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INTERACTION OF HAEMOGLOBIN AND CYTOCHORME *C* WITH ALIPHATIC ALCOHOLS AS STUDIED BY ^{1}H NMR SPECTROSCOPY*

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Association of haeme proteins, haemoglobin and cytochrome *c*, with eight aliphatic alcohols (methanol, ethanol, two isomeric propanols and four butanols) was studied by ^{1}H NMR spectroscopy.

NMR spectra of alcohols were monitored at 60 MHz at increasing concentration of the proteins. Selective broadening of the NMR signals of individual segments of alcohols was observed only in the case of alcohol-haemoglobin systems. Its quantitative evaluation and interpretation in terms of formation of low affinity intermolecular alcohol — protein complexes led to the conclusion that haemoglobin associates with alcohol molecules in a way depending on the length and isomeric branching of the alkyl chains; in particular, the methylene and methine groups vicinal to the hydroxyl are subject to stronger immobilization than the terminal methyls or other groups. Thus, the model of hydrophobic complexes stabilized by hydrogen bonds described previously for association of bovine serum albumin with alcohols (Lubas *et al.*, Biochemistry, **18**, 4943-4951, 1979) seems to apply also to haemoglobin association.

In the case of cytochrome *c* association, ^{1}H NMR data alone are insufficient for structural evaluation of the mechanism of formation of the alcohol — cytochrome *c* complexes.

The role of aliphatic alcohols as proteins denaturants has been extensively studied. By the use of the classical spectrophotometric and spectropolarimetric techniques it was found [1, 2, 3] that the denaturing effectivity of alcohols increases with the length of their apliphatic chains and decreases with the increase of isomeric branching. To explain these effects it was suggested that intermolecular hydrophobic complexes were formed between the molecules

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of alcohols and the protein macromolecules. The association constants of these complexes were theoretically calculated in 1962 [4] to be of the order of 10^{-2}-10^{-3} M^{-1}. However, it was only as late as in 1975 that Brill *et al.* [5], by the use of equilibrium dialysis and EPR spectroscopy, presented experimental evidence for actual formation of low affinity complexes of methanol and ethanol with proteins.

In this situation we made an attempt to study the mechanism of binding of alcohols to proteins by ^{1}H NMR spectroscopy. Our approach was generally based on the concept that the mode of binding should find a reflection in the NMR spectra of the alcohols as the ligands. First experiments were carried out for the systems: alcohols-bovine serum albumin. Analysis of the observed selective broadening of the individual NMR lines enabled us to construct a model [6] of the alcohol-protein association in which the hydrophobic alcohol-albumin complexes are stabilized by hydrogen bonds between hydroxyl groups of the alcohols and peptide linkages of the protein. Comparison of the ^{1}H NMR data: (a) for monovalent alcohols with those for glycols [6]; (b) for the ternary systems: alcohol-albumin--urea [7]; (c) for alcohol-albumin-sulphonamide systems [8] led to the conclusion that, in the alcohol-protein complexes, the hydrophobic association plays the primary role, and hydrogen bonds – the secondary role.

However, serum albumin is a protein which shows an especially high binding capacity towards various ligands. Therefore, to answer the question how universal and generally applicable is the mixed model of alcohol-protein association, similar NMR experiments with other proteins were necessary. In this paper an analysis of model systems containing two haeme proteins, haemoglobin and cytochrome *c*, is reported.

Haeme proteins were selected for the experiments in view of the fact that they were the first proteins found to form complexes with alcohols [5].

Our ^{1}H NMR studies were carried out with each of the simplest eight alcanols (methanol, ethanol, isomeric propanols and isomeric butanols) in the presence of increasing concentration of haemoglobin or cytochrome *c*. The suggested structure of the alcohol-protein complexes, as well as the details of the ^{1}H NMR procedure used, were described previously [6].

MATERIALS AND METHODS

The proteins used in all experiments were commercial products from the Sera and Vaccines Plant Biomed (Kraków, Poland) and were used without additional purification. Human haemoglobin (series 1/11/79), purified and lyophilized, contained 95% of oxyhaemoglobin. Cytochrome *c* prepared from pig hearts, was dialysed and lyophilized.

