Preferable methods for immobilized biocatalysts in enzyme electrode construction

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Introduction

The essential point of all immobilization procedures is the sensitivity of the enzyme used. By respective folding of the polypeptide chains an optimal spatial arrangement with high specificity in the active centre of the enzyme can be achieved. This structure can be stabilized by several bond types:

a) hydrogen bonds
b) van der Waals' – forces
c) hydrophobic interactions
d) ionic bonds of residue of some amino acids
e) covalent disulfide bonds with some SH – proteins

Even if only one of these is affected during the coupling the three-dimensional structure may be more or less influenced, which may alternate the activity distinctly.

For most of the immobilization procedures only those functional groups of the enzyme are taken into account which can enter ionic bonds according to the pk_a – value (1).

ε – amino groups of lysine, pk_a= 10,53 and α – amino groups of N – terminal amino acids, pk_a= 9,0 – 9,9;
β - and δ – carboxylic groups of aspartic and glutamic acid, pk_a= 3,86; 4,07 and α – carboxylic groups of C – terminal amino acids, pk_a= 1,8 – 2,4;
phenol groups of tyrosine, pk_a= 10,07;
mercapto groups of cysteine, pk_a= 8,27;
imidazole groups of histidine, pk_a= 6,10;
guanidine groups of arginine, pk_a= 12,48;
hydroxyl groups of serine and threonine;
indole groups of tryptophan.

For the common covalent binding of enzymes the first four groups are most interesting for practical purposes.

The pk_a – values of several ionizable groups within an enzyme may deviate from those of the corresponding free amino acids due to neighbor effect in the peptide chain. The pH – value at which the total charge of the enzyme equals zero is called "isoelectric point". At lower pH – values additional protonation leads to systems with an excess of positive charge dominate. Thus the reaction medium determines the protein giving rise to attraction or repulsion in the presence of an ionic carrier. As it will be later discussed absorption phenomena are also influenced.

The adjustment of charge by use of the pH – value of the reaction mixture may allow performing a reaction more selectively. Normally the pk_a – value of α – amino groups of N – terminal amino acids is lower than of ε – amino groups of lysine, i.e. at smaller pH – values α – amino groups will be less charged than ε – amino groups. If in such cases the reactive groups of the carrier react preferentially with neutral amino groups, these should be α – amino groups. The selectivity of the enzyme coupling should ensure that no functional groups of the active centre are involved nor those domains which are essential for the biological activity.
If necessary it can be tried to modify the enzyme with respect to the reactive group carrier. Polytrosyl chains could be incorporated into the enzyme in the case of the coupling of trypsin to diazonium carrier (2) or enzymes can be thiolated using N – acetyl homocysteine thiolactone (3) for the couling to thiol – containg carrier.

For a better characterization of the coupling properties of a carrier investigation should be done under standardized or at least comparable conditions. This should be done independently of the selected aims for this special carrier in order to obtain an efficient assignment of the coupling variables. An enzyme should be chosen which is well characterized with respect to its composition, molecular weight, three – dimensional structure, spatial distribution of the relevant functional groups and its kinetically parameters. The use of products with high purity should guarantee these specifications.

Immobilization methods

The immobilization methods can be either of physical or chemical nature or of a mixed type:

a) cross linking of enzyme with bi-functional reagents;
b) adsorption of enzymes on polymeric or porous solids (a subsequent cross linking with bi-functional reagents is possible);
c) entrapment of enzyme in polymeric gel lattices (an additional cross linking with bi-functional reagents is possible);
d) entrapment of enzymes in microcapsules (micro-encapsulation) or membrane reactors (flat membranes or hollow fibers);
e) covalent binding of enzymes to polymeric or porous carriers (grain, films, tubes, fibers, microgels, soluble polymers) via reactive functional groups or with the aid of activation reagents which from active intermediates.(4-8)

As a special case the so – called "protein copolymerization" is mentioned, in which enzymes are covalently bound to reactive vinyl monomers and subsequently copolymerized with other vinyl monomers (9).

