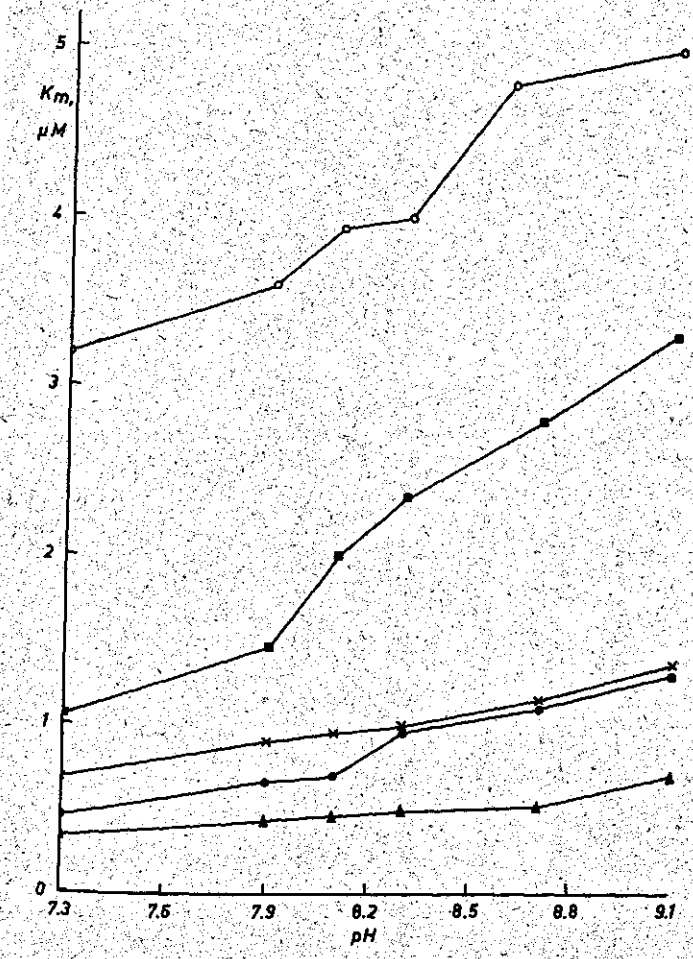


Figure 6.5 Michaelis constant K_m of xanthine oxidase for 7-p-X-phenyl) pteridin-4-ones as a function of pH at 20°C. (x) X = OMe; (●) X = H; (○) X = H, immobilized xanthine oxidase; (▲) X = Br; (■) X = NO_2.

not necessarily a disadvantage, since milk xanthine oxidase is commercially available at a relatively cheap price, or can easily be isolated from milk. Also stabilization by means of special immobilization techniques is possible[3a]. The instability has no consequences for the kinetic data, since the assay time (1-2 min) is short comparing to the half life and consequently the change in activity is insignificant.

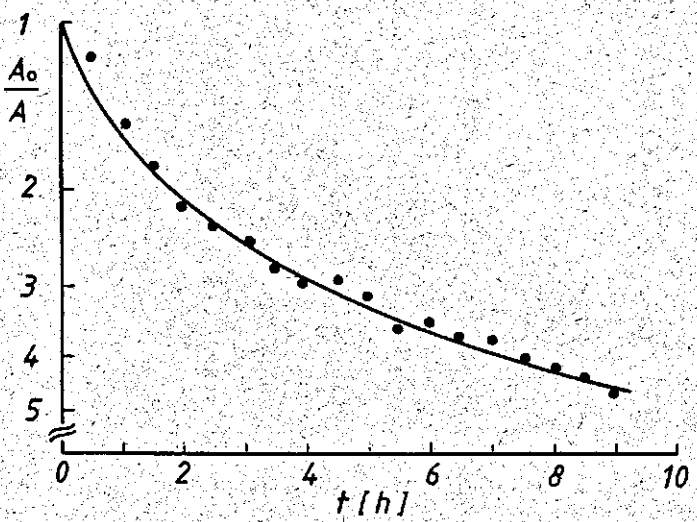


Figure 6.6 Stability of immobilized xanthine oxidase under continuous operation at 20°C (constant flow rate: 46 ml/hr). 1.5 mg xanthine oxidase bound to 0.5 ml octyl-substituted Sepharose 4B was packed in a column (0.5 cm i.d.) and fed with 200 μM 7-phenylpteridin-4-one in Tris-HCl buffer (pH 8.7; I = 0.01; 0.01 mM EDTA). The ratio of the conversion at t = 0 (A_o) and t(A) is plotted.

6.5. EXPERIMENTAL

Melting points are uncorrected. The IR spectra were recorded on a Perkin-Elmer model 237, the mass spectra on an AEI MS-902 instrument. The ^{1}H- and ^{13}C-NMR spectra were recorded with a Hitachi Perkin-Elmer R24B and a Varian XL-100-15 spectrometer, respectively, the latter operating at 25.2 MHz and equipped with a pulse unit and a 620 L-16K online computer system. The measurements were performed under conditions described previously[10]. The ionization constants of the 7-(p-X-phenyl)pteridin-4-ones were determined by the spectrophotometric procedure of Albert and Serjeant[13]. As result of poor solubility at low pH, only low concentrations could be used to determine the molar extinction coefficient of the molecular species. The spread in some of the values is, therefore, slightly larger than the recommended $\pm$ 0.06[13].

6.5.1. Synthesis of the recorded pteridines

6.5.1.1. 7-p-X-phenylpteridines (6a-e)

The 7-(p-X-phenyl)pteridines (6a-e) were obtained in almost quantitative yield by condensation of 4,5-diaminopyrimidine and the appropriate phenyl glyoxal in accordance with the procedure described for the parent substance 6a[14]. The structural characterization of these pteridines was established by ^{13}C-NMR[9] and further characterized by their microanalytical data and melting points (see Table 6.II).

6.5.1.2. 7-p-X-phenylpteridin-4-ones (4a-e)

A solution of 2 mmoles of the pertinent 7-(p-X-phenyl)pteridin 6a-e, dissolved in 200 ml of $CHCl_3$, was refrigerated to 0-5°C. A cooled solution of 2.2 mmoles (1.1 eqs.) of m-chloroperbenzoic acid (~ 70%) dissolved in 20 ml of $CHCl_3$, was then added in one lot. The resulting dark yellow-red solution, is kept at 0-5°C for 24 hours. The precipitate was collected by filtration, washed with $CHCl_3$, ethanol and ether. Reprecipitation from a dilute NaOH solution by acetic acid and recrystallization from DMF-H_2O afforded the analytically pure compounds (see Table 6.II).

6.5.1.3. 7-phenyl[^{2}H-2]pteridin-4-on (8)

450 mg (2 mmoles) of compound 4a and 10 ml of D_2O were heated at 120°C in a sealed tube for a period of 100 h. The ice-cooled contents of the tube were filtered and reprecipitated from a 1 M NaOH solution by a slight excess of acetic acid to yield 110 mg (25%) of dry crystalline material. The ^{2}H-2 content (65%) was established by ^{1}H-NMR and mass spectrometry.

6.5.1.4. 7-p-X-phenyllumazines (7a-c)

Condensations of the hydrochloride of 5,6-diaminouracil and the corresponding phenyl glyoxal afforded the required lumazines. The reaction conditions and purification procedure were the same as reported in the literature[14] for the parent 7-phenyllumazine. In order to obtain analytically pure samples several reprecipitations from alkaline solutions were needed. The lumazines 7b-e are insoluble in water and only sparingly soluble in DMF. The ^{13}C-NMR spectrum of 7-phenyllumazine was easily recorded for a DMSO-d_6 solution. The data are mentioned in Table 6.II.

