Heme Sulfuric Anhydrides. II. Properties of Heme Models Prepared from Mesoheme Sulfuric Anhydrides*

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ABSTRACT: This paper describes spectral studies on a series of mesoheme derivatives which contain histidine and methionine covalently linked to the propionic acid side chains of the heme. All possible derivatives were examined, including the mono- and bishistidine derivatives, the mono- and bismethionine derivatives, and the mixed-histidine–methionine derivative of mesoheme. The covalently bound amino acids are capable of coordination to the heme iron. Histidine is a stronger ligand to the oxidized form of the heme than to the reduced form, whereas the opposite is true of methionine. The spectrum of monohistidine mesoheme is very similar to that of mesoheme myoglobin, while the spectrum of bishistidine mesoheme is similar to that of cytochrome *c*. These synthetic heme derivatives should be of use as model compounds to aid in the interpretation of physicochemical studies on heme proteins.

The preparation of the mono- and bissulfuric anhydrides of mesoheme opens a route for the synthesis of heme models in which the fifth and sixth coordination positions of the iron atom can be specifically occupied by known amino acid ligands (Warme and Hager, 1970). We report in this paper on the spectral and electrochemical properties of the mono- and bishistidine mesoheme derivatives, the mono- and bismethionine mesoheme derivatives and the mixed-histidine–methionine mesoheme derivative. Conditions for obtaining monomeric solutions of these heme peptides are described and their value as models for interpretation of the spectra of heme proteins is discussed.

Experimental Section

Materials. The synthesis, purification, and characterization of the various heme derivatives have been reported in the preceeding paper (Warme and Hager, 1970). Dioxane stabilized with diethyl dithiocarbamate was obtained from J. T. Baker. In several cases peroxide contamination in the dioxane was detected by the potassium iodide test. Since hemes and heme derivatives are rapidly decolorized by peroxides, it was necessary to purify such samples of dioxane by passage through an alumina column in the manner recommended for removal of peroxides from ether (Dasler and Bauer, 1946). A column prepared from 80 g of alumina was sufficient to remove the peroxides from 100 to 200 ml of dioxane. The *N*-acetyl-L-methionin amide used in these experiments was obtained from Cyclo Chemical Corp.

Preparation of Heme Solutions. About 1 mg of each of the heme derivatives was dissolved in 50 μl of 0.1 N NaOH and immediately diluted with 0.95 ml of distilled water. The stock solutions were kept at 0–4° during use and at −20° when not in use. The solutions were discarded after a total exposure of 24 hr at 0–4° in order to avoid aggregation effects which were noticeable in solutions of monohistidine mesoheme, as mentioned in the text.

Spectral Measurements. Spectral measurements were made on a Cary Model 15 spectrophotometer at room temperature (23°) in 1.0-cm path-length cells except as noted. The wavelength scale was calibrated against the hydrogen emission lines at 486.1 and 656.2 mμ.

Results

Conditions for Disaggregation. Aggregation is a common problem encountered in work with hemes and heme derivatives. It is usually manifested in the visible absorption peaks, which are broadened and diminished in intensity when the heme is aggregated. In the spectral studies on heme derivatives described in this paper, it was necessary to establish conditions for disaggregation, since aggregation could lead to intermolecular coordination of a ligand from one heme peptide molecule to the heme iron of another molecule. Addition of 10% dioxane to a solution of bishistidine mesoheme in aqueous solution buffered at pH 7 causes a 27% increase of the extinction coefficient of the Soret band maximum at 403 mμ as shown in Figure 1. Further increases of the dioxane concentration cause a negligible increase of the absorption intensity but result in a slight red shift of the maximum until at 40% (v/v) dioxane, the Soret maximum is at 405 mμ. The results of the dioxane titration illustrated in Figure 1 are summarized in Figures 2 and 3 along with the results of similar titrations performed on mesoheme, mesoheme plus imidazole, and the monohistidine, monomethionine, bismethionine, and histidine–methionine derivatives of mesoheme. The oxidized and reduced Soret bands of all these derivatives are sharpened and intensified by addition of dioxane. The extent of change and the shapes of the titration curves vary, but the extinction coefficient of each derivative increases to a maximum and then either decreases or plateaus. At dioxane concentrations below the concentration required

