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Interaction mechanisms between insulin and N-acetylneuraminic acid in affinity chromatography

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Abstract

Silica beads are coated with dextran carrying a calculated amount of positively charged diethylaminoethyl (DEAE) groups in order to neutralize negatively charged silanol groups at the silica surface and in this way to minimize non-specific interactions between silica and proteins in solution. Dextran-coated silica supports are potentially excellent stationary phases for high-performance liquid chromatography of proteins. These supports combine the advantages of polysaccharide phases with the excellent mechanical characteristics of silica. These supports [silica–dextran–DEAE (SID)] are easily functionalized by grafting N-acetylneuramic acid (NANA), extracted from edible birds' nests, using conventional coupling methods. The performance of supports bearing NANA was studied by high-performance liquid affinity chromatography of insulin, the hypoglycaemic peptide hormone of the human organism. The study showed that these supports exhibit a reversible and specific affinity towards insulin and allow separations with high purification yields. The influence of different physico-chemical parameters (pH, temperature and insulin concentration) on insulin retention on the support was studied. This allowed the optimization of the conditions of adsorption and a better understanding of the interaction mechanisms between insulin and NANA as a biospecific ligand.

1. Introduction

The adsorption of proteins at solid–liquid interfaces has been the subject of extensive theoretical [1] and experimental studies [2]. This phenomenon results from interactions between, on the one hand, the protein and the surface and, on the other, between the protein and the solvent. The importance of the latter interaction depends essentially on the chemical nature of the individual protein.

In the case of insulin and according to the nature of the solvent, structural modifications occur, which can modify its biological activity. Insulin is the hypoglycaemic peptide hormone of the human organism; it is constituted of 51 amino acids contained within two peptide chains: an A chain with 21 amino acids and a B chain with 30 amino acids [3]. The chains are connected by two disulfide bridges. Endogenous insulin circulates predominantly in the monomeric state in the bloodstream. Nevertheless, insulin has a strong tendency to dimerize in solution with a dimerization constant of $1.5 \cdot 10^4$ [4,5]. The dimer is the prevalent species in most systems; the main driving force for its formation is hydrophobic interactions. At the C-terminus of the B chain, a high population of non-polar amino acids is dominant; by forming an anti-parallel B-sheet with a neighbouring insulin

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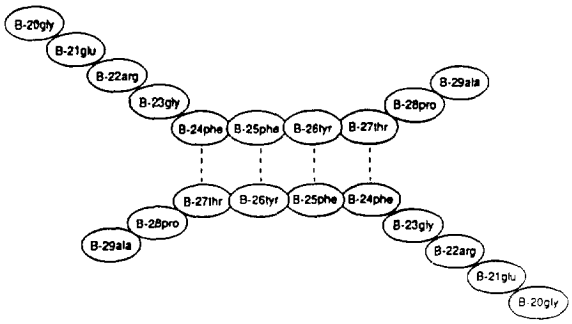

Fig. 1. Schematic representation of insulin dimer interactions [6].

monomer, the insulin monomers form a strongly bound dimer (Fig. 1). The dimerization phenomenon depends on numerous parameters [6]. The dimerization constant decreases sixfold over the pH range 2–11.2. From pH 11.2 to 12.7, this constant decreases another twenty-fold. In this latter range, electrostatic repulsion occurs, resulting from the B-22 arginine ionization, which is located in the centre for dimerization and bioactivity.

Insulin aggregation depends also strongly on its concentration. The aggregation phenomena appear more rapidly when the concentration exceeds 10 mg/ml in aqueous solutions. pH has an influence on the insulin solubility. Insulin is relatively insoluble in aqueous medium from pH 4 to 7 [6].

A rise in temperature triggers off insulin deamidation [7]. Deamidation of insulin is a natural phenomenon that occurs in insulin solutions. The deamidation does not affect the biological activity of insulin. Deamidation is very temperature dependent. Over the temperature range 4–25°C and a time frame of 2 years, it was shown that the deamidation only amount to a few percent in neutral solution at 4°C. The deamidation at 25°C was 10% after 3–4 months and about 25% after 1 year. At 37°C, in both neutral and acidic solutions, deamidation is rapid. However, in acidic medium covalent aggregates are formed, causing a significant loss in the biological activity of insulin.