In the following we focus our attention on the immobilization by covalent binding. With this method – taking into account certain criteria – in the most cases a good stabilization of the enzymes can be achieved. The comprehensive variability of this coupling procedure enables the researcher to perform further optimization. With respect to application also the other methods have their respective favorable field of use.

a) The cross linking of enzymes only with bi-functional reagents is of less economical importance but there is still some use in connection with the adsorption respectively entrapment method. Some characteristic bi-functional reagents are summarized in table 1 (10).

b) The adsorption of enzymes on solid surfaces (7, 12) as e.g. charcoal, alumina, silica, kaolinite, collagen, cellulose, collodion or synthetic ion exchangers is an easy but rather unspecific method. The binding forces between support and enzyme are in most cases relatively weak. Interaction with the substrate or charges of ph and ionic strength can lead to desorption. On the other hand this variation may be used to regenerate the biocatalyst by recharging.

A cross linking between the enzyme molecules themselves or between enzyme and carrier can improve the fixation (7).

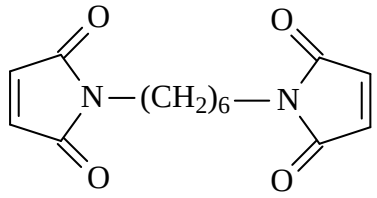
c) The entrapment of enzymes in polymeric lattices (7) as polyacrylamide, silastic gel, collagen, poly(vinyl alcohol) by copolymerization of appropriate comonomers or by cross linking of soluble polymers in the presence of enzymes leads to better fixed biocatalysts. In many cases a leakage of enzyme caused by the broad distribution of pore sizes in the gels (too large pores) cannot be prevent end. If smaller pores are achieved the particle interior is only accessible to substrates of relative low molecular weight.

By the entrapment method the kinetically properties of the enzyme itself are not affected except for a possible deactivation during the polymerization by occurring radicals.

d) By the entrapment of enzymes in microcapsules (13), which consist of thin, spherical, semi-permeable nylon – or cellulose nitrate – membranes by appropriate techniques a good free

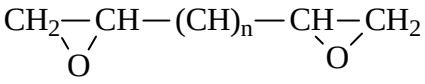
Table 1

Some bifunctional reagents	Preferred coupling groups of the protein
Glutardialdehide	
$OHC – (CH_2)_3 – CHO$	NH_2
Hexamethylene diisocyanate	
$OCN – (CH_2)_6 – NCO$	NH_2
N, N’ – hexamethylene bismaleimide	



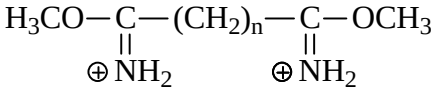
SH

Bisoxirane



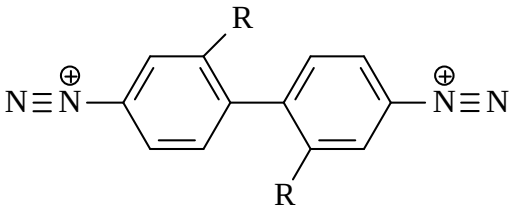
NH_2, OH

Bisimidate

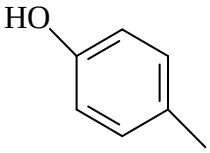


NH_2

Bisdiazobenzidine -2,2' – disulfonic acid



(R = $SO_3^{\ominus}$)

