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Experimental studies of pressure/temperature dependence of protein adsorption equilibrium in reversed-phase high-performance liquid chromatography

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Abstract

The effect of the average pressure and temperature of the column on the adsorption equilibrium of insulin variants on a C_8 bonded silica was studied in isocratic reversed-phase HPLC. Analytical injections of samples of four different insulins (bovine, porcine, Lys–Pro and human recombinant) were carried out at constant flow-rate but under increased average pressure. The temperature dependence of the retention parameters over the range 25–50 °C was studied under two different average column pressures (47 and 147 bar). Substantial increases of the retention time (up to 300%) were observed when the pressure and/or the temperature were increased. Similar adsorption-induced changes in the partial molar volume at constant temperature ($\Delta V_m \approx 102$ ml/mol) were found for all the variants studied. Furthermore, ΔV_m was revealed to be practically independent of the temperature, which suggests that the temperature has no or very little influence on the mechanism of the pressure induced perturbations in the molecular structure of the solute. This conclusion was also derived from the observed temperature dependence of the logarithm of the retention factor (k) measured under different pressures. The relation between the temperature and ln k was nonlinear with a parabolic shape. Moreover, the shapes of the plots corresponding to the low and high pressures were found to be exactly the same, except that the curves were vertically shifted, due to the difference between the two average column pressures. These results indicate that pressure and temperature affect the retention behavior of insulins in a different and separate way.

Keywords: Temperature effects; Adsorption isotherms; Pressure effects; Hydrophobic surfaces; Proteins; Insulin

1. Introduction

Pressure and temperature are two major external parameters (thermodynamic variables) that influence the adsorption processes involved in liquid chromatography. For example, a precise control of the temperature in any chromatographic experiment, including in the reversed-phase (RP), hydrophobic

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interaction, or ion-exchange modes, is of great practical importance for any separation of biomolecules such as proteins or peptides [1–7]. Sometimes, changes in the retention behavior induced by an appropriate variation of the temperature make possible the separation of closely related proteins, e.g., insulin variants [8]. A general picture emerging from high-performance liquid chromatography (HPLC) studies of proteins and peptides proves that adsorption equilibrium in such systems is extremely sensitive to external parameters such as the pH, the temperature or the organic modifier content of the mobile phase [7,9,10]. However, although, manipulations of the temperature have long been recognized to be very useful for the optimization of separation processes [5,11–18], the influence of pressure on adsorption equilibria in liquid chromatography remains generally disregarded.

Theoretical considerations of the influence of pressure began more than 30 years ago, when Giddings et al. [19,20] developed supercritical fluid chromatography. A complete and rigorous mathematical formulation of the pressure/temperature equilibrium shifts in chromatographic separations was proposed later by Martire [21,22] in the framework of the unified molecular theory of chromatography (UMTC). Unfortunately, the practical meaning of the UMTC theory and of other microscopic models [23–26] seems rather limited when considering the influence of the pressure on the solute retention behavior in HPLC. This is mainly because liquid solutions are normally treated as incompressible in the conventional LC pressure regime ($<$350 bar). In consequence, pressure induced shifts in the interaction equilibria that influence the solute retention such as adsorption, ionization, complexation, and hydrophobic interactions are usually assumed to be negligible.

There is, however, a growing number of experimental investigations reported in the literature that make this usual approach questionable. For example, McGuffin and Evans [27] observed a substantial increase (from 9.3 to 24.4%) in the retention factor (k) of derivatized fatty acids adsorbed on octadecyl silica as the pressure was changed from about 100 to 350 bar. Also, in the case of chromatographic separations of chiral compounds, such as ibuprofen, propranolol or benzoin, a significant change in k (from -20 to $+12$%) was observed when the pressure was increased by ~240 bar [28]. At the same time, the pressure was found to have a large influence on the chromatographic efficiency, since, in one case, the theoretical plate height changed by up to 240%. Similar results were reported for nitrophenol isomers on β-cyclodextrin-based stationary phases [29]. In this case, the observed changes in k were exclusively negative, with decreases of up to 35%. Surprisingly, big shifts in the retention factor, hence in the molecular volume of the solute, have been observed also for macromolecules such as porcine insulin [30] or, recently, for chicken egg white lysozyme [31]. For these proteins the calculated decrease in V_m upon adsorption was about 100–130 ml/mol, which is much higher than what was found in the case of the relatively small molecules studied before [27–29,32].

Changes in k can be explained in terms of classical thermodynamics, which states that, in a closed system at equilibrium, an increase in the pressure favors transformations that cause a reduction of the volume of the system (Le Chatelier's principle). The adsorption process is governed by changes in the Gibbs free energy, which may involve possible changes of the solute molar volume when the molecules passes from the solution to the stationary phase. Thus, by measuring changes in the molar volume of the solute caused by adsorption, one can obtain quantitative information about the average degree of structural perturbations of the retained molecules. As one may expect, ΔV_m should be small in the case of small molecules with relatively rigid structures while it may be larger for molecules composed of long and flexible chains. Such a conclusion can be supported by experimental results reported by McGuffin and Evans [27] and by the simple thermodynamic considerations proposed by Guiochon and Sepaniak [32]. For example, it was found that, in a homologous series, ΔV_m increases linearly with the number of carbon atoms in the aliphatic chain. Another factor that can influence ΔV_m is the degree of solvation of retained molecules by the molecules of the mobile phase. Most proteins in aqueous solutions are surrounded by a tightly bound hydration layer [33]. In this layer, the water molecules remain less mobile and more highly ordered than in the bulk water. Thus, contact with the

stationary phase can, possibly, change the effective molar volume of the protein by disturbing its hydration layer.

