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# CIRCULAR DICHROISM STUDY OF THE INTERACTION BETWEEN CONFORMATIONALLY ALTERED HUMAN SERUM ALBUMIN AND TESTOSTERONE

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**Summary**—Binding ability of testosterone (TEST) on conformationally altered human serum albumin (HSA) in the presence of several concentrations of NaSCN, urea and KCl at various pH was examined qualitatively on the basis of the rotational strength at 303, 273 and 208 nm by means of circular dichroism (CD). The values of the binding index expressed as a ratio of $[\theta]_{303}/[\theta]_{273}$ at each rotational strength in the presence of various concentrations of salt at pH 7.4 were inversely proportional, parallel, and independent of the α-helix content based on the peptide backbone alteration of HSA by urea, KCl and NaSCN, respectively. The values in the presence of a constant concentration of 1.0 M urea or KCl at various pH were dependent on the salt, showing a significant effect of these salts on the binding sites of the amino acid chain rather than the peptide backbone of the protein. It was generally observed that the decrease in α-helix content caused by pH changes tends to decrease the binding ability of TEST to HSA. The decreased binding index value observed in 40 mM NaSCN causing a low α-helix content, suggests that the secondary conformational changes caused by the salt might not be related to the binding ability, in contrast to the results of urea or KCl at different pH. It was clearly demonstrated that the binding ability of TEST to HSA is closely associated with skeletal conformational alterations as well as changes in the binding sites of the amino acid side chains of the protein.

## INTRODUCTION

Hofmeister[1] was the first to recognize specific ion effects on macromolecules, and gave the name "chaotropic salt" to the ions which specifically lead to unfolding, extension and dissociation of proteins [2]. More recently, Sawyer and Puckridge[3] have investigated the use of chaotropic salts, which are known to increase the solubility of nonpolar molecules in water, in studies on protein dissociation. They specifically showed that NaSCN works well at low concentrations which do not cause major shifts in protein conformation. Consequently, in the place of agents like urea, detergent, and guanidine hydrochloride; NaSCN, and KCl have been employed preferably and successfully in the study of the quantitation of nuclear binding sites by a single-step method involving the extraction of estradiol–receptor complex from nuclei and concomitant exchange of exogenous hormone with labeled estradiol [4, 5]. The same salts have been previously used in an exchange assay of estradiol–receptor complex in calf uterus cytosol and have also been used to elute estradiol from an affinity column [6], but failed in the assay of androgen receptor [7]. In addition to these experiments, the interactions or binding of steroids to the receptor protein, as well as an aggregation of the protein itself [6], have been extensively studied not only for their theoretical interest but also for their importance in understanding mechanisms of steroid hormone action. It is generally supposed that the development of steroid action is closely related to the intensity of the interaction of the steroid and the protein. For example, the analogy of androgen [8], estrogen [9], progesterone [10, 11] and glucocorticoid [12] have shown that with few exceptions relative binding affinity corresponding to each receptor protein paralleled steroidal activity. For the examination of this intensity, steroid binding potency has been studied in different ionic strength solutions in the presence of various salts. To clarify the effects of the salts on the interaction of the protein and steroid, it would be very useful to obtain information on the relation between steroid binding ability with conformationally altered protein, as well as side chain and conformational alteration of the protein caused by changes of salt or hydrogen ion concentration. The equilibrium dialysis [13], solubility [14], fluorescence quenching [15] and gel filtration [16] methods described previously, however, are inadequate for determining the effects of the salts on the conformational alteration which is supposed to closely correlate to steroid binding ability. Thus, there is little information available on the relation between conformational alterations and steroid binding ability to the protein. Although circular dichroism appears to be more suitable for monitoring conformational alterations in protein it has not been used for investigation of this complex prior to our study except for some limited measurements on a system containing steroid and serum albumin [17], for

*Abbreviations*: CD: circular dichroism. HSA: human serum albumin. TEST: testosterone.

which the extrinsic Cotton effect had not been reported. HSA was used in this study, because it is extremely difficult to obtain sufficient amounts of the receptor protein.