Finally, the activity and the stability of insulin in solution depend on its concentration in addition to the pH and temperature. Hence, it appears important to study the influence of these parameters on insulin behaviour in affinity chromatography. Insulin deamidation will not be considered, as insulin solutions are prepared prior to being used.

The influence of different physico-chemical parameters on insulin–surface interactions can be observed by high-performance liquid affinity chromatography (HPLAC). HPLAC allows us to study adsorption and desorption phenomena under hydrodynamic conditions. These phenomena are correlated with several conjugated physico–chemical effects whose resultant determines the specificity and bioaffinity of the functional supports.

The presence of sugars similar to sialic acid surrounding the insulin receptor structure allows one to assume that sialic acid may probably lead to the formation of specific interactions which can be used in a procedure for separation by affinity chromatography. For this purpose, we synthesized chromatographic supports based on silica coated with dextran–DEAE and functionalized by N-acetylneuraminic acid. The interaction of these active phases and their biospecificity for insulin in solution were studied.

2. Experimental

2.1. Synthesis of silica–dextran–DEAE (SID)–NANA chromatographic support

The synthesis of coated silica supports was carried out as reported previously [8]. The preparation of the affinity support is performed in two steps. First, silica beads are coated with dextran substituted by a calculated amount of DEAE functions to hide negative charges at its surface. Second, ligands are immobilized using a coupling agent (Fig. 2).

Coating of silica beads with dextran substituted by DEAE

The substitution of dextran T70 (68 000 g/mol) (Pharmacia, Bois d'Arcy, France) by 2-

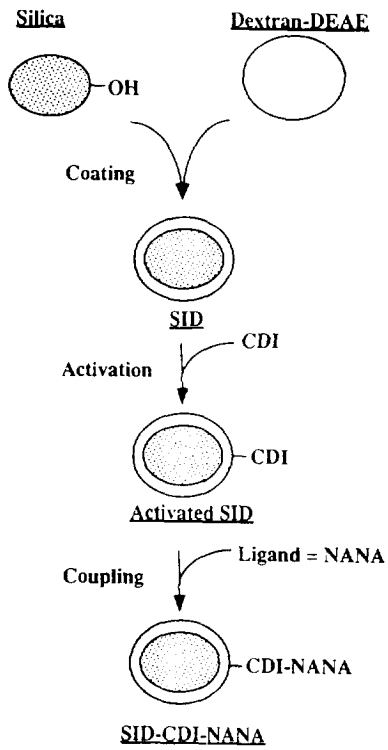

Fig. 2. Schematic representation of the synthesis of the chromatographic support.

chloro-N,N-diethyl-aminoethane (Janssen Chemica, Noisy le Grand, France) was performed in a very alkaline medium at 55°C for 30 min. The substitution rate of dextran with DEAE was determined by elemental analysis of nitrogen. The conditions for dextran modification were previously determined [9,10] to obtain a proportion of dextran units carrying DEAE groups (Dx–DEAE) varying from 4 to 13%.

Silica beads (particle size 15–25 μm, porosity 1000 Å), kindly provided by Biosepra (Villeneuve la Garenne, France), were impregnated with a modified dextran solution (8 g of DEAE–dextran in 100-ml) adjusted to pH 11. Dextran-coated silica was cross-linked with 1,4-butanediol diglycidyl ether (BDGE) (Sigma, La Verpillière, France). The amount of Dx–DEAE covering the silica beads was determined by a spectrophotometric assay of the sugar units after hydrolysis and by elemental analysis of carbon.

Coupling of NANA on SID

Extraction of NANA. N-Acetylneuraminic acid belongs to the sialic acid family and rarely exists in the free state; it is generally associated with glycoconjugates [11,12] and it is also present in insulin receptor subunits [13–15]. Hence, it is necessary to have sialic acid in a pure state for coupling to the chromatographic support. For this purpose, sialic acid was extracted from edible birds' nests, which consist of sialyl glycoproteins containing up to 9% by mass of NANA.