Unfortunately, the purely thermodynamic approach described above gives no information on the detailed mechanism of interactions involved in the retention process considered at the microscopic level. One should remember that the value of ΔV_m is a lumped parameter which can be correlated with several associated phenomena taking place during the adsorption process. It should be expected, especially in the case of macromolecules, that changes in V_m may originate from different sources, e.g., variations in the energy of molecular interactions, solvation, aggregation or changes in the energy density of these interactions [34]. These effects play, for example, a key role in the retention of peptides or proteins. Conformational changes induced by pressure, besides affecting V_m directly, can also modify the surface hydrophobicity of the molecule. In consequence, the creation, disappearance or change in the spatial distribution of certain hydrophobic patches or sites can occur within proteins or at their surface. This phenomenon can substantially contribute to the measured value of ΔV_m. The unfolding (or spreading) of protein molecules upon adsorption, leading to the exposition of their hydrophobic core is a well-known phenomenon observed experimentally [8,34–39] as well as modeled by means of analytical approximations [40,41] or by computer simulations including molecular dynamics [42,43] and Monte Carlo methods [44–48]. Despite numerous experimental and theoretical studies devoted to protein adsorption in RP-HPLC, such intriguing problems as the influence of the pressure and the temperature on retention mechanisms remain incompletely understood.

The cells of many UV detectors can now be operated under relatively high pressures, in the order of several hundred bar, making easy the experimental investigation of the pressure dependence of retention factors in all modes of HPLC. The main objective of this work is to clarify the present picture of this phenomenon, to report on the results of new systematic measurements of the influence of the pressure on the adsorption of four closely related protein molecules, and to suggest possible correlations between the influence of the pressure and the temperature on the retention parameters of model proteins in RP-HPLC.

2. Theory

Changes in the solute molar volume upon adsorption can easily be determined by means of basic classical thermodynamic relationships. Let the concentrations of the solute in the mobile and the stationary phase at equilibrium be C_m and C_s, respectively. We can write:

$$K = \frac{C_s}{C_m} \tag{1}$$

and

$$\Delta G = -RT \ln K = \Delta H - T\,\Delta S = \Delta E + p\,\Delta V_m - T\,\Delta S \tag{2}$$

where R is the universal gas constant, K the equilibrium constant of adsorption, and ΔG, ΔH, ΔE, ΔV_m, and ΔS denote the associated changes in Gibbs free energy, enthalpy, internal energy, partial molar volume and entropy of the system [32]. The remaining variables are the temperature, T and the pressure, p (external parameters). The influence of the pressure on the equilibrium constant can be estimated from the pressure dependence of the change in the Gibbs free energy. To that purpose it is sufficient to differentiate Eq. (2) with respect to p:

$$\Delta V_m = \frac{\partial \Delta G}{\partial p} = -RT \cdot \frac{\partial \ln K}{\partial p} = V_a - V_b \tag{3}$$

where V_a and V_b are the molar volumes of the solute in the adsorbed and the bulk liquid phase, respectively. The measured value of the retention factor is related to the equilibrium constant through the following relationship:

$$k = \beta K \tag{4}$$

where β is the phase ratio or volumetric ratio of the stationary and mobile phase. Taking into account the above relationship, Eq. (2) can be rewritten in the following form:

$$\ln k = -\frac{\Delta E}{RT} - p \cdot \frac{\Delta V_m}{RT} + \frac{\Delta S}{R} + \ln \beta \tag{5}$$

Providing that p is independent of the pressure,

ΔV_m can be determined from a plot of the first derivative of ln k versus the pressure, at constant temperature.

3. Experimental

3.1. Equipment

A HP 1100 liquid chromatograph (Hewlett-Packard, now Agilent Technologies, Palo Alto, CA, USA) was used for all the experimental determinations. This instrument was equipped with an isocratic solvent delivery system, a manual sample injector with a 10 μl loop, a variable-wavelength detector with a high-pressure UV cell (up to 400 bar), a thermostated column compartment, and a computer system. A capillary restrictor [0.0025 in. polyether ether ketone (PEEK) tubing, Upchurch, Oak Harbor, WA, USA; in. = 2.54 cm], cut to desired length, was placed downstream the UV detector, to allow an easy adjustment of the average column pressure (ACP). The reproducibility of the flow-rate delivered by the pump of the HP 1100 is characterized by a relative standard deviation better than 0.1% [49]. The long term stability of the flow-rate was tested periodically as part of the validation tests of the equipment recommended by the manufacturer.

3.2. Materials

3.2.1. Mobile phase and chemicals

The mobile phase was a mixture of a phosphate buffer at pH 2.2–acetonitrile (74:26, v/v). Sodium dihydrogenphosphate (99% pure) was purchased from Aldrich (Milwaukee, WI, USA). Orthophosphoric acid (85% concentrated) was from Mallinckrodt (Phillipsburg, NJ, USA). Acetonitrile and water, used for the preparation of the buffer, were HPLC-grade solvents from Fisher Scientific (Fair Lawn, NJ, USA). Bovine insulin was purchased from Sigma (St. Louis, MO, USA) while Lys–Pro, human and porcine insulins were gifts from Eli Lilly (Indianapolis, IN, USA). The purity of the variants used, estimated with HPLC, was greater than 95% (bovine) and 99% (Lys–Pro, human and porcine).

