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Effect of Inorganic Salts on Hemochromogen Aggregation[1]

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The absorbance and difference absorbance spectra of pyridine–hemochromogen at different concentrations showed the presence of two different types of pyridine–hemochromogens, one unstable form (compound III) at low concentration and a more stable form (compound II) at higher concentrations, suggesting that there is an interaction between hemochromogen molecules at high concentration. In the presence of inorganic salts, the unstable compound III is converted to stable compound II. The effect of various inorganic ions on the formation of compound II has been studied. The order of increasing effectiveness of the anions on compound II formation was $HPO_4^{2-} > SO_4^{2-} > F^- > Cl^- > Br^- > NO_3^- > SCN^-$ and that of cation was $Li^+ > Na^+ > K^+$. The results are discussed on the basis of the effect of these ions on the structure of water. It appears compound II is an aggregate of compound III and the aggregation is due to hydrophobic interaction.

INTRODUCTION

During the titration of heme with pyridine in alkaline aqueous medium, the existence of three types of pyridine–hemochromogens was reported by Smith (*1*). A detailed study of the conditions for the optimum development of each of these compounds and their spectral characteristics was carried out by Gallagher and Elliott (*2*). At higher pyridine concentration, the classical pyridine–hemochromogen (compound I) was formed as the final product of the titration, whereas, at very low concentration of the ligand, in a medium of low ionic strength, an unstable pyridine–hemochromogen (compound III) was formed, which, on standing, gradually changed to another compound (compound II) with different spectral characteristics from those of compound III. Gallagher and Elliott (*2*) suggested that compound II is an aggregate of compound III, as the formation of compound II is enhanced and becomes almost instantaneous under conditions favorable to hydrophobic bonding. According to Kauzmann (*3*), the structure of water in the close proximity of the apolar groups of molecule, in an aqueous medium, plays an important role in the adherence of these groups. This type of aggregation is mainly due to the formation of hydrophobic bonds, rather than the van der Waals forces between the groups. Changes in the structure of water can be created by adding inorganic ions which decrease the polarity of surrounding water. Hanstein et al. (*4*) have demonstrated the use of a series of inorganic ions in the destabilization of biomembranes and multiprotein complexes. They have arranged these ions

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in the order of their increasing ability to disorder water, i.e., their chaotropic potency. This consideration prompted us to use inorganic salts in the study of aggregation of pyridine–hemochromogen molecules as an additional argument favoring (or disfavoring) hydrophobic bonding as the basis for compound II formation.

EXPERIMENTAL

Materials and Methods

Hematin was obtained from Sigma Chemicals. Fresh stock hematin solutions (10^{-4} *M*) were prepared every 4 hr in 0.02 *M* NaOH. All concentrations are given on the basis of a dimeric heme (2 atoms of iron/mol of heme). Compound III was prepared (*2*) by reducing alkaline hematin with the minimum possible amount of sodium dithionite and then adding pyridine to give a final concentration of 0.02 *M*.

Final concentration of the salt being added in each experiment was kept at 0.01 *M* unless otherwise stated. Absorption spectra were recorded in quart cuvettes by use of a recording spectrophotometer (Cary 14, Varian Instruments).

RESULTS

Preparation of Compound III and Compound II

Compound III is prepared by reducing 10 μM alkaline hematin (in 0.02 *N* NaOH) with the minimum possible amount of sodium dithionite and then adding pyridine to give a final concentration of 0.02 *M*. On standing 10 min, this compound gradually changes to compound II, but on addition of a small amount of LiCl, compound II formation becomes instantaneous (Fig. 1), shifting the α peak from 555 to 562 nm and the Soret peak from 415 to 432 nm.

FIG. 1. Conversion of compound III into compound II. Curve A represents freshly formed compound III, and curve B was obtained with the same sample 10 min later. Curve C shows the spectrum of same sample immediately after the addition of 0.01 *M* LiCl.

Concentration Effect

Absorbance spectra of pyridine–hemochromogen were taken at concentrations of 5–50 μM using cells of path lengths 10–1 mm and keeping the product of concentration and path length constant. Figure 2 shows that at low concentrations only compound III is formed, and, as the concentration of hemochromogen is increased, there is

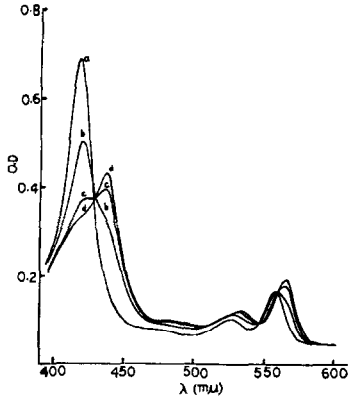

FIG. 2. Absorbance spectra of pyridine–hemochromogen at different concentrations. The concentrations of pyridine–hemochromogen and path lengths of the cells were: (a) 5 μM and 10 mm; (b) 10 μM and 5 mm; (c) 25 μM and 2 mm; (d) 50 μM and 1 mm.

an increase in the formation of the more stable form of pyridine–hemochromogen, compound II. The difference absorbance curve in Fig. 3 shows the magnitude of deviation from Beer's Law due to the change in aggregation.