Table 6.II

		m.p. (°C)	Found C%	Found H%	Required C%	Required H%
7-(p-X-phenyl)pteridine	X					
6a	H	158-160	69.3	4.1	69.22	3.87
6b	OCH_3	210-211	65.4	4.1	65.53	4.23
6c	Br	216-217	50.0	2.4	50.19	2.46
6d	NO_2	>247[a]	56.9	3.1	56.92	2.79
6e	CN	255[a]	67.1	2.7	66.94	3.03
7-(p-X-phenyl)pteridin-4-one	X					
4a	H	295[a]	64.2	3.6	64.28	3.60
4b	OCH_3	320[a]	61.6	4.0	61.40	3.96
4c	Br	280[b]	47.4	2.4	47.54	2.33
4d	NO_2	290[b]	53.3	2.6	53.53	2.62
4e	CN	295[b]	62.3	3.0	62.65	2.83
7-(p-X-phenyl)lumazine[c]	X					
7b	OCH_3	> 330	270.0753		270.0753	
7c	Br	> 330	317.9760		317.9753	
7d	NO_2	> 330	285.0491		285.0498	
7e	CN	> 330	265.0591		265.0586	

a. Decomposition
b. Subl.; m.p. >330°C
c. Exact mass measurements are given instead of microanalytical data. The lumazines were found by mass spectroscopy to contain variable amounts of water; however, this could not be removed even after drying these compounds under high vacuum at 100°C over P_2O_5 for 48 h.

6.5.1.5. Enzymic oxidation of the 7-p-X-phenylpteridin-4-ones into 7-p-X-phenyllumazines

A. The derivatives with X = Br, CN and NO_2.

One ml of xanthine-oxidase suspension (Boehringer; 10 mg protein per ml, ∿ 0.03 mmole ml^{-1}) was dissolved in 100 ml buffer of pH 8.6, containing 0.1 mM EDTA (tetrasodium ethylenediaminetetraacetate). One-tenth mmole of the 7-(p-X-phenyl)-pteridin-4-one was dissolved in a small excess of alkali and diluted to 100 ml with distilled water. The latter solution was slowly added (in ∿ 6 h) to the stirred enzyme solution and the reaction continued overnight. During the addition the pH was maintained at 8.6 by the addition of 2 M HCl as needed. The precipitated lumazines were filtered, washed with water and dried. The yield (> 90%) could be slightly augmented by first lowering the pH to about 6.5. Care must then be taken that no coprecipitation of the enzyme occurs by making the pH lower than 6.

B. The derivatives with X = OCH_3 and H.

Immobilized xanthine oxidase was prepared by adsorption of xanthine oxidase to *n*-octyl-Sepharose 4B, prepared as previously described[3a].

a. Immobilized enzyme containing 10 mg of protein is packed in a small column. One-tenth mmole of the 7-(p-X-phenyl)pteridin-4-one dissolved in a small excess alkali and diluted with 1 L buffer pH 9.1, containing 0.1 mM EDTA, was slowly ($2 ml.min^{-1}$) passed through the column and recirculated overnight. The effluent was acidified (pH 3), evaporated to about 100 ml and the precipitated lumazines then filtered, washed with water and dried.

b. One-tenth mmole of the 7-(p-X-phenyl)pteridin-4-one was dissolved in a small excess of alkali and diluted to 50 ml with distilled water. This concentrated substrate solution is slowly added (in ∿ 8 h) to 100 ml of a stirred suspension of immobilized xanthine oxidase (corresponding to 10 mg of protein). The suspension was buffered at pH 9.1 (and adjusted as needed) and contained 0.1 mM EDTA. After the addition of all the substrate the reaction was allowed to continue overnight. The suspension was filtered and the filtrate acidified with concentrated acetic acid. The product was filtered, washed with water and dried.

6.5.2. Determination of kinetic constants

All the values of V, V', K_m and K'_m (Table 6.I; Fig. 6.2 and 6.5) were estimated by the method of Halwachs[15]. This method was used because the initial rates of all the compounds 4a-e were obscured by a small lag phase, which was proportional

to the substrate concentration. The assays were performed by the method described earlier[3a]. The change in absorbance was followed at the appropriate wavelength (Table 6.I) until the reaction was completed. The concentrations used in the calculations were determined from these absorbance-time curves. The pH profiles (Fig. 6.2 and 6.5) were determined by this method starting with a low concentration of substrate (5 μM), since the poor solubility at low pH did not allow higher concentrations. No lag phase was observed at this low concentration, but it is not excluded that an initial increase in this case may be obscured by a decrease in rate as result of working with a substrate concentration near or lower than the K_m value. The more detailed studies at pH 9.1 were started with a concentration of 40 μM.

6.6. ACKNOWLEDGEMENT

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7. DIRECT IMMOBILIZATION OF MILK XANTHINE OXIDASE IN MILK PROTEIN WITH AND WITHOUT ADDITION OF GELATIN

J. Tramper, H.C. van der Plas and F. Müller

7.1. SUMMARY

Milk or a solution of gelatin and milk or commercial xanthine oxidase were lyophilized and the powdered freeze-dried materials crosslinked with glutaraldehyde. The resulting preparations of immobilized xanthine oxidase have a good stability, are highly active and well suited for organic synthesis.

7.2. INTRODUCTION

In recent papers[1-4] we have described the properties of xanthine oxidase immobilized under a variety of conditions. The aim of these studies was to search for a preparation suitable for use in organic synthesis. The operational stability of both free and immobilized xanthine oxidase is, however, poor[1,5] and limits the efficiency in synthesis. Therefore, we have further explored this area of research and now report on a modified improved procedure of entrapment of xanthine oxidase in gelatin. The modification consists of lyophilization of the enzyme-gelatin solution prior to the crosslinking with glutaraldehyde. The resulting preparation has a good mechanical stability, shows no protein leakage, and the yield of active immobilized xanthine oxidase is high and stable. The same simple procedure can be used for direct immobilization of unpurified xanthine oxidase in milk protein, with or without addition of gelatin. Whole milk, without any preceding treatment, or skimmed milk can be used as starting solution. Consequently, the financial advantage is dramatic: the cost is reduced about 2000 times.

7.3. MATERIALS

Milk xanthine oxidase (E.C. 1.2.3.2) was purchased from Boehringer, Mannheim (fl.300/100mg). Fresh whole milk was obtained from the University farm. Skimmed milk was prepared as follows. The whole milk was first stored overnight at 5°C to accomplish the dissociation of xanthine oxidase from the fat-globule membranes[6] and then centrifugated at low speed. Xanthine (puriss) and glutaraldehyde (25% in water) were from Fluka A.G., Buchs S.G., Switzerland. All other materials used were of reagent grade. All solutions were prepared using glass-distilled water.

7.4. METHODS

7.4.1. Immobilization procedure

The following stock solutions (A-E) were used for the synthesis of the various immobilized xanthine-oxidase preparations:

A. A gelatin/xanthine-oxidase solution, prepared by dissolving 5 g of gelatin in 25 ml of hot water to which, after cooling to room temperature, 0.5 ml of a suspension of commercial xanthine oxidase (10 mg protein.ml^{-1}) was added.
B. 50 ml of fresh whole milk.
C. 2.5 g of gelatin dissolved (at 60°C) in 50 ml of fresh whole milk.
D. 50 ml of skimmed milk.
E. 2.5 g of gelatin dissolved (at 60°C) in 50 ml of skimmed milk.
F. Commercial xanthine oxidase was also immobilized by adsorption to *n*-octyl-substituted Sepharose 4B as described previously[1].

