Amino acid	I'	I"	II
Asp	10.3	10.0	10.9
Tre	5.0	8.5	4.7
Ser	8.7	6.1	8.7
Glu	18.6	14.8	25.8
Pro	6.3	Tr	4.9
Gly	8.5	14.5	8.2
Ala	8.5	9.4	6.3
Val	8.5	10.2	7.5
Met	0.6	Tr	Tr
Ile	5.5	5.1	3.6
Leu	6.3	7.3	4.4
Tyr	1.0	1.5	2.0
Phe	4.0	3.6	4.9
His	2.1	2.2	2.6
Lys	3.3	6.5	2.5
Arg	11.9	4.0	9.4
Carbohydrates	–	–	3%
Trp (number of moles per mole of protein)	+	4	1

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THE PREPARATION OF BIOSPECIFIC SORBENTS BY THE ACTIVATION OF SEPHAROSE WITH p-BENZOQUINONE

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The method usually used for obtaining biospecific sorbents is the activation of Sepharose by cyanogen bromide followed by the addition of a ligand at the amino group [1], this method being associated with the use of an extremely toxic reagent. Recently, Porath et al. [2] have proposed a method of immobilizing enzymes which is based on the activation of Sepharose with p-benzoquinones and the addition of the protein to the activated Sepharose. It appeared important to study of the possibility of using this method for adding amines and obtaining biospecific sorbents.

The activation of Sepharose was performed in accordance with Porath's procedure: 10 g of washed and settled Sepharose 4B in 6 ml of 0.1 M $NaHCO_3$ (pH 8) was mixed with a solution of 0.108 g (1 mmole) of p-benzoquinone in 4 ml of ethanol. The suspension was stirred at room temperature for 1 h, and the activated Sepharose was washed with 20% ethanol, water, 1 M NaCl, and water again. The ligands used were N-2,4-

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dinitrophenyl(DNP)hexamethylenediamine hydrochloride, the hydrochloride of the methyl ester of ε-aminocaproyl-L-phenylalanyl-D-phenylalanine, the antibiotic gramicidin C, and a number of model peptides (see below).

The p-benzoquinone-activated Sepharose was carefully stirred with an equal volume of a solution of the ligand in 0.1 M $NaHCO_3$, pH 10, or in 50% dimethylformamide in 0.1 M $NaHCO_3$, pH 10, at room temperature for 24 h. The final concentration of peptide in the solution was 0.5 M. Mono-DNP-hexamethylenediamine was dissolved in a 3.5-fold volume of 50% dimethylformamide in 0.1 M $NaHCO_3$, pH 10. The final concentration of the amine was 0.3 M. Dark colored sorbents were obtained, the color remaining after they had been washed free from excess of the ligand.

For the peptide sorbent the amount of ligand in 1 ml of moist sorbent was calculated from the results of an amino-acid analysis of hydrolyzates of the sorbents (5.7 M HCl, 107°C, 24 h). The concentration of the ligand in the Sepharose-DNP-hexamethylendiamine could not be determined, since a hydrolyzate of this sorbent under the action of trifluoroacetic acid [3] had no absorption maximum at 360 nm characteristic for the DNP group but had a maximum at 282 nm.

Below we give information on the addition of the ligands to the p-benzoquinone-activated Sepharose 4B ($Ahx = NH_2(CH_2)_5CO-$):

Ligand	*pH*	Amount of peptide added, μmole/ml
Ahx-Gly-Gly-OMe	10	0.47
	11	0.42
	12	0.32
H-Gly-Phe-OH	10	0.29
N-p-Tos-Ser-Tyr-OH	"	0
H-His-Leu-OH	"	0.24
H-Gly-Leu-OH	"	0.17
Ahx-L-Phe-D-Phe-OH	"	0.56

In the Sepharose derivatives obtained, the concentration of ligands was 0.2-0.5 μmole/ml, which is approximately an order of magnitude lower than for the activation of Sepharose with cyanogen bromide [4]. To check the method we repeated Porath's experiment on the immobilization of chymotrypsin and obtained a result agreeing with his; the concentration of protein in the sorbent was 0.07 μmole/ml. While in the case of immobilized enzymes such a concentration is quite sufficient, to use Sepharose modified with small organic molecules as an affinity sorbent a higher degree of substitution is desirable.

To evaluate the reactivity of the various functional groups in the protein, we treated a series of model peptides (see above) with the p-benzoquinone-activated Sepharose. The results obtained show that the amino group is the most reactive. The hydroxy groups of serine and tyrosine do not take part in the reaction at all, and histidine occupying the N-terminal position is no more active than other amino acids. Gramicidin C does not react with p-benzoquinone-activated Sepharose.

The Sepharose 4B-DNP-hexamethylenediamine synthesized with the aid of the p-benzoquinone activation of the sorbent was used for the chromatography of porcine pepsin in 0.05 M glycine HCl buffer, pH 2.1 (the isoelectric point of the pepsin). When 1 mg of the protein was deposited on 1 ml of sorbent, 80% of the enzyme was eluted on washing with the initial buffer, and only 18% was sorbed and was then eluted by 20% of isopropanol in 1 M NaCl. The specific activities of the two fractions were identical with the initial activity. The sorbent Sepharose 4B-ε-aminocaproyl-L-phenylalanyl-D-phenylalanine, obtained similarly, contains a peptide forming an analog of the substrate for pepsin. It is known that the enzyme is strongly sorbed on columns with the same ligand obtained by the activation of Sepharose with cyanogen bromide [4]. On chromatography in 0.05 M glycine-HCl buffer, pH 2.1, with a load corresponding to 1 mg of protein per ml of sorbent, 52% of the pepsin in terms of protein and 47% in terms of activity was eluted when the column was washed with the initial buffer, and 23% in terms activity and 34% in terms of protein could be desorbed only by 20% isopropanol in 1 M NaCl.

Thus, the activation of Sepharose with p-benzoquinone does not permit the preparation of sorbents with a sufficiently high capacity for affinity chromatography. However, it is not excluded that the method may be used for ligands having a very high affinity for enzymes.

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