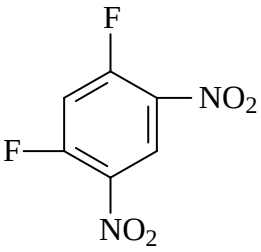


Divinylsulfone

$H_2C = CH – SO_2 –CH = CH_2$

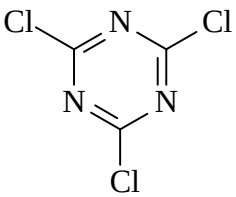
NH_2

1,5 – difluoro – 2,4 – dinitrobenzene



NH_2,

2,4,6 – trichloro – s - triazine



NH_2, OH

Further bi-functional reagents can be found in the literature (7, 10, 11) mobility, i. e. full enzymatic activity of the enzyme, is retained. The uniform pore size in the membranes prevents a leakage of enzyme or other toxic substances and thus allows potential medical applications (e. g. treatment of inborn enzyme defects).

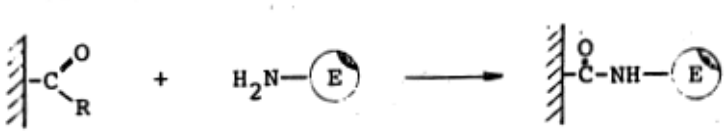
In the case of self – digesting enzymes, e. g. some hydrolases, there is no stabilizing effect by this immobilization procedure except if an additional cross linking is done.

e) In the future probably the most promising applications for the immobilization of enzymes will be performed by covalent coupling to polymeric and / or porous matrices (4, 5, 7, 8, and 14). The large variability of the polymeric carriers and their reactive respectively functional groups and the design of special coupling procedures should allow finding a tailored process for the field of use.

In the following the main coupling methods shall be presented (in the order the modification step at the enzyme). For better clarity we specify only the reactive respectively functional groups, as the influence of the carrier matrix. So, we remember only that the functional groups may be parts of different polymeric matrices.

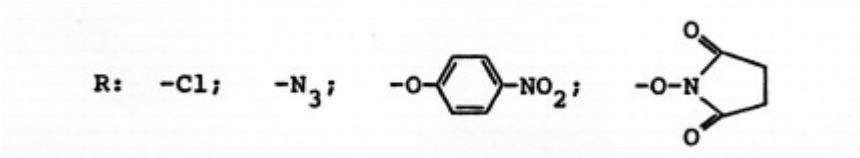
Acylating reactions

The reaction of reactive acid derivative with enzymes leads preferentially to acid amides.



To a smaller extent also thiol and hydroxyl groups of the protein can react.

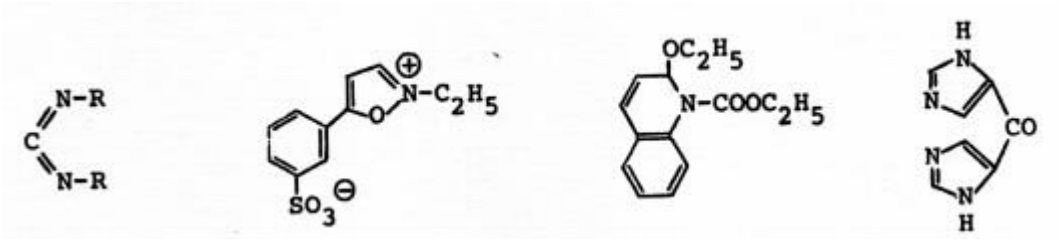
Acylation reactions with acid chlorides (5, 15), azides (16 – 18) at pH 8 – 9.



Higher pH – values might be favorable for the nucleophilic attack of the enzyme groups. Nevertheless they often are avoided due to the enhanced possibility of irreversible denaturation of the enzyme and fast hydrolysis of the reactive groups at higher pH – values. The use of nitrphenil esters may be attractive, as the liberated nitrophenolate allows an optical control (at 405 nm) of the coupling reaction.