Each of these solutions (A-E) was rapidly frozen in liquid nitrogen, lyophilized and the freeze-dried materials thoroughly grounded in a mortar. 1 g of the powder was then added to a vigorously stirred glutaraldehyde solution - 0.5 ml of 25% glutaraldehyde, 12 ml of water and 12.5 ml acetone - and the stirring continued for 30 min at room temperature. Finally, the crosslinked preparations were exhaustively washed with Tris-HCl buffer pH 8.2 (I = 0.02; 0.1 mM EDTA).

7.4.2. Determination of the activity and stability

The washed preparations were immediately packed in a column (1 cm i.d.) and substrate passed through it. The substrate solution consisted of 200 μM xanthine in

Tris-HCl buffer pH 8.2 (I = 0.02; 0.1 mM EDTA). The flow rate (LKB peristaltic pump) was 14 ml h^{-1} and the temperature 5°C. Fractions of 21 ml were collected and the optical density measured at 290 nm^{1}. Two L of substrate were pumped through all the reactors and the total amount of product determined.
All the effluents were assayed for protein by the Biuret method.

7.5. RESULTS AND DISCUSSION

7.5.1. The immobilization

It is necessary to make a well mixed solution of gelatin and the enzyme prior to lyophilization, in order to prepare a gelatin matrix in which xanthine oxidase is homogeneously distributed. The commercial solution of concentrated xanthine oxidase is best mixed with a gelatin solution, which is cooled to a temperature where it is still liquid. The whole-milk and skimmed-milk gelatin solutions are best prepared by dissolving the gelatin at 60°C in the respective liquids. At this temperature the denaturation of xanthine oxidase is sufficiently slow that the loss in activity is negligible during the time required for the dissolving of gelatin. The solutions are then immediately frozen in liquid nitrogen.
The addition of acetone to the glutaraldehyde solution is necessary to prevent milk protein from redissolving when the freeze-dried powder is added to this solution for crosslinking. A concentration of acetone of 50% (v/v) is sufficient and causes no significant loss in activity of xanthine oxidase. Vigorously stirring of the glutaraldehyde solution prevents coagulation when the freeze-dried powder is added.

7.5.2. Mechanical properties of the supports

A reaction time of 30 min for crosslinking in a 0.5% glutaraldehyde solution (25 ml per gram of freeze-dried material) at room temperature yields a mechanical stable support. Protein assays of the effluents showed that no protein leakage occurs. The flow properties of the preparations with gelatin are good. The pressure drop is low and the flow rate remained constant during the time of operation (∿ 1 week), irrespective of upflow or downflow was used. The flow rate of the reactors containing the milk preparations without gelatin gradually decreased with downflow, even though a pump was used. Reversing the flow largely eliminated this decrease; only a small decrease was observed with the whole-milk reactor as re-

sult of fine particles packing at the top of the column. The results described here were obtained from reactors with upflow and the data for the whole-milk reactor are corrected for the small change in flow rate. No differences in up and downflow were, however, observed when corrections for decreasing flow rates were made.

Table 7.I Properties of various preparations of immobilized milk xanthine oxidase[a].

Preparation[b]	Highest reaction rate observed ($\mu moles.h^{-1}.L^{-1}$)[c]	Specific activity ($\mu moles.h^{-1}.mg^{-1}$)	Half life $t_{\frac{1}{2}}$ (days)	μmoles of substrate converted in $t_{\frac{1}{2}}$[c]
A	1.0[d]	1.0	4.2	85[e]
B	269	5.4[f]	4.5	23000
C	394	7.9[f]	4.3	34000
D	145	2.9[f]	3.4	9000
E	118	2.4[f]	5.0	11000
F	1.4[d]	1.4	0.8	29[e]

a. Substrate: 200 μM xanthine in Tris-HCl buffer, pH 8.2 (I=0.02; 0.1 mM EDTA); 5°C. Flow rate: 14 ml.h^{-1}.

b. Letter corresponding to the stock solution used for immobilization (see Methods).

c. μmoles oxidized by an amount of material corresponding to 1 L of milk.

d. $\mu moles.h^{-1}.mg^{-1}$.

e. μmoles converted per mg protein.

f. Based on an average xanthine oxidase content of 50 $mg.L^{-1}$ milk[7].

7.5.3. Activity of the preparations

Column 2 of Table 7.I lists the highest reaction rates observed for the various preparations, expressed as μmoles of substrate converted in one hour with material corresponding to 1 L of milk or 1 mg of protein of the commercial enzyme. Immobilization of commercial xanthine oxidase in gelatin by the procedure now described gives a retention of activity of about 65%, while we found previously[1] less than 10%. These data are based on comparison with xanthine oxidase adsorbed to *n*-octyl-substituted Sepharose 4B with a retention of activity of 90%[1].

The average xanthine-oxidase content in milk is about 50 mg per litre[7]. If we use this figure, a high specific activity, especially with whole milk, is calculated for all the milk preparations (Column 3 of Table 7.I). In case of the whole milk, the addition of gelatin considerably enhanced the specific activity.

7.5.4. Stability of the preparations

Some results of the stability experiments are shown in Figure 7.1. The logarithm of the ratio of the conversion at t = 0 and t = t is plotted as a function of time. The time at which the reactors reached the highest rate of conversion of substrate (column 2 of Table 7.I) is defined as t = 0. This rate was achieved after about 15 h in all cases except the reactor containing xanthine oxidase adsorbed to *n*-octyl-substituted Sepharose 4B, which reached the highest rate of con-

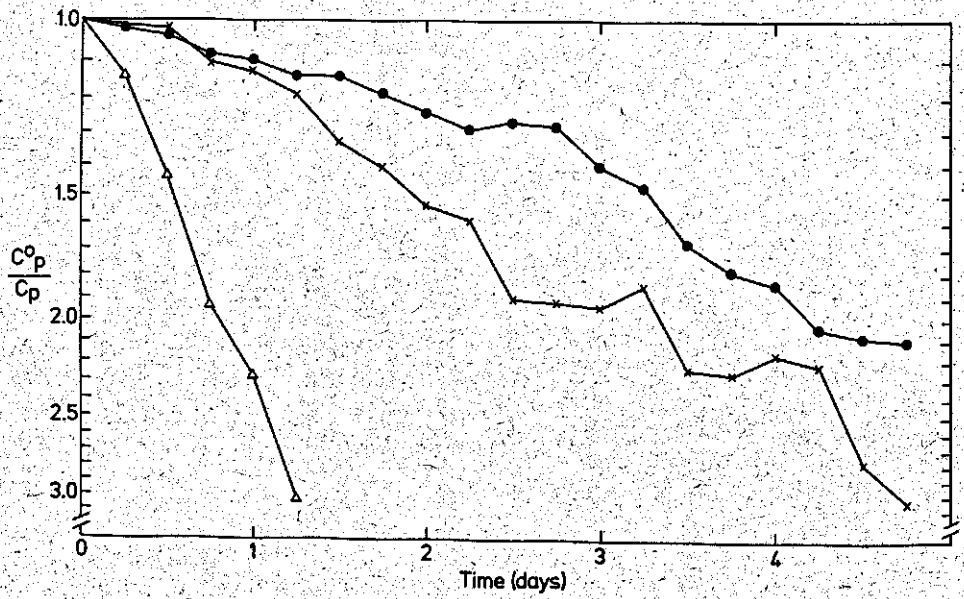

Figure 7.1 Stability of immobilized milk xanthine oxidase under continuous operation of 5°C. Substrate: 200 μM xanthine in Tris-HCl buffer pH 8.2 (I = 0.02; 0.1 mM EDTA). Flow rate: 14 ml.h^{-1}. (●) C; (x) D; (Δ) F. Letters corresponding to the preparations described in METHODS.

version almost immediately after the start of the reaction. The average of 4 fractions (6 h) is given in Fig. 7.1. Scanning the complete UV-spectra of various fractions of the effluents, which showed sharp isosbestic points at the appropriate wavelengths, demonstrated that the decrease in absorbance at 290 nm with time was the result of decreasing conversion of substrate.