The present paper addresses the above subject on the basis of the detailed CD spectral behavior of TEST–HSA complex in solutions where pH, and/or concentrations of NaSCN, KCl, and urea are altered. The effects of low NaSCN concentration on secondary protein structure and the binding ability of TEST to HSA were determined.

## EXPERIMENTAL

### *Reagents*

Redistilled, deionized water was used throughout this work. All the other chemicals used were analytical grade reagents and were employed without further purification. Essential fatty acid free human serum albumin and testosterone were obtained from Sigma, St Louis, U.S.A. The albumin concentrations were 13.1 $\mu$M and all solutions contained 0.1% ethyl alcohol. Dilutions of 100 $\mu$M testosterone were made with 1/15 M phosphate buffer and adjusted to the desired pH with 1.0 M HCl or 1.0 M NaOH.

### *Circular dichroism measurements*

CD measurements were made at 25°C with a Union Giken Mark II spectropolarimeter calibrated with (+)-D-camphorsulfonic acid. All spectra were recorded in a square quartz cell with 1.0 or 10.0 nm path length for short or long wavelength using a full scale deflection of 0.02 or 0.2, respectively, and a spectral band width of 5.0 nm. Results are expressed as molar ellipticities, $[\theta](\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1})$, calculated with reference to the HSA concentration, using a mol. wt of 69,000. Each CD spectrum reported is the average of 20 scans for short or 10 scans for long wavelength. The binding index was calculated by the following equation; Index $= [\theta]_{303}/[\theta]_{273}$. Where $[\theta]_{273}$: the molecular ellipticity of 13.1 $\mu$M HSA bound to TEST at 273 nm, $[\theta]_{303}$; the molecular ellipticity of unbound TEST in the presence of 13.1 $\mu$M HSA at 303 nm of CD spectrum. $\alpha$-Helix content was estimated from the molecular ellipticity of the 208 nm band as described elsewhere by Greenfield and Fasman[18]. In observations of extrinsic Cotton effects, it has been reported that no difference larger than the experimental error was observed among the various samples of HSA with respect to CD between monomeric or the unchromatographed HSA, although there have been differences reported in rat and rabbit for their binding affinity [13, 17]. Therefore, essentially fatty acid free HSA from Sigma Chemical Co. was used throughout the experiments without any further treatment.

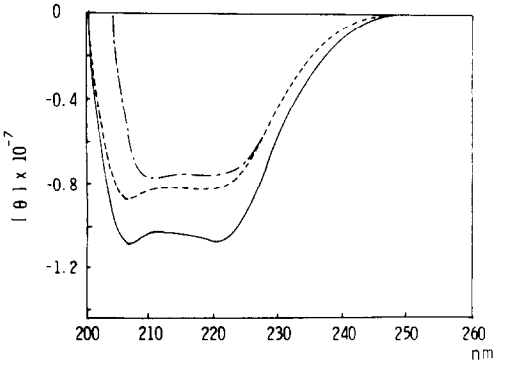


Fig. 1. CD spectra of HSA in 1/15 phosphate buffer in the 200–260 nm region at various pH. 12.0; (—·—), 3.0; (– – – –) and 7.4; (——). CD spectra of HSA at pH 7.4, 4.8, 8.5 and 10.0, and of HSA in the presence of 100 $\mu$M TEST were quite similar to that of 7.4.