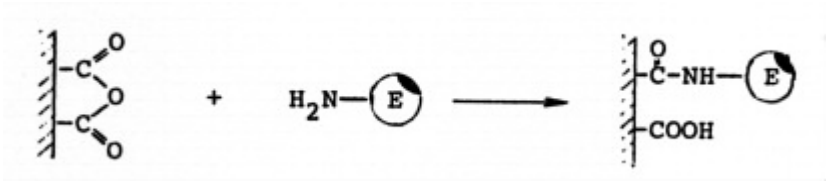
N – hydroxysuccinimide ester derivatives react even at ph 5 – 6 (5), thus allowing a selective coupling with α – amino groups of N – terminal amino acids.

The polymeric carboxylic carriers can be coupled directly with enzymes in the presence of activation reagents like water soluble carbodiimide (5, 20, 21, see also 4.1.2). Woodward's reagent K (22, 23), N – ethoxycarbonyl – ethoxy – 1,2 – dihidroquinoline (24), or carbonyldiimidazole (25).

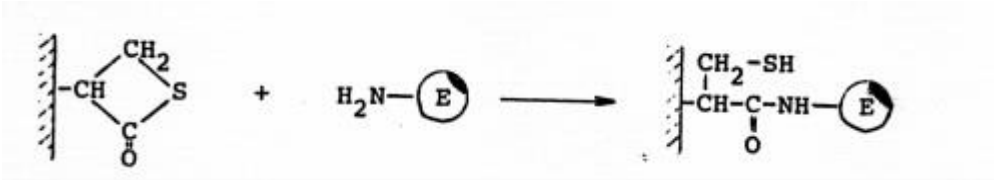


The slightly acidic coupling conditions (e.g. ph 4,75 for the carbodiimide coupling) should be favorable for enzymes, which tent to denaturate at higher ph – values (e.g. trypsin). If the activation reagent is given to the carrier / enzyme – mixture the side reaction of enzyme / enzyme – binding cannot be excluded.

Polymeric acid anhydrides (18, 26, 27) give always rise to a polyionic matrix during the coupling reaction (formation of carboxylic groups).



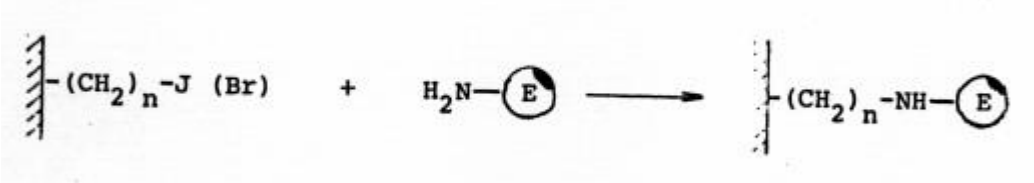
Acylation products also should be formed from polythiolactones and enzymes at pH 6 (3, 14).



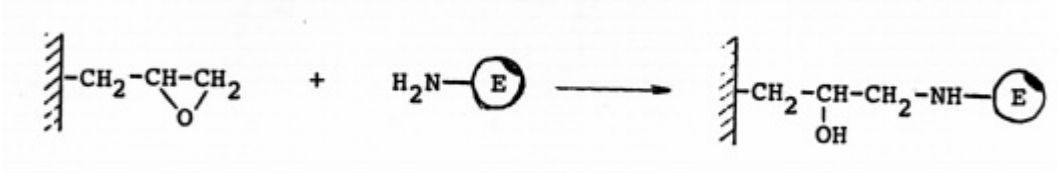
Also with the later discussed "Ugi – reaction" acylation products can be obtained.

Alkylation reaction

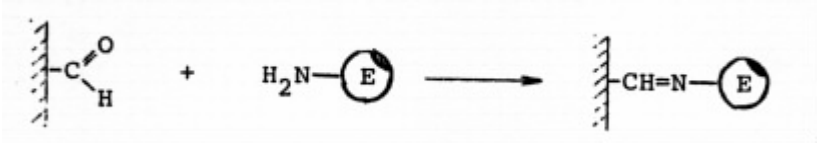
Stable alkylation products are obtained by the reaction between enzymes and side chains of the alkyl halogenides (28 – 30) of different chain length at pH 8 – 8,5. Also thiol groups of proteins can react.