The alcohols used were high purity products from POCh (Gliwice, Poland), except 2-butanol (VEB-Chemie, Berlin, G.D.R.) and t-butanol (Reanal, Hungary). ^2H_2O (isotopic purity of 99.8%; Institute of Nuclear Research, Świerk, Poland) was used as solvent for the proteins. The concentration of proteins and alcohols was expressed as percentage, w/v and v/v, respectively.

^1H NMR measurements were carried out on a Varian EM-360 spectrometer at the resonance frequency of 60 MHz. The data presented in Figs. 1, 2, 3 and 4 are average values of 15 measurements (3 individually prepared samples, 5 registrations each).

The sample preparation, choice of NMR signals analysed and linewidth measurements were carried out as described previously [6]. Molecular weight of haemoglobin (65000) for concentration recalculation was taken from Dickerson & Geis [9].

RESULTS AND DISCUSSION

The typical spectral effect of the increasing concentration of protein in aqueous solution on the ^1H NMR spectra of the aliphatic alcohols is a high increase of the linewidth, with no changes in the line positions and the coupling constants. Such an effect, first described for bovine serum albumin [6], was now also observed with haemoglobin and cytochrome *c*.

The alcohol - haemoglobin systems

The dependence of the linewidth of the individual alcohol lines (at alcohol concentration of 2%, v/v) on the concentration of haemoglobin, increasing up to 8% (w/v), is presented for methanol, ethanol and two isomeric propanols in Fig. 1 A - D, and for four isomeric butanols in Fig. 2 A - D. Due to very strong broadening of the lines for 1-butanol and 2-butanol, quantitative analysis of their linewidth had to be limited to haemoglobin concentrations not exceeding 3% (w/v), (Fig. 2, C and D).

The dependence of the increase in broadening of the individual lines of alcohols in the analysed binary systems with haemoglobin was generally very similar to that described previously for albumin [6]. The increase of the linewidth of the alcohols in these systems was consistently strongly dependent on the length of the alkyl chains and on their isomeric branching. For example, in the presence of 8% haemoglobin, the broadening of the methyl proton lines increased in the order:

$$\text{methanol} \sim \text{ethanol} < \text{1-propanol} < \text{isobutanol} \qquad (1)$$

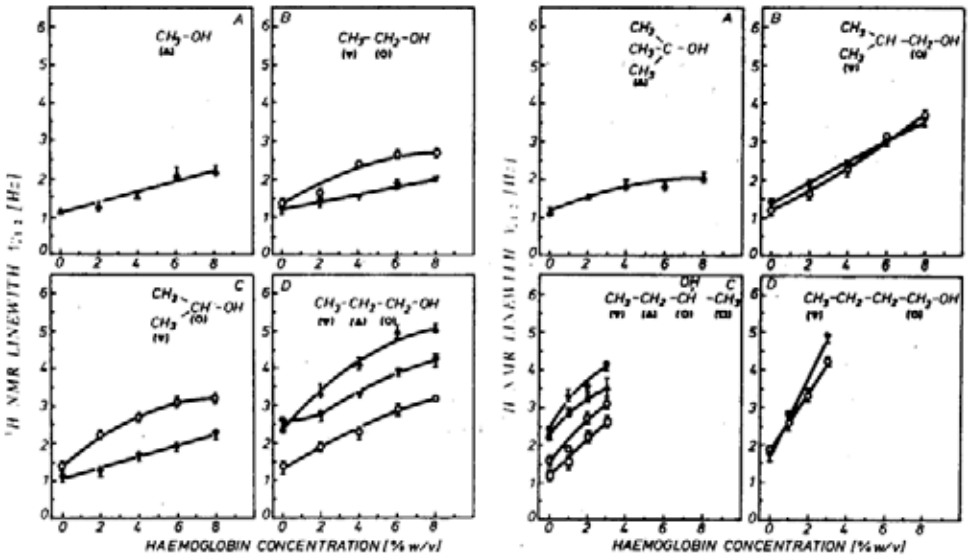

Fig. 1. The increase of halfwidth of the ^1H NMR lines of aliphatic alcohols (2%, v/v) in function of the increasing haemoglobin concentration in ^2H_2O solution, pH 7.0 ± 0.1. A, Methanol; B. ethanol; C, 2-propanol; D, 1-propanol.