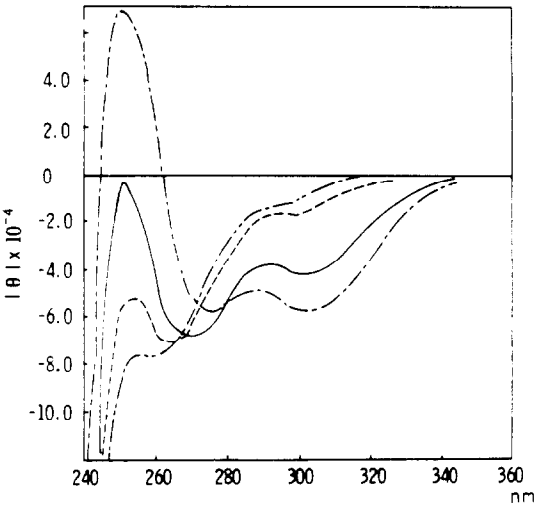


Fig. 2. CD spectra of HSA in the presence of various TEST concentrations in the 240–360 nm region at pH 7.4. The concentration of TEST were 0 $\mu$M; (—·—·—), 20 $\mu$M; (-----), 50 $\mu$M; (——) and 100 $\mu$M; (—·—).

## RESULTS

The interaction and binding of TEST to conformationally altered HSA caused by the changes of several concentrations of NaSCN, urea and KCl at various pH was followed qualitatively by CD spectropolarimeter on the basis of HSA spectra in the wavelength regions of 260–360 nm and of 200–260 nm. CD spectra of HSA alone [19] or in the presence of TEST showed a negative peak with $[\theta] = -1.1 \times 10^{-7}$ of approximately the same rotational strength at 208 nm that is attributable to $\alpha$-helix conformation of the protein (Fig. 1). However, the CD spectrum of the HSA solution in the presence of TEST was composed of two new negative bands with maxima at 273 and 303 nm, in addition to the above 208 nm band. When TEST binds to the protein, the new extrinsic Cotton effect due to the absorption at 273 nm was first observed by this author in the current work, and provided evidence on the basis of CD for a molecular interaction between HSA and TEST. The above is based on the following evidence: Fig. 2 shows CD spectra for HSA alone and

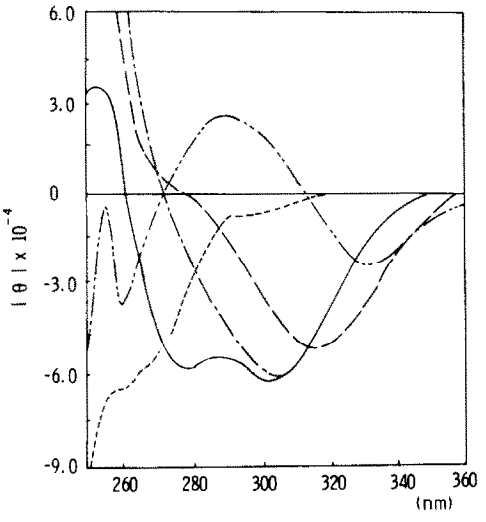


Fig. 3. CD spectra of HSA, TEST and their subtraction in the 250–360 nm region. HSA only; (-----), 100 $\mu$M TEST in MeOH; (——), HSA in the presence of 100 $\mu$M TEST; (——), subtraction of (——) – (-----); (—·—) and of (——) – (—·—); (—··—).