3.2.2. Column

A 150 × 3.9 mm Waters Symmetry column (Waters, Milford, MA, USA), packed with a C_8-bonded silica as the stationary phase, was used for all the determinations and experiments. The mean particle size of the packing material was 5 μm with the average pore diameter of the silica being 9 nm. The total column porosity, measured by injecting unretained uracil, was 0.62. The hold-up time was 1.11 min. The efficiency of the column measured with the same compound was approximately 3000 theoretical plates.

3.3. Measurement of experimental data

The column temperature was varied over the range 25–50 °C, the column temperature being measured with an accuracy of 0.1 °C. In order to measure accurately the column temperature, an additional sensor, connected with a digital thermometer (HFT-82, Aritsu Meter, Franklin Lakes, NJ, USA), was placed inside the column compartment.

All the experimental data were measured with a 1.0 ml/min mobile phase flow-rate. The corresponding pressure drop was 94 bar at 25 °C and 60 bar at 50 °C. The average column pressure was monitored within the interval 30–250 bar with an accuracy of 1 bar. In chromatography, it is usually assumed that the column permeability is independent of the position and that the liquid compressibility is negligible. If this is true, the pressure decreases linearly from the column inlet to its outlet. Accordingly, the average value of a parameter (e.g., ΔG, Eq. (2), ΔV_m in Eq. (3) is assumed to be independent of the pressure, as a first approximation) that varies linearly with the pressure is equal to the value of this parameter at the average pressure. Additional measurements were carried out to confirm the validity of this assumption. To this purpose, the retention volume of bovine insulin was measured at different flow-rates (0.25–1 ml/mm) but under constant average column pressure (ACP = 47 bar). The retention volume was found to be practically independent of the flow-rate, which proves the linear behavior of the local pressure.

Fresh insulin samples were filtrated through 0.20 μm PTFE filters (Nalgene, Rochester, NY, USA). Concentrations of all the insulin variants in the

samples injected were always the same and equal to 0.5 mg/ml. The peak profiles were recorded with the UV detector, the signal being monitored at 210 nm. The reproducibility of the retention times in the case of all the insulin variants was typically characterized by a RSD lower than 1%.

4. Results and discussion

4.1. Pressure dependence at constant temperature

Two series of experiments were carried out in order to study the influence of the average column pressure on the retention time of the insulin variants. In the first series, analytical injections were performed at 25 °C, in the second series, at 50 °C. In both series, the ACP was gradually decreased (by cutting short section of the restrictor tubing) from ~200 bar down to the natural column backpressure at the column temperature (95 bar at 25 °C and 60 bar at 50 °C, respectively). The order of elution of the insulin variants (B<L<H<P) was found to be independent of the pressure and of the temperature. A series of elution profiles obtained for different insulins, at two different average pressures illustrates these results in Fig. 1. The retention times of the L, H and P insulin variants were much closer to each other than those of B and L insulin. For example, under conventional operating conditions (inlet pressure, 95 bar at 25 °C), the retention times were 4.16 min (B), 5.25 min (L), 5.83 min (H) and 6.28 min (P). At both temperatures, for all the variants, we observed a substantial increase of the retention time when the pressure was increased by approximately 150 bar. This increase was typically 75% at both temperatures. At the same time, we observed a large increase in the retention time of all the insulin variants, by up to 300%, when the temperature was increased by 25 °C at constant pressure. Surprisingly,

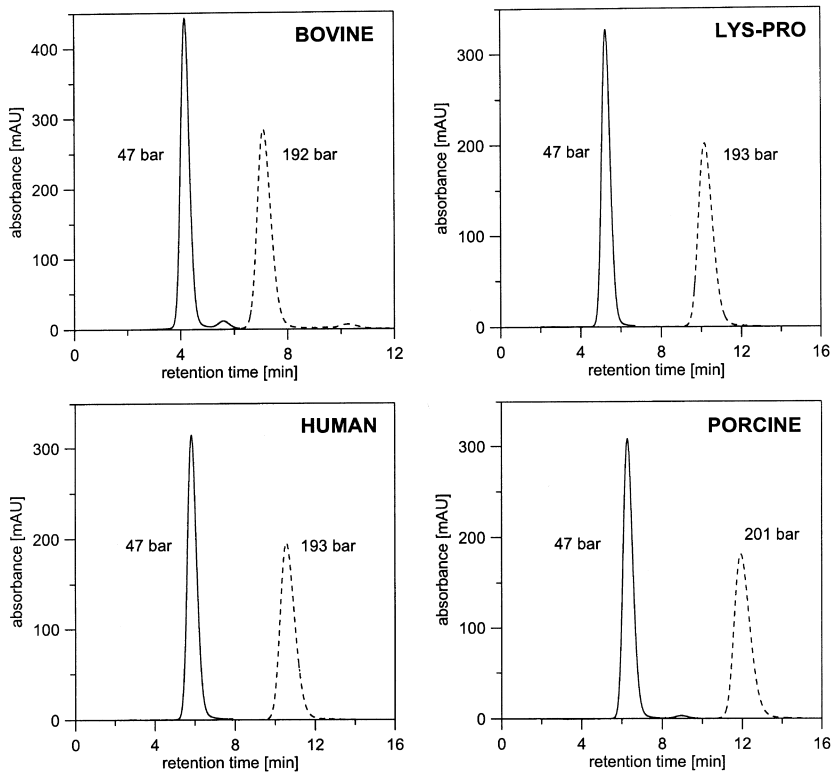

Fig. 1. Elution profiles of different insulin variants at constant flow-rate and constant temperature, under low and high average column pressure.

the relative shift in the retention time caused by the temperature increase was practically the same for all the variants and it did not change much with the pressure. The observed increase of the retention time of insulin with increasing temperature is in agreement with experimental findings obtained for other proteins, e.g., cytochrome *c* [38,50], bovine serum albumin (BSA) [49], or for smaller polypeptides [51,52]. It will be discussed later.