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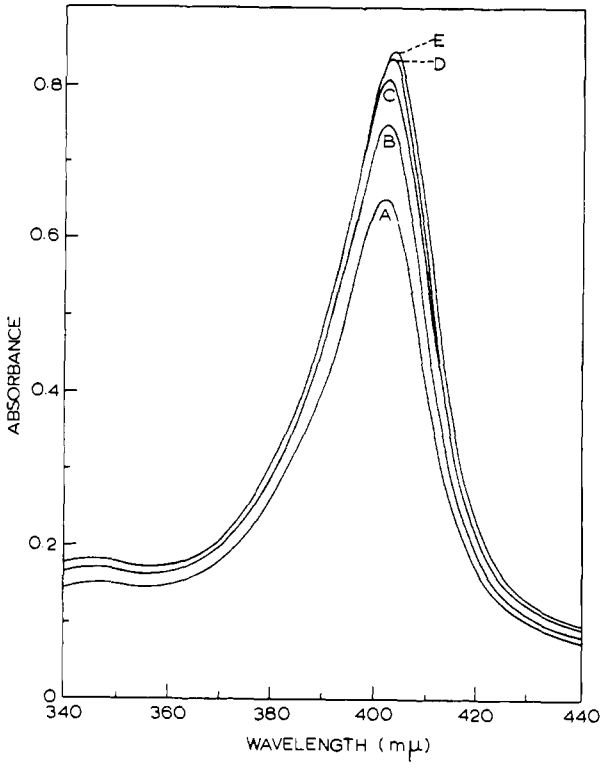

FIGURE 1: Effect of dioxane concentration on the oxidized soret spectrum of bishistidine mesoheme. These curves were obtained at a 6.7×10^{-6} M concentration of bishistidine mesoheme in dioxane–potassium phosphate buffer, pH 7, with varying concentrations of dioxane; curve A, 0%; curve B, 2.5%; curve C, 5%; curve D, 10%; curve E, 20% dioxane.

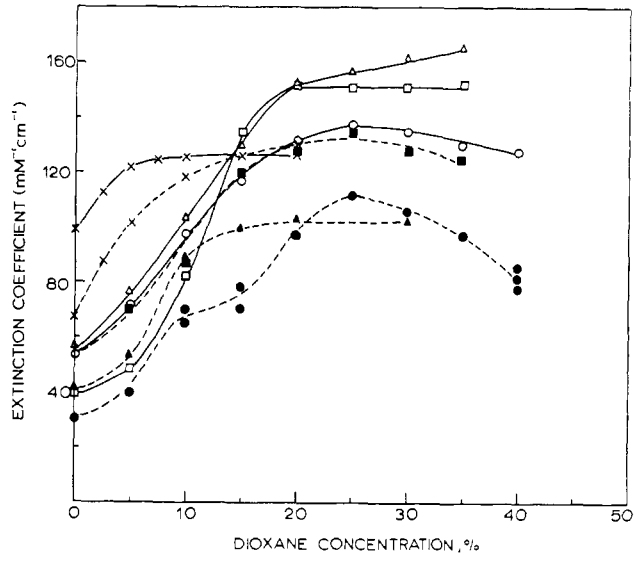

FIGURE 2: Effect of dioxane concentration on extinction coefficients of heme derivatives (I). Heme derivatives were tested at the indicated concentrations and Soret maxima in both the oxidized and reduced form: mesoheme, 7.6×10^{-6} M, ϵ_{391} oxidized (○—○), ϵ_{406} reduced (●---●); monohistidine mesoheme, 5.8×10^{-6} M, ϵ_{393} oxidized (△—△), ϵ_{413} reduced (▲----▲); bishistidine mesoheme, 6.7×10^{-6} M, ϵ_{403} oxidized (×—×) ϵ_{418} reduced (×----×); mesoheme plus imidazole, 7.6×10^{-6} M, ϵ_{402} oxidized (□—□), ϵ_{410} reduced (■----■). The required amount of dioxane was diluted with 0.1 M potassium phosphate buffer, pH 7.0, or 0.1 M imidazole–HCl buffer, pH 7.0.

for attainment of the maximal extinction coefficient, the curves were not exactly reproducible. However, the spectra at the optimal dioxane levels were generally reproducible. In most cases, a time dependent increase of the absorbance was observed after adding the heme derivatives to the dioxane–buffer mixtures. Therefore, the spectrum was scanned repeatedly until the absorbance change was less than 0.002 absorbance unit between successive scans (generally less than 5 min after mixing).

The higher wavelength absorption bands of the heme peptides are also sharpened and intensified by addition of dioxane. For example, in the absence of dioxane, both the monohistidine and bishistidine derivatives have bands at ~570 mμ in the reduced form. In 20% dioxane, this high-wavelength band disappears and the α and β bands are intensified. Thus, the 570-mμ band is probably due to heme–heme interaction in the aggregates which are undoubtedly present in aqueous solution. The titration of reduced bishistidine mesoheme with dioxane is illustrated in Figure 4. In this case, a higher dioxane concentration was required to achieve the maximal extinction coefficient than was required in the Soret region because the concentration of the heme derivative was six times higher.