HSA in the presence of TEST at three different concentrations. The intensity of the 273 nm band was not effectively influenced by a concentration of TEST up to 100 $\mu$M, while that at 303 nm was dependent on TEST concentrations in the range from 20–100 $\mu$M. At the lowest concentration (20 $\mu$M) of TEST tested, all TEST in the solution might be essentially bound to the binding site of HSA, and the observed ellipticity was slightly decreased in the 273 nm wavelength region. However, when the concentration was increased to almost 2.5-fold, the ellipticity at 273 nm was almost at the same value, in contrast to that at 303 nm which demonstrated a remarkable negative increase. Furthermore, there was no direct proportional relation between this increase and decrease on rotational strength of HSA with concentrations of TEST greater than 20 $\mu$M. The absorption peak of the protein solution in the presence of 50 $\mu$M of TEST, shifted from 273 nm to the longer wavelength side by about 6.0 nm, in comparison with that at 20 $\mu$M, but kept almost the same ellipticity. On the other hand, the 2.6-fold difference of rotational strength of HSA in the presence of 20 or 50 $\mu$M TEST was observed in the same absorption band at 303 nm. Therefore, it might be concluded that the bands at 273 and 303 nm could be respectively assigned to absorption attributable to a HSA–TEST interaction and free TEST which was left in the solution after the saturation of the binding site on HSA. This assignment of two new absorptions was further confirmed by the difference spectra between HSA alone, HSA with TEST, and TEST in MeOH as shown in Fig. 3. The TEST has positive and negative absorptions in polar solvents like MeOH at 250 and 315 nm, respectively, and the spectrum seen can not be fully ascribed to the extrinsic Cotton effect obtained when TEST was bound to the protein surface. The observed Cotton effect also apparently consisted of two transitions, some of which may cause overlap with same negative signs. The changes produced can be better seen on the difference spectra presented in Fig. 3. Here, the HSA spectrum was subtracted from the spectrum obtained with HSA in the presence of 100 $\mu$M TEST in phosphate buffer. The resulting Cotton effect is roughly superimposable to a CD curve of TEST of the same concentration in MeOH, if we assume that a 12.0 nm shift between both peaks is due to solvent differences [20, 21]. However, for more detailed comparison of differences of the ellipticities between the subtracted and measured curves in MeOH, the above curve was again subtracted from the CD curve of TEST in MeOH, with the resulting band being composed of positive and negative peaks at 290 and 332 nm, respectively. The amplitude at 290 nm is obviously larger than that at 332 nm. This suggests strongly that the CD curve obtained by subtracting HSA from TEST–HSA solution spectra contains the portion of the ellipticity due to the interacting band with HSA and TEST. Evidently, TEST gave a negative Cotton effect with a maximum at 273 noted when bound to the binding site on HSA, and another negative band was attributable to the free TEST absorption as previously mentioned. Figure 3 shows the CD spectrum of HSA in the presence of 100 $\mu$M TEST measured at 240–360 nm at pH 7.4. As was mentioned earlier, this CD curve at pH 7.4 was composed of two negative extrema at 273 and 303 nm which were assigned to the absorptions of interacted HSA–TEST and free TEST respectively. Accordingly, the rotational strengths of 273 and 303 nm bands may be used for the calculation of the binding ability of TEST to HSA. Thus, the index calculated from the ratio of 273 vs 303 nm of each molecular ellipticity might reflect the binding degree of TEST on the HSA molecule having a constant content of $\alpha$-helix under the respective conditions. That is to say, the index suggests the proportion of binding or removal of TEST on HSA may correspond to $\alpha$-helix content. As the basic research for the examination of the relation between $\alpha$-helix content and binding ability of TEST on the conformationally altered HSA, CD measurements of HSA in the presence of 100 $\mu$M TEST in the range 260–360 nm were performed under identical conditions as in the case of the $\alpha$-helix content obtained from the ellipticity at 208 nm as shown in Figs 4–6. Also, the plot of the index calculated from curves in Figs 4–6 and of $\alpha$-helix content vs pH at a constant concentration of salts are presented in Fig. 7. In Fig. 7, the binding index at various pH can be compared with $\alpha$-helix content in the absence or presence of 40 mM NaSCN, 1.0 M urea or 1.0 M KCl. The index obtained in the absence of salt showed a constant value just like the $\alpha$-helix content at the region in pH 4.8–10.0, whereas under more acid or alkaline conditions, it was inversely

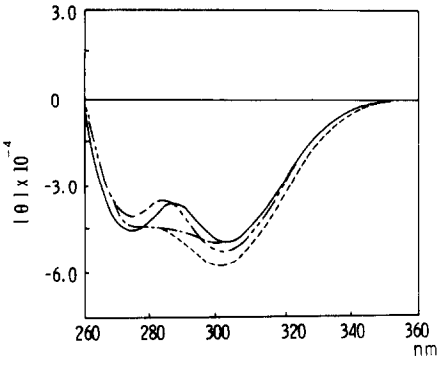


Fig. 4. CD spectra of HSA in the presence of 100 $\mu$M TEST and 40 mM NaSCN in the 260–360 nm region at various pH. pH conditions of each curve were the same as in Fig. 1 except for pH 4.8; (– – –). CD spectra of pH 8.5 and 10.0 were quite similar to that of pH 7.4.

