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Some Spectral Properties of the Human Hemoglobin–Haptoglobin Complex*

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ABSTRACT: Circular dichroism spectra, electron spin resonance spectra, and the action of dithionite to the heme of hemoglobin–haptoglobin (Hb–Hp) complex are described. A negative circular dichroism band at the Soret region which is observed in oxygenated, ferric, and deoxygenated derivatives of adult hemoglobin (Hb) is absent in the corresponding derivative of the Hb–Hp complex. The positive maximum of the circular dichroism spectra at the Soret region of the oxygenated and carbomonoxy Hbα–Hp complex is located at a wavelength longer than that of the corresponding derivative of Hbα chains. The electron spin resonance spectrum of the ferric Hb–Hp complex is indistinguishable from that of ferric Hb. After treatment of the oxygenated or ferric Hb–Hp complex with dithionite under aerobic conditions, an absorption band at 633 mμ and roughly a 10% decrease in absorption at 557 mμ of reduced pyridine–protochemochromogen are observed. Under the same conditions, both oxygenated and ferric adult Hb do not show an absorption band at around 630 mμ. The possibility that the absorption band at 633 mμ is due to the formation of a choleglobin-like substance is discussed.

The data suggest that the heme environments of HbA and Hbα subunits are altered by the binding of Hp and that the heme in the Hb–Hp complex is more labile to the action of dithionite under aerobic conditions than the heme in HbA.

Haptoglobins (Hp) are plasma glycoproteins which have the properties of binding Hb stoichiometrically *in vivo* and *in vitro* (Jayle and Moretti, 1962). All three common genetic types of human Hp consist of an equal number of α and β chains linked by interchain disulfide bond (Smith *et al.*, 1962; Connell *et al.*, 1962) and can bind Hb in the ratio of half a molecule of Hb to one α and one β chain (Hamaguchi, 1969). In the formation of the Hb–Hp complex the β chain of Hp appears to be involved with the globin moiety of Hb (Gordon and Bearn, 1966; Gordon *et al.*, 1968; Giblett, 1968) and the kinetic measurements indicate that the interaction between Hb and Hp is very rapid (Nagel and Gibson, 1967). Most of the properties of the Hb–Hp complex to which heme moieties are essential are similar to those of the isolated α and β subunits of HbA rather than to those of HbA: the Hb–Hp complex shows a high affinity for oxygen, no heme–heme interaction, no Bohr effect (Nagel *et al.*, 1965), broad and flat absorption band at the Soret region of deoxy form (Chiancone *et al.*, 1966), and a high combination rate of reaction with carbon monoxide (Nagel and Gibson, 1966). However, the Hb–Hp complex has much higher peroxidase activity than HbA and isolated Hb chains (Jayle and Moretti, 1962; Smith and Beck, 1967).

In order to obtain information on the local environmental structure of the heme chromophore and the spin state of ferric iron electrons of heme in the Hb–Hp complex, circular dichroism spectra of various derivatives of the Hb–Hp complex and electron spin resonance spectra of ferric derivatives of the Hb–Hp complex were examined with particular reference to comparison with those of Hb. The action of dithionite on the heme of the Hb–Hp complex was also studied.

Preliminary report of these studies has appeared (Hamaguchi *et al.*, 1969).

Materials and Methods

Unhemolyzed adult human blood containing acid–citrate–glucose was used as the starting material. Whole blood

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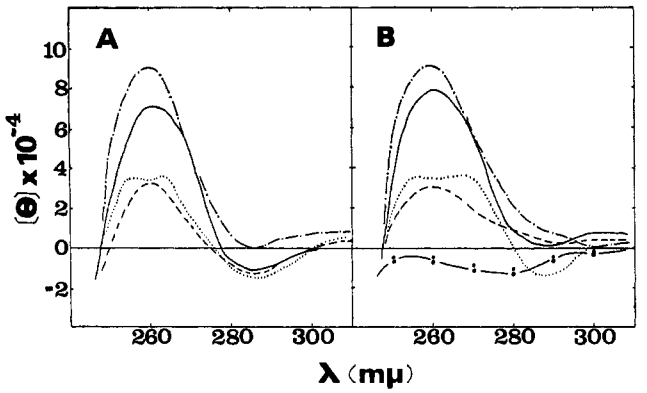

