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Formation of Products of the 5,6-Dihydroxydihydrothymine Type by Ultraviolet Light in HeLa Cells[†]

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ABSTRACT: The formation of products of the 5,6-dihydroxydihydrothymine-type (t^{UV}) and cyclobutane-type pyrimidine photodimers (TT) and tritiated water (3H_2O) by monochromatic light at 240, 265, 280, and 313 nm (6-nm half-bandwidth) was investigated in HeLa S-3 cells which were labeled in their DNA with [*methyl*- 3H]thymine. The efficiency of the formation of all three products was maximal at 280 nm and dropped towards longer and shorter wavelengths. The efficiency of TT formation dropped more strongly towards longer and shorter wavelengths than the efficiency of t^{UV} formation (comparison at a dose of $5 \times 10^3 \text{ J m}^{-2}$). Total monomeric thymine ring saturation (t_{sat}) was estimated from the t^{UV} data.

Cyclobutane-type pyrimidine dimers are the major lesions produced in the DNA of bacteria and mammalian cells by far-ultraviolet light (far-UV, $\lambda < 300 \text{ nm}$). They are responsible for most of the killing and mutagenic action of far-UV. The formation of other lesions, in particular, of nondimeric ring-saturation products of thymine and cytosine, has been observed. Wacker (1963) described the formation of products other than photodimers in DNA irradiated at 254 nm and one of these products was later identified as 5,6-dihydrothymine (Yamane et al., 1967). The formation of small amounts of thymine photohydrate, 6-hydroxy-5,6-dihydrothymine, by 265-nm irradiation (Fisher and Johns, 1973) and of deoxycytidine photohydrate, 6-hydroxy-5,6-dihydrodeoxycytidine, in oligonucleotides and DNA at 254 nm (Vanderhoek and Cerutti, 1973) has been documented. Photooxidation of thymine to 5,6-dihydroxydihydrothymine by light of 184.9 and 254 nm to 5-hydroxymethyluracil, 5-formyluracil, and uracil has been investigated by Daniels and Grimison (1967) and by Alcantara and Wang (1965), respectively.

It was calculated that 0.06 t_{sat} was formed for each TT at 280 nm, but 0.73 t_{sat} per TT at 313 nm. It follows that monomeric thymine ring saturation represents a minor photochemical reaction relative to pyrimidine dimerization in the far-ultraviolet but a major reaction in the near-ultraviolet. The formation of 3H_2O by ultraviolet light from [*methyl*- 3H]thymidine-labeled HeLa cells most likely indicates the attack of hydroxyl radicals on the cellular DNA; 5-methylenuracil radicals formed as a consequence of the reaction may be important intermediates in ultraviolet-induced DNA-DNA and DNA-protein cross-linking.

The photochemistry of DNA and its building blocks in the near-ultraviolet region (near-UV, 300-380 nm) is less well understood. Near-UV light is of particular interest to the environmental and medical sciences, since it is contained in the sun spectrum reaching the surface of the earth in contrast to far-UV which is mostly absorbed by the terrestrial ozone shield. Cyclobutane-type pyrimidine dimers are formed by near-UV but with much lower efficiency than by far-UV light (Tyrrell, 1973). Near-UV has been shown to produce alkali-labile bonds in DNA in situ in the cell but these lesions have not yet been chemically characterized (Tyrrell et al., 1974). Irradiation of thymine in solution in the presence of H_2O_2 with near-UV light leads to the formation of hydroperoxy derivatives of 5,6-dihydrothymine (Hahn and Wang, 1974).

We have undertaken a study of the formation of thymine photodimers and monomeric, ring-saturated thymine lesions of the 5,6-dihydroxydihydrothymine type (t^{UV}) in human carcinoma HeLa S3 cells upon irradiation with monochromatic light at 240, 265, 280, and 313 nm under aerobic conditions. We have also measured the formation of tritiated water from [*methyl*- 3H]thymidine-labeled cells which gives a measure of the photochemical reactivity of the methyl group of thymine. It was found that products of the 5,6-dihydroxydihydrothymine type represent minor lesions relative to pyrimidine photodimers in the far-UV but major lesions in the near-UV.