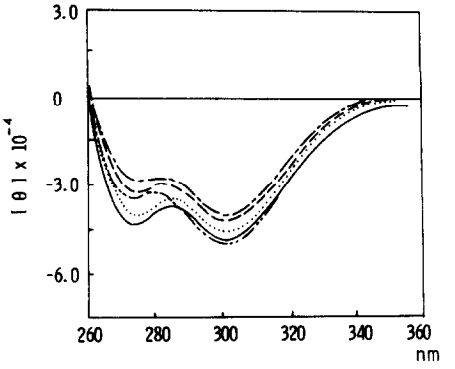


Fig. 6. CD spectra of HSA in the presence of 100 $\mu$M TEST and 1.0 M KCl in the 260–360 nm at various pH. pH conditions of each curve were the same as in Fig. 5 except for pH 8.5; (· · · · · · · ·).

proportional to the α-helix content. That is to say, the titration curves of both α-helix content and the binding index vs pH are highly symmetrical in this case, suggesting a relationship of inverse proportionality on secondary structure and the binding ability of TEST on the protein. On the other hand, the binding index in the presence of 1.0 M urea or KCl was significantly more dependent on the hydrogen ion concentration than the effect of those salts on the HSA conformation in the respective pH. However, the index curve of NaSCN at 40 mM exhibited remarkably low values above pH 7.4 and moderately low below pH 7.4, indicating TEST binding on the protein, in spite of α-helix content as low as one half, as compared to the case of no-salt in all ranges of pH. This effect of NaSCN on the index might not relate to the α-helix content of the HSA as seen in no-salt, because in the TEST–HSA solution in the presence of up to 6.0 M salt the extrinsic Cotton effect based on the interaction of TEST and HSA was still observed clearly at 273 nm. Additionally, to make sure that NaSCN of low concentration modifies the conformation of HSA, the effect of NaSCN on α-helix structure was investigated using poly-L-lysine at pH 11.0, at which conditions it demonstrates one hundred percent α-helix conformation. The effect of millimolar concentrations of the salt on conformation as contrasted to urea and KCl will be presented in a separate paper. Therefore, it seems reasonable to assume that the low value of the binding index in the presence of NaSCN may not be responsible for the decreased α-helix content. The CD curves of HSA in the presence of 100 $\mu$M TEST with NaSCN or urea of different concentrations at a constant pH of 7.4 are presented in Figs 8 and 9. The salt concentrations were varied from 0 to 6.0 M for NaSCN and urea respectively. By increasing the NaSCN or urea concentrations, the ellipticity at 273 nm decreased progressively with a corresponding negative increase at 303 nm. However, there was a quite different effect of salts on the α-helix content as shown in Fig. 10. Both the plots of the binding index and α-helix

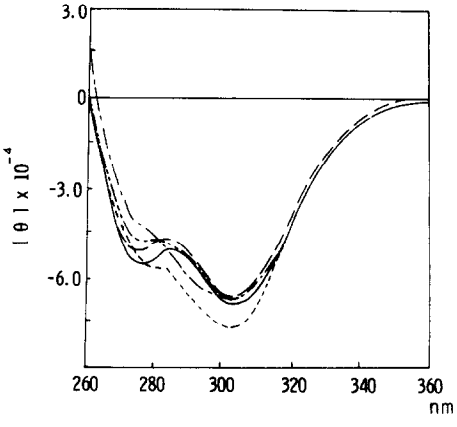


Fig. 5. CD spectra of HSA in the presence of 100 $\mu$M TEST and 1.0 M urea in the 260–360 nm at various pH. pH conditions of each curve were the same as in Fig. 4 except for pH 10.0; (– –). CD spectrum of pH 8.5 was quite similar to that of pH 7.4.