An alternate method for dissociating heme aggregates is to carry out experiments at very low concentrations. As shown in Figure 5, dilution of a bishistidine mesoheme solution from 6.6×10^{-5} to 6.6×10^{-7} M causes a 28% increase in the extinction coefficient at the Soret band. It is possible that higher dilution would cause a further increase in the intensity, but practical considerations made measurements at higher dilution difficult. However, addition of 10% dioxane at the lowest concentration caused a slight decrease in extinction coefficient instead of an increase which should

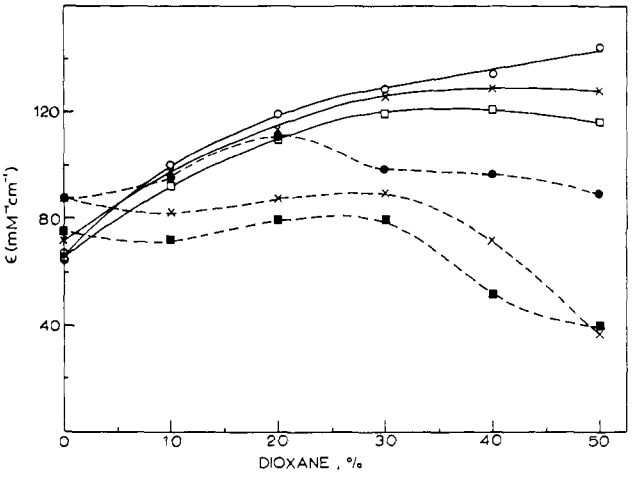

FIGURE 3: Effect of dioxane concentration on extinction coefficients of heme derivatives (II). All concentrations were 5.0×10^{-6} M in 0.1 M potassium phosphate buffer, pH 7.0, diluted with the required amount of dioxane: histidine–methionine mesoheme, ϵ_{395} oxidized (○—○), ϵ_{415} reduced (●--●); bismethionine mesoheme, ϵ_{394} oxidized (×—×), ϵ_{417} reduced (×---×) monomethionine mesoheme, ϵ_{394} oxidized (□—□), ϵ_{417} reduced (■---■).

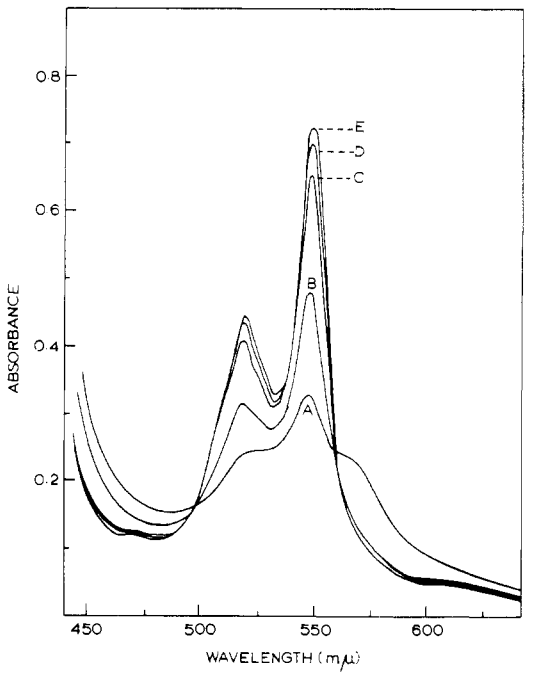

FIGURE 4: Effect of dioxane on the reduced α–β spectrum of bishistidine mesoheme. These curves were obtained at a 4.0×10^{-5} M concentration of bishistidine mesoheme in dioxane–potassium phosphate buffer, pH 7.0: curve A, 0%; curve B, 5%, curve C, 10%; curve D, 15%; curve E, 20% dioxane.

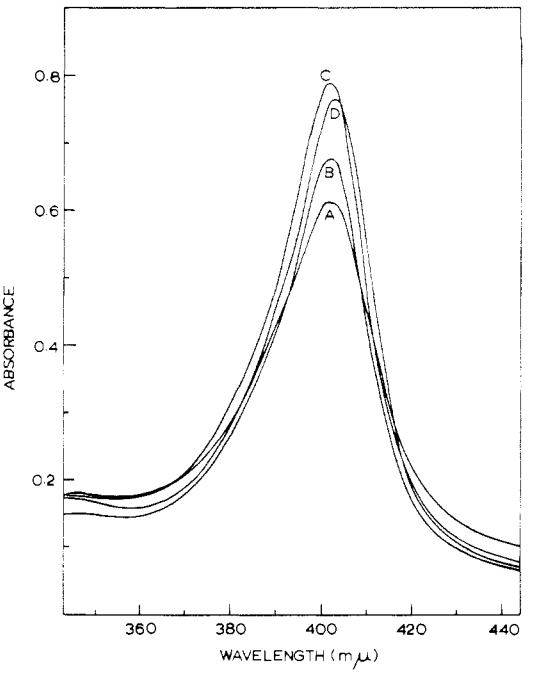

FIGURE 5: Effect of dilution on the Soret spectrum of bishistidine mesoheme: curve A, 6.65×10^{-5} M bishistidine mesoheme (0.1-cm light path); curve B, 6.65×10^{-6} M (1-cm light path); curve C, 6.65×10^{-7} M (10-cm light path), all in 0.1 M potassium phosphate buffer, pH 7.0. In curve D, a 10-cm light path cuvet contained 6.65×10^{-7} M bishistidine mesoheme in 10% dioxane–potassium phosphate buffer.

have occurred if aggregated forms persisted at this concentration.