Fig. 2. The increase of halfwidth of the ^1H NMR lines of isomeric butanols (2%, v/v) in function of the increasing haemoglobin concentration in ^2H_2O solution, pH 7.0 ± 0.1. A, t-Butanol; B, isobutanol; C, 2-butanol; D, 1-butanol.

with the maximum value of 3.1, whereas the analogous pattern for isomeric butanols at the haemoglobin concentration of 2% (Fig. 2 A - D) was of the order:

$$\text{t-butanol} < \text{isobutanol} < \text{2-butanol} < \text{1-butanol} \qquad (2)$$

with the maximum value of 1.9. Furthermore, analysis of the data for individual aliphatic groups of alcohols (CH_3 and CH_2) points to a strong dependence of broadening selectivity on the location of the respective group with respect to hydroxyls. For the methylene and methine groups located in the closest neighbourhood of $-OH$ groups, selectively stronger broadening was noted for all alcohols except 1-butanol and 2-butanol.

For more precise interpretation of the phenomena observed, the results of relative broadening were recalculated and plotted in function of the alcohol - protein molar ratio (Fig. 3). The obtained plots are presented for three proton groups, according to their location with respect to the $-OH$ groups, i.e.: A, protons in methylene and methine groups vicinal to the hydroxyl; B, protons in terminal methyl groups, mostly separated from the hydroxyl; and C, protons in CH_3 or CH_2 groups other than A and B. An analogous analysis was previously carried out for the alcohol - albumin systems [6].

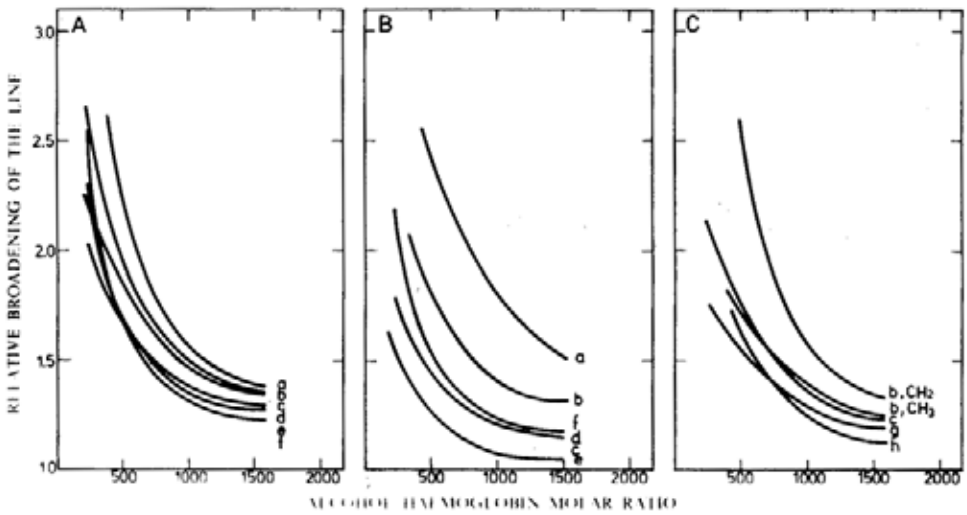

Fig. 3. The dependence of the relative broadening of the ^{1}H NMR lines of aliphatic fragments of alcohols in function of the alcohol - haemoglobin molar ratio. A. Protons in methylene and methine groups vicinal to $-OH$; B. protons in terminal methyl groups mostly separated from $-OH$; C. protons in CH_3 or CH_2 groups other than A and B. a. 1-Butanol; b. 2-butanol; c. 1-propanol; d. ethanol; e. 2-propanol; f. isobutanol; g. t-butanol; h. methanol.