The extraction was carried out according to the protocol described by Le Gallic [16]. Acid hydrolysis was performed on 10 g of ground bird's nests by adding 300 ml of 0.2 *M* sulfuric acid (Carlo Erba, Reuil-Malmaison, France) and heating for 30 min at 100°C. The mixture was cooled and adjusted to pH 5 with concentrated $Ba(OH)_2$ (Fluka, Saint Quentin Fallavier, France) solution. The mixture was then filtered with a filter system composed of a 4-in. coarse sintered Buchner funnel, celite (4 g) (Prolabo, Bondoufle, France) and another filter (0.7 μm) (glass microfibre, Whatman) to remove non-solubilized bird's nest fragments and the precipitate of $BaSO_4$ formed during the neutralization step. The filtrate was separated on a cation-exchange column (Amberlite IR 120 H^+) (Fluka), which retained positively charged molecules, and finally on an anion-exchange column (Amberlite IR 400 formate), (Fluka), which was first converted from the Cl^- to the formate form by stirring overnight in 2.5 *M* formic acid solution (300 ml of solution for 70 ml of resin). The resin was then washed with doubly distilled water to eliminate excess acid. Adsorbed sialic acid was eluted by using a gradient of formic acid (0 to 2 *M*). The fractions containing sialic acid (positive colorimetric test) were pooled and lyophilized.

The yield of the eluted sialic acid was determined by a spectrophotometric assay [17] that allows one to calculate the sialic acid concentration of the different eluted fractions by means of a calibration graph established from known concentrations of commercial NANA (Sigma).

A qualitative evaluation of the purification was made by analysis of the fractions by NMR

spectrometry (Hitachi R-1200 NMR spectrometer) and comparison with the NMR spectrum of commercial sialic acid.

Ligand immobilization on SID (SID–NANA). The immobilization of sialic acid on the stationary phase requires the use of a coupling agent to create covalent bonding between the ligand and the support. In this study, the coupling agent used was carbonyldiimidazole (CDI) (Sigma) [8]. Activation of dextran-coated silica was carried out with CDI in 1,4-dioxane (Carlo Erba, Reuil-Malmaison, France) solution. NANA was coupled to the activated support in carbonate buffer for 48 h. The amount of ligand fixed on the support was determined by a spectrophotometric assay on the coupling solution supernatant using a periodate–resorcinol method [17].

2.2. Determination of affinity constants

For the determination of adsorption isotherms, a calculated amount of insulin was incubated with a fixed amount of support (SID–NANA). The insulin concentration varied from 5 to 100 μg/ml; 100 μl of a support suspension (50–100 mg/ml) were incubated with 200 μl of insulin solution at various concentrations (in a polystyrene tube) for 30 min at different temperatures (4, 20 and 37°C). The amount of residual insulin was determined, after incubation, using a radioimmunoassay (RIA) kit (CIS Bio International, Gif sur Yvette, France) and the amount of adsorbed protein on the support was deduced by subtraction. Adsorption isotherms were obtained; the affinity constant of insulin for the support and the capacity of the active support were determined using the Langmuir model [18].

2.3. HPLAC of insulin

The HPLC apparatus consisted of a pump (L-6210; Merck) monitored by a programmer and equipped with an injection valve (Model 9126, Rheodyne), connected to an UV–visible spectrophotometric detector (L-4000; Merck), an integrator (D-2520 GPC integrator; Merck) and a fraction collector (Model 203, Gilson).

The insulin used in HPLAC, kindly provided by Diosynth (AKZO, Eragny-Sur-Epte, France), was a mixture of porcine insulin with 6% bovine insulin. A 100-μl amount of the insulin was injected on to the column containing the SID–NANA support at a flow-rate of 0.5 ml/min. The fractions corresponding to elution and desorption peaks were collected and analysed by the Bradford assay to calculate the purification yield.

3. Results and discussion

3.1. Synthesis of SID–NANA

Passivation of silica beads by Dx–DEAE

The synthesis of a support based on silica beads coated with polysaccharides permits one to combine the advantages of polysaccharides in chromatography, in particular their hydrophilic feature and their ability to be grafted by bioactive ligands, with the mechanical resistance properties of silica. Dextran substitution was carried out in order to confer a weak anion-exchange capacity by DEAE groups into glycosidic units. The T70 dextran substitution rate by DEAE groups was 5%. The adsorption of Dx–DEAE on silica beads results from interactions between anionic groups on the silica surface and DEAE groups carrying positive charges. The coverage is further reinforced by cross-linking dextran chains with BDGE, which leads to the formation of ether-type bonds with polysaccharide hydroxyl groups. The conditions for the optimization of the passivation were determined previously [10]. The polymeric coverage on the silica supports was 35 mg of Dx–DEAE per gram of silica.

