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Note

Immobilization of ligands for affinity chromatography

Coupling on a spacer arm gel with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as condensation agent: study of coupling conditions by means of a radioimmunologic method

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When ligands have a low molecular weight, the interposition of a spacer arm is specially important in affinity chromatography in respect of the steric enhancement[1,2]. Several gels with six-carbon spacer arms and terminal carboxyl or amine groups are commercially available. Generally, ligands with a primary amine or carboxyl function are coupled on these supports by means of a condensation agent, such as water-soluble carbodiimides[3,4].

This report studies the coupling reaction with EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline), which has been employed in peptide synthesis[5]. EEDQ is particularly interesting because the coupling agent yields are comparable with those obtained with water-soluble carbodiimides[6] and there is no need to monitor the pH during the reaction. Also, the medium aqueous ethanol has an advantage because of the slightly water-soluble properties of some ligands such as testosterone hemisuccinate (TH). Moreover, EEDQ is available at low cost.

In many cases, ligands are expensive and/or unstable at certain temperatures, so we studied the reaction to determine the best operational conditions. Relative amounts of immobilized ligands were determined by a radioimmunological method.

EXPERIMENTAL

The chromatographic support AH Sepharose 4B was obtained from Pharmacia. EEDQ and TH were purchased from Sigma. The whole antibody anti-rabbit Ig iodinized from donkey (1.85 MBeq/μg) was obtained from Amersham. The whole antibody anti-testosterone from rabbit was generously supplied by the medical biophysic laboratory of the hospital of Tours.

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Immobilization of testosterone hemisuccinate on AH Sepharose 4B with EEDQ

In this experiment the coupling reaction was studied as a function of temperature, of concentration of ligand and of incubation time to determine the best immobilization conditions of TH on the support with respect to the economy and stability of the reagent. So 3.3 g of AH Sepharose 4B were swelled in 1 *M* sodium chloride, which gave *ca.* 12 ml of gel. This was washed on a sintered glass funnel with 1 *M* sodium chloride, followed by deionized water. The gel was then divided into six aliquots of 2 ml. Three aliquots were each mixed with 23.3 mg of TH in ethanol–water (75:25) and 15 mg of EEDQ in ethanol to a final volume of 4.8 ml; the three were stirred at 4°C, 15°C, and 25°C, respectively, for 10 h. A fourth aliquot was mixed with 11.7 mg of TH and 7.5 mg of EEDQ in aqueous ethanol to the same final volume and stirred at 15°C for 10 h. The fifth aliquot was treated similarly, except that the concentrations were 58.3 mg of TH and 37.5 mg of EEDQ. The last aliquot was kept as a reference without any ligand.

A 50-μl volume of each aliquot was tested by a radioimmunological method at different times: 0.5 h, 1 h, 2 h, 5 h, and 10 h.

Relative determination of the immobilized ligand

The sample (50 μl of coupled gel) was stirred for 1 h at room temperature with 200 μl of antibody antitestosterone 1/100 in bovine serum albumin (BSA) (50 g/l). After two washings with 5 ml of phosphate buffer, 0.025 *M*, pH 7.4, containing 0.5 *M* sodium chloride, 100 μl of anti-rabbit Ig (^{125}I) in BSA (50 g/l) were stirred with the sample for 1.5 h at room temperature. Then two washings were carried out with the same buffer, and centrifugation removed the excess of iodinizated anti-rabbit Ig. Pellets were counted on an LKB 1282 Compugamma.

This test was first evaluated with respect to specificity, proportionality and reproducibility (see Table I). The averages were calculated twice for each assay on five experiments for which similar results were obtained. The comparison between controls and assays indicates a good specificity. The values obtained for three volumes of gel show that the assay is proportional.

TABLE I

VALUES OBTAINED WITH THE RADIOIMMUNOLOGICAL METHOD

Incubation for 24 h under rotative stirring at 15°C and 15 μmol/ml gel of TH.

	Volume of gel (μl)	*Amount of immobilized TH (cpm)*	
		cpm	*cpm Ass. − cpm cont. 1*
Assays:	50	17,900	9900
ligand + anti T + anti Ig (^{125}I)	75	24,350	15,000
	100	31,200	19,900
Control 1:	50	8000	–
ligand + anti Ig (^{125}I)	75	9350	–
	100	11,300	–
Control 0:	50	8150	–
anti T + anti Ig (^{125}I)	75	9300	–
	100	11,100	–

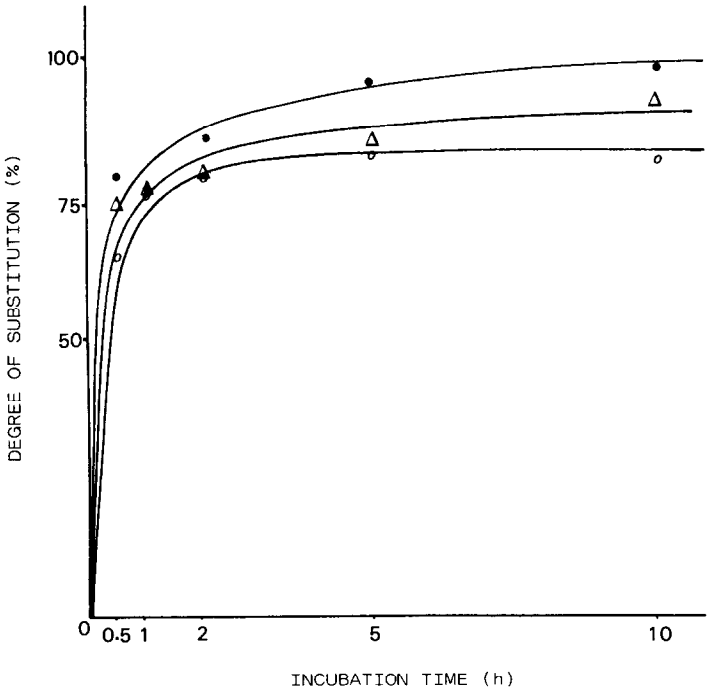

Fig. 1. Coupling of TH on AH Sepharose 4B by means of EEDQ as a function of incubation time and temperature (● = 25°C; △ = 15°C; ○ = 4°C).