In conclusion, the influence of a large pressure increase on the retention behavior of the insulin variants studied was very similar. Fig. 2 shows the influence of the ACP on the relative increase of the retention factor of the insulin variants ($\Delta k/k$), measured at 25 and 50 °C. The experimental data corresponding to the B, L, H and P variants at a given temperature overlay almost exactly. This demonstrates that the influences on the retention time of changes in either the temperature or the pressure are considerably larger than those of differences in the amino acid sequence of the peptide chain of the insulin variants studied. This chain structure itself has almost no influence on the relationship between $\Delta k/k$ and the pressure or the temperature.

Insulin is a relatively small polypeptide consisting of 51 amino acid residues of which 21 belong to the A-chain while the remaining 30 form the B-chain. Crystallographic studies [53] have demonstrated that the insulin molecule contains three α-helices. Two of them reside in the A-chain, at positions A2–A8 and A13–A20 while the third one is located in the B-chain, at positions B9–B19. The differences between the variants studied here are in the nature of the amino acid residues in positions A8, A10, B28, B29, and B30 of the polypeptide chain. Table 1 indicates the residues at which the variants differ. The physical structure of the variants is, thus, very similar (same length of the peptide chain, very similar molecular mass and volume). Our results show that the relative changes in *k* induced by changes in the pressure are nearly insensitive to the microscopic details of the molecular structure. On the other hand, they may depend on global features of the protein molecule, such as the chain length, the electrostatic charge, or the ability of the chain to unfold/deform upon adsorption. As one can also notice, at low pressures, the two sets of points in Fig. 2 are horizontally shifted by approximately 17 bar. This merely comes from the difference between the reference ACPs (i.e., the natural column backpressures) at 25 and 50 °C.

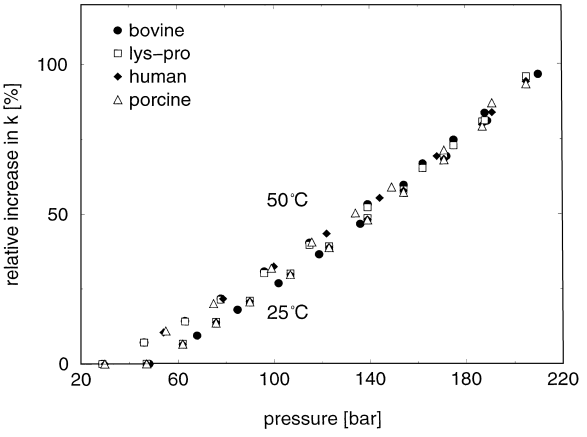

Fig. 2. Influence of the average column pressure on the relative increase in the retention factor of different insulin variants.

Another fact which can be explained on the basis of the information given in Table 1 is the order of elution of the variants. It follows from the table that the primary structures of the L, H and P insulin differ only by minor changes in the amino acid sequence of the B chain but these three variants have close retention times. By contrast, bovine and porcine insulin, that have markedly different retention times, differ also by only two residues (Ala or Thr in A8, Val or Lle in A10). Thus, minor changes in the sequence of amino acid residues in the A-chain seem to cause larger differences in the retention behavior than similar changes in the B-chain [8]. This could be explained by an adsorption process involving more strongly the former chain.

The similarity between the behavior of the differ-

Table 1
Major differences between primary structures of the insulin variants used in this work

Insulin variant	M_r	Amino acid at position				
		A8	A10	B28	B29	B30
Bovine	5734	Ala	Val	Pro	Lys	Ala
Lys–Pro	5808	Thr	Ile	Lys	Pro	Thr
Human	5808	Thr	Ile	Pro	Lys	Thr
Porcine	5778	Thr	Ile	Pro	Lys	Ala

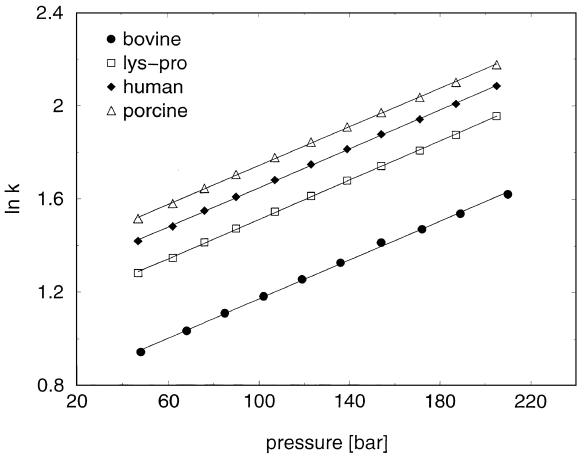

Fig. 3. Pressure dependence of ln k in the case of different insulins, measured at 25 °C.