Materials and Methods

Growth of Cells and Radioactive Labeling. HeLa S-3 cells were grown at 37 °C, in spinner cultures and in Joklik's modified minimal essential medium, supplemented with cold thy-

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¹ Abbreviations used are: UV, ultraviolet light; t^{UV} , products of the 5,6-dihydroxydihydrothymine type; t_{sat} , total monomeric, ring-saturation and ring-destruction products of thymine; TT, cyclobutane type pyrimidine photodimers containing one or two thymine moieties; 5'-TMP, thymidine 5'-monophosphate.

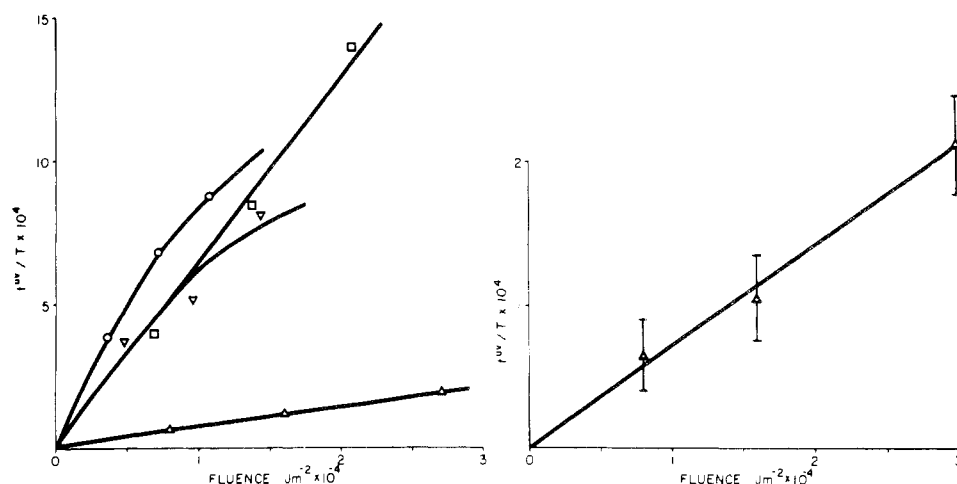


FIGURE 1: Formation of products of the 5,6-dihydroxydihydrothymine type (t^{UV}) in HeLa S-3 cells by ultraviolet light from 240 to 313 nm. For experimental procedures, see under Materials and Methods. Left side: (\square) irradiation at 240 nm; (∇) irradiation at 265 nm; (\circ) irradiation at 280 nm; (Δ) irradiation at 313 nm. Right side: data at 313 nm plotted on an expanded scale.

midine to a final concentration of 10^{-6} M, 3.5% calf serum, 3.5% fetal calf serum, and antibiotics. The cells were labeled in their DNA with [*methyl*- 3 H]thymidine in two steps for a total of 24 h (8 μ Ci/mL, specific activity 40–60 Ci/mmol), followed by cell growth in fresh nonradioactive medium for 1 hour. The cells were then washed twice in cold Hank's balanced salt solution (pH 7.4) and resuspended in cold Hank's balanced salt solution at 5×10^5 cells/mL.

Irradiation Conditions and Dosimetry. HeLa cells were irradiated in suspension, in a plastic dish maintained at 0 °C, at a concentration of 5×10^5 cells/mL. Usually each sample consisted of 2-mL cell suspensions in cold Hank's buffer (pH 7.4), the depth of the suspension being 0.5 cm. The light source was a 1000-W xenon–mercury high-pressure Hanovia lamp. Monochromatic light was obtained with a Schoeffel GM250 high-intensity quarter meter grating monochromator (1180 grooves/mm). The monochromatic light (6-nm half-bandwidth), emerging from the exit slit, was reflected by a mirror so as to be incident on the dish containing the cell suspension. The wavelengths used for the present study included both the near- and far-ultraviolet regions from 240 to 365 nm. The incident fluence of ultraviolet light, up to 280 nm, was measured using a Blak-ray meter J-225 (Ultraviolet Products Inc.) and for wavelengths beyond 280 nm a long-wavelength UV meter, J-221, was used. The incident fluence on the surface of the 2-mL cell suspension ranged from 3 to 1600 kJ m^{-2} . For some experiments involving UV irradiation of thymidine 5'-monophosphate, a germicidal lamp was used as light source.