FIGURE 1: Circular dichroism spectra between 245 and 310 mμ of the Hb–Hp complex and adult Hb. (A) Hb–Hp complex: oxygenated derivative (type 2-1), ———; carbomonoxy derivative (type 2-1), —·—; ferric derivative (type 2-1), – – – –; deoxygenated derivative (type 2-2), ·····. (B) Adult Hb: oxygenated derivative, ———; carbomonoxy derivative, —·—; ferric derivative, – – – –; deoxygenated derivative, ·····. Hp: type 2-1, —○—; type 2-2 —●—.

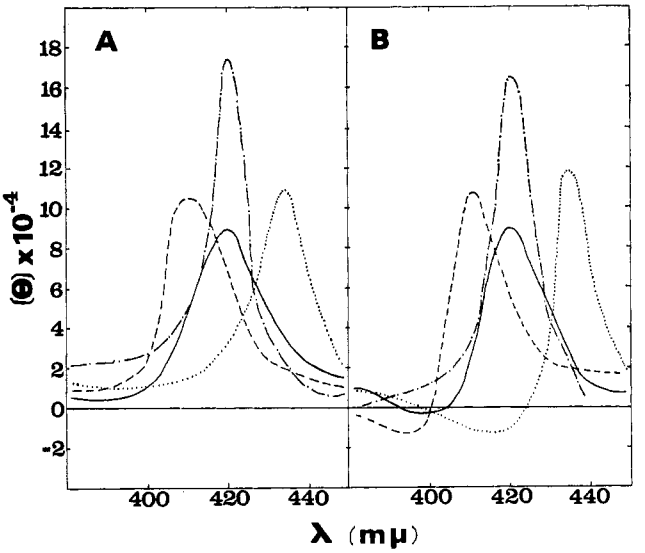

FIGURE 2: Circular dichroism spectra in the Soret region of the Hb–Hp complex (A) and adult Hb (B). The type of Hp used is same as in Figure 1. Oxygenated derivative, ———; carbomonoxy derivative, —·—; ferric derivative, – – – –; deoxygenated derivative, ·····.

was allowed to stand for several days at 4° until the cells were well settled, and the plasma was aspirated. Human adult oxygenated Hb was prepared by the method of Drabkin (1946), but without employing $AlCl_3$. Human Hp 2-1 and 2:2 and the oxygenated Hb–Hp 2-1 and 2-2 complex were purified as described elsewhere (Hamaguchi, 1969). These samples were dialyzed against 50 mM phosphate buffer (pH 7.0) for 12 hr at 4°. Isolated oxygenated Hbα chains were obtained through the courtesy of Dr. Hwang of the Department of Physiology, School of Medicine, Osaka University. The oxygenated Hbα–Hp 2-2 complex was obtained by the addition of the oxygenated Hbα chains to the purified Hp 2-2 to 50% saturation of its Hb binding capacity (HbBC). Carbomoxy derivatives were prepared by bubbling a gentle stream of carbon monoxide gas through the oxygenated derivative solution for 5 min. Ferric derivatives were obtained from the oxygenated derivatives by the addition of a fivefold excess of potassium ferricyanide followed by exhaustive dialysis against 50 mM phosphate buffer at pH 6.5 at 4°. Deoxygenated derivatives were prepared by stirring the oxygenated solution under a stream of nitrogen gas at room temperature, and deoxygenated solution thus obtained was slowly injected into a sealed cuvet previously flushed with nitrogen gas. It took at least 3 hr to obtain the deoxygenated Hb–Hp complex by this method.