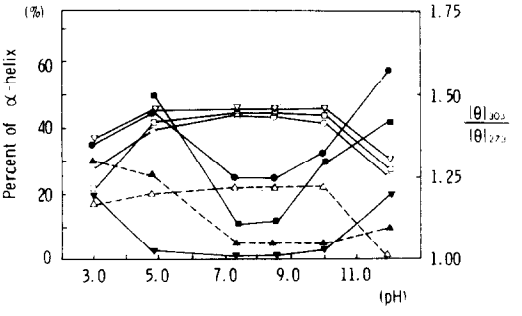


Fig. 7. The plots of α-helix content and the binding index vs various pH. Left ordinate; α-helix content (%) calculated by the elliptical strength of HSA at 208 nm in the absence or presence of salt. No-salt; (▽), 1.0 M urea; (○), 40 mM NaSCN; (△) and 1.0 M KCl (□). Right ordinate; the binding index estimated by a ratio of $[\theta]_{303}/[\theta]_{273}$ at respective ellipticity in the coexistence of 13.1 $\mu$M HSA and 100 $\mu$M TEST under various salt. no-salt; (▼), 1.0 M urea; (●), 40 mM NaSCN; (▲) and 1.0 M KCl; (■).

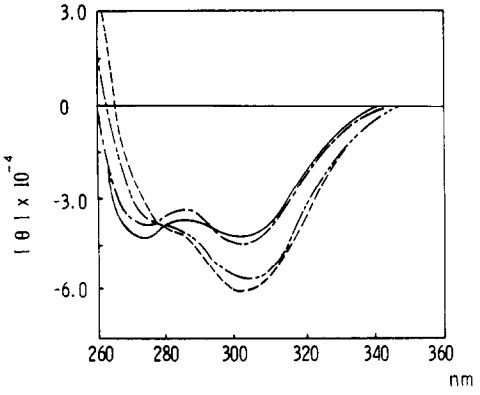


Fig. 8. CD spectra of HSA in the presence of 100 $\mu$M TEST and various urea concentrations in the 260–360 nm region at pH 7.4. 0 M; (——), 1.0 M; (—·—), 3.0 M; (——··——) and 6.0 M; (-----).

content vs NaSCN concentration did not display any relationship between TEST binding ability and conformational alteration of HSA. A dramatic decrease of α-helix content was demonstrated with slight increases of the salt, but the presence of urea revealed the removal of TEST from the binding site on the protein to be inversely proportional to the decrease in α-helix content caused by increasing concentrations of the salt. In contrast to the cases of NaSCN and urea, the titration curves of the α-helix content and the binding index were not at all dependent on KCl concentration.

## DISCUSSION

Sodium thiocyanate, a chaotropic salt, known to inhibit receptor aggregation [6], increased turnover of estrogen receptor complex at 4°C [5]. This effect was successfully applied to the exchange assay of estrogen receptor at low temperature [4]. The concentrations used of NaSCN in their studies were mainly the ranges from 0.1 to 0.5 M. However, the destructive effect of millimolar concentrations (Figs 7 and 10) of NaSCN on the conformation of HSA was quite different from the results of Sawyer *et al.*[3] showing that the salt did not cause a major conformational alteration of a protein to be grounded the reason on their receptor investigations. Among these salts tested in the present study, the distortion power of NaSCN of 40 mM on the conformational stability of the peptide backbone was undoubtedly several hundred times stronger than that of the other salts (Fig. 10). Therefore, binding ability of TEST on conformationally altered HSA in the presence of several concentrations of NaSCN at various pH was qualitatively examined and compared with urea and KCl on the basis of the rotational strength of CD spectra. The values of the binding index of TEST and HSA at pH 7.4 were independent, inversely proportional and parallel to the α-helix content based on the conformational alteration of HSA by NaSCN, urea and KCl, respectively. α-Helix content of HSA in the presence of 40 mM NaSCN at pH 7.4 dropped to about a half as compared with no-salt, but the effect of the conformational changes on the binding index was not observed under the same conditions (Fig. 7). On the other hand, the binding index in the presence of 320 mM NaSCN or above increased gradually with the increase in the concentration of the salt (Fig. 10). From the facts described above, it became evident that NaSCN concentrations of a range from 0.1 to 0.5 M used in the receptor investigations were closely associated with the conformational alterations as well as the binding ability of TEST on HSA, and that the conformation is an important factor of the interaction of HSA and TEST.