Postirradiation Procedures. Following ultraviolet irradiation at any particular wavelength, 1.8 mL of each sample was withdrawn and treated with 0.2 mL of 50% cold trichloroacetic acid. The acid precipitate was analyzed for products of the 5,6-dihydroxydihydrothymine type (t^{UV}) according to Hariharan and Cerutti (1974), and the acid-soluble fraction was analyzed for its content in tritiated water as described by RotiRoti and Cerutti (1974). The remaining 0.2 mL of the original sample was treated separately with trichloroacetic acid and the precipitate was analyzed for its content in cyclobutane-type pyrimidine dimers (TT) according to Goldman and Friedberg (1973).

Results

Formation of Products of the 5,6-Dihydroxydihydrothymine Type (t^{UV}) from Thymidine 5'-Monophosphate by

254-nm Light. The amount of t^{UV} formed from 5'-[*methyl*- 3 H]TMP by 254-nm irradiation (unfiltered mercury low-pressure lamp) was determined by the alkaline–acid degradation assay (Hariharan and Cerutti, 1974) and compared to the loss of absorbance at 260 nm which is a measure of total thymine ring saturation. Linear dose–response curves were obtained and the assay yield relative to ring saturation was 22%. This value is close to that obtained for products of the 5,6-dihydroxydihydrothymine-type formed by γ irradiation of 5'-TMP or osmium tetroxide oxidation of poly(dA-T) (Hariharan and Cerutti, 1974). No photodimerization of 5'-TMP could be detected by the chromatographic procedure of Goldman and Friedberg (1973).

As mentioned above, the formation of 5,6-dihydrothymidine in DNA was demonstrated upon irradiation with ultraviolet light (Yamane et al., 1967). The possibility that 5,6-dihydrothymidine might interfere with the alkaline–acid degradation assay for t^{UV} was considered. Therefore, an authentic sample of 5,6-dihydro[*methyl*- 3 H]thymidine was prepared by catalytic reduction of [*methyl*- 3 H]thymidine according to Green and Cohen (1957) and further purified by thin-layer chromatography as described by Cadet and Téoule (1974). Treatment of this material under the standard assay conditions for the determination of products of the 5,6-dihydroxydihydrothymine type (Hariharan and Cerutti, 1974) did not yield a positive reaction.

Formation of Products of the 5,6-Dihydroxydihydrothymine-Type (t^{UV}) and Cyclobutane-Type Pyrimidine Dimers (TT) by Ultraviolet Light in the DNA in HeLa S-3 Cells. The formation of t^{UV} and TT in the DNA in HeLa S-3 cells upon irradiation in air-saturated salt solution at 0 °C by monochromatic light (6-nm half-bandwidth) at 240, 265, 280, and 313 nm was determined. On the right side of Figure 1 are shown the dose–response curves for the formation of t^{UV} , and it is evident that the efficiency of t^{UV} formation is maximal at 280 nm and decreases towards shorter and longer wavelengths. The data for t^{UV} formation at 313 nm are replotted on the right half of Figure 1 on an expanded scale in order to give an indication of the reproducibility of the data at this wavelength. The left half of Figure 2 contains the data for the production of TT which was measured in aliquots of the same samples which were used for t^{UV} determination. The dose–response curves for TT at 240 and 265 nm are nonlinear and level off towards high fluences. Similar to t^{UV} formation, photodimerization