In order to ascertain that the stationary phase used for coupling of the ligand will not undergo non-specific interactions with standard proteins, these proteins were eluted on the SID support under high-performance size-exclusion chromatographic (HPSEC) conditions [8]. The results showed that Dx–DEAE covers all accessible protein areas of the support, which prevents

non-specific interactions in the affinity process after ligand coupling.

Functionalization of SID by NANA

Sialic acid extracted from edible birds' nests was eluted on a cation-exchange column by the use of a linear gradient of formic acid. The sialic acid concentrations of the eluted fractions were different (Fig. 3). Fractions of similar concentration were pooled and lyophilized. The content of sialic acid was determined by a spectrophotometric assay using a periodate–resorcinol method and finally they were lyophilized. The results are presented in Table 1. The extraction yield for 1 g of sialic acid per gram of hydrolysed birds' nest was 10.4% from a nest weighing 9.80 g.

In order to confirm the purity of sialic acid, highly purified extracts were analysed by ^{1}H NMR spectrometry. The spectra are shown in Fig. 4, and demonstrate the similarity between extracted NANA and commercial NANA. These extracts are pure enough to be coupled to chromatographic supports.

During the SID support activation by CDI, hydroxyl functions of the polysaccharide coating on the silica surface are transformed into carbonyldiimidazole groups. In a second step, the latter groups react with the NANA hydroxyl function probably located in position 1 (Fig. 5). The amount of NANA coupled on SID was 8 mg per gram of SID, i.e., the coupling yield was 40%.

Table 1
Purification yields of different fractions

Extract	Fractions	Amount (mg)	Purification yield (%)
NANA 1	9–10–11–12	675	80
NANA 2	13–14–15–16	219	100
NANA 3	17–18–19	51	75
NANA 4	8–20–21–23	78	57

3.2. *Effect of temperature*

The effect of temperature on insulin adsorption was studied under static (batch) conditions from the adsorption isotherms of SID–NANA supports at different temperatures (4, 20 and 37°C) using the Langmuir model. The adsorption isotherms (Fig. 6) show that the amount of insulin adsorbed increases with increase in the amount of insulin initially present until it reaches a saturation plateau corresponding to the maximum adsorption capacity of the active support,

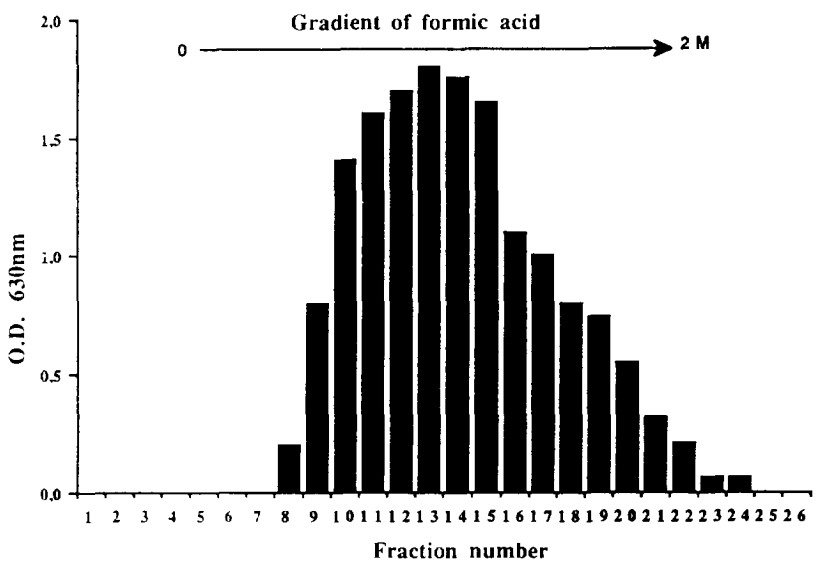

Fig. 3. Elution profile of N-acetylneuraminic acid.