The advances in both the instrumentation for CD and the interpretation of ellipticity have reached a stage where this technique becomes very useful in probing the conformation of macromolecule [22–24], as well as the interaction between the protein and drugs in the solution [25–28]. The CD, thus, is a convenient and efficient technique for studies of the interaction of TEST with conformationally altered

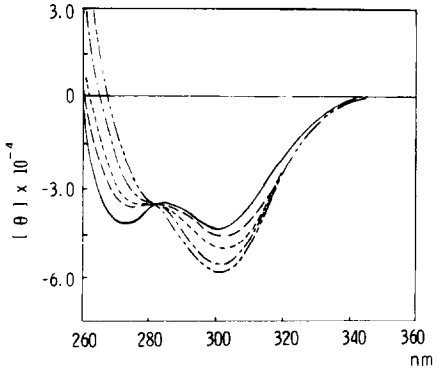


Fig. 9. CD spectra of HSA in the presence of 100 $\mu$M TEST and various NaSCN concentrations in the 260–360 nm region at pH 7.4. 0 M; (——), 0.32 M; (——), 1.0 M; (-----), 3.0 M; (—·—) and 6.0 M; (——··——).

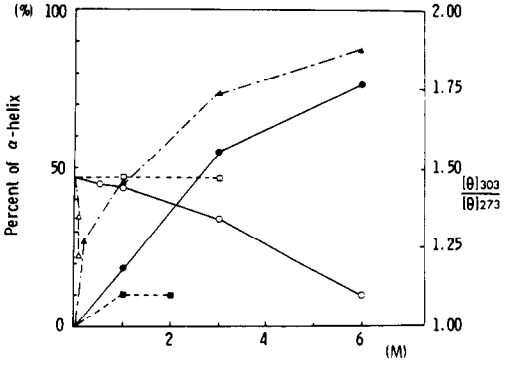


Fig. 10. The plots of α-helix content (%) and the binding index vs various salt concentrations at pH 7.4. Representations of left and right ordinate and symbols were same as in Fig. 7.