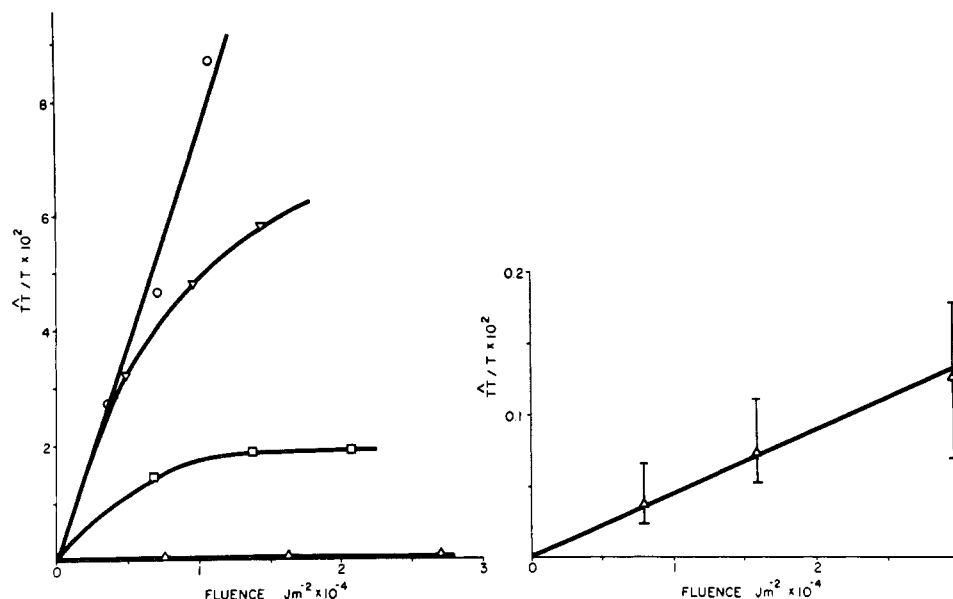


FIGURE 2: Formation of cyclobutane-type pyrimidine dimers (TT) in HeLa S-3 cells by ultraviolet light from 240 to 313 nm. For experimental procedures, see under Materials and Methods. Left side: (\square) irradiation at 240 nm; (∇) irradiation at 265 nm; (\circ) irradiation at 280 nm; (Δ) irradiation at 313 nm. Right side: data at 313 nm plotted on an expanded scale.

occurs with higher efficiency at 280 nm than at shorter and longer wavelengths. The right half of Figure 2 gives the 313-nm data on an expanded scale.

Formation of Tritiated Water from [methyl- ^3H]Thymidine in DNA in HeLa S-3 Cells upon Irradiation with Ultraviolet Light. The formation of $^3\text{H}_2\text{O}$ from [methyl- ^3H]thymidine in the DNA in HeLa-S-3 cells upon irradiation with light at 240, 265, 280, and 313 nm was determined. This reaction is a measure of the photochemical reactivity of the methyl thymine group and, presumably, the formation of 5-methyleneuracil radicals which result from hydrogen abstraction by hydroxyl radicals. $^3\text{H}_2\text{O}$ was measured in the acid-soluble portion of the same samples which were used for t^{UV} and TT determination. The procedure for $^3\text{H}_2\text{O}$ determination has been described previously for analogous experiments carried out with ionizing radiation (RotiRoti and Cerutti, 1974). As shown in Figure 3, the dose-response curves were linear with the possible exception of the data at 313 nm. The efficiency of $^3\text{H}_2\text{O}$ formation was maximal at 280 nm and lower both at shorter and longer wavelengths as was observed for t^{UV} and TT formation.