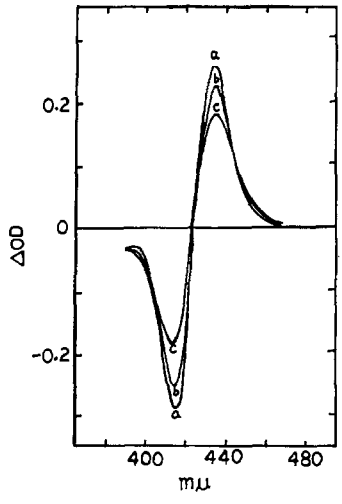

FIG. 3. Difference absorbance curves on inversely varying concentration and path length. The reference beam was 1 μm × 50-mm path length. The sample beams were: curve a, 50 μM × 1-mm path length; curve b, 25 μM × 2-mm path length; curve c, 10 μM × 5-mm path length.

Effect of Inorganic Salts

It is evident from Fig. 1 that the presence of an inorganic salt in solution favors the formation of compound II. The effect of a series of inorganic salts on compound II formation has been studied. The order of increasing effectiveness of the anions on compound II formation is shown in Fig. 4 (only the Soret region of the spectra is shown for convenience) as $HPO_4^{2-} > SO_4^{2-} > F^- > Cl^- > Br^- > NO_3^- > SCN^-$. In Fig. 5, the

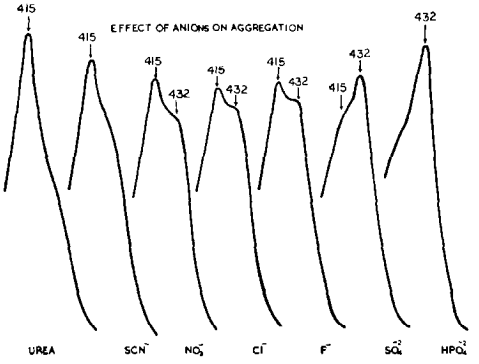

FIG. 4. Effect of anions on compound II formation. In each case the potassium salt concentration was 0.01 *M*.

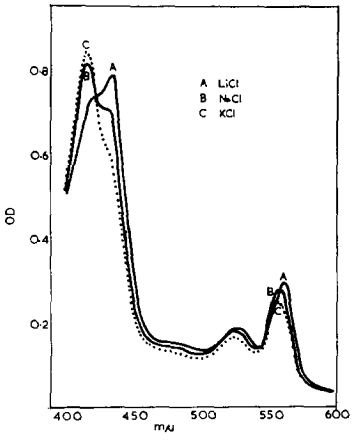

FIG. 5. Effect of cations on compound II formation. In each case the salt concentration was 0.01 *M*.

effect of cations on compound II formation is shown; Li^+ ion favored compound II formation most, whereas K^+ favored it the least. The compound II formation was also observed to increase with increasing salt concentration. Figure 6 shows the effect of potassium fluoride concentration on compound II formation.

DISCUSSION

The concentration effect, as followed by direct recording of the difference in absorbance, indicates that there is an interaction between the hemochromogen molecules at

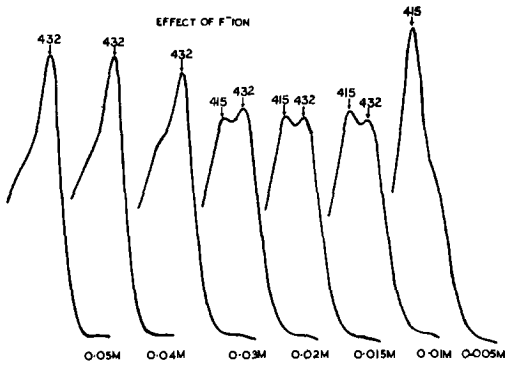

FIG. 6. Effect of concentration of KF on compound II formation.

high concentrations resulting in the formation of a more stable form of pyridine–hemochromogen in agreement with the findings of Gallagher and Elliott (*2*) based on absolute spectra. We have observed that certain inorganic ions have a considerable effect on compound II formation, which may be explained on the basis of the chaotropic potency of these ions. Generally, large monovalent ions with low charge densities are chaotropic, whereas small or divalent ions with high charge densities are "antichaotropic." Unlike chaotrops (SCN^-, NO_3^- etc.), the antichaotropic ions (HPO_4^{2-}, SO_4^{2-}, F^- etc.) have a very strong salting-out effect on nonelectrolytes (*5*) and have very small entropies of aqueous ions (*6*) and are considered as the water-structure-forming ions. The instantaneous conversion of compound III into compound II by antichaotropic ions seems to be due to the ordering effect of these ions on aqueous phase and consequent strengthening of hydrophobic attraction between the hemochromogen molecules. Our observations of cationic effect may also be explained on the above basis that Li^+, being small with high charge density, is more water-structure-forming than large K^+ with low charge density. Our results are in agreement with the findings of Davis and Hatefi (*7*) on the effects of chaotropic ions on the resolution and antichaotropic ions on the reconstitution of water insoluble multiprotein complexes. Therefore, the action of chaotropic ions on the aggregation of hemochromogen molecules is another argument that the stabilizing force for hemochromogen aggregates is hydrophobic bonding.

It is considered unlikely that μ-oxo dimer formation has occurred in these experiments as no evidence for the formation of oxidized products such as parahematin has been noted in the spectra. Therefore, the dithionite-produced anaerobiosis has prevented or minimized μ-oxo dimer formation in these experiments.

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