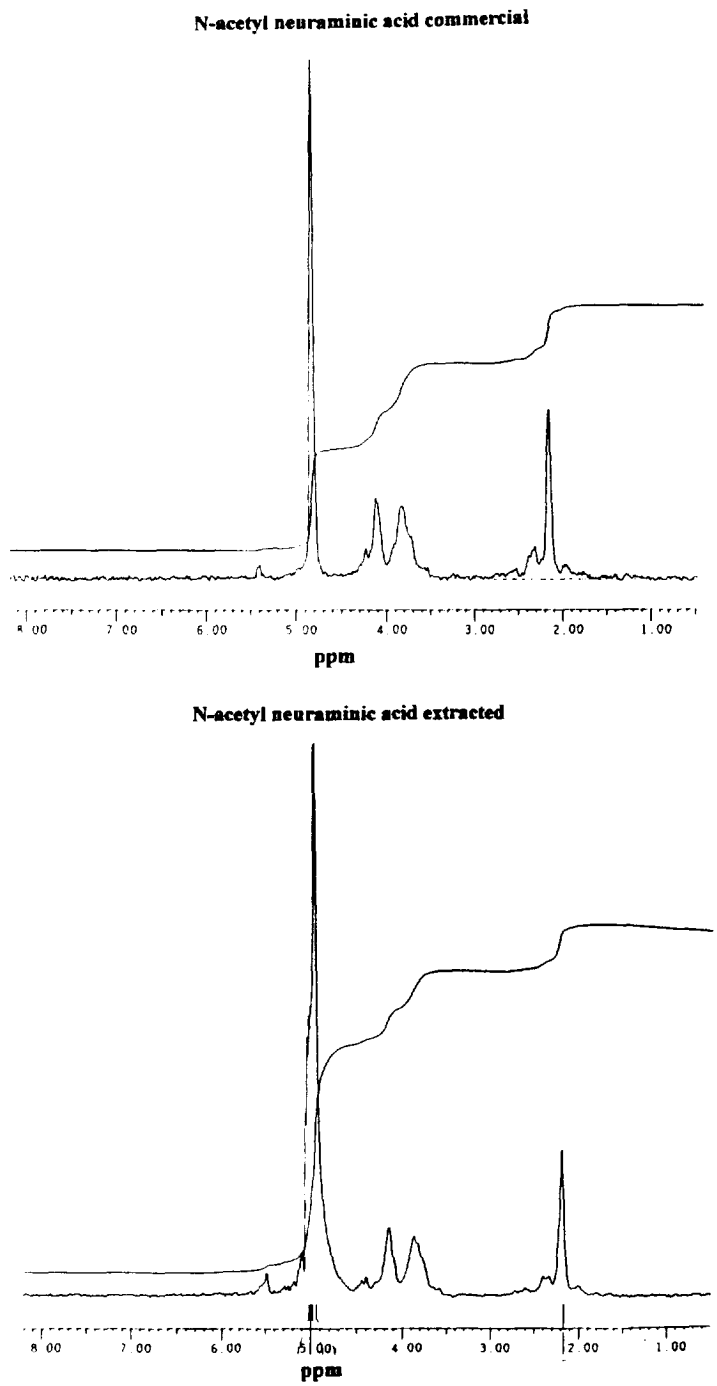

Fig. 4. ^{1}H NMR spectra of N-acetylneuraminic acid.

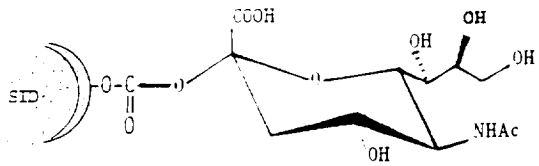

Fig. 5. Structure of affinity support (SID–NANA).

which is about $1.6 \cdot 10^{-6}$ M/g of silica for the three temperatures (Table 2). The amount of adsorbed insulin at saturation corresponds to the total number of sites accessible to the protein. From these adsorption isotherms, the affinity constants of insulin for SID–NANA were determined at each temperature.

It can be noted that the interaction force between insulin in solution and the SID–NANA support increases with increase in temperature until it reaches a plateau at about 20°C, whereas the maximum adsorption capacity of the support remains constant (Table 2).

In the temperature range 4–37°C and in basic solutions, temperature does not affect the insulin structure during the short time of the experiment. Hence, it seems that the temperature affects the whole of the insulin–SID–NANA interaction. Indeed, a rise in temperature leads to agitation of the reaction medium favouring release of ions and electrons from each system component, and therefore it improves and reinforces the stability of insulin–ligand interactions.