Discussion

The following considerations make it very likely that the t^{UV} lesions possess the structural features of products of the 5,6-dihydroxydihydrothymine type. For mechanistic reasons, it is expected that treatment with mild alkali and acid under the conditions of our assay leads to the release of a small, radioactively labeled fragment from products of the 5,6-dihydroxydihydro[methyl- ^3H]thymine type but not from cyclobutane-type thymine dimers, thymidine photohydrates (6-hydroxy-5,6-dihydrothymidine), or 5,6-dihydrothymidine labeled in the methyl group. This prediction was substantiated by the following experiments. The significant differences in the dose-response curves for t^{UV} and TT eliminate the possibility that the alkaline-acid degradation assay inefficiently measures cyclobutane-type photodimers. Incubation of irradiated samples of DNA at 37 °C for 24 h in 5×10^{-3} M phosphate buffer, pH 7.4, i.e., conditions leading to the reversion of thymine photohydrates (Fisher and Johns, 1973),

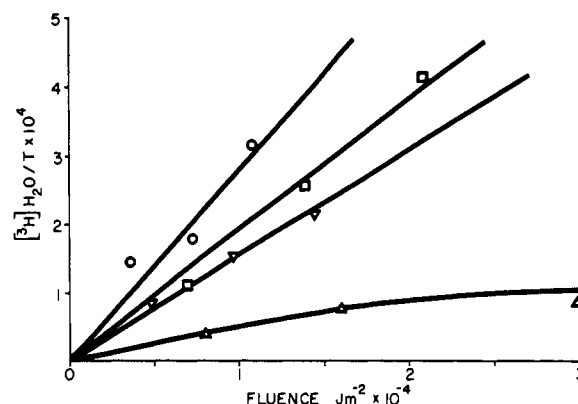


FIGURE 3: Formation of tritiated water in HeLa S-3 cells labeled in their DNA with [methyl- ^3H]thymine by ultraviolet light from 240 to 313 nm. For experimental procedures, see under Material and Methods. (\square) Irradiation at 240 nm; (∇) irradiation at 265 nm; (\circ) irradiation at 280 nm; (Δ) irradiation at 313 nm.

did not result in a significant decrease in the amount of t^{UV} measured by the assay. Treatment of authentic 5,6-dihydro-[methyl- ^3H]thymidine under the conditions of the alkali-acid degradation assay did not yield a positive reaction.

Since part of the dose-response curves for t^{UV} and TT formation are nonlinear and low dose data cannot be obtained because of the limited sensitivity of the biochemical assays, it was not possible to derive reliable values for initial rates for all wavelengths. Estimates of the amounts of t^{UV} and TT formed at an equal dose of $5 \times 10^3 \text{ J m}^{-2}$ are listed in Table I instead. Table I also contains estimates for the total amount of thymine ring saturation (t_{sat}) due to the formation of monomeric, saturated thymine derivatives. The values for t_{sat} are calculated from t^{UV} by multiplication with a factor of 4.5. This factor corrects for the yield of 22% in the alkaline-acid degradation assay relative to loss of absorbance at 260 nm. The right-hand column of Table I contains values for the ratios of t_{sat} over TT. It is evident that the amount of t_{sat} formed relative to TT is lowest in the 260–280-nm region and increases to shorter and longer wavelengths. The high value of 0.12 for t_{sat}/TT at 240

TABLE I: Amounts of t^{UV} and TT Formed in HeLa Cells at a Dose of $5 \times 10^3 \text{ J m}^{-2}$ as a Function of Wavelength.

Wave-length ^a (nm)	$t^{UV}/T \times 10^4$	$t_{sat}/T \times 10^4$ ^b	TT/T $\times 10^4$	t_{sat}/TT
240	3.2	14.4	102	0.12
265	3.4	15.3	325	0.05
280	5.0	22.5	375	0.06
313	0.35	1.6	2.2	0.73

^a Monochromatic purity: 6-nm half-bandwidth. ^b t_{sat} , total monomeric thymine ring-saturation products. The amount of t_{sat} is obtained from t^{UV} data by multiplication with a factor of 4.5. This factor was derived at from the yield of 22% in the alkaline-acid degradation assay relative to loss of ring aromaticity (see text).

