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Study of Vinyl Sulfonyl Reactive Dye Intermediates in the Coimmobilization of Enzymes and Coenzymes

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ABSTRACT

p-*Aminobenzenesulfonylethyl-CL agarose was activated by diazotization, and with condensation with* CS_2 *and glutaraldehyde to give the diazonium salt, isothiocyanate or aldehyde respectively. These were then used for the immobilization and coimmobilization of NAD, alcohol dehydrogenase and lactate dehydrogenase. The NAD immobilization ratio and activity recovery of immobilized enzymes were determined. The NAD regeneration ability of the site-to-site coimmobilized enzyme and coenzyme system was investigated.*

1 INTRODUCTION

Some intermediates useful in the synthesis of reactive dyes have been used as bifunctional agents for covalent coupling both with carrier matrix and enzyme. The most widely used are cyanuric chloride[1] and vinyl sulfone sulfuric ester.[2] Usually, one active group of these reagents reacts with the hydroxyl or amino group of the carrier matrix, and the other is used for reaction with the —NH_2, —COOH, —SH, phenolic or imidazole groups of the amino acid residue of the protein. In this present paper 4-(β-sulfatoethylsulfonyl)aniline (SESA) was initially fixed by cross-linking to agarose, giving aminobenzenesulfonyl-CL agarose (ABSE-agarose), and the phenylamino group of the matrix intermediate was then activated by converting it into the diazonium salt (**1**). In a similar way, the phenylamino

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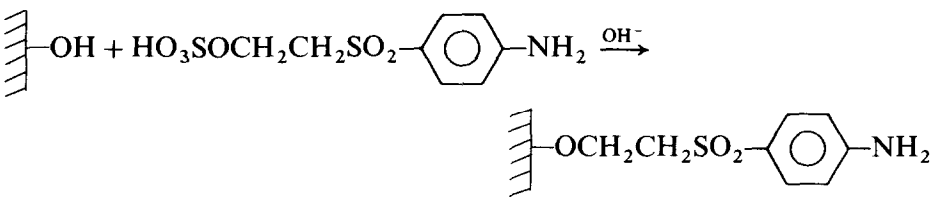
compound was reated with CS_2 in presence of DCC to form the isothiocyanate (**2**), or was condensed with glutaraldehyde to form compound **3**. Compounds **1**, **2** and **3** were then used to couple with alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH) and NAD. ADH, LDH and NAD were thus immobilized and coimmobilized site-to-site on these activated matrices.

2 EXPERIMENTAL

2.1 Activation of matrix

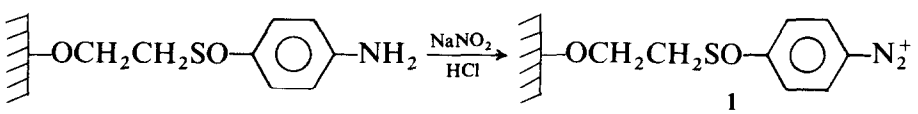
2.1.1

SESA (3 g) was dissolved in 100 ml water, and 10% Na_2CO_3 solution was added to give pH 7. After filtering, a clear solution of SESA was obtained. This solution was added to 50 g agarose (wet) and the mixture was stirred and heated to 80°C; 2 g Na_2CO_3 was then added. After reacting for 1 h, the liquor was filtered and the product washed with water to give ABSE-agarose.



2.12

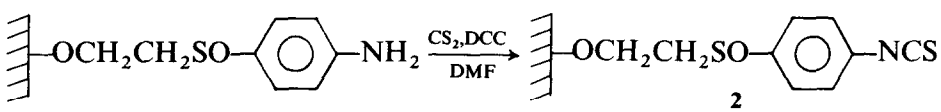
To a stirred mixture of 10 g ABSE-agarose in 20 ml water and 2 ml 1 M-HCl at 0–5°C was added 1 ml 5% $NaNO_2$. After 30 min, the activated agarose diazonium salt was collected, washed with cold water and then used for coupling with the enzyme.



1

2.1.3

10 g ABSE-agarose (ethanol-washed, water-free) was added to 20 ml *N,N'*-dimethylformamide (DMF) and 0·1 g dicyclohexylcarbodi-imide (DCC). Carbon disulphide (1 ml) was dropped into the stirred mixture and after 4 h the activated agarose isothiocyanate was collected, washed by ethanol and water and then coupled with the enzyme.



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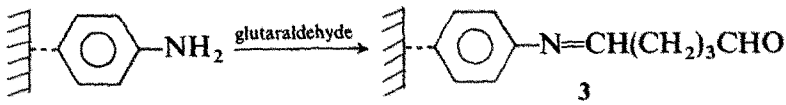
2.1.4

10 g ABSE-agarose was added to 20 ml water and 3 ml 25% glutarealdehyde and the mixture was stirred for 30 min. The product was washed with water, and then coupled with enzyme.