Circular dichroism measurements were performed at 20° on a Jouan dichrograph. The heme concentration of the sample varied from 50 to 150 μM. A cuvet having a path length of 1 mm was used for the measurements in the near-ultraviolet region and in the Soret region, and a cuvet having a path length of 10 mm was used in the 500–600-mμ region. Molar ellipticities were given on a heme basis. The ellipticity of Hp was calculated from the HbBC of Hp. Electron spin resonance measurements were taken with a Varian Model 4500 spectrometer equipped with a 100 kc field modulation and a variable-temperature attachment.

The experiments to test the action of dithionite to the Hb–Hp complex and adult Hb were carried out at room temperature as follows. In the case of aerobic conditions, a few milligrams of dithionite was added to 2 ml of the oxygenated or ferric samples, and the reaction mixture was shaken gently in a test tube exposing it to atmospheric oxygen for 1 min. In the case of anaerobic conditions, the oxygenated or ferric Hb–Hp complex was placed in a Thunberg-type cuvet and evacuated for a few minutes. The ligand states of the samples were the same as before evacuation as evidenced by their spectral properties. A few milligrams of dithionite was added to the evacuated sample, and the reaction mixture was shaken gently for 1 min. Optical spectra were taken with a Cary Model 14 spectrophotometer.

All measurements were performed in a 50 mM phosphate buffer (pH 7.0) except in the case of ferric derivatives which were tested at pH 6.5. After conversion to reduced pyridine–hemochromogen in 0.1 N NaOH, the concentration of heme was calculated from the absorbance at 557 mμ using the millimolar extinction coefficient for the reduced pyridine–hemochromogen of 34.7 (Paul *et al.*, 1953). The HbBC was determined by polyacrylamide gel electrophoresis on a mixture of increasing amounts of Hb maintaining constant volumes in the Hp samples. A benzidine stain was used.

Results

Circular Dichroism Spectra. Figure 1A shows the near-ultraviolet circular dichroism spectra of oxygenated, ferric, carbomonoxy, and deoxygenated derivatives of the Hb–Hp complexes and Figure 1B shows those of the corresponding derivatives of adult Hb and Hp 2-1 and 2-2. The circular dichroism spectrum of the Hb–Hp complex is similar to the sum of the circular dichroism spectra of the corresponding derivative of adult Hb and Hp excepting that the intensity of the negative band near 285 mμ of the deoxygenated Hb–Hp complex is much smaller than the sum of those of deoxygenated Hb and Hp.

Figure 2 presents the circular dichroism spectra in the Soret region of oxygenated, ferric, carbomonoxy, and deoxygenated derivatives of the Hb–Hp complex (Figure 2A) and adult Hb (Figure 2B). The circular dichroism curves of adult Hb described here are similar to those of HbA reported by Geraci and Li (1969). The only pronounced difference is that the maximum intensity of the deoxy form reported previously (Geraci and Li, 1969) is much stronger

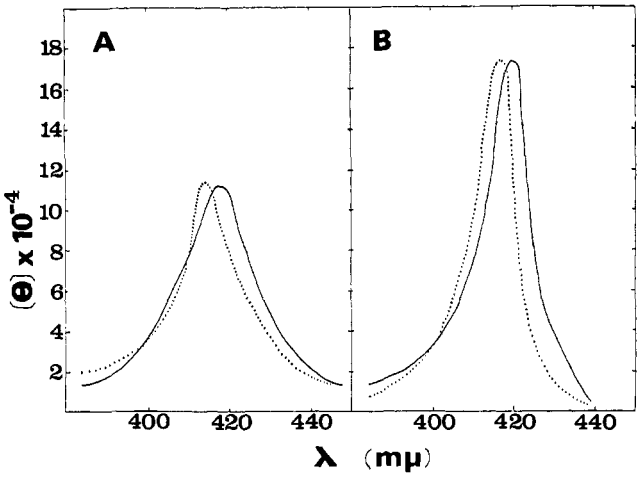

FIGURE 3: Circular dichroism spectra in the Soret region of the Hbα–Hp 2-2 complex (———) and Hbα chains (·····). (A) Oxygenated derivative; B, carbomonoxy derivative.