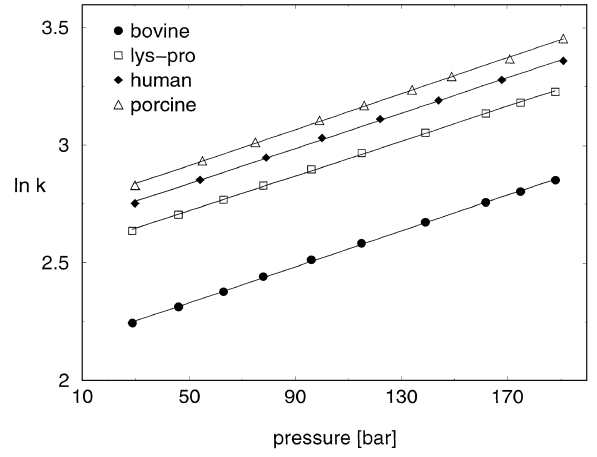

Fig. 4. Pressure dependence of ln k in the case of different insulins, measured at 50 °C.

ent variants can also be observed when comparing the pressure dependence of ln k. Figs. 3 and 4 show plots of ln k versus the ACP, at 25 °C and 50 °C, respectively. The symbols represent the experimental data, the lines are the results of a linear regression of these experimental data. At both temperatures, the experimental data points are on almost perfectly parallel straight lines, with slopes of about 0.0042 (25 °C) and 0.0038 (50 °C). The difference between the two slopes is relatively small, suggesting that, as mentioned earlier, the temperature has only a small influence on the pressure dependence, at least in this interval. Note also, when comparing Figs. 3 and 4, that the data sets in the two figures are vertically shifted by approximately 1.3 units, a value directly related to the temperature effect. In this case, for each insulin variant, we observe that, independently of the ACP, a temperature increase of 25 °C induces a constant increase of 1.3 in ln k.

The change in partial molar volume associated with adsorption from the solution can be derived from a plot of the first derivative of ln k versus the pressure, at constant temperature. In our case, this procedure involves the determination of the slope of the straight lines shown in Figs. 3 and 4. The results of the calculation are summarized in Table 2. The values of ΔV_m corresponding to the different insulin variants are very close to each other and, in general, agree with other experimental data obtained for different proteins (e.g., up to −128 ml/mol for porcine insulin [30] and −97 ml/mol for lysozyme [31]). Since, for all the variants, the relative difference between ΔV_m at 25 °C and at 50 °C is less than 4%, it is obvious that the temperature does not affect seriously the mechanism that controls the effect of the pressure on the adsorption equilibrium. More-

Table 2
Changes in the molar volume of different insulins caused by adsorption on C_8 stationary phase

Insulin variant	ΔV_m at 25 °C (ml/mol)	RSD^{25} (%)	ΔV_m at 50 °C (ml/mol)	RSD^{50} (%)	$\Delta\Delta V_m$ (%)
Bovine	−102.3	1.23	−101.0	0.72	+1.27
Lys–Pro	−103.0	0.59	−98.9	0.81	+3.98
Human	−102.2	0.59	−100.0	1.09	+2.15
Porcine	−101.5	0.61	−101.6	0.89	−0.09

The RSD of the changes of molar volume associated with the retention mechanism are provided by the numerical program used to fit the experimental data to Eq. (5).

over, the sum of the relative standard deviations listed in Table 2, $RSD^{25}+RSD^{50}$, is comparable to $\Delta\Delta V_m$ itself which indicates that the observed shifts in ΔV_m could as well originate from an accumulation of the experimental errors. In other words, the effects of the pressure and the temperature do not seem to be correlated via changes in ΔV_m. Because ΔV_m is independent of the temperature, it is possible to predict easily the effect of the temperature on the pressure dependence of ln k. At any temperature between 25 and 50 °C, the slope of ln k plotted against the pressure can be calculated from ΔV_m determined at any other temperature within the interval. Then the slope is $-\Delta V_m/RT$.

4.2. Temperature dependence at constant pressure

The results described in the previous section, the lack of correlation between the effect of temperature and pressure, demand a discussion of the converse relationship, i.e., of the possible influence of the pressure on the temperature dependence of the insulin retention times. To this purpose, we measured the retention time of the B, H, L and P variants as functions of the temperature in the range 25–50 °C under two constant values of the average column pressure, 47 and 147 bar. The results of these experiments are shown in Fig. 5. They demonstrate the rather large influence of the temperature on k under constant pressure. Alike in the study of the pressure influence (Fig. 2), the behavior of all the variants is very similar. The data measured under constant pressure for the four different insulin variants are overlaid again. This proves also that the mechanism of the thermal deformation/denaturation of the insulin variants is but weakly affected by differences in the primary molecular structure or by changes in the column pressure. This is not surprising since the denaturation of insulin is limited because the disulfide bridges are unlikely to be broken under the mild experimental conditions used in this work.

We are of the opinion that the observed dependence of ln k on the temperature is most interesting. Figs. 6 and 7 show plots of ln k as functions of the temperature under ACPs of 47 and 147 bar, respectively (symbols, experimental data). Obviously, the dependence of ln k on the temperature is nonlinear.

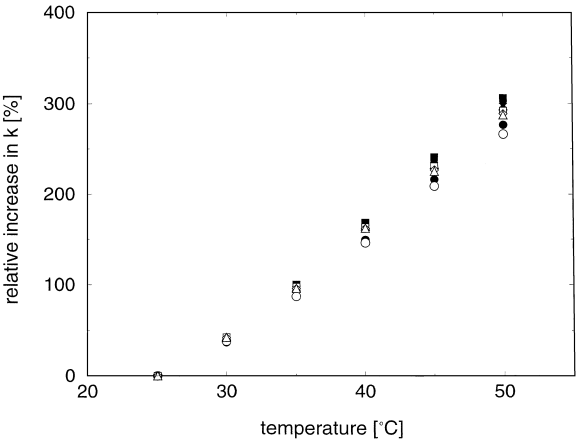

Fig. 5. Influence of temperature on the relative increase in the retention factor of different insulin variants. The data corresponding to different insulin variants are denoted by circles (B), squares (L), diamonds (H) and triangles (P). The filled symbols denote the data measured for ACP = 47 bar while the open ones represent the data measured for ACP = 147 bar.