It is clear that entrapment of xanthine oxidase in protein enhances the stability considerably (column 3 of Table 7.I). Their is no pronounced difference in stability of the various milk preparation, except maybe for the skimmed-milk material. Apparently, the kind of inert protein used to entrap xanthine oxidase is not crucial.

7.5.5. *Economic considerations*

Column 4 of Table 7.I shows the amount of substrate converted in one half time by an ammount of material corresponding to 1 L of milk, or 1 mg of protein of commercial xanthine oxidase, under the conditions used. The price of 1 mg of commercial xanthine oxidase is about 5 times that of 1 L of milk. Consequently, the difference in cost of conversion of 1 mole of substrate by commercial xanthine oxidase entrapped in gelatin compared to the whole-milk gelatin preparations is about a factor of 2000. The difference in cost with the xanthine oxidase adsorbed to the *n*-octyl-substituted Sepharose 4B is even greater, both because the labor involved in the preparation and the cost of the support material are much higher and because of the lower conversion of substrate per mg of protein.

7.6. CONCLUSIONS

Direct immobilization of milk xanthine oxidase in whole-milk protein enriched with gelatin yields a highly active and stable preparation with good mechanical stability and flow properties. The method is very simple and can be performed in any laboratory equipped with a freeze-drying apparatus. Moreover the costs are very low. Consequently, this immobilized xanthine-oxidase preparation is very suitable for application in organic synthesis. Since the described immobilization procedure is very mild it would appear to be applicable to many enzymes.

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8. XANTHINE-OXIDASE ACTIVITY OF ARTHROBACTER X-4 CELLS IMMOBILIZED IN GLUTARALDEHYDE-CROSSLINKED GELATIN

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8.1. SUMMARY

Cells of Arthrobacter X-4 were immobilized by entrapment in gelatin crosslinked with glutaraldehyde. The xanthine-oxidase activity and stability were determined at various temperatures. In comparison with bovine-milk xanthine oxidase the bacterial enzyme is more stable and has a different substrate specificity. 1-Methylxanthine was oxidized on a preparative scale.

8.2. INTRODUCTION

Since 1975 we study the application of immobilized enzymes in organic synthesis. Xanthine oxidase was chosen as model enzyme, because it catalyzes the oxidation of many azaheterocyclic compounds, which chemistry is a main objective in the laboratory of Organic Chemistry (for reviews see van der Plas[1,2]). Hitherto the enzymes from bovine milk[3,4,5] and from chicken liver[4,6] were studied. Recently we have reported the direct immobilization of the milk enzyme by means of entrapment in glutaraldehyde-crosslinked gelatin, yielding a cheap, highly active and relatively stable xanthine-oxidase preparation[7]. In the present study we have further improved the material properties of the gelatin support and extended the method to the immobilization of whole bacterial cells, *i.e.*, those of Arthrobacter strain X-4 that was isolated from soil and shown to exhibit high xanthine oxidase activity.

Arthrobacter was chosen for the following reasons. As the result of a survey of about 50 diverse strains of bacteria containing xanthine-oxidase activity, Woolfolk and Downard[8] observed that 2 strains of Arthrobacter gave by far the highest specific activities using molecular oxygen as terminal electron acceptor. Furthermore, in contrast to the milk enzyme, Arthrobacter xanthine oxidase shows substrate activation rather than substrate inhibition and has a specific activity of about 50 times that of the most active preparations of the milk enzyme.

We have immobilized the whole cells without any purification, for it is known[9] that the next enzyme in the purine-oxidative-pathway sequence, uricase, is in contrast to xanthine oxidase highly specific and only oxidizes uric acid. Since we are interested in the oxidation of other substrates than xanthine, thus leading to other products than uric acid, we expected that no further breakdown after the first oxidation would occur and that therefore the whole cells would suffice. In this paper we wish to present the initial results of this study.

8.3. MATERIALS AND METHODS

8.3.1. Growth of cells

The xanthine/agar medium[10] used for the isolation and purification of soil Arthrobacter strains contained per litre 0.1 M potassium-phosphate buffer pH 7.2: 100 mg of yeast extract, 100 mg of xanthine (Merck, for biochemical purposes), 200 mg of magnesium sulfate, 100 mg of calcium chloride, 0.5 mg of ferrous sulfate and 20 g of agar. Portions of 30 ml were poured in Petri dishes of 12 cm internal diameter and supplied with an overlay of 3 ml of the same medium supplemented with 10 g of xanthine per litre.

Cells of strain X-4 were grown in ethanol/yeast-extract medium that contained per litre 0.1 M potassium-phosphate buffer pH 7.2: 5 g of yeast extract, 10 g of ethanol (added after sterilization), 2 g of xanthine, 100 mg of sodium sulfate, trace elements, magnesium chloride and calcium chloride[11]. Portions of 100 ml of this medium were contained in 300 ml conical flasks, sterilized for 20 min at 120°C, inoculated with a loopful of strain X-4 and incubated at 30°C in a rotary shaker. Cells were harvested by centrifugation after 48 h of growth and washed with 0.05 M potassium-phosphate buffer pH 7.5. Adaptation of these cells to xanthine was performed at 36°C in a 0.1 M potassium phosphate buffer pH 7.5 containing 1 g of xanthine per litre for about 8 h, while air was passed through The xanthine concentration was determined at intervals. The cells were then washed with Tris-HCl buffer pH 8.5 (I=0.01) until no xanthine was detected in the wash liquid, and immobilized. Cells grown for 48 h in 500 ml of medium were collected, adapted to xanthine, washed, resuspended in a small amount of buffer, divided in 15 portions of 1 ml (corresponding to 90 mg of freeze-dried cell material) and stored frozen (-20°C) until the immobilization.

8.3.2. Immobilization procedure

The cells were suspended in a gelatin solution, lyophilized, and crosslinked with glutaraldehyde, analogous to the procedure described previously for milk xanthine oxidase[7]. Instead of grinding the freeze-dried material in a mortar, a Gulatti analysis mill containing a sieve was used. Particles with nominal diameter of 0.5, 0.7 and 1 mm were investigated. In order to determine the effect of the gelatin concentration in the initial solution on the ultimate properties of the support, concentrations of 0.5, 2, 5, 10, 11, 12.5, 15 and 20% were made. Preparations consisting of particles with a nominal diameter of 1 mm, 10% gelatin, and a dry-weight ratio of cells and gelatin of 1, were used routinely.