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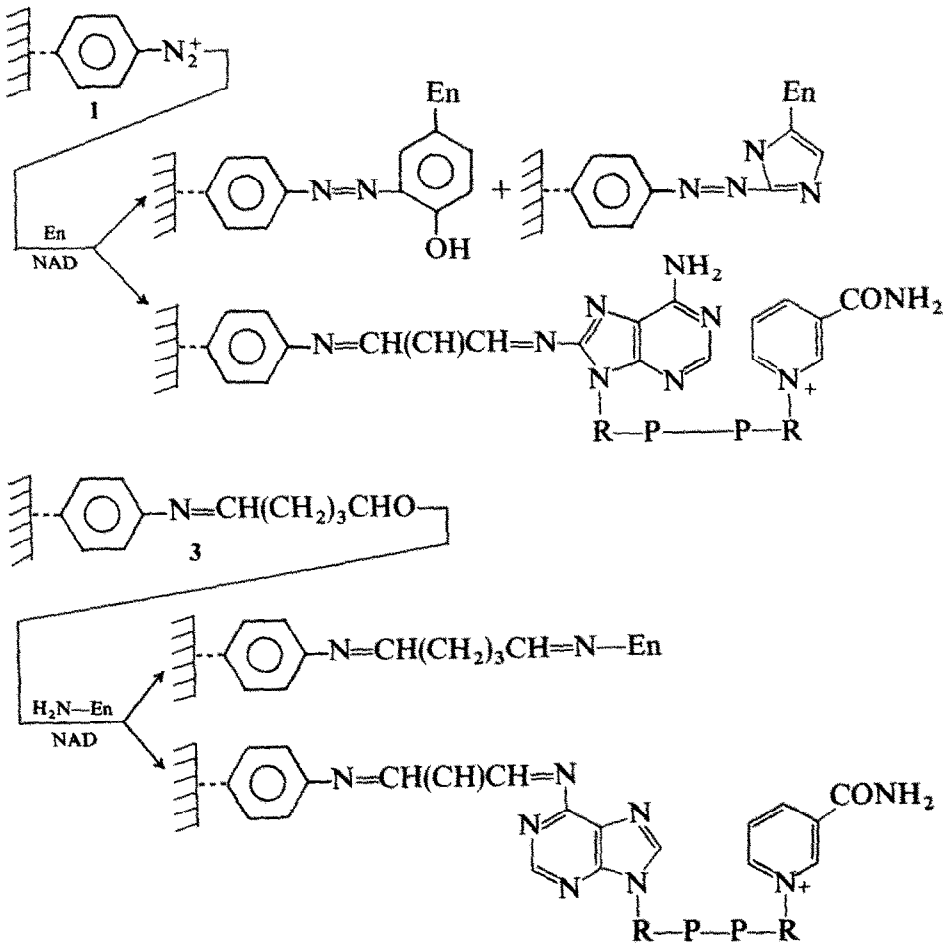
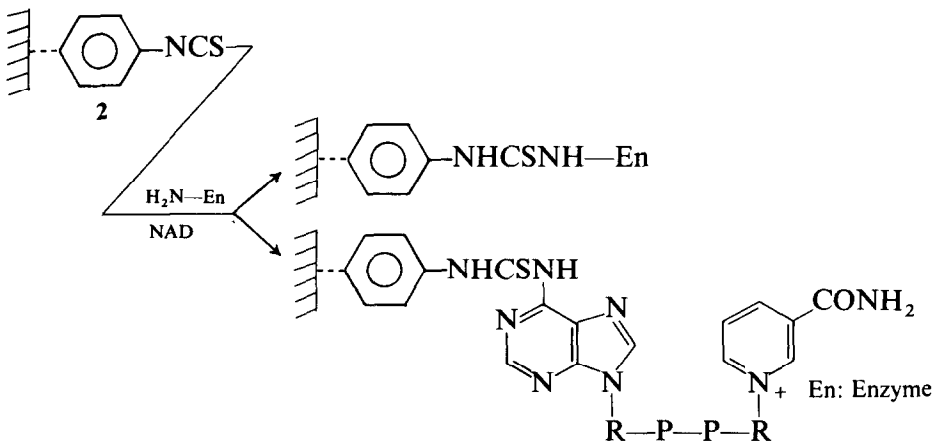
2.2 Immobilization of ADH, LDH and NAD

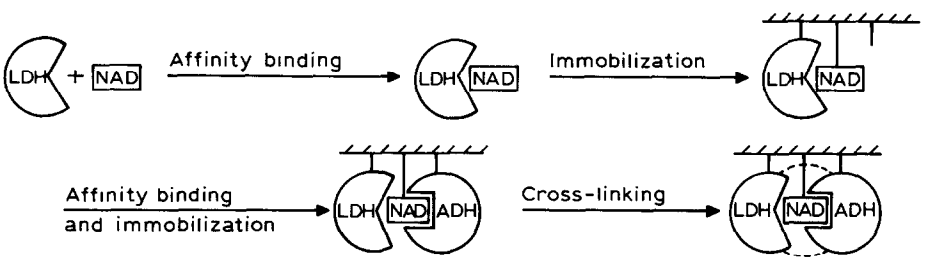
To 1 g of each of the three activated agarose derivatives was added 2 ml ADH, LDH or NAD solution ($\frac{1}{15}$M-phosphate buffer, pH 5, 5·5, 6, 6·5, 7, 7·5, 8), and the mixture was allowed to react overnight at 4°C. The resultant immobilized ADH, LDH and NAD were washed with 0·5M-NaCl and water and then assayed for enzyme activity.