The lines shown in the figures are the results of quadratic fits of the experimental data. The parabolic shape of the curves suggests that there exists a maximum in ln k, hence in the retention time, at temperatures somewhat higher than 50 °C [54]. Such a curvilinear behavior of ln k is not unique to proteins. It was reported in the case of various polypeptides like bombesin, glucagon, β-endorphin [51] or for enantiomeric pairs of polypeptides [52]. However, the main difference between the results

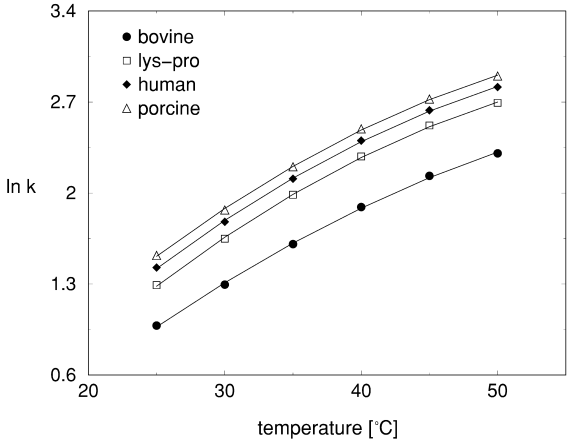

Fig. 6. Temperature dependence of ln k in the case of different insulins, measured for ACP = 47 bar.

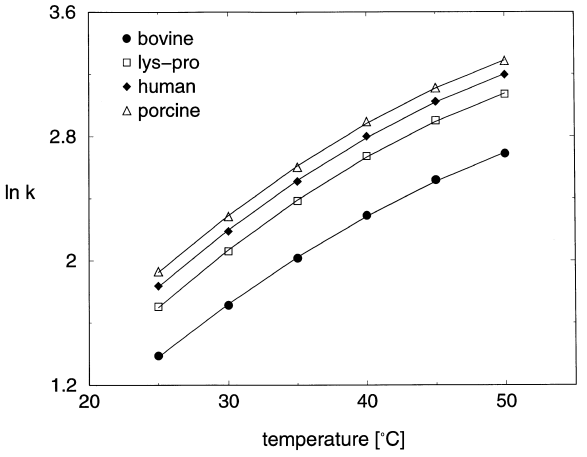

Fig. 7. Temperature dependence of ln k in the case of different insulins, measured for ACP = 147 bar.

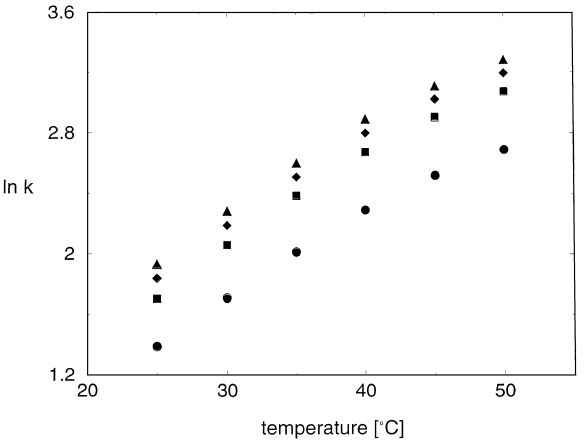

Fig. 8. Overlaid plots of the data displayed in Figs. 6 and 7 (see the text for details). The data corresponding to different insulin variants are denoted by circles (B), squares (L), 24 diamonds (H) and triangles (P). The filled symbols denote the data measured for ACP = 47 bar while the open ones represent the data measured for ACP = 147 bar.

presented here and the data reported in the literature [51,52] is the fact that the latter were measured under an average column pressure that was varying with the temperature because, for the sake of experimental convenience, they were carried out under constant flow-rate. Since the mobile phase viscosity decreases markedly with increasing column temperature, the head pressure, hence the average pressure, decreases in the same time. However, a correct thermodynamic analysis of the system behavior at different temperatures requires that measurements be made under constant pressure. Careful satisfaction of this condition reveals to be necessary, especially in cases like the adsorption of insulin on hydrophobic stationary phases [54].

Regarding the temperature effects, there is an obvious similarity between the retention behavior of different variants. The sets of points corresponding to 47 and 147 bar are vertically shifted in the same amount for each of the variants (compare Figs. 6 and 7). Our earlier results suggest that, at a given temperature, an increase in the pressure by Δp should induce an increase in ln k by $-\Delta p\ \Delta V_m/RT$, at least over the range of temperatures studied, 25 to 50 °C. To verify this conclusion, we assumed an average value of ΔV_m equal to -102 ml/mol and calculated the corresponding vertical shift of the temperature dependence of ln k at 47 bar, $124.3/T$ for $\Delta p = 100$ bar. The two sets of experimental results, the one at 147 bar and the one at 47 bar corrected for the pressure shift, are overlaid in Fig. 8. These two sets of data match perfectly. This, again, proves that while both pressure and temperature affect considerably the retention mechanism of insulins, they do so in different and independent fashions.