8.3.3. Activity and stability assays

The preparations of immobilized whole cells were packed in a column (i.d. 0.7 cm; bed about 1 cm high), thoroughly washed with Tris-HCl buffer pH 7.9 (I=0.01; 0.1 mM EDTA) for about 24 h, and 100 μM substrate (1-methylxanthine, puriss, Fluka) pumped through it (18 ml h^{-1}). Fractions of 18 ml were collected and the conversion calculated from the absorbance at 290 nm, using $\Delta\varepsilon$ = 10.3 mM^{-1} cm^{-1}.

8.3.4. Specificity assays

In addition to 1-methylxanthine the activity was determined with 8-phenylpurin-6-one (preparation to be published), 7-phenylpteridin-4-one[5] and 6-phenylpteridin-4-one (preparation to be published), and compared with those of milk xanthine oxidase. The concentration used was 100 μM for all substrates and the change in absorbance measured at 280, 325 and 310 nm, respectively. The rates were calculated using $\Delta\varepsilon$ = 4.5, 1.7 and 2.2 mM^{-1} cm^{-1}, respectively.

8.3.5. Oxidation of 1-methylxanthine on a preparative scale

Fifty mg of 1-methylxanthine were dissolved in a minimal amount of NaOH-solution (4M) and diluted to 300 ml with Tris-HCl buffer pH 7.9 (I=0.01; 0.1 mM EDTA). This solution was recycled through a column containing immobilized Arthrobacter cells (270 mg of cell material) until complete conversion was reached (after about a week) and then acidified to pH about 5 with HCl (2M). The solution was evaporated *in vacuo* to a small volume, cooled (0°C), filtered and the residue extensively washed with distilled water.

8.4. RESULTS AND DISCUSSION

8.4.1. Strain selection and growth of cells

Arthrobacter cells which are able to grow at the expense of purines are very abundant in soil[12]. A xanthine-dissimilating Arthrobacter strain was isolated by streaking a suspension of garden soil on xanthine/agar plates, without preceding enrichment culture. After 3 days at 30°C numerous colonies were surrounded with clear zones which indicated xanthine dissimilation. Bacterial colonies were streaked on the same medium to obtain pure cultures. The most active xanthine-dissimilating ones were examined microscopically after growth on nutrient agar. One strain (X-4) showing the rod coccus transformation in senescent cultures, which is characteristic of Arthrobacter, was chosen and maintained on nutrient agar slants.

Strain X-4 is Gram-positive, non-motile, strictly aerobic and produces abundant slime in glucose/yeast-extract medium. Young cultures on nutrient agar consisted of rods that were occasionally branched and transformed into ovate cocci in senescent cultures. Cells growing at the expense of xanthine were coccoid[10]. Growth yields on xanthine as the sole source of carbon and energy were low due to the low energetic value of this substrate. For this reason cells were pre-grown on ethanol/yeast-extract medium and subsequently adapted to xanthine prior to immobilization. After 24 h of growth in this medium branched rods predominated that showed moderate xanthine-oxidase activity after adaptation and immobilization (Table 8.I). The coccoid growth phase was reached after about 40 h. Cells harvested after 48 h showed a satisfactory enzyme activity after adaptation and immobilization (Table 8.I). After 72 h of growth in ethanol/yeast-extract medium the capacity of xanthine-oxidase induction is apparently lost (Table 8.I). Storage at -20°C and lyophilization did not affect the ultimate activity.

Time of growth (h)	Cell morphology	Relative immobilized activity (%)
24	branched rods	66
48	predominantly cocci	100
72	cocci	0

Table 8.I Influence of time of growth of the culture on the ultimate activity of immobilized xanthine oxidase.

8.4.2. Immobilization of cells

When a concentration of 10% of gelatin in the starting solution was used, grinding was easy and the particles formed had good flow properties. When the particles were thoroughly washed before utilization, no protein was detected in the effluent during catalysis. Concentrations of gelatin much lower than 10% resulted in a weak and gelatinous support. At much higher concentrations a very hard support resulted which was difficult to grind. The milling and sieving step was introduced to obtain a more homogeneous distribution of particle sizes. Agglomeration of the particles with a nominal diameter of 0.5 and 0.7 mm occurred when suspended in the crosslinking solution and particles with a nominal diameter of 1 mm were therefore used.

8.4.3. Activity and stability

When xanthine was used as the substrate, uric acid was formed as intermediate product, but eventually disappeared completely. As expected, if 1-methylxanthine was used as the substrate, the specificity of uricase prevented further oxidations and 1-methyluric acid was the end product. The activity at various temperatures is given in Table 8.II. From these data an activation energy of about 22 kJ mol^{-1} is calculated being close to the one of immobilized xanthine dehydrogenase[6]. The half life ($t_{\frac{1}{2}}$) and the amount of substrate converted in the first half time at these temperatures are also given in Table 8.II. At a temperature of about 20°C immobilized Arthrobacter xanthine oxidase performs optimally.

T(°C)	$t_{\frac{1}{2}}$(days)	Total conversion in $t_{\frac{1}{2}}$ in mg/ 100 mg of column material	Initial activity µmoles/min/100 mg of column material
4	9.5	14.3	6.4
17	8.4	26.1	9.9
28	4.2	16.5	16.3
36	2.4	10.4	20.4

Table 8.II Temperature dependency of xanthine-oxidase activity and stability of immobilized Arthrobacter cells, and amount of 1-methylxanthine converted in the first half time per 100 mg of column material (dry weight).

8.4.4. Specificity

Four substrates were tested with immobilized Arthrobacter cells and the relative initial activities compared with those of the bovine-milk enzyme (Table 8.III). It is clear that the bacterial enzyme has a substrate specificity different from that of milk xanthine oxidase.

8.4.5. Synthesis

1-Methylxanthine was converted on a small preparative scale by immobilized Arthrobacter cells. Starting with 50 mg of substrate the yield of analytically pure 1-methyluric acid was 33 mg (60%). UV and mass spectra were identical to those of authentical material.

Substrate	Immobilized Arthrobacter		Milk xanthine oxidase	
	units/100 mg of column material	%	units x 10^3/ mg protein	%
1-methylxanthine	10.7	100	240	100
8-phenylpurin-6-one	0.8	7.5	1.9	0.8
7-phenylpteridin-4-one	2.9	27	65	27
6-phenylpteridin-4-one	7.0	66	5.8	2.4

Table 8.III Specific activities of milk xanthine oxidase and immobilized Arthrobacter cells with various substrates at 20°C (1 unit = 1 μmole min^{-1}).

8.5. CONCLUSIONS

Immobilized Arthrobacter X-4 cells have a high xanthine-oxidase activity and are suitable for application in organic synthesis in a continuous fashion. The specificity of the bacterial enzyme is different from that of milk xanthine oxidase, thus the spectrum of substrates that can be oxidized efficiently, is widened.

8.6. REFERENCES

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9. GENERAL DISCUSSION

In Chapter 2 it is concluded that for a particular application an evaluation of various supports is necessary. So far we have only discussed agarose and gelatin as a support for the immobilization of xanthine oxidase or xanthine dehydrogenase, but in the course of our study several other supports have been investigated on their merits as a support for these enzymes and the results are shortly discussed here.