than that described here. This difference may be ascribed to strong absorption by Hb in this region. The corresponding derivatives of the Hb–Hp complex and adult Hb have a similar magnitude of positive peak at almost the same wavelength: approximately 420 mμ in the case of oxygenated and carbomonoxy derivatives, approximately 411 mμ in the case of ferric derivatives, and approximately 434 mμ in the case of deoxygenated derivatives. However, a negative trough which is observed in oxygenated, ferric, and deoxygenated derivatives of adult Hb is absent in the circular dichroism spectra of corresponding derivatives of the Hb–Hp complex. The circular dichroism spectra of the Hb–Hp complex described here are rather similar to the mean circular dichroism spectra of the isolated Hb chains reported by Geraci and Li (1969), but there is still some difference between

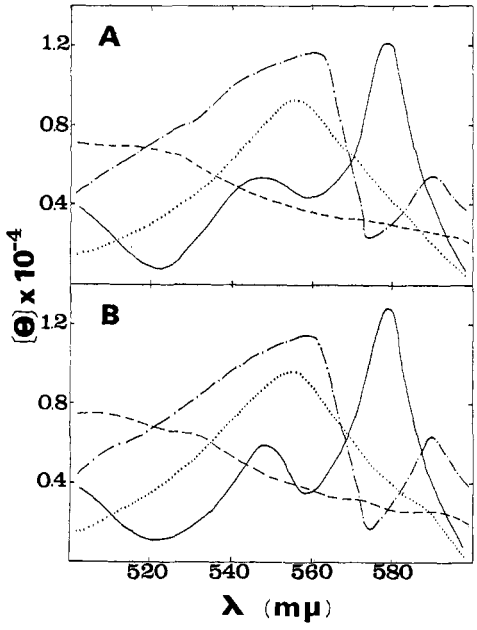

FIGURE 4: Circular dichroism spectra between 500 and 600 mμ of the Hb–Hp complex (A) and adult Hb (B). The type of Hp is same as in Figure 1. Oxygenated derivative, ———; carbomonoxy derivative, —·—; ferric derivative, – – – –; deoxygenated derivative, ·····.

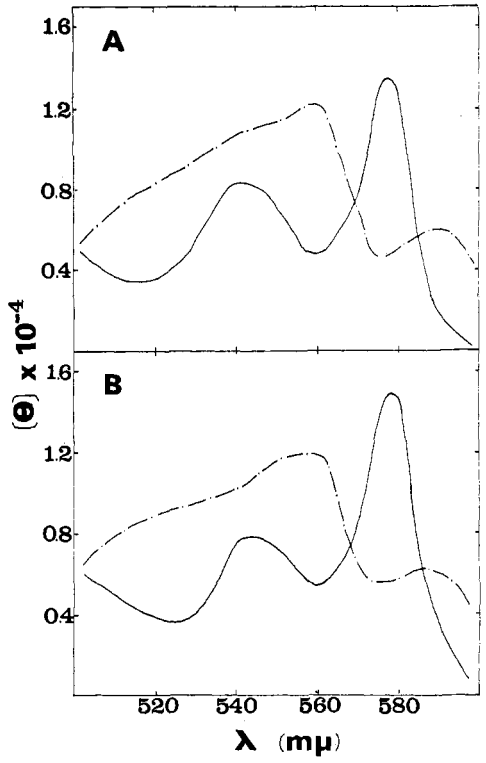

FIGURE 5: Circular dichroism spectra between 500 and 600 mμ of the Hbα–Hp 2-2 complex (A) and Hbα (B). Oxygenated derivative, ———; carbomonoxy derivative, —·—.