There are two possible practical consequences of the findings reported here. First, it is necessary precisely to control the pressure during the chromatographic separation of macromolecules exhibiting secondary/ternary structures. Pressure effects may be beneficial for the separation in certain cases. They may also interfere with temperature effects. Second, a correct understanding of the thermodynamics of adsorption cannot be achieved if the pressure and the temperature influence on the adsorption equilibrium constant are not determined separately, by carrying measurements in which only one does vary. A change in the column temperature induces a change in the mobile phase viscosity, hence in the system pressure. This effect can mask or alter the true temperature dependence of k. Fortunately, the converse is not true, changes in pressure have little effect on the viscosity of liquids [55]. As a consequence of the temperature effect on liquid viscosity, values of ΔH and ΔS derived from experimental data acquired at constant mobile phase

flow-rate, following the usual Van't Hoff plot method, can be inaccurate. This problem will be discussed later in more detail [54].

4.3. Possible mechanism of retention under different external conditions

While both the pressure and the temperature have a large influence on the adsorption equilibrium constant of the different insulin variants in RP-HPLC, it seems that the pressure effect is less complex than the temperature effect. In the former case between 25 and 50 °C, ln k is a linear function of the pressure with a slope proportional to $1/T$. This slope is also proportional to the change in the protein molar volume upon adsorption. This result is a consequence of the retention mechanism. The observed reduction of the molar volume is due to a change in the environment of the molecule probably arising from the creation of new contact points between the solid interface and the protein surface. The decrease in molar volume of insulin (102 ml/mol) is relatively small compared to its molar volume in the solid state (a few percent). These facts support previous findings [36] proving that the interaction of insulin with hydrophobic surfaces, even at moderate temperatures, leads to conformational perturbations of the protein.

Surprisingly, the degree of deformation connected with ΔV_m is weakly dependent on the temperature. The origin of this phenomenon is, however, hard to interpret. It is possible that, due to a multistep kinetics of unfolding, in the very initial stage of adsorption, the insulin molecules reside on the surface in their still folded globular or ellipsoidal form. This first contact causes the molecules to change their conformation to some fixed extent. The kinetics of this unfolding process of the protein molecules depends on the temperature. In consequence, when the column temperature is increased, the contact area between the molecule and the stationary phase becomes larger which explains why the retention times of the insulin variants increase. This suggestion is supported by the results shown in Fig. 4. On the other hand, our results indicate also that ΔV_m is practically independent of the temperature while the contact area seems to increase with increasing temperature. This phenomenon can originate from a possible flattening of the globular protein molecule upon adsorption. Thus, although the contact area between the molecule and the adsorbent increases, the volume of the protein remains nearly constant as long as it does not denature.

This would lead to the following possible mechanism of insulin retention at different temperatures. Since the effect of temperature on ln k is qualitatively similar for all the insulin variants, we take porcine insulin as an example. Fig. 9 shows the Van 't Hoff plot (VHP) determined for this variant, as well as its best fit obtained with a quadratic regression, according to the following equation:

$$\ln k = +aT^{-2} + bT^{-1} + c \tag{6}$$

where a, b and c are the best fit parameters. An obvious qualitative feature of the curve in Fig. 9 is its downward convexity ($a<0$ in Eq. (6)), which means that the slope of the tangent decreases in proportion to the reciprocal of the temperature while the intercept increases. From a physical point of view, such a dependency involves a decrease in ΔH and in ΔS with increasing temperature. This temperature dependence of the slope and the intercept of the local VHP can be quantitatively related to ΔH and ΔS by means of Eq. (6). Thus, by comparison of Eqs. (2), (5) and (6), we obtain from the slope:

$$\Delta H = -R(2aT^{-1} + b) \tag{7}$$

and from the intercept:

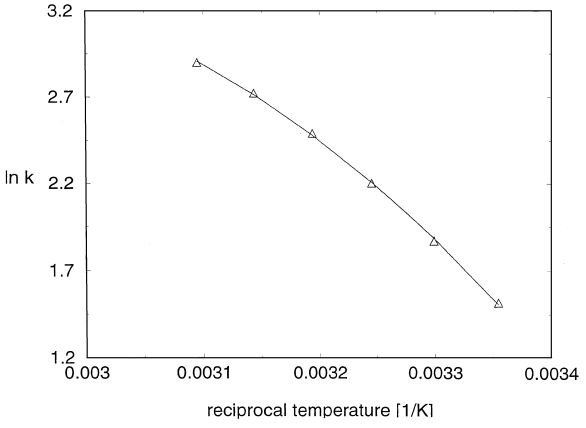

Fig. 9. Van't Hoff plot in the case of the porcine insulin.

$$\Delta S = R(c - aT^{-2}) - R \ln \beta \quad (8)$$

Changes in the enthalpy and the entropy of the system induced by a temperature variation can be seen in Fig. 10, where we displayed ΔH and ΔS calculated from Eqs. (1) and (2), respectively, with $\beta = 0.61$, as estimated by measuring the breakthrough volume of a sodium nitrate dilute solution. Although there still exists possibility of changes in β caused by temperature, it has been shown [52] that neglecting these effects has no serious consequences for the interpretation of the thermodynamics of adsorption.

An explanation of the observed trend in the system behavior can be based on a molecular model of protein adsorption. It is generally accepted that their adsorption from solutions is associated with their displacing (or desorbing) solvent molecules, residing on the surface. In the case of a protein, solvent molecules are also released from their surface [33]. When a protein molecule adsorbs, the solvation layer coating its surface is highly disturbed or even, possibly, lost. This process is enhanced when the molecule unfolds and exposes new segments. The major factors accelerating the unfolding (or denaturation) kinetics are the temperature and the presence of an adsorbing hydrophobic surface [8,34–39]. Despite the fact that, in some cases (e.g., immunoglobulin G [39]), both factors taken separately influence the final conformation of the protein molecule in a different way, they certainly help the molecule to find energetically favorable geometry. Thus, the surface-mediated unfolding is sometimes considered as a catalytic process with the substrate (catalyst) lowering the activation energy and allowing the molecules to dissipate the strain energy of their folded state [38]. In consequence adsorption itself involves a negative change in the enthalpy. However, from the results presented here it clearly follows that the insulin adsorption on C_8 bonded phase is an endothermic process with positive values of the adsorption enthalpy and entropy.