1. *Controlled-pore glass (CPG).* The γ-aminopropyltriethoxysilane derivative of CPG (p.41) was developed concurrently with agarose. It was activated in four different manners: *i.* reaction with glutaraldehyde (p.45); *ii.* reaction with s-trichlorotriazine (p.43); *iii.* using a carbodiimide (p.42); and *iv.* preparation of the diazo derivative (p.41). Only the glutaraldehyde-activated CPG appeared to be useful. The binding capacity of this material is comparable with that of agarose substituted with *n*-octylamine, but the retention of activity is about half On the other hand the flow properties of CPG are better when applied in a column. Therefore, the operational stability of various xanthine-oxidase/CPG preparations was tested, *i.e.*, xanthine oxidase coimmobilized with superoxide dismutase and/or catalase. Results were obtained comparable with those of the xanthine-oxidase/agarose preparations, except that the stability was about twice as high. Thus the loss in activity as result of immobilization is compensated for by the increase in stability. Therefore, about similar amounts of substrate can be converted with the same amount of soluble enzyme used for immobilization. That eventually the agarose preparations were preferred was due to the ease of the assay possible with these preparations, *i.e.*, directly in the cuvet in the same way as the soluble enzyme. This was very convenient for testing new substrates, one of our goals. The flow properties of the agarose preparations in a column were also sufficient for our purposes. Further, CPG has the disadvantage that it gradually dissolves at the optimum pH (∿ 8.7) of xanthine oxidase. To circumvent the latter problem, controlled-pore titania, which is more stable in weak-alkaline solutions, was investigated as well. A nominal pore diameter of 45 nm was chosen for the following reason. This diameter is about twice that of the largest dimension of the enzyme and allows easy access, has an optimal binding capacity and the greatest probabi-

lity of stabilizing the enzyme. The latter can be explained on the basis of confinement of the enzyme into a space allowing the conformational changes necessary for catalysis still to occur, but preventing conformational changes leading to inactivation. Xanthine oxidase was immobilized to the controlled-pore titania by adsorption and by the method of salt-bridge formation as described for urease by Messing[1]. Adsorption resulted in a moderate-active preparation having a stability comparable with that of the CPG preparations. When xanthine oxidase was bound by salt-bridges, all the activity was lost. The latter method was chosen because it was expected that multiple salt-bridge formation would stabilize the active conformation, but with maintenance of the flexibility of the enzyme necessary for catalysis.

2. *Activated carbon (AC).* The application of AC was investigated because it is known[2] that AC catalyzes the decomposition of hydrogen peroxide, a by-product of the catalysis. We showed that the rapid inactivation of xanthine oxidase during catalysis is at least partly due to denaturation by hydrogen peroxide. Accumulation of this by-product is likely to occur in the microenvironment of the enzyme where it is produced. Further, as result of the high surface area, AC is conducive to large amount of protein binding so that it was expected that the substrate optimum of xanthine oxidase could be chosen at will by varying the amount of enzyme immobilized (Chapter 4). However, no xanthine oxidase could be bound at all, either by adsorption or covalent binding by means of glutaraldehyde or carbodiimide.

3. *Other supports.* Carboxymethylcellulose, polyacrylamide, Enzacryl AA and AH, Phenyl and Octyl-Sepharose Cl-4B and thiolated gelatin, all have been investigated as a support for xanthine oxidase or xanthine dehydrogenase for various reasons. Either almost no binding occurred or the retention of activity was low, making these materials unsuitable.

Figure 4.1 shows the pH optima of free and immobilized xanthine oxidase. In Chapter 4 as a possible explanation for the acid shift upon immobilization was mentioned the positive charge of the support creating a microenvironment of the enzyme with a pH different from that measured in the bulk, a phenomenon extensively discussed in the literature[3]. When this is indeed the case, this shift should disappear when a sufficiently high salt concentration is applied. However, in a solution with a high salt concentration no cancellation of the shift was observed, showing that this explanation is not applicable in our case. The substrate concentration used in the experiment of Figure 4.1 is about equal to the K_m' value found later at the optimum pH of the immobilized enzyme. A concentration not too much

above the K_m of the free enzyme was chosen because it is known[4] that even at low substrate concentrations inhibition can occur. To determine the actual cause of the shift the complete concentration profiles were determined as a function of pH (Fig. 9.1) under the conditions of the assays as described in Chapter 4. This figure (9.1) clearly shows that the observed shift at low concentrations of substrate is solely due to the higher K_m' than K_m, which in turn is caused by the internal-diffusion limitation in the immobilized-enzyme system as described in Chapter 4.

In addition to the 7-(p-X-phenyl)pteridin-4-ones described in Chapter 6, several other series of substrates have been synthesized and investigated, *i.e.*, the 6-(p-X-phenyl)pteridin-4-ones with X = OCH_3, CH_3, H, Cl, and Br, and 8-(p-X-phenyl)purin-6-ones with X = $N(CH_3)_2$, OCH_3, CH_3, H, Cl, Br, CN, and NO_2. They turned out to be very poor substrates but strong inhibitors of xanthine oxidase. This indicates that the affinity of the enzyme for these compounds is large, but that as result of the different orientation of the phenyl group, these compounds apparently fit in the wrong orientation in the active site of the enzyme. These result induced us to study the inhibitory properties of these compounds in detail for the following reasons. *i.* In the pharmaceutical area much research is directed to find new inhibitors to replace the anti-gout drug allopurinol, which is known to have several disadvantages. *ii.* Immobilized enzymes are better models of enzymes *in vivo* than highly purified, water-soluble enzymes. Therefore, we want to introduce immobilized xanthine oxidase as model system for testing new inhibitors and study possible differences between the free and immobilized enzyme. *iii.* To determine if the influence of X on the inhibitory capacity of these compounds can be described by a linear Hammett relation.
The preliminary results of these studies are: *i.* above-mentioned purine derivative are better inhibitors than allopurinol, having an I_{50}-value three orders of magnitude smaller than allopurinol; *ii.* the difference between the free and immobilized enzyme depends on the substrate used; *iii.* no linear Hammett relationship exists, neither for the purine nor for the pteridine derivatives.

Based on a small number of substrates investigated, Woolfolk and Downard[5] conclude that xanthine oxidase from Arthrobacter is more specific than the milk enzyme. However, our results - see Table 8.II - show actually the opposite. Evident is, that the specificity of these enzymes is different. This is, however, not surprising. Although the similarities in the absorption spectrum of the enzymes suggest that the prostetic group content is very similar (not yet proved), the