them. For example, the positive maximum of the mean circular dichroism spectra of the isolated oxyhemoglobin chains is at a wavelength shorter than the positive peak of the oxygenated Hb–Hp complex. The similar findings are observed between the circular dichroism spectra of the Hbα–Hp complex and Hbα chains (Figure 3). The positive peak of the oxygenated Hbα–Hp 2-2 complex is situated at around 418 mμ, while oxygenated Hbα chains exhibit a peak at 414 mμ (Figure 3A). The positive maximum of the carbomonoxy Hbα–Hp 2-2 complex is located at around 420 mμ and does not coincide in spectral location with that of carbomonoxy-Hbα chains which is at around 417 mμ (Figure 3B).

Figures 4 and 5 show the circular dichroism spectra in the 500–600-mμ region of oxygenated, ferric, carbomonoxy,

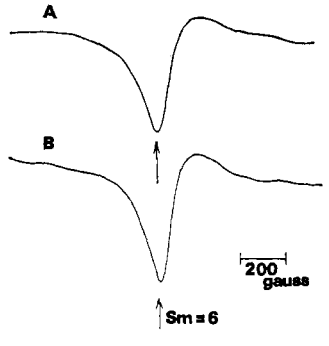

FIGURE 6: Electron spin resonance spectra of the ferric Hb–Hp 2-2 complex (A) and ferric Hb (B). 3.5 × 10^{-4} M samples were measured in 0.05 M phosphate buffer (pH 6.5). Modulation amplitude, 8 G; microwave power, 10 db; magnetic field, 470–1790 G; gain, ×50; temperature, near-liquid nitrogen.

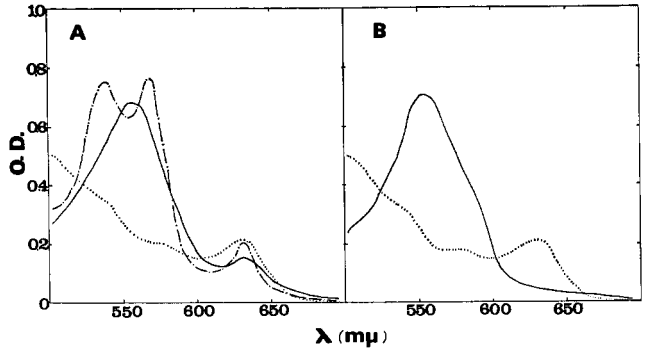

FIGURE 7: Absorption spectra of the Hb–Hp 2-2 complex (A) and adult Hb (B) after reduction of ferric derivatives with dithionite under aerobic conditions. Ferric derivative, ·····; after reduction with dithionite, ———; saturation with CO gas after reduction with dithionite, —·—.

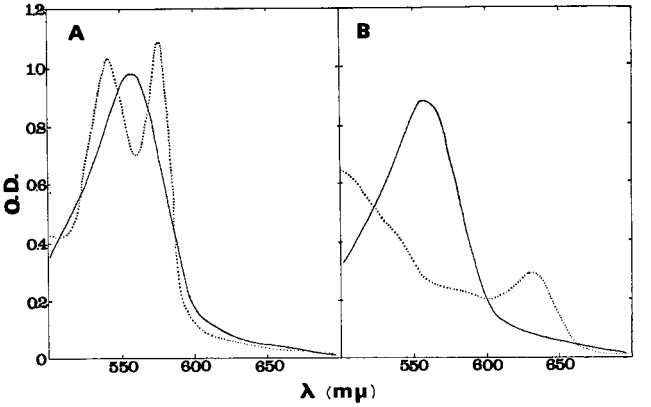

FIGURE 9: Absorption spectra of the Hb–Hp 2-1 complex after treatment of oxygenated derivative (A) and ferric derivative (B) with dithionite under anaerobic conditions. Oxygenated and ferric derivatives, ·····; after treatment with dithionite, ———.

and deoxygenated Hb–Hp complexes (Figure 4A) and corresponding derivatives of adult Hb (Figure 4B), and oxygenated and carbomonoxy Hbα–Hp 2-2 complex (Figure 5A) and corresponding derivatives of Hbα chains (Figure 5B). The circular dichroism spectra of the Hb–Hp complexes and the Hbα–Hp complex are similar to those of adult Hb and Hbα chains in corresponding derivatives, but a shoulder around 590 mμ of deoxygenated Hb is absent in the deoxygenated Hb–Hp complex.