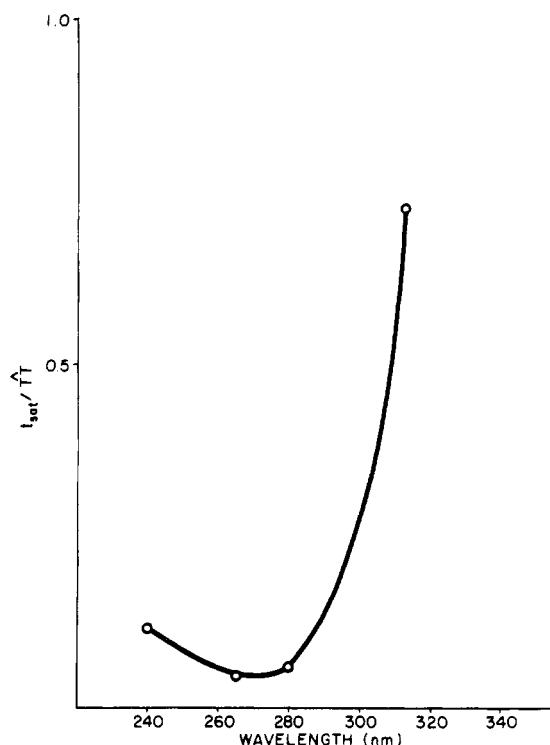


FIGURE 4: Ratio of the amount of monomeric, ring-saturation products of thymine (t_{sat}) over cyclobutane-type pyrimidine photodimers (TT) formed by ultraviolet light from 240 to 313 nm in HeLa S-3 cells.

nm may be a reflection of the photostability of t^{UV} , the photoreversibility of TT, and the low photostationary state of dimerization at this wavelength. Therefore, this value may be considerably lower at physiological doses. The apparent linearity of the dose-response curves at 313 nm and the fact that they extrapolate to zero gives the value of 0.73 for t_{sat}/TT at this wavelength more significance, on the other hand. From the t_{sat}/TT data it can be concluded that monomeric, ring-saturated thymine lesions represent minor lesions relative to pyrimidine photodimerization in the far-UV but major lesions in the near-UV.

The presence of t^{UV} in the DNA of HeLa cells was also demonstrable following irradiation with very high doses of light at 365 nm, but no reliable data for the efficiency of t^{UV} formation could be obtained at this wavelength. For technical reasons, we were not able to obtain meaningful data for the relative efficiency of t^{UV} and TT formation in the 300–305-nm region.

Enzymological evidence for the formation of DNA lesions other than cyclobutane-type photodimers has been obtained by Bacchetti and collaborators (Bacchetti et al., 1972; Bacchetti and Benne, 1975). These authors characterized an endonuclease from calf thymus which recognizes lesions in fully photoreactivated, far-UV-irradiated DNA. It was estimated that these lesions are formed 35 times less efficiently than photodimers in free DNA by irradiation at 254 nm. A comparable value of 20 was obtained for the ratios of TT over t_{sat} in intact HeLa cells upon irradiation at 265 nm in our work (see Table I). The relationship between the lesions which are recognized by this endonuclease and t_{sat} remains to be elucidated. A related enzyme has also been identified in rat liver (VanLancker and Tomura, 1974). Further evidence for the presence of UV lesions other than cyclobutane-type pyrimidine dimers also comes from the work of Feldberg and Grossman (1976) who discovered a protein in human cells which lacks endonuclease, exonuclease, and glycosidase activity but selectively binds to far-UV-irradiated DNA, regardless of whether photodimers were removed by photoreactivation or not (Feldberg and Grossman, 1976). It should be mentioned that extracts from xeroderma pigmentosum skin fibroblasts contained normal levels of the endonuclease described by Bacchetti et al. (1972) and possessed normal capabilities for the removal of t^{UV} from exogenous DNA (Hariharan and Cerutti, 1976).

Epidemiological data indicates that sunlight plays a major role in the etiology of skin cancer in man (see, e.g., Epstein, 1970). No experimental data is available which allows the definition of an action spectrum for skin cancer. However, from the sun spectrum at the surface of the earth and an action spectrum for ultraviolet-induced DNA damage and its lethal and mutagenic effects in bacterial systems, Setlow (1974) speculated that light in the 300–305-nm region should possess maximal effectiveness for the induction of skin cancer. It is interesting to note in this context that the ratio of t^{UV} over TT increased dramatically in this region of the spectrum.