Spectral Studies on the Heme Derivatives. The spectra of mesoheme, monohistidine mesoheme, and bishistidine mesoheme are compared in Figure 6. These measurements were made at dioxane concentrations sufficient to produce maximal extinction coefficients. In the Soret region, there is a shift of the maximum to longer wavelengths with increased histidine content of the derivatives in both oxidized and reduced forms. The monohistidine mesoheme is distinguished by its high extinction coefficient in the oxidized form. However, in the reduced form, this derivative has a much broader band than either mesoheme or bishistidine mesoheme, perhaps reflecting only partial coordination of the single histidine. This interpretation is also suggested by the fact that imidazole is a stronger ligand in the oxidized form than in the reduced form, as will be discussed below.

In the α–β region of the spectra, the oxidized maxima also show a shift to higher wavelength with increased histidine content of the derivatives. However, the reduced α and β maxima show a slight shift in the opposite direction with increased histidine content. All three reduced spectra have weak shoulders at about 605 mμ, although this band of the bishistidine derivative is significantly weaker than the other two. This 605-mμ band is not generally observed in reduced heme protein spectra, and probably reflects a certain amount of high-spin character of the reduced form by analogy to the 620-mμ charge transfer band of oxidized high-spin heme spectra.

The spectrum of the imidazole complex of mesoheme is very similar to that of bishistidine mesoheme, as shown in Figure 7. The principal differences are that the entire bishistidine mesoheme spectrum is shifted about 2 mμ to higher wavelengths and the extinction coefficients are about 20% lower overall than mesoheme plus imidazole. However, the shapes of the curves and the ratios of the extinction coefficients are nearly identical (Table I). The spectrum of the imidazole complex has a dependence on dioxane concentration similar to that of the heme–amino acid derivatives (see Figure 2).

The spectra of the bismethionine, monomethionine, and histidine–methionine derivatives of mesoheme are shown in

TABLE I: Extinction Coefficient Ratios of Reduced Heme Derivatives.

Mesoheme Derivative	α/β	α/min	Soret/α
Mesoheme	0.96	1.05	12.0
Monomethionine mesoheme	1.10	1.39	5.6
Bismethionine mesoheme	1.12	1.57	5.6
Histidine–methionine mesoheme	1.33	1.88	6.7
Monohistidine mesoheme	1.22	1.12	8.9
Bishistidine mesoheme	1.59	2.16	6.7
Mesoheme + 0.1 M imidazole–HCl	1.60	2.31	5.9
Cytochrome c^a	1.74	3.85	4.7

[a] From Margoliash *et al.* (1959).

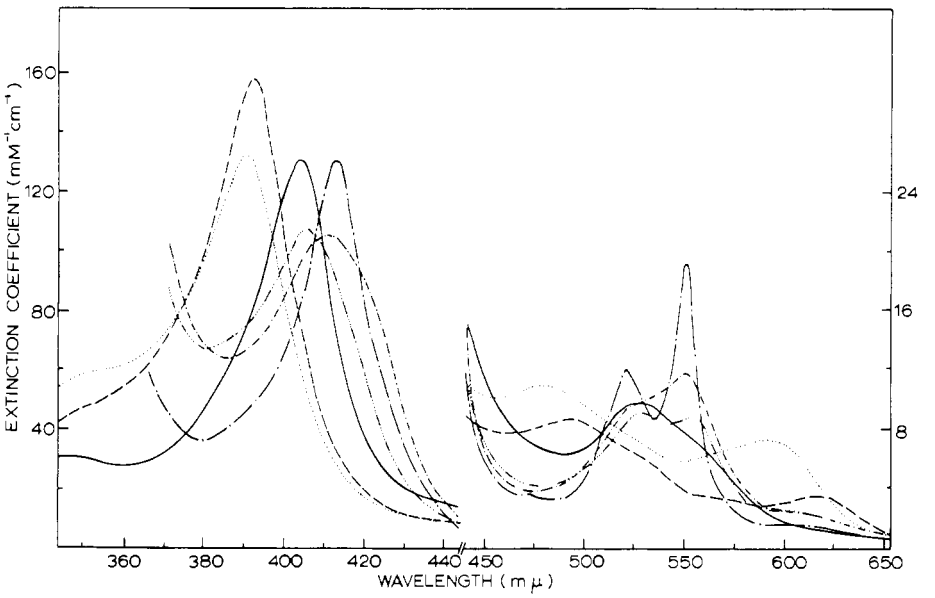

FIGURE 6: Spectra of mesoheme, monohistidine mesoheme, and bishistidine mesoheme. For the Soret band measurements, the heme derivative concentrations were 5.0×10^{-6} M in 25% dioxane–potassium phosphate buffer, pH 7.0. For the α–β band measurements, concentrations were 2.5×10^{-5} M in 40% dioxane–potassium phosphate buffer, pH 7.0: (···) mesoheme oxidized; (···—···) mesoheme reduced; (------) monohistidine mesoheme oxidized; (·—·) monohistidine mesoheme reduced; (—) bishistidine mesoheme oxidized; (·——·) bishistidine mesoheme reduced.