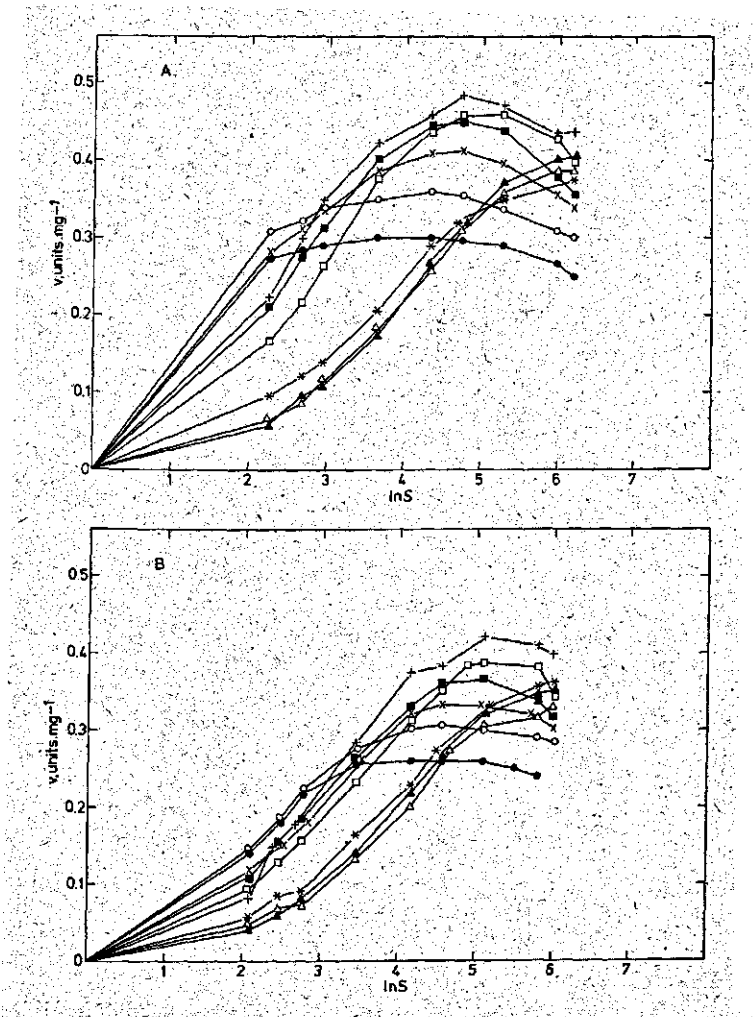

Figure 9.1 Oxidation rate v as a function of the xanthine concentration S (μM) by free (A) and immobilized (B) xanthine oxidase at various pH's: (●) 7.9, (○) 8.2, (x) 8.5, (■) 8.8, (+) 9.0, (□) 9.1, Tris-buffer systems; (*) 9.1, (▲) 9.4, (Δ) 9.6, borate-buffer systems.

absorption ratio A_{550}/A_{450} (Fe/S:FAD) resembles more closely that of aldehyde oxidase (an enzyme similar to xanthine oxidase, but with a different specificity for azaheterocycles) and xanthine dehydrogenase. The ratio A_{280}/A_{450} is also different, about half that of the milk enzyme, which may be connected with the fact that the bacterial xanthine oxidase has a molecular weight of about half that of milk xanthine oxidase and thus about one half as much protein per prosthetic center. It is therefore likely that some chemical and physical properties are altered as compared to milk xanthine oxidase.

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SUMMARY

The objective of the study described in this thesis was to show that enzymes, especially immobilized enzymes, can be advantageously used in synthetic organic chemistry.

In Chapter 1 enzymes are introduced and the advantages discussed when these highly active and specific biocatalysts are immobilized, *i.e.*, attached to a solid support. Criteria for general acceptation of a specific enzyme as a routine catalyst in the organic-chemistry laboratory are stated. The reasons for the choice of xanthine oxidase as a model enzyme and some relevant properties are given.

The solid support used for the immobilization determines to a large extent the ultimate characteristics of the immobilized enzyme. In Chapter 2 properties of an ideal support and guidelines for the evaluation of a specific support are discussed. Various supports are treated in some detail.

The mode of immobilization is also very important, especially with respect to the eventual specific activity of the immobilized enzyme. In Chapter 3 the various methods of immobilization are discussed. Just as with the supports (Chapter 2), special attention is given to the ones investigated in this study.

In Chapter 4 various properties of immobilized milk xantine oxidase are described, as well as the stabilization by coimmobilization with protein, superoxide dismutase and catalase. It is concluded that conversion of substrate on a preparative scale can be performed conveniently, but that for efficient application a more stable enzyme preparation is desired.

Chicken-liver xanthine dehydrogenase is an enzyme very similar to milk xanthine oxidase. In Chapter 5 the reasons are given why it was expected that the liver enzyme would be more stable and practical for synthetic application. The immobilization and the properties of the free and immobilized enzyme are described. Analogous to the milk enzyme, the operational stability of xanthine dehydrogenase is much lower than the storage stability.

In order to determine the substrate limits of xanthine oxidase more systematically, so that more-reliable predictions would be possible, series of substrates were synthesized for this purpose. In Chapter 6 the synthesis of 7-(p-X-phenyl)-pteridin-4-ones and the oxidation by free and immobilized xanthine oxidase are

presented. As X becomes more electron withdrawing, the rate of oxidation decreases. It is therefore concluded that the electron donation from substrate to enzyme must be the rate-limiting step.

Convenient oxidation of the above substrates and easy product isolation was possible on a small preparative scale. The limited stability of xanthine oxidase demanded however a relatively large amount of enzyme. In Chapter 7 an easy and mild immobilization procedure, using gelatin as support and glutaraldehyde as cross-linking agent, is presented. Whole milk can be used as starting enzyme solution and no isolation is required. The resulting preparation of immobilized xanthine oxidase is very cheap, highly active, relatively stable and suitable for application in organic synthesis in a continuous manner.

In Chapter 8 the improvement of the immobilization method with gelatin is described and the extension to the immobilization of whole cells, *i.e.*, Arthrobacter cells containing xanthine-oxidase activity. These cells were chosen because of their high specific activity, their substrate activation instead of inhibition and their expected higher stability. Immobilized Arthrobacter xanthine oxidase is indeed highly active, more stable and has a different substrate specificity than milk xanthine oxidase. Therefore, a wider spectrum of substrates can be conveniently and efficiently oxidized.

In the final Chapter 9 additional information, mostly gathered at a later stage than the time of publication, is given and discussed.

The end conclusion is: Xanthine oxidase, either from whole milk or Arthrobacter, when immobilized in glutaraldehyde-crosslinked gelatin, can be conveniently used for the oxidation of a wide range of substrates in a continuous fashion.

SAMENVATTING

Het doel van het beschreven onderzoek was aan te tonen dat enzymen, vooral geïmmobiliseerde enzymen, met profijt in de preparatieve organische scheikunde gebruikt kunnen worden.

In hoofdstuk 1 worden enzymen ingeleid en de voordelen besproken wanneer deze zeer actieve en specifieke biokatalysatoren geïmmobiliseerd worden door hechten aan een vaste drager. Maatstaven voor algemene aanvaarding van een bepaald enzym voor dagelijks gebruik in het scheikundig laboratorium worden opgesomd. Verder worden in dit hoofdstuk de redenen gegeven voor de keus van xanthine oxidase en enige ter zake dienende eigenschappen van dit enzym.

De vaste drager die gebruikt wordt voor de immobilisatie bepaalt in belangrijke mate het uiteindelijk karakter van het geïmmobiliseerde enzym. In hoofdstuk 2 worden de eigenschappen van een ideale drager besproken evenals richtlijnen voor de evaluatie van een bepaalde drager.

De wijze waarop het enzym geïmmobiliseerd wordt, is eveneens zeer belangrijk, vooral wat betreft de uiteindelijke specifieke activiteit van het geïmmobiliseerde enzym. In hoofdstuk 3 worden de verschillende manieren van immobilisatie besproken. Evenals bij de dragers (hoofdstuk 2) wordt speciale aandacht geschonken aan de methoden die door ons onderzocht zijn.

In hoofdstuk 4 worden verscheidene eigenschappen van geïmmobiliseerd melk xanthine oxidase beschreven en de stabilisatie door coimmobilisatie met eiwit, superoxide dismutase en catalase. De conclusie is dat omzetting van substraat op preparatieve schaal op eenvoudige wijze mogelijk is, maar dat voor efficiënte toepassing een stabieler enzym preparaat gewenst is.

Xanthine dehydrogenase uit kippelever is een enzym dat grote overeenkomsten vertoont met xanthine oxidase uit koeiemelk. In hoofdstuk 5 wordt beredeneerd waarom verwacht werd dat dit enzym stabieler zou zijn en praktischer voor preparatieve toepassingen. De immobilisatie en de eigenschappen van het vrije en geïmmobiliseerde enzym worden in dit hoofdstuk beschreven. Analoog aan het melk enzym is de operationele stabiliteit van xanthine dehydrogenase veel lager dan de opslagstabiliteit.