Acknowledgments

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Linear Electric Field Effect in Electron Paramagnetic Resonance for Two Bisimidazole-Heme Complexes, Model Compounds for B and H Hemichromes of Hemoglobin and for Cytochrome b_5 [†]

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ABSTRACT: Bisimidazole-ferric heme is considered to be the structure at the heme site of cytochrome b_5 and two different low spin ferric hemochromes spontaneously formed from ferric hemoglobin. The addition of strong base to bisimidazole-ferric heme in organic solvents alters the optical and magnetic properties of this compound. With the use of the linear electric field effect in the electron paramagnetic resonance, we dem-

onstrate that addition of base does not lead to the exchange of hydroxide anion for ligated imidazole and that the bisimidazole structure is retained. Analysis of optical titrations indicates that 2 equiv of base react reversibly with bisimidazole-ferric heme. It is suggested that the two hemichromes formed from hemoglobin differ in structure from one another by the state of protonation of N-1 in the bound imidazoles.

The slow, spontaneous denaturation of high-spin ferric hemoglobin A, as well as the more rapid denaturation of ferric β or ferric α chains (Rachmilewitz et al., 1971), lead to the formation of a variety of low-spin ferric heme compounds collectively called "hemichrome" (Peisach and Blumberg, 1971; Peisach et al., 1972). One of these, designated "H hemichrome" (Blumberg and Peisach, 1971), can be reversibly renatured to functional hemoglobin (Rachmilewitz et al., 1971) and is thought to represent the ligation of histidine imidazole E7 to heme (Rachmilewitz, 1969) displacing water which is normally bound to the iron in the ferric protein. With time, H hemichrome is converted to a second product, termed "B hemichrome," which does not renature to functional hemoglobin.

An attempt has been made to identify the chemical nature of the axial ligands for both of these hemichromes observed in denatured hemoglobin using the EPR¹ technique (Blumberg and Peisach, 1971). The g values and the crystal field parameters for B hemichrome (Table I) were found to be similar to those observed for cytochrome b_5 (Bois-Poltoratsky and Ehrenberg, 1967; Ikeda et al., 1974) and for the model com-

pound bisimidazole-ferric heme (Peisach et al., 1973). X-ray crystallographic analysis shows that the latter two compounds have the same heme ligand structure (Mathews et al., 1971). On the other hand, the g values of H hemichrome were close to those obtained for the compound which is formed by adding strong base to bisimidazole-ferric heme (Peisach et al., 1973) or cytochrome b_5 (Bois-Poltoratsky and Ehrenberg, 1967; Ikeda et al., 1974).

Recently, Peisach et al. (1973) proposed that imidazole bound to heme can exist in two forms which are stable in nonaqueous medium. In one form, the N-1 nitrogen atom is protonated and in the other, not. It was suggested, on the basis of EPR of model compounds, that B hemichrome contains protonated imidazole ligands, and H hemichrome, deprotonated imidazole ligands, deprotonation being brought about in the model compound by the addition of strong base (Peisach et al., 1973). It remained, however, to demonstrate that the changes observed in the model compound did not involve the removal of an imidazole ligand and, in particular, that they did not correspond to the substitution of hydroxide anion. Moreover, it was necessary to determine experimentally the number of equivalents of base required to react with bisimidazole-ferric heme in order to effect the transformation from the ligand structure of B hemichrome to that of H hemichrome. According to the above assumption, this should be one or two, depending on the number of imidazole ligands undergoing deprotonation.

The considerable difference between the g values for hemoglobin or myoglobin hydroxide (Table I) (Gurd et al., 1967), where the ligands are assumed to be imidazole and hydroxide anion, and the g values of the other heme centers discussed above already affords some evidence against the

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¹ Abbreviations used: EPR, electron paramagnetic resonance; LEFE, linear electric field effect; g_{\max} , the low-field end g value of the EPR spectrum; g_{\min} , the high-field end g value of the EPR spectrum.