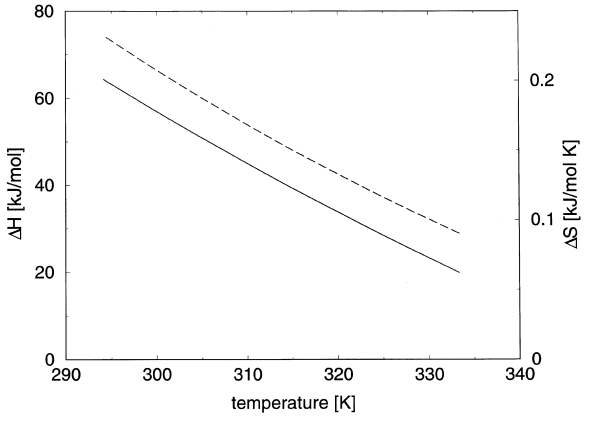

Fig. 10. Changes in enthalpy (solid line) and entropy (dashed line) of adsorption as a function of temperature, calculated for the porcine insulin.

The origin of this behavior can be explained as follows. At moderately low temperatures, the insulin molecule resides on the surface in a partially unfolded state but the rate of unfolding is low. Thus, its adsorption does not induce any dramatic changes in the secondary structure of the protein. Since unfolding is a multistep reaction with several metastable states, in which the molecule may be trapped, complete opening of the insulin molecule can be slowed. On the other hand, since insulin is a large molecule with many contact points, adsorption causes the displacement of many solvent molecules present either on the substrate or at the protein surface. Desorption of these solvent molecules gives rise to an increase of both ΔH and ΔS. The adsorption of the insulin molecule involves also the dissipation of its strain energy, that gives a negative contribution to ΔH. However, this enthalpy change of the system cannot compensate the positive change arising from the desorption of the solvent molecules. Because the overall or TOTAL ΔH is positive, a large increase in the system entropy guarantees that $\Delta G < 0$. In other words, in this step, adsorption is strongly entropically driven. When the temperature is increased, the insulin molecule undergoes continuous unfolding which involves the dissipation of energy and a stronger immobilization of the protein chain. This phenomenon causes negative changes in the entropy and the enthalpy. However, in this temperature range (25–50 °C), the global changes, ΔH and ΔS, still remain positive since the contributions of the desorption of the solvent molecules overweight that of the protein adsorption. It is also probable that a further increase in the temperature would cause a complete denaturation of the insulin molecule and would reduce or stop any further changes in its

conformation. This may lead to a transition of the behavior of the system, from endothermic to exothermic [54].

This hypothesis can be only a simplification, which neglects other possible phenomena associated with adsorption, like the aggregation of insulin molecules, possible multilayer adsorption, steric exclusion, molecular association or the formation of long range interactions [34,56]. More detailed studies of the simultaneous influence of the pressure and the temperature on the retention behavior of proteins in RPLC should provide further tests of the validity of this proposed mechanism.

5. Conclusions

The adsorption of insulin on hydrophobic surfaces is a complex phenomenon which is much affected by external parameters like pressure and temperature. Both the pressure and the temperature affect the adsorption equilibrium and modify significantly the retention behavior of the insulin molecules. Our study proves that the retention times of the different insulin variants increases with increasing pressure. Despite the differences between the primary structures of the variants, the relative influence of the pressure on their retention parameters is nearly the same. It seems justified to admit that the pressure-induced shifts in the adsorption constant are but weakly sensitive to the particular amino acid sequence in the protein chain but that they depend much more on global features of the protein, like its molecular mass/volume ratio, solvation effects, or the surface tension. The observed effect of the pressure is directly related to a negative change in the protein molar volume upon adsorption on a hydrophobic substrate.

Besides the practical independence of the relative pressure-induced change of the retention time on the nature of all the variants studied here, this change is also practically independent of the temperature. The lack of correlation between ΔV_m and the temperature suggests that the adsorption of the insulin molecule involves a first step during which the molecule changes its structure to some limited extent which is slightly (or not at all) affected by the temperature. The results presented here show also that the temperature has a considerable influence on the retention time of insulin. The relative change of k due to an increase of the temperature by 25 °C is up to 300% for all the variants. This influence is, however, more complex than that of the pressure, in which case, ln k is a simple, linear function. The temperature dependence of ln k under constant pressure displays an obviously nonlinear behavior, associated with decreases of the adsorption enthalpy and entropy with increasing temperature.

The endothermic character of the adsorption process of insulin on C_8-bonded silica is the global result of two simultaneous processes with opposite trends. The first one is the desorption of the solvent molecules adsorbed on the substrate (displacement by the protein molecule) or associated with the protein surface. It causes the ΔH and ΔS of the system to increase. The second one involves such phenomena as the opening or spreading of the protein molecule, the dissipation of its strain energy, the exposure of its hydrophobic core to interactions with the surface and/or the solvent molecules, and the immobilization of the protein chain against the surface. this causes both ΔH and ΔS to decrease. However, it seems that, at least in the range of temperatures studied here, the energetic/entropic effects of the first process still overweight the contributions of the latter process. In consequence, positive adsorption enthalpy and entropy are observed.

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