Generally, in the case of haemoglobin the relative line broadening in function of the alcohol - protein molar ratio (Fig. 3) was the higher the longer was the alkyl chain of alcohol, and decreased for more branched isomers. The signals of protons of group A were significantly more strongly broadened as compared with those of group B and C. However, this does not hold for 1-butanol and 2-butanol. Such a picture is, as a whole, essentially similar to that observed earlier for bovine serum albumin [6]. The highest broadening noted for the signals of 1-butanol and 2-butanol is also consistent with the earlier results. Thus, the structural model of the alcohol - protein complexes, proposed previously for bovine serum albumin, seems to be valid also in the case of haemoglobin. The unique quarternary structure of haemogloin, with its hydrophobic pockets promotes the hydrophobic association with alcohols; it seems that this association can be additionally strengthened by hydrogen bonds.

The alcohol - cytochrome c systems

The gradual broadening or the ^{1}H NMR signals of alcohols at cytochrome *c* concentrations increasing over the range 0 - 12% (w/v). (Fig. 4 A - D. Fig. 5 A - D) was generally distinctly lower as compared with the systems

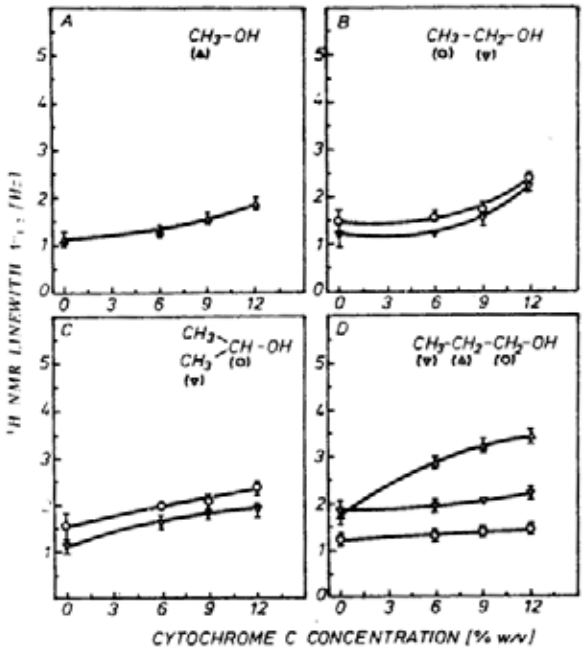

Fig. 4. The changes in linewidth of the ^1H NMR lines of aliphatic alcohols (3%, v/v) in function of the increasing concentration of cytochrome *c* in ^2H_2O solution, pH 6.2 ± 0.1. A. Methanol; B. ethanol; C. 2-propanol; D. 1--propanol.

containing haemoglobin. However, also in the case of this protein, the highest increase of the linewidth was observed for the two analysed lines (CH_3 and CH_2) of 1-butanol. Line broadening for branched butanols was rather lower than for alcohols with shorter aliphatic chains. For methanol ethanol and 2-propanol relative broadening of the lines showed a similar pattern, and at cytochrome *c* concentration of 12% (w/v) reached values of only 1.5 - 1.87.

The data presented in Table 1 can be used for precise comparison of broadening of the selected lines for particular alcohols, in the analysed range of the alcohol - cytochrome *c* molar ratios. As it may be seen, the broadening for protons of group A, i.e., located in the segments vicinal to the hydroxyls, does not appear higher as compared with that noted for protons of other aliphatic fragments.

Generally the broadening of the ^1H NMR lines of alcohols observed in the alcohol - cytochrome *c* systems was much lower than in the systems with haemoglobin. However, except for propanols (Fig. 4 C and D), also in the case of this protein the dependence of line broadening on the length of alkyl chain and isomeric branching was observed. This reflects obviously the restricted mobility of alcohol molecules associated with the protein.