2.3 Site-to-site coimmobilization of ADH, LDH and NAD

To 5 ml of a cold phosphate buffer (0·05M, pH 6·0) were added NAD and LDH, and the mixture was equilibrated for 30 min at 4°C, 4 g each of the appropriate activated agarose derivatives were added and the reaction continued for 4 h. After washing with 0·5M-NaCl and water (5 ml), cold phosphate buffer (0·05M, pH6·0) and ADH were added. After a further 2 h and washing with cold water, 5 ml of cold PB and 5 ml of 25% glutaraldehyde were added and cross-linking continued for 2 h. The products were finally washed with 0·5M NaCl and water. The coimmobilized LDH–NAD–ADH multienzymes thus prepared were evaluated with respect to their ability to NAD.



Scheme 1

2.4 Assay

The enzyme activity of ADH, LDH–NAD was determined by known methods[3] and the NAD regeneration ability of the coimmobilized multienzymes was determined by Mansson's method.[4]

3 RESULTS AND DISCUSSION

3.1 Immobilization of ADH, LDH and NAD

The recovery of activity after immobilizing ADH with many covalent coupling proceedings, such as with BrCN and glutaraldehyde, is poor. In this present investigation the recovery using diazonium salt and glutaraldehyde coupling was also very low (well below 1%) but when using the agarose isothiocyanate, the recovery was raised to 7% (see Table 1). For immobilization of LDH, the isothiocyanate matrix was found to be the best and glutaraldehyde the worst (Table 2). From Tables 1 and 2 it can be seen that pH plays an important role in the immobilization of the enzymes. This is

TABLE 1
Activity Recovery of Immobilized ADH (%)

pH of phosphate buffer / *Activated agarose*	*5·0*	*5·5*	*6·0*	*6·5*	*7·0*	*7·5*	*8·0*
Diazonium salt	0	0	0	0	0	0	0
Isothiocyanate	5·2	—	5·1	5·7	7·0	6·5	5·4
Aldehyde	—	0·1	0·1	0·1	0·2	0·1	0·1

—, undetected.

TABLE 2
Activity Recovery of Immobilized LDH (%)

pH of phosphate buffer / *Activated agarose*	*5·0*	*5·5*	*6·0*	*6·5*	*7·0*	*7·5*	*8·0*
Diazonium salt	—	4·7	5·2	5·7	6·5	5·4	4·9
Isothiocyanate	4·4	9·6	9·6	7·4	7·4	8·1	—
Aldehyde	—	2·9	3·6	1·8	0·8	0·6	0·5

—, undetected.

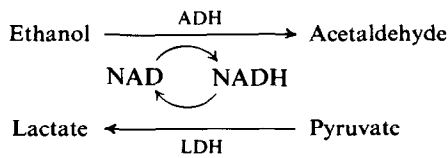
TABLE 3
NAD Immobilization Ratio (%)

pH of phosphate buffer / *Activated agarose*	*5·0*	*5·5*	*6·0*	*6·5*	*7·0*	*7·5*
Diazonium salt	18·2	21·1	13·7	14·1	14·1	14·1
Isothiocyanate	6·2	8·4	7·1	4·7	4·7	4·7
Aldehyde	10	7·8	8·0	8·0	8·0	7·8

because, in addition to the covalent coupling between the enzymes and the matrix being dependent on the pH of the reaction media, enzymes also have an optimum pH at which they show the highest activity, and at pH values displaced from this optimum, they may denature to some extent.

Table 3 shows the agarose diazonium salt is better than the other two activated agarose derivatives, and that in the weakly acidic condition the NAD immobilization ratio is higher.

3.2 Coimmobilization of ADH, LDH and NAD



Scheme 2. Mechanism of NAD regeneration.

Table 4 shows that the NAD regeneration ability of the three coimmobilized systems is higher than that of the free system. This may be due to the NAD, ADH and LDH being more closely bonded in the site-to-site coimmobilized system than in the free system. Thus, the immobilized dehydrogenases can catalyse the NAD regeneration more rapidly than the free enzymes, which must diffuse through transformation and fit site-to-site once again. Furthermore, the spacer length of the activated agaroses also affects the NAD regeneration. The longer the spacer links, the greater the freedom of the coimmobilized multienzymes, and hence the NAD regeneration rate is higher. The low NAD regeneration of the diazo salt coupling system is due to the very poor activity recovery of ADH immobilization.

TABLE 4
Comparison of NAD Regeneration Ability between Coimmobilized Enzymes and Free Enzymes System

	Free system	*Coimmobilized system*		
		Diazonium	*Isothiocyanate*	*Aldehyde*
NAD regeneration rate (cycles h^{-1})	5·7	15	23	29

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