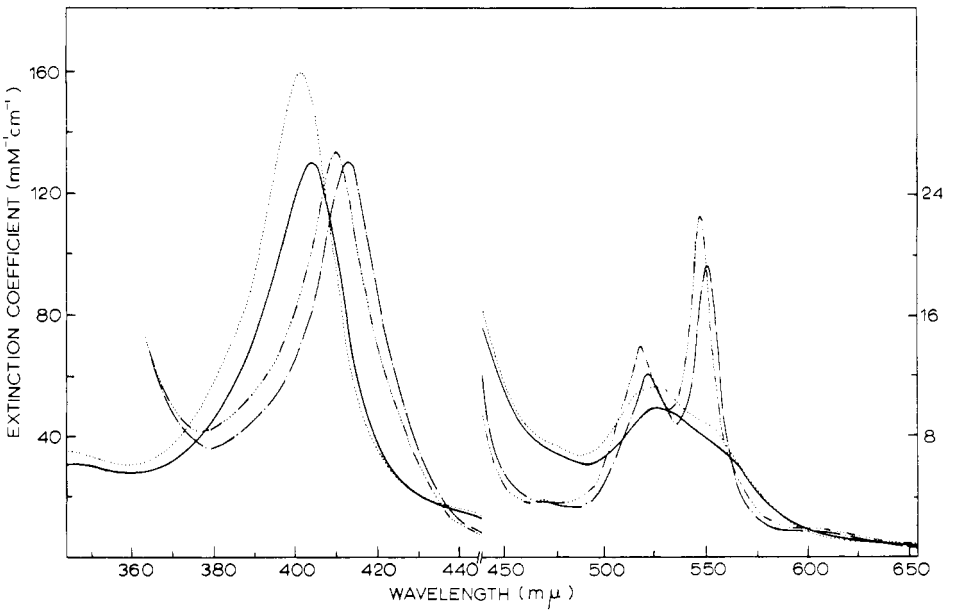

FIGURE 7: Spectral comparison of bishistidine mesoheme with mesoheme plus excess imidazole. Concentrations for the Soret spectra were 5.0×10^{-6} M in 25% dioxane–potassium phosphate buffer, pH 7.0, and 25% dioxane–0.1 M imidazole-HCl buffer, pH 7.0. For the α–β spectra, concentrations were 2.5×10^{-5} M in 40% dioxane–0.06 M potassium phosphate buffer, pH 7.0, and 40% dioxane–0.10 M imidazole–HCl buffer, pH 7.0: (——) bishistidine mesoheme oxidized; (·—·) bishistidine reduced; (····) mesoheme + imidazole oxidized; (···—···) reduced.

Figure 8. These spectra were run in 30% dioxane–phosphate buffer, pH 7.0, because of the pronounced decreases of the extinction coefficients of the bismethionine and monomethionine derivatives in the reduced form at higher dioxane concentrations. These decreases probably reflect aggregation of the methionine derivatives when the positive charge on the iron is lost on reduction. The oxidized spectra of bismethionine and monomethionine mesoheme are very similar to the spectrum of mesoheme in Figure 6. This similarity suggested that the methionine might be very weakly coordinated to the heme or not coordinated at all. A sample of bismethionine mesoheme in 30% dioxane buffer, pH 7.0, was