Electron Spin Resonance Spectra. Figure 6 shows the electron spin resonance spectra of the ferric derivatives of the Hb–Hp complex and adult Hb. No significant differences are observed in the electron spin resonance spectra between them.

Action of Dithionite under Aerobic Conditions. When a minimum amount of dithionite is added to the oxygenated or ferric derivatives of the Hb–Hp complex under aerobic conditions, a new absorption band always appears at 633 mμ along with the usual characteristic band for deoxygenated Hb at 556 mμ (Figures 7A and 8A). Under these conditions both ferric and oxygenated adult Hb do not show a peak at around 630 mμ (Figure 7B). Carbon monoxide increases the extinction at 633 mμ (Figure 7A) and roughly a 10% decrease in absorption at 557 mμ of reduced pyridine–protochemochromogen was observed after the treatment of the oxygenated Hb–Hp complex with dithionite under aerobic conditions, suggesting that the band at 633 mμ is attributable to a degradation of heme in the Hb–Hp complex. Figure 8 shows that alkali shifts the band to 618 mμ, and that carbon monoxide reacts with the alkaline solution of the reaction mixture shifting the absorption band from 618 to 629 mμ. In the presence of 0.5 M NaOH and 20% pyridine, the band persists at around 617 mμ along with the usual characteristic α and β bands for the reduced pyridine–protohemochromogen. No peak appeared at 633 mμ by the treatment of the oxygenated and ferric Hb–Hp complex with dithionite under anaerobic conditions (Figure 9), by the treatment of carbomonoxy Hb–Hp complex with dithionite under aerobic conditions, or by the treatment of the oxygenated and ferric Hb–Hp complex with ascorbate under aerobic conditions.

Another spectral finding is that no shoulder at 590 mμ is observed in the absorption spectra of the deoxygenated Hb–Hp complex (Figure 9).

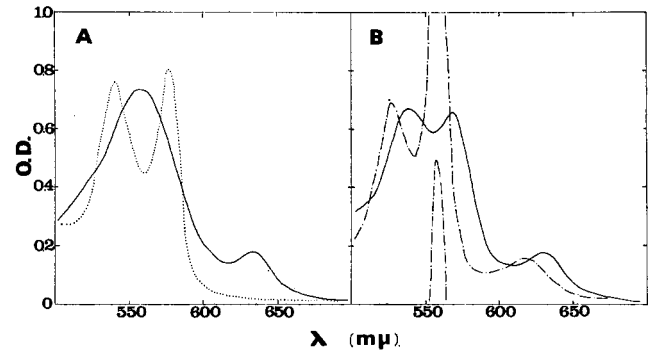

FIGURE 8: Absorption spectra of the Hb–Hp 2-1 complex after treatment of oxygenated derivative with diothinite under aerobic conditions. (A) Oxygenated derivative, ·····; after treatment with dithionite, ———. (B) In 2% NaOH after treatment with dithionite, —·—; saturation with CO gas in 2% NaOH after treatment with dithionite, ———.