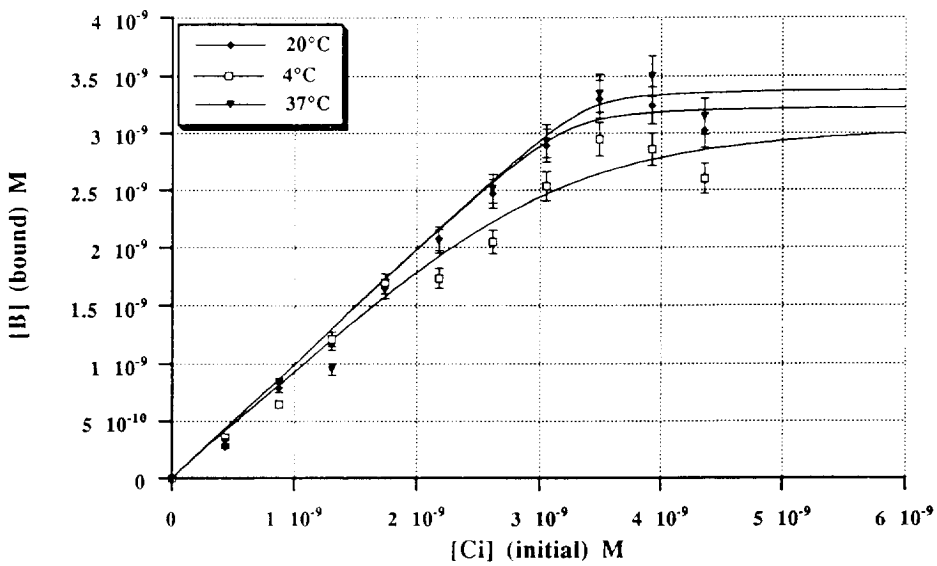

Fig. 6. Adsorption isotherms of insulin on SID–NANA supports at different temperatures.

Table 2
Affinity constants and binding capacities of SID–NANA at different temperatures

Temperature (°C)	Affinity constant (l/mol)	Binding capacity (mol/l · g)
4	$5.9 \cdot 10^{8}$	$1.6 \cdot 10^{-6}$
20	$10.7 \cdot 10^{9}$	$1.6 \cdot 10^{-6}$
37	$9.5 \cdot 10^{9}$	$1.7 \cdot 10^{-6}$

3.3. Effect of pH

Insulin is a very pH-dependent protein. The study of its affinity for the SID–NANA support according to the pH is important for analysing the influence of pH on the interaction mechanisms of insulin on this support.

The elution profiles of insulin (porcine + 6 bovine) dissolved in an adsorption buffer composed of water–methanol (95:5, v/v) at different pH values are presented in Fig. 7.

The fractions corresponding to the different adsorption and desorption peaks were collected and analysed by the Bradford assay to determine the protein yield. Fig. 8 shows a plot of the amount of adsorbed protein versus pH. At acidic pH, no insulin retention occurs, whereas at basic pH the amount of adsorbed protein increases with increase in pH. Nevertheless, at pH 5, as shown in Fig. 7, no significant peak appears. The use of a 2 *M* NaCl–50 m*M* phosphate buffer allowed the insulin fixed on the support to be released after 10 min. This result can be explained by the fact that insulin, forming aggregates, becomes insoluble and remains bound to the head of the column. This phenomenon probably results from a mechanism different from the interaction with the ligand.

The non-retention of insulin at low pH can be explained by the acid nature of this protein, causing a repulsion between the protein and the free carboxylic functions present on the surface of the stationary phase, and thus inhibiting adsorption. Moreover, in the pH range 4–7,