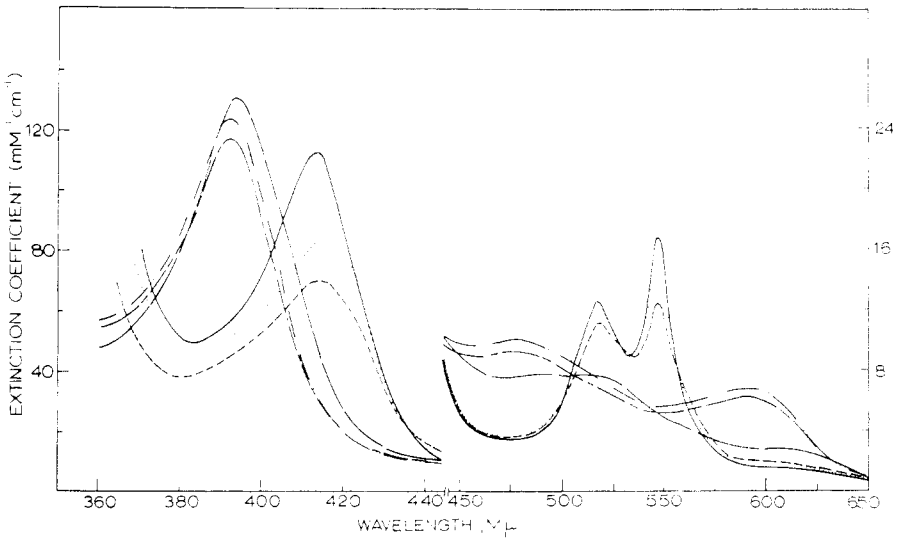

FIGURE 8: Spectra of histidine–methionine mesoheme, monomethionine mesoheme and bismethionine mesoheme. Concentrations are 5.0×10^{-6} and 2.5×10^{-6} M in 30% dioxane–potassium phosphate buffer, pH 7.0, for the Soret band and α–β band spectra, respectively; (— —) histidine–methionine mesoheme oxidized; (——) histidine–methionine mesoheme reduced; (— — —) monomethionine mesoheme oxidized; (-----) monomethionine mesoheme reduced; (· · ·—· · ·) bismethionine mesoheme oxidized; (· · · ·) bismethionine mesoheme reduced.

titrated with 1.0 M α-*N*-acetylmethioninamide, but even at a level of 0.45 M, the spectrum had changed very little, although the extinction coefficients had increased by 25 to 40% between 450 and 600 mμ. Thus, an extremely high concentration of methionine would be required in order to achieve full complexation if the fully complexed form gives a low-spin-type spectrum.

The reduced spectra of the methionine derivatives in Figure 8 show significant differences. Perhaps the most significant difference is found in the α to β ratio of the extinction coefficients. Table I lists this ratio for each of the synthetic heme-amino acid derivatives. The values suggest the generalization that the α to β ratio increases with increasing histidine content and decreasing methionine content for the bisamino acid derivatives. The rather slight differences between the spectra of the mono- and bismethionine derivatives can be explained in part by contamination of the monomethionine derivative with substantial amounts of bismethionine mesoheme, as mentioned in the preceding paper (Warme and Hager, 1970). In the Soret region, the peaks of both the monomethionine and bismethionine derivatives have shoulders at 420 mμ. In this connection, it is relevant to note that the sample of bismethionine mesoheme in 0.45 M acetylmethioninamide mentioned above gave a reduced Soret band at 420 mμ with a shoulder at about 416 mμ. In the α–β region, this sample had a reduced spectrum which was quite similar to the monomethionine mesoheme spectrum. If the 420-mμ-reduced Soret band is characteristic of a true bismethionine complex, it follows that the methionine does not fully coordinate even in the reduced form of the synthetic heme derivatives. The resulting mixed spectrum could account for the broad bands and low extinction coefficients of the methionine derivatives.

Imidazole Titrations. In order to establish that the histidine of monohistidine mesoheme is coordinated to the heme iron under the conditions of the spectral studies, both mesoheme and monohistidine mesoheme were titrated with imidazole–HCl, pH 7.0. The titration curve of monohistidine mesoheme conforms closely to the theoretical curve for association of a single ligand with $pK_{Im} = 3.9$ as shown in Figure 9. This figure also shows the experimental points for the titration of mesoheme with imidazole, bracketed between the theoretical curves for binding of a single ligand (curve A) and for simultaneous binding of two ligands (curve B) with $pK_{Im} = 3.0$. The experimental points conform fairly closely to curve B at low imidazole concentrations but fall between the theoretical curves at higher imidazole concentrations. Thus, the binding of imidazole to mesoheme is a stepwise process, although the first and second association constants must be only slightly different in value. The imidazole concentration required for complete coordination of imidazole with mesoheme is considerably higher than is required for monohistidine mesoheme.

Nearly constant isosbestic points were observed during both imidazole titrations, but the wavelengths of the isosbestic points were different. The spectra during the mesoheme titration had isosbestic points at 451, 505, and 563 mμ, while the monohistidine spectra had isosbestic points at 471, 506, and 584 mμ. The fact that almost constant isosbestic points were observed in the mesoheme titration is probably fortuitous, but this again suggests that the first and second association constants must have nearly the same value. Furthermore, at intermediate concentrations of imidazole, no spectrum similar to that of monohistidine mesoheme was observed, which indicates that at no point is the mono-imidazole intermediate present at appreciable levels.