proteins. The asymmetric conformation around the chromophore is changed by the interaction, which theoretically can lead to differences in the extrinsic Cotton effect. TEST gave rise to a negative extrinsic Cotton effect at 273 nm when bound to a side chain on HSA. This is the first report demonstrating an extrinsic Cotton effect of different rotational strength when the concentration of TEST or salt changes. The differences in strength make it possible to study the specific events taking place on HSA. However, the lesser effects of TEST on the helical conformation of HSA at 208 nm (Fig. 1) agreed essentially with spectra reported for BSA by Atallah N. *et al.*[15], and Ronald T. *et al.*[17] giving fluorescence quenching data and CD spectra, respectively. Ronald *et al.* also suggested that there is no change in the asymmetry of the molecular environment of the steroid even in its new, less polar environment, at least to the extent detectable by CD measurement. The effect of TEST on $\alpha$-helix content was ignored in the calculation of binding index. The conformation of the polypeptide main chain in the case of no-salt is not altered by TEST as mentioned above, but the environmental changes induced by the interaction of the steroid with the amino acid side chain of HSA is attributable to the extrinsic Cotton effect at 273 nm, since the difference spectra of u.v. has shown that the $\alpha$, $\beta$-unsaturated ketone group of TEST has bound to a less polar environment in the BSA molecule, and the fluorescence quenching data suggested a close approach of the steroid to a tryptophan residue in BSA [15]. The high values of the binding index correspond to the removal of bound TEST on HSA and this is observed on both sides of isoelectric point, showing inverse proportionality to $\alpha$-helix content as shown in Fig. 7. The lack of any detectable differences in the value at pH 12.0 and 3.0 indicates that a potently interacting group with TEST, such as an aromatic residue, is perturbed equally by the rupture of intramolecular hydrogen bonds formed between amino and carboxyl groups at both pH. However, the index was remarkably dependent on pH in the presence of KCl, and not on the concentration (Figs 7 and 10). The different effect of KCl on the binding ability is due to the number of neutralized groups which might drive out TEST from the binding site of HSA, since an increase of these groups will give KCl closer access to the interacting amino acid moiety on the side chain of the protein, or to a hydrophilic site of TEST. In contrast to the case of KCl, the binding value in the presence of urea is dependent on both the concentration and pH. The large binding index as compared with no-salt is due to side chain alteration rather than the conformational change of the peptide back bone in HSA, since pH-dependent $\alpha$-helix content is quite similar in magnitude to the no-salt value.

The binding ability is inversely proportional to $\alpha$-helix content with increasing salt concentration. This means that the binding ability of TEST to HSA is closely related to secondary conformational alteration in the protein. High urea concentration suggests a conformational change which exposes more hydrophobic surfaces thus increasing the interactive possibilities and it would explain the effect of urea on the binding index. The exposure of the hydrophobic binding sites in the unfolded protein may compete with TEST for urea at these sites. Atallah N. A.[15] showed that the marked decrease of fluorescence quenching of albumin by TEST in 6.0–8.0 M urea may be attributed to interaction with urea, to complete displacement of the steroid molecules by urea, to an alteration in the hydration state of the protein, or a combination of the above.

The results obtained with NaSCN present quite a different pattern than the cases of KCl and urea. That is to say, the changes in the $\alpha$-helix content caused by the remarkable change in the peptide back bone of the HSA molecule due to 40 mM NaSCN is a steady state phenomenon regardless of pH changes, and is almost one half the value in comparison to the others. In spite of this lesser $\alpha$-helix content, the binding index is very low except under acidic conditions which serve to create a bulky complex like $NH_3^+SCN^-$ leading to conformational disturbance [29]. This suggests that the exposure of the hydrophobic moiety induced by the conformational alteration of the peptide back bone of HSA is not directly correlated with the binding ability. $\alpha$-Helix content dropped dramatically with slight increases in salt concentration, and was almost 0 at 40 mM. Nevertheless, the plots of the binding index corresponding to NaSCN concentration used for a $\alpha$-helix estimation exhibit a plateau line (data not shown) indicating continuous good attachment of TEST on HSA. There appears to be a preferential effect of NaSCN on the skeletal peptide rather than on the side chain, such as the binding sites of the protein. This characteristic effect of low concentrations of NaSCN might be useful in estrogen receptor assays and as probe in other steroid receptor research. The effect of the salt concentration on the binding index is quite similar to that of urea, indicating only an action upon the TEST binding sites of the protein.

Generally, the binding index between TEST and HSA in the presence of various salts is less dependent on pH at higher salt concentrations, but is dependent on pH at lower concentrations of these salts .

These results show that CD is well suited for investigations of the relationship between the steroid and the conformationally altered protein caused by varying salt concentrations. Studies on the interaction of the binding of TEST on conformationally altered HSA caused by pH changes in the presence of various concentrations of those salts, has provided useful information in the several respects. It was especially useful in determining which conformational changes of the side chain or skeletal peptide were mainly responsible for the TEST–HSA interactions.

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