On the other hand, analysis of the line broadening for individual segments of alcohols, taking into account their location with respect to

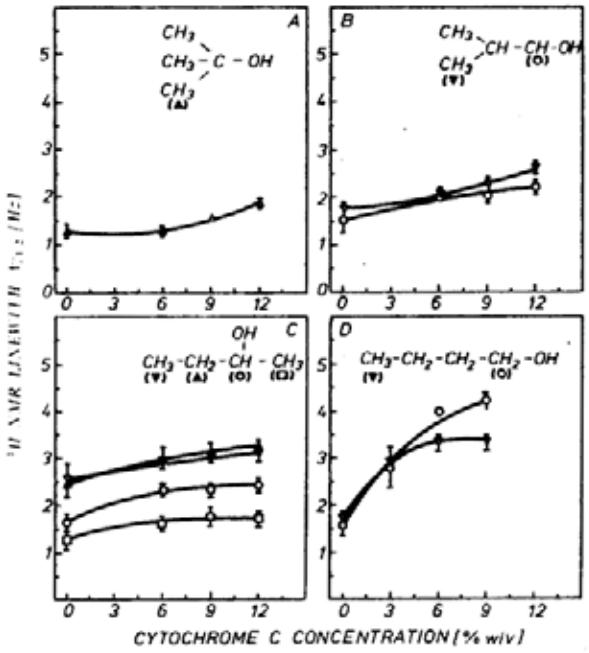

Fig. 5. The changes in halfwidth of the ^{1}H NMR lines of isomeric butanols (3%, v/v) in function of increasing cytochrome *c* concentration in ^2H_2O solution. pH 6.2 ± 0.1. A. t-Butanol; B. iso-butanol; C. 2-butanol; D. 1--butanol.

the −OH group, gave a picture differing distinctly from that observed both for albumin and haemoglobin. In the case of 1-propanol, 2-propanol and ethanol the highest broadening was observed for the signals of groups non-vicinal to −OH, whereas for isobutanol and 2-butanol the differences in broadening between groups A, B and C were very small. Thus, the results seem to suggest formation of a number of non-specific complexes in which hydrogen bonds are probably not involved in stabilization of the hydrophobic association.

The ^{1}H NMR data alone seem to be insufficient for determination of the structure of these complexes.

To summarize, we conclude that the model of the low affinity alcohol - protein complexes proposed previously for bovine serum albumin [6] holds also in the case of haemoglobin. The association between this protein and alcohols also seems to have a hydrophobic character, with additional stabilization by hydrogen bonds. This suggests that such a specific interaction of alcohols with a protein may exist in the case of large globular proteins, with a rather high content of the hydrophobic amino acids. On the other hand, elucidation of the structure of the complexes formed between alcohols and smaller proteins, such as cytochrome *c*, appears to require further experiments on model systems, possibly by techniques differing from ^{1}H NMR spectroscopy.

Table 1

Interpolated values of the relative broadening of the ^1H NMR lines of the aliphatic alcohols for selected molar ratios alcohol/cytochrome c.*

Characteristics of the aliphatic fragments	Molar ratio alcohol/ protein	Methanol	Ethanol	2-Propanol	1-Propanol	t-Butanol	Isobutanol	2-Butanol	1-Butanol
A	40		—	—	—		1.34	1.43	2.68
Methylene and	50		1.87	1.42	1.14		1.30	1.42	2.63
methinine groups	75		1.15	1.29	1.09		1.27	1.41	2.37
vicinal to the	100		1.06	1.23	1.04		1.21	1.38	1.78
hydroxyl group	125		—	—	—		—	—	—
B	40		—	—	—		1.26	1.15	2.03
Terminal methyls	50		2.35	1.63	1.06		1.21	1.12	2.01
mostly distant	75		1.23	1.47	1.02		1.17	1.09	1.88
from the	100		1.04	1.38	1.00		1.14	1.09	1.65
hydroxyl	125		—	—	—		—	—	—
C								CH_2 CH_3	
Group CH or CH_2	40	—			—	1.21		1.37 1.28	
being	50	—			1.42	1.09		1.32 1.25	
intermediates	75	1.65			1.28	1.07		1.20 1.13	
between A and B	100	1.41			1.19	1.00		1.11 1.06	
	125	1.27			—	—		— —	

* From plots of relative broadening *versus* protein concentration analogous as in the case of haemoglobin (Fig. 3)

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