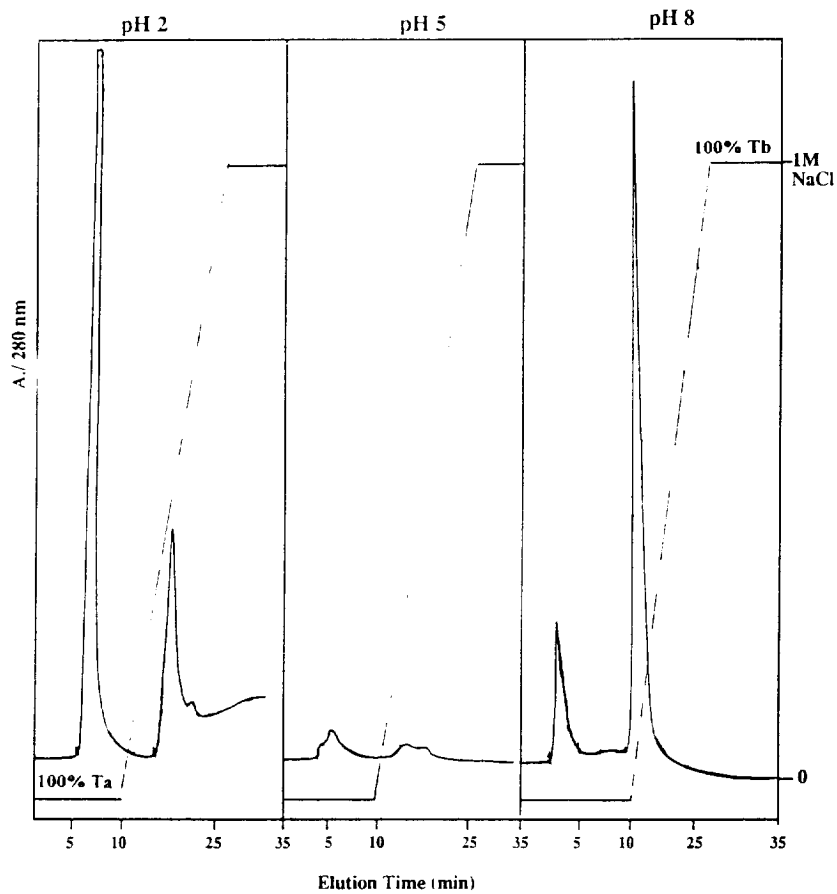

Fig. 7. Elution of 100 μl of insulin (1 mg/ml) on SID–NANA. Column, 12.5 × 0.4 cm I.D.; flow-rate, 0.5 ml/min; eluents, Ta = 5% methanol at different pH and Tb = 0.05 *M* phosphate buffer–1 *M* NaCl (pH 7.4).

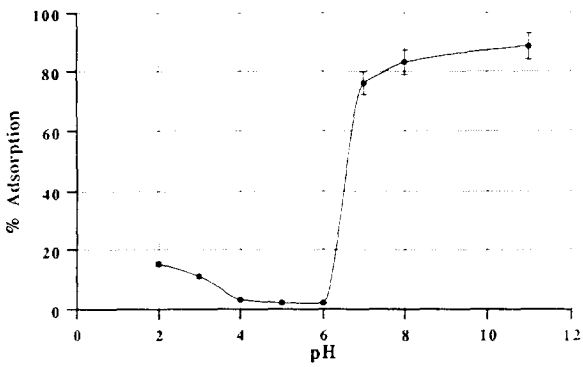

Fig. 8. Influence of eluent pH on chromatographic adsorption of insulin on SID–NANA support.

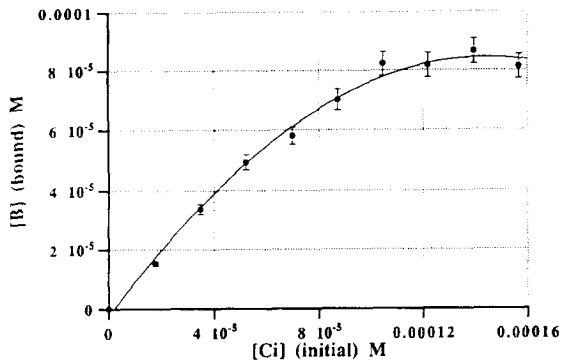

Fig. 9. Influence of insulin concentration on chromatographic adsorption of insulin on SID–NANA support.

insulin is relatively insoluble in aqueous medium and it has a strong tendency to dimerize. Insulin dimerization prevents its retention on the SID–NANA support.

We assume that the association of two or several insulin monomers inhibits the interaction site with the support. Further, the region responsible for insulin dimerization is known and has been sequenced [6], and it can be suggested that insulin interacts with NANA via this region, composed of the following amino acids: Phe B24–Phe B25–Tyr B26–Thr B27.

At basic pH, an increase in insulin retention on the support occurs. In this pH range, insulin exhibits a negative global charge (pH >pHi, insulin pHi = 5.3), and therefore it should not be retained on the support carrying free carboxylic groups. This interaction depends on the monomeric state of insulin, which governs the accessibility to the active sites.