Om de substraatgrenzen van xanthine oxidase meer systematisch vast te leggen, zodat betrouwbaarder voorspellingen gemaakt kunnen worden, werden met dit oogmerk

verscheidene series substraten gesynthetiseerd. In hoofdstuk 6 wordt de bereiding beschreven van 7-(p-X-phenyl)pteridin-4-ones en de oxidatie van deze verbindingen onder invloed van vrij en geïmmobiliseerd xanthine oxidase. Hoe electronenzuigender X, hoe lager de oxidatiesnelheid en de conclusie is daarom dat de electronoverdracht van het substraat naar het enzym de snelheidsbepalende stap moet zijn.

Bovengenoemde verbindingen konden op eenvoudige en gemakkelijke wijze geoxideerd worden in kleine preparatieve hoeveelheden. De beperkte stabiliteit van xanthine oxidase benodigde echter een betrekkelijk grote hoeveelheid enzym. In hoofdstuk 7 wordt een gemakkelijke en milde immobilisatie methode gepresenteerd die gebruik maakt van gelatine als drager en glutaaraldehyde als crosslinkingsreagens.

Verse volle melk kan direct als enzym oplossing gebruikt worden en enzymzuivering is overbodig. Het op deze wijze verkregen preparaat van geïmmobiliseerd xanthine oxidase is erg goedkoop, katalytisch zeer actief, relatief stabiel en goed bruikbaar voor toepassing in organische synthesis op een continue manier.

In hoofdstuk 8 wordt de verbetering beschreven van de methode met gelatine en de uitbreiding tot de immobilisatie van hele cellen, en wel, Arthrobacter cellen die xanthine-oxidase activiteit bevatten. Deze cellen werden gekozen vanwege hun hoge specifieke activiteit, hun substraat activering in plaats van remming en hun verwachte grotere stabiliteit. Geïmmobiliseerd Arthrobacter xanthine oxidase is inderdaad actiever, stabieler en heeft een andere substraat specificiteit dan melk xanthine oxidase. Een breder spectrum van substraten kan daarom op efficiënte en eenvoudige wijze geoxideerd worden.

In het slothoofdstuk (9) wordt aanvullende informatie, hoofdzakelijk verzameld op een later tijdstip dan de tijd van publicatie, gegeven en besproken.

De eind-conclusie luidt: Xanthine oxidase, hetzij uit volle melk, hetzij uit Arthrobacter, kan, wanneer geïmmobiliseerd in gelatine dat gecrosslinkt is met glutaaraldehyde, gebruikt worden voor oxidatie van een breed spectrum van substraten op een continue wijze.

CURRICULUM VITAE

Op 18 mei 1949 werd ik te 's-Gravenpolder in Zeeland geboren. Na het behalen van het HBS-B diploma aan het Goese Lyceum te Goes in 1966, nam mijn studie in de Scheikundige Technologie aan de TH Delft een aanvang. Een van de hoogtepunten vond ik mijn praktisch werk aan de universiteit van Tel Aviv in Israël. Mijn afstudeeronderwerp was de konservering van hout met behulp van reaktieve fungiciden en dit onderzoek werd onder leiding van Prof.Dr.P.M.Heertjes uitgevoerd. In april 1973 rondde ik mijn studie met lof af. In augustus 1973 vertrok ik naar de VS en liet me inschrijven aan Purdue University in Lafayette, Indiana. Naast student was ik daar "teaching assistent". In december 1974 behaalde ik het MS diploma in "Chemical Engineering" bij Prof.Dr.A.H.Emery en Prof.Dr.H.C.Lim. Mijn onderzoeksonderwerp was de immobilisatie van het enzym glucose isomerase. Een dergelijk biotechnologisch onderwerp kon ik voortzetten in februari 1975 als promovendus bij de vakgroep Organische Chemie van de Landbouwhogeschool in Wageningen. Prof.Dr.H.C.van der Plas en Dr.F.Müller begeleidden deze studie. De biotechnologie blijft mijn belangrijkste werkterrein bij de vakgroep Levensmiddelentechnologie, sectie Proceskunde, eveneens van de LH, waar ik op 1 september 1979 in dienst trad.

6

Bij de hydrogenering van nitraat met een palladium katalysator hebben Lopatin *et al.* geen rekening gehouden met de aktiverende werking van geringe hoeveelheden andere metalen.

V.L. Lopatin *et al.*, Kinet.Katal., *20*, 373-379 (1979).

7

De NMR gegevens die Sato en Stammer rapporteren voor N-hydroxythioureum, zijn niet in overeenstemming met hun bewering dat het een eenthiol en thion tautomeer mengsel betreft.

M. Sato en C.H. Stammer, J.Med.Chem., *19*, 336-337 (1976).

8

Van der Molen bestrijdt het gebruik van alternatieve landbouwmethoden op een te eenzijdige en subjectieve wijze.

H. van der Molen, 19nu, *15*(4) 26-29 (1979); Economisch-Statistische Berichten, 4 juli 1979, 666-669.

9

De hoge ziekenhuiskosten worden zeker niet veroorzaakt door te hoge salarissen van leerling-verpleegkundigen; gezien hun grote verantwoording en hun zeer onregelmatige diensten worden ze zelfs onderbetaald.

J. Tramper 19 december 1979

Oxidation of Azaheterocycles by Free and
Immobilized Xanthine Oxidase and Xanthine Dehydrogenase

STELLINGEN

1

Naoi *et al.* presenteren onvoldoende experimentele gegevens om hun conclusies met betrekking tot de stabilisering door immobilisatie van D-aminozuuroxidase tegen de inwerking van proteasen, te rechtvaardigen.

M. Naoi *et al.*, FEBS Lett., *88*, 231-233 (1978).

2

Mukkerjee en Srere geven diffusie limitatie van het substraat als één van de mogelijke oorzaken voor de grotere K_m van geïmmobiliseerd citraatsynthase in vergelijking met die van het vrije enzym; de resultaten van hun experimenten sluiten deze mogelijkheid echter uit.

A. Mukkerjee en P.A. Srere, J.Solid Phase Biochem., *3*, 85-94 (1978).

3

De door van Velzen gevolgde methode om het systeem invertase geïmmobiliseerd in "crosslinked" gelatine te sterilizeren met behulp van een 2% glutaardialdehyde oplossing, is aan bedenkingen onderhevig.

A.G. van Velzen in Industrial Enzymes, Recent Advances (J.C. Johnson, ed.) 1977, NDC, Oak Ridge (NJ) p. 45.

4

De verhouding tussen 1-alkyl-6-oxo- en 1-alkyl-4-oxo-nicotineamide, gevormd bij de oxidatie van 1-alkylnicotineamide chloride met behulp van aldehyde-oxidase, is zeer waarschijnlijk afhankelijk van de beginconcentratie van het substraat.

R.L. Felsted *et al.*, J.Biol.Chem., *248*, 2580-2587 (1973).

5

De mogelijkheid dat de ringopening in de aminering van phenyl-1,3,5-triazine in vloeibare ammoniak met kaliumamide via een ongeladen open-keten intermediair verloopt, wordt door de experimenten niet uitgesloten.

Gy. Simig en H.C. van der Plas, Recl.Trav.Chim (Pays-Bas) *95*, 125-126 (1976).