A sample of bismethionine mesoheme in 30% dioxane buffer, pH 7.0, was also titrated with imidazole–HCl, pH 7.0, and a $pK_{Im} = 3.0$ was evaluated. The isosbestic points during this titration were at 453, 507, and 568 mμ, which correspond closely to those found during the mesoheme titration. This finding supports the conclusion that the methionine is very weakly coordinated, if at all, in the oxidized form. However, at the final concentration of imidazole in this titration (0.015

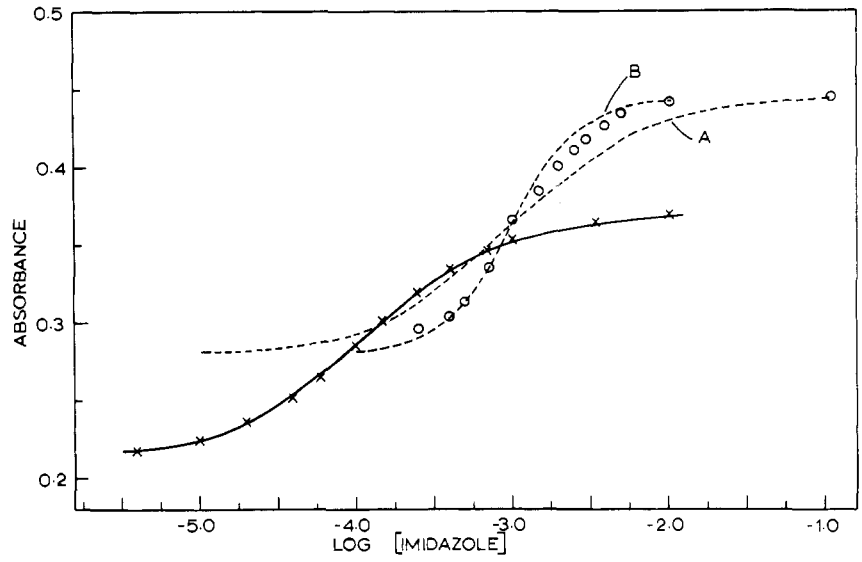

FIGURE 9: Titration of mesoheme and monohistidine mesoheme with imidazole. The lower curve (×—×) shows the experimental points for titration of 2.5×10^{-5} M monohistidine mesoheme with imidazole–HCl, pH 7.0, fitted by a theoretical dissociation curve with $pK = 3.9$. The experimental points for titration of 3.8×10^{-5} M mesoheme (○) are superimposed on theoretical dissociation curves for binding of a single ligand (A) and two ligands (B) with $pK = 3.0$. The absorbance changes were recorded at 525 mμ.

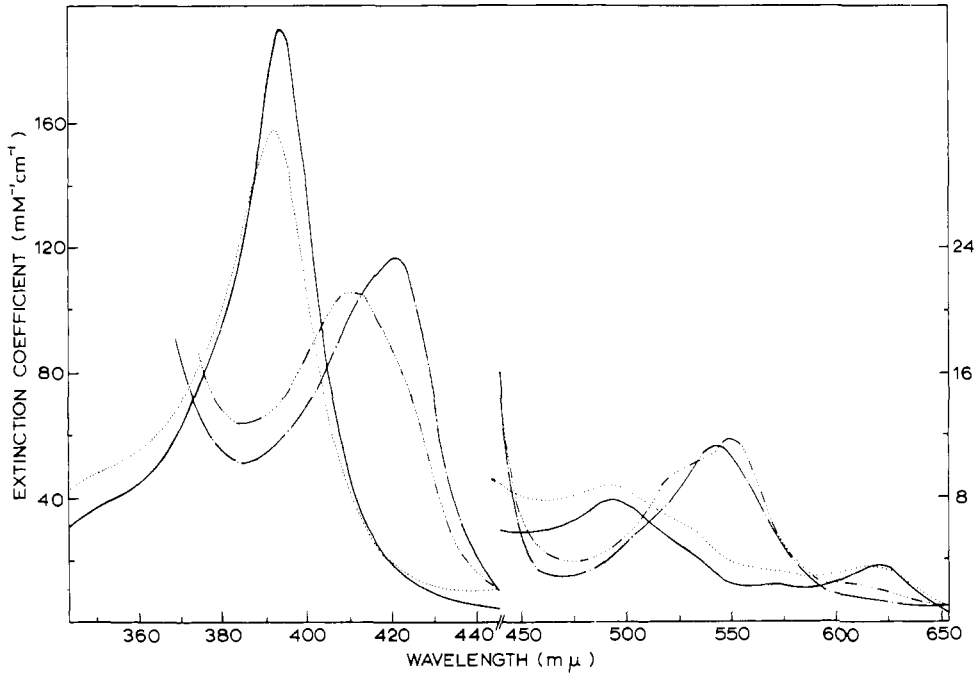

FIGURE 10: Spectral comparison of monohistidine mesoheme and mesoheme myoglobin. The Soret band concentrations were 5.0×10^{-6} M and the α–β band concentrations were 2.5×10^{-5} M. Monohistidine mesoheme (· · · · ·) oxidized, and (· · ·—· · ·) reduced in 25 and 40% dioxane–potassium phosphate buffer, pH 7.0. Mesoheme myoglobin (——) oxidized, and (·—·) reduced in 0.1 M potassium phosphate buffer, pH 7.0.