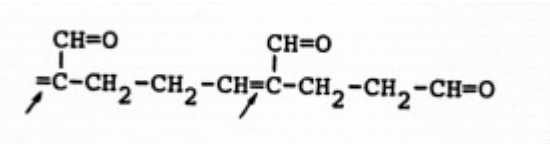
Carrier with oxirane groups alkylate enzymes at pH 7 – 8 (31 – 33,



It the last time a photo – coupling method was developed leading to an alkylating attack of the hydrocarbon part of glycine in the enzyme (33).
Another alkylation reaction is the azomethine formation with polymeric aldehydes at pH 5 – 7 (5, 34).

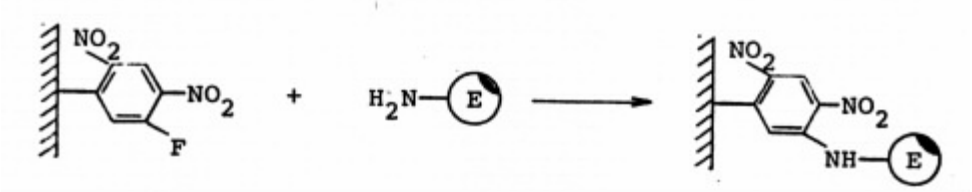


It is a disadvantage of this reaction, that the azomethine bridge is rather unstable and might be cleaved reversibly. This may be inhibited by reduction with sodium borohydride. Precautions should be taken if other reducible groups are within the enzyme (e. g. disulfide bridges). It might be of interest, that the coupling of carrier and enzyme by the biofunctional reagent glutardialdehyde (especially in the alkaline), leads to stable bonds (35, 36). From model experiments it could be concluded, that these are other bonding that azomethine bonds. Glutardialdehyde may give aldol condensation products. Subsequently the enzyme might react with the double bonds of these oligomers (37).

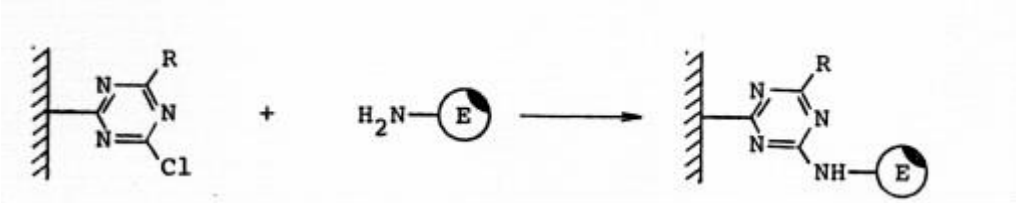


Arylation reactions

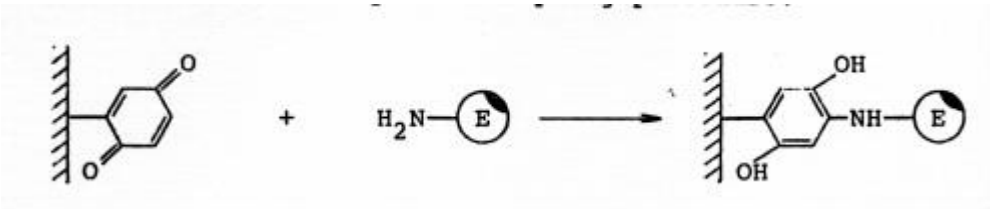
Here we will mention there examples. Two of them are reactions with activated halogen compounds, which reacted with enzymes at pH 8 -9. The first method used activated fluoro compounds (38 – 41).



The second method used a polymeric chloro – s – triazine derivative (42 – 44).