3.4. Effect of insulin concentration

The study of insulin affinity for a given support according to its concentration permits one to measure both the maximum adsorption capacity of the support and the affinity constant of insulin under dynamic conditions. The elution of 100 μl of insulin at different concentrations was performed as described previously (Table 3). The amount of adsorbed insulin increases with increase in the initial insulin concentration until it reaches a saturation plateau corresponding to the maximum adsorption capacity of the active support, which is about $8 \cdot 10^{-5}$ M/g of silica (Fig. 9). The apparent affinity constants determined

Table 3
Concentration of insulin adsorbed on SID–NANA

Initial concentration (mol/l)	Insulin bound on SID–NANA (mol/l)	Free concentration (mol/l)
0	0	0
$1.74 \cdot 10^{-9}$	$1.52 \cdot 10^{-9}$	$2.20 \cdot 10^{-10}$
$3.48 \cdot 10^{-9}$	$3.37 \cdot 10^{-9}$	$1.10 \cdot 10^{-10}$
$5.23 \cdot 10^{-9}$	$4.94 \cdot 10^{-9}$	$2.90 \cdot 10^{-10}$
$6.97 \cdot 10^{-9}$	$5.81 \cdot 10^{-9}$	$1.16 \cdot 10^{-9}$
$8.72 \cdot 10^{-9}$	$7.02 \cdot 10^{-9}$	$1.70 \cdot 10^{-9}$
$1.04 \cdot 10^{-8}$	$8.22 \cdot 10^{-9}$	$2.24 \cdot 10^{-9}$
$1.22 \cdot 10^{-8}$	$8.17 \cdot 10^{-9}$	$4.03 \cdot 10^{-9}$
$1.39 \cdot 10^{-8}$	$8.64 \cdot 10^{-9}$	$5.31 \cdot 10^{-9}$
$1.56 \cdot 10^{-8}$	$8.13 \cdot 10^{-9}$	$7.56 \cdot 10^{-9}$

under dynamic conditions are about $3 \cdot 10^5$ l/mol and far weaker than the affinity constants obtained under static conditions. This difference in the affinity constants obtained under static conditions. This difference in the affinity constants probably reflects the influence of kinetic parameters on the adsorption or desorption process under the chromatographic conditions. Under dynamic conditions insulin adsorption consists of four successive steps: protein transport towards the support (surface), adsorption, structural rearrangement of the protein and finally desorption.

For a low insulin concentration, transport towards the support surface depends mainly on convection and diffusion movements of molecules. For a high insulin concentration, interactions between insulin and the support become the limiting factor. Evaluation of the amount of adsorbed insulin is made after insulin desorption. Under static conditions, insulin is in contact with the support so that fewer parameters are implicated and the developed surface area is greater. The determination of the amount of adsorbed insulin is a direct evaluation. Many workers have observed such phenomena with other proteins [19–21].

4. Conclusion

The behaviour of insulin towards supports functionalized with NANA was studied by affinity chromatography and the results obtained allowed the analysis of the influence of several physico-chemical parameters on insulin–ligand interactions.

As demonstrated previously [8], the ligand–insulin complex is formed by different attractive forces of various natures whose resultant determines the stability of this complex and leads to an affinity interaction. Hydrophilic, hydrophobic and weak ionic interactions are probably involved in the overall interaction mechanism.

Different parameters (pH, temperature and insulin concentration) influence the mechanisms of separation in HPLAC and demonstrate the complexity of these interactions inherent to adsorption and desorption processes in addition to those due to affinity mechanisms. An increase in temperature improves the stability of the ligand–insulin complex; the influence of insulin concentration allows us to determine the maximum adsorption capacity of the support and to improve the purification yield. pH determines the insulin solubility and the protein ionization. Both pH and insulin concentration influence the retention of insulin on the functional support.

From the results, we can conclude that insulin interacts with the NANA ligand via the amino acids sequence Phe B24–PHE B25–Tyr B26–Thr B27 of the B chain. The interactions between insulin and immobilized sialic acid residues lead to an affinity mechanism. This affinity can be utilized to separate or purify insulin by affinity chromatography on dextran-coated silica supports grafted with NANA. Nevertheless, the conditions of insulin adsorption on SID–NANA chromatographic supports show little selectivity. Hence further experiments would be necessary in order to improve the yield and the selectivity of the support. This can be carried out by grafting specific ligands other than sialic acid on the support. These ligands will be chosen so as to mimic the insulin receptor structure. Finally, the aim would be the development of an "insulin receptor-like" support. Such work could also contribute to a better understanding of the interaction mechanisms of insulin with its receptor.

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