Discussion

A few phenomena have been observed which suggest a difference in the environment of heme between Hb and the Hb–Hp complex. First the Hb–Hp complex has higher peroxidase activity than HbA and isolated Hb chains (Jayle and Moretti, 1962; Smith and Beck, 1967), and second the exchange of heme among hemoglobins and between Hb and albumin is blocked by the prior binding of Hb to Hp (Bunn and Jandl, 1968). The circular dichroism spectral analysis of the Hb–Hp complex will provide useful information on the local environmental structure of the heme chromophore of the Hb–Hp complex. The circular dichroism spectra in the Soret region of the Hb–Hp complex presented here are different from those of adult Hb. The positive peak of the circular dichroism spectra at the Soret region of the oxygenated and carbomonoxy Hbα–Hp complex is located at a wavelength longer than that of the corresponding derivative of Hbα chains. The data suggest that the heme environments of HbA are changed by the binding of Hp, and that the heme environments of Hbα–Hp complex are also different from those of Hbα subunits. It is not determined from the data presented here whether or not the heme environments of Hbβ chains are altered by the binding of Hp to HbA.

In addition to the circular dichroism spectral difference at the Soret region (Geraci and Li, 1969; Nagai *et al.*, 1969), several spectral differences in deoxygenated derivatives have been reported between HbA and its isolated subunits. The molar absorptivities in the Soret region of both the deoxygenated Hb α and β chains are lower than that of deoxygenated Hb (Antonini *et al.*, 1965). The absorption spectrum of deoxygenated Hb shows a shoulder at 590 mμ, which is absent in the isolated Hb chains (Brunori *et al.*, 1968). A negative circular dichroism band at 285 mμ of deoxygenated Hb is absent in the deoxygenated Hb subunits (Beychok *et al.*, 1967). In these respects the deoxygenated Hb–Hp complex behaves similarly to the deoxygenated Hb subunits. The broad and flat band at the Soret region of the deoxygenated Hb–Hp complex has already been reported (Chiancone *et al.*, 1966). A shoulder at 590 mμ is absent in the absorption spectrum of the deoxygenated Hb–Hp complex. A negative circular dichroism band at 285 mμ in the deoxygenated Hb–Hp complex is much smaller than the sum of circular dichroism at 285 mμ of adult Hb and Hp. Further, the circular dichroism spectrum of the deoxygenated Hb–Hp complex lacks a shoulder at 590 mμ which is present in the circular dichroism spectrum of deoxygenated HbA. These spectral findings indicate that intrinsic properties of Hb subunits are manifested by the binding of Hp to HbA.

There are no significant differences in electron spin resonance spectra between the ferric Hb–Hp complex and ferric adult Hb. The data suggest that the spin state and ligand field of the heme iron in the ferric Hb are not changed by the binding of Hp.

The spectral characteristics of choleglobin are as follows. (1) The absorption band of reduced choleglobin is at 628–632 mμ. (2) Carbon monoxide increases the extinction at 628 mμ. (3) Alkali shifts the band of reduced choleglobin to 618–622 mμ transforming it into denatured globin–cholehemochromogen. (4) Carbon monoxide reacts with the denatured globin–cholehemochromogen shifting the band from 618 to 628 mμ. (5) If the pyridine is added to the denatured globin–cholehemochromogen, there is the band of cholehemochromogen at 618–620 mμ (Lemberg *et al.*, 1941). The spectral properties of the substance produced from the Hb–Hp complex by dithionite under aerobic conditions are very similar to those of choleglobin described above. The data presented in this paper suggest that the absorption band at 633 mμ is due to the formation of a choleglobin-like substance from a part of the Hb–Hp complex. An absorption band similar to that of choleglobin can be observed after repeated treatments of oxygenated Hb with dithionite, following reoxygenation (Lemberg and Legge, 1949). In the case of the oxygenated and ferric Hb–Hp complex, one treatment under aerobic conditions is enough to produce a choleglobin-like substance. Under the same conditions, both oxygenated and ferric adult Hb do not produce a choleglobin-like substance. These data indicate that the heme of the Hb–Hp complex is more labile to the action of dithionite under aerobic conditions than the heme of HbA. The mechanism of the reaction is unknown. Conformation of heme environments of the Hb–Hp complex different from HbA might be related to the phenomenon.

Acknowledgments

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