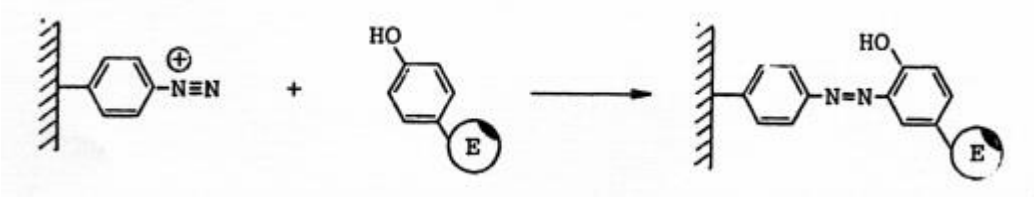


With the third method a reactive benzoquinone derivative leads within a broad ph – range from 3 to 10 to a hydroquinone coupling product (32, 45). Also thiol groups and phenolic groups should be involved by coupling procedure.



Azo coupling

A frequently used immobilization method can be performed with polymeric diazonium salts, which seldom are isolated prior to use (41, 46 – 49). Commonly a polymeric amine is diazotized, washed in the cold to a neutral reaction and coupled at pH 8.

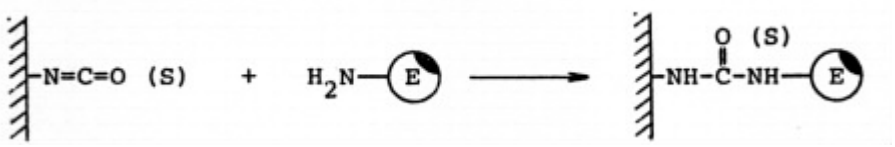


By the reaction with ε – amino groups of lysine at lower ph – values also diazoamino derivatives (- N = N – NH -) might occur, but they are labile to acids.

Of special interest are the findings, that the azo – coupling of trypsin to a neutral carrier gave inactive conjugates, whereas ionic carriers gave highly active enzyme conjugates, i. e. there must be certain selectivity (7).

Carbamylation and thiocarbamylation reactions

Many polymeric isocyanates (50) and isothiocyanates (41, 48, 51 -54) being of aromatic or aliphatic nature can be reacted with enzymes at ph 9 or lees. Stable products with urea respectively thiourea structure are obtained.

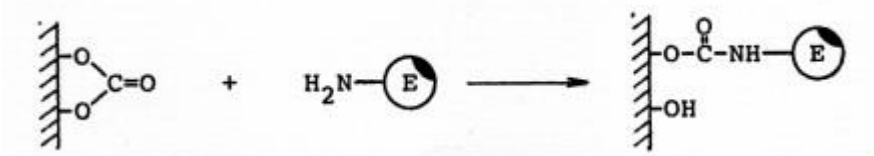


Model experiments have shown that less stable bondings are also formed with thiol, hydroxyl and carboxyl groups. These bonds are labile to alkali.

Due to the high sensitivity of the isocyanato groups for hydrolysis the isothiocyanato derivatives proved more useful.

Coupling with cyclic carbonates

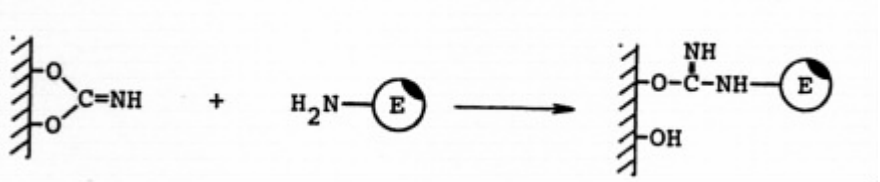
The coupling of cellulose – trans – 2,3 – cyclic carbonates with the amino groups of the enzymes gives rise to polymeric urethanes (55).



The same reaction can be performed with synthetic poly (allylcarbonates) (56).

Cyananogen bromide method

The BrCN – method is one of the most popular approaches for carrier on polysaccharide basis (32, 57). Although the reactions of the reactive intermediate – imidocarbonate – are ambiguous (see also chapter 2.1), due to its feasibility this method is extensively use for the immobilization and also for the affinity chromatography.