RESULTS

The degree of substitution was estimated for five incubation times as function of temperature and concentration of ligand. It was expressed as a percentage by calculating the ratio

$$\frac{\text{immobilized ligand (cpm)}}{\text{immobilizable ligand (cpm)}}$$

where the amount of immobilizable ligand is calculated assuming a horizontal asymptote. The results are shown in Figs. 1, 2 and 3.

DISCUSSION

It appears that ligand concentrations of *ca.* 14 μmol/ml of gel (about twice that of the gel in amine groups) are sufficient to obtain acceptable degrees of substitution, compared with the usual concentration of 45 μmol/ml of gel[6]. Therefore, the cost of experiments decreases, which is particularly advantageous when expensive ligands must be used. Then we can consider that 2 h of incubation are sufficient for coupling most of the ligands (90%) that can be immobilized. If the temperature increased, the mass transfer coefficient increase as the concentration of coupled li-

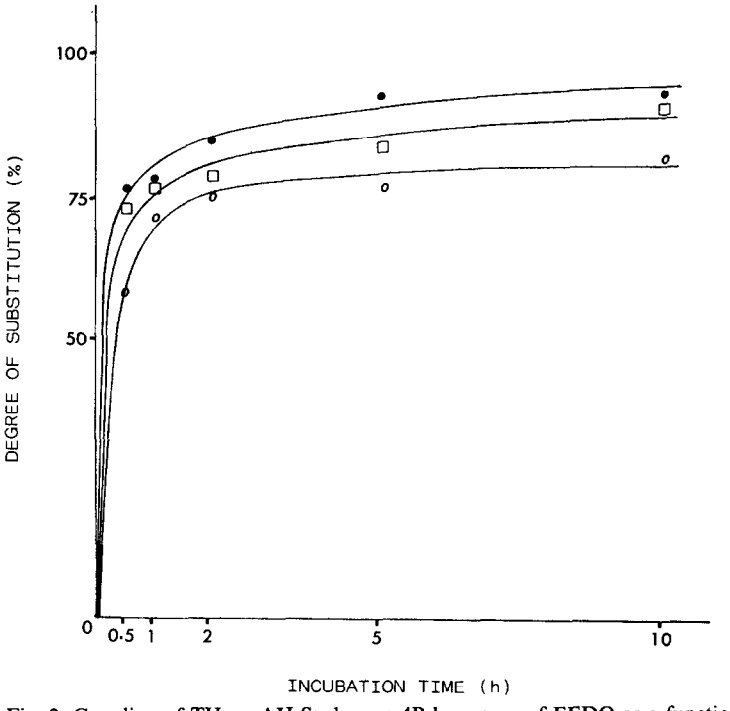

Fig. 2. Coupling of TH on AH Sepharose 4B by means of EEDQ as a function of incubation time and ligand concentration at 15°C (● = 31.3 μmol/ml gel; □ = 12.5 μmol/ml gel; ○ = 6.3 μmol/ml gel).

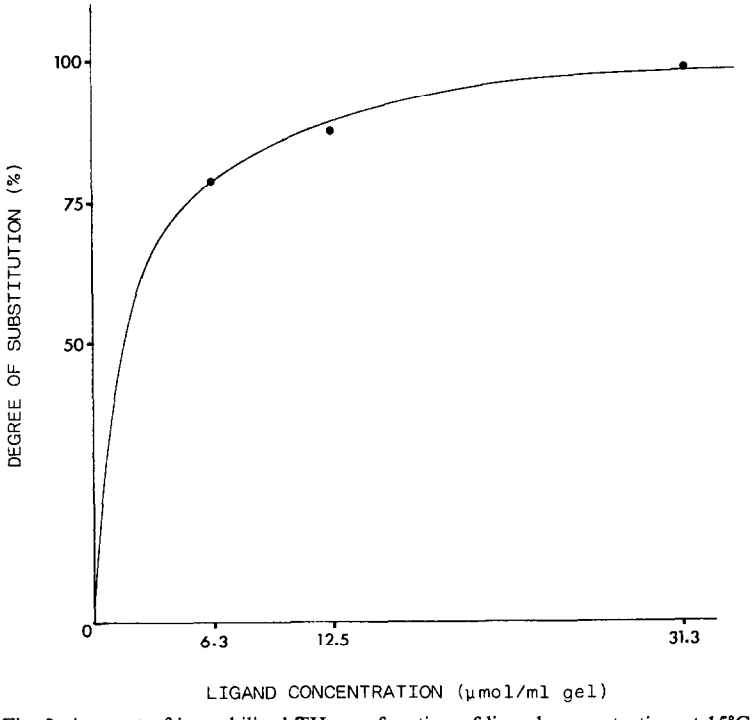

Fig. 3. Amount of immobilized TH as a function of ligand concentration at 15°C for 5 h of incubation.

gand. But it is better to achieve the reaction in less time and at a lower temperature by rotative stirring, which preserves the stability of ligands.

The radioimmunological method presented in this report constitutes a suitable alternative to conventional methods[7–10] for testing the coupling reaction: practicable, rapid, and with good specificity and sensitivity. It is usable for many systems when the primary antibody is available and allows quantitative determination in relation to a reference method[11].

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