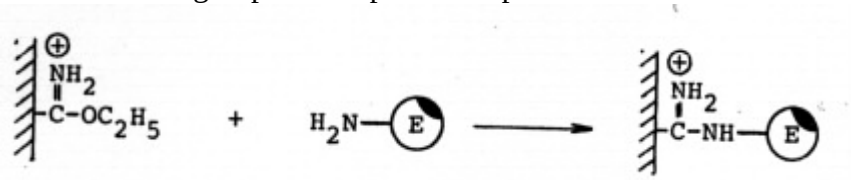


The main product should be a N – substituted isoureea. Model experiments with low mol. Weight amines demonstrated a high pk_a – value, which could be expected for a basic amidine structure.

The BrCN – activation can also be used for synthetic polymers with hydroxyl groups (19, 21, 58). In this case no ringformation is possible and the reactive intermediate is an cyanic acid ester instead of the cyclic imidocarbonate.

Amidination reactions

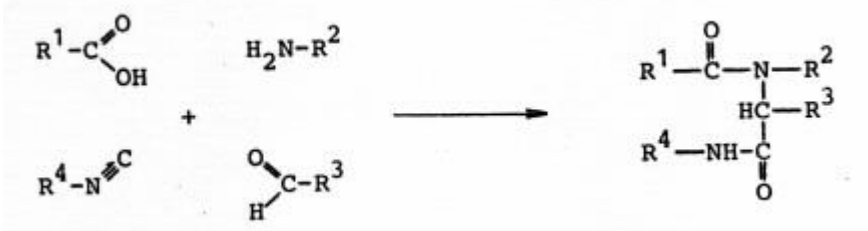
At weak alkaline conditions carriers containing imido ester groups react selectivity with α – amino or ω - amino groups of the protein to products amidine structure (59, 60).



As mentioned in chapter 2.1 the imido ester groups may be part of the side chain or of the polymer backbone. According to the literature (60) the positive charge at the basic amidine structure is favorable for the conservation of the enzymatic activity. Thereby the loss of charge due to the coupling via the basic lysine residue is compensated. This should help to maintain the structure. A disadvantage of this bond is the labiality against alkali.

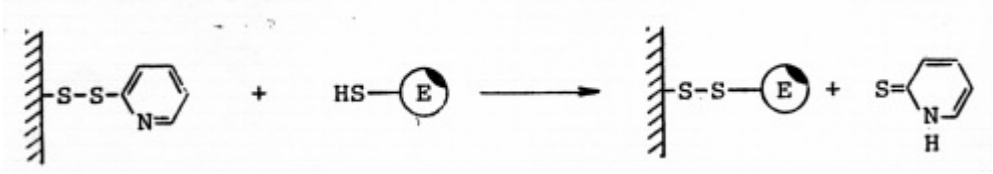
Four – component condensation ("Ugi reaction")

An enormous variability with respect to the polymeric carrier offers the "Ugi reaction". It is a four – component condensation, involved carboxylic, amino, isocyanic and aldehyde groups. The enzyme supplies the amino or carboxyl group. The carrier must contain one of missing groups (61, 62). The further two components are low – molecular compounds. The coupling can be performed at neutral pH – values.



Disulfide coupling

Enzymes with thiol groups which are not essential for their activity can be coupled to carriers with thiol groups by a thiol / disulfide interchange reaction. In most cases such carriers are activated with 2 2' – dipyridyl disulfide prior to the coupling reaction (63).



During the coupling 2 – thiopyridone is liberated, which can be measured photometrically. Under non – reducing conditions such immobilized enzymes are stable. The disulfide bond can be cleaved by thiol reagents thus splitting off the bound enzyme. The interchange effect is used for the so – called "covalent chromatograpy" (64).

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