M), the reduced spectrum had strong α and β peaks, in contrast to mesoheme which gave hardly a trace of hemochrome bands at this imidazole concentration. Furthermore, the α to β ratio was 1.25, indicating that the predominant form is the bismethionine complex (*cf.* Table I). In 0.1 M imidazole–HCl, reduced mesoheme is essentially completely complexed with imidazole, but reduction of bismethionine mesoheme gave an α to β ratio of 1.46, which falls between the ratios for histidine–methionine and bishistidine mesoheme. Thus, at imidazole concentrations between 0.01 and 0.1 M, the covalently bonded methionine can effectively compete with imidazole for the ligand sites of reduced heme.

Comparison with Heme Protein Spectra. In Figure 10, the spectrum of monohistidine mesoheme is compared with the

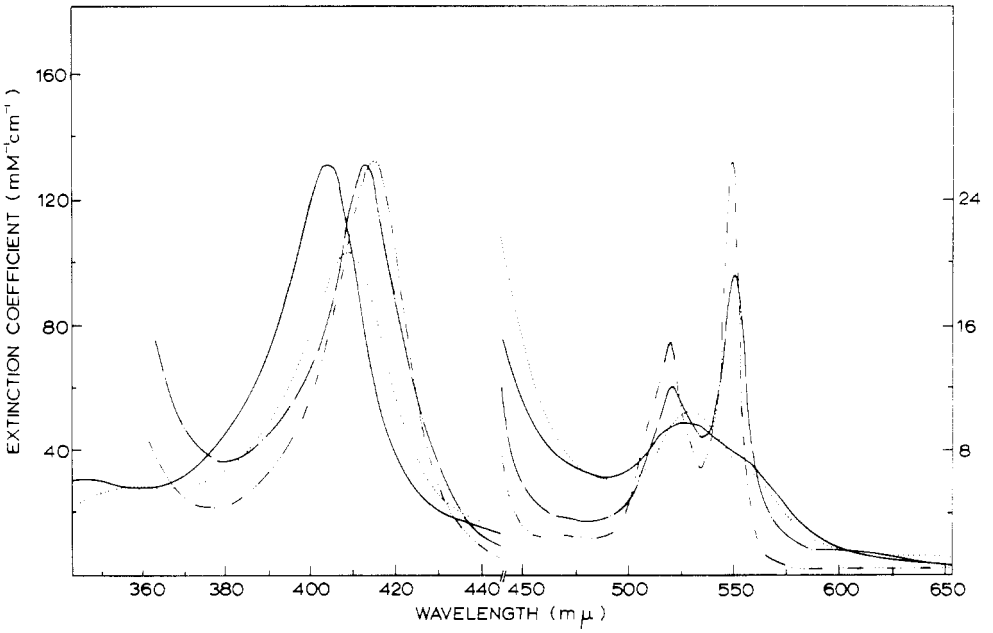

FIGURE 11: Spectral comparison of bishistidine mesoheme and cytochrome *c*. Soret band concentrations were 5.0×10^{-6} M and α-β band concentrations 2.5×10^{-5} M. Bishistidine mesoheme (——) oxidized and (· — ·) reduced in 25 and 40% dioxane-potassium phosphate buffer, pH 7.0, respectively. Cytochrome *c* (· · · ·) oxidized and (· · · — · · ·) reduced in 0.1 M potassium phosphate buffer, pH 7.0.

TABLE II: Wavelengths (mμ) and Extinction Coefficients of Heme Derivatives.

Mesoheme Derivative		Oxidized				Reduced			
		CT[a]	α′	β′	Soret	α	Min	β	Soret
Mesoheme	λ	591		480	391	554	542	529	407
	ε	(7.3)		(11.0)	(127)	(8.8)	(8.4)	(9.2)	(106)
Mono-methionine	λ	592		479	393	548	534	518.5	415
	ε	(6.2)		(9.4)	(116)	(12.4)	(8.9)	(11.2)	(70)
Bismethionine	λ	592		479	393	548	534	518.5	415.5
	ε	(6.8)		(10.1)	(122)	(14.8)	(9.4)	(13.2)	(82)
Monohistidine	λ	617	526	493	393.5	551	536	523	412
	ε	(3.5)	(6.3)	(8.8)	(155)	(12.7)	(10.4)	(9.6)	(104)
Mesoheme myoglobin	λ	621	526	494	395	543.5			422.5
	ε	(3.5)	(4.9)	(7.8)	(174)	(11.2)			(118)
Histidine-methionine	λ	606	516	489	394.5	548	533	518.5	414
	ε	(2.6)	(7.7)	(7.9)	(130)	(16.7)	(8.9)	(12.6)	(112)
Bishistidine	λ		552	529	405	551	536	521	414
	ε		(7.7)	(9.8)	(129)	(19.2)	(8.9)	(12.1)	(129)
Mesoheme + imidazole	λ	600	551	524.5	403	549	532	519	416
	ε	(2.0)	(8.9)	(11.3)	(159)	(22.4)	(9.7)	(14.0)	(133)
Cytochrome *c*[b]	λ		566.5	528	410	550.2	535	520	416
	ε		(7.8)	(11.2)	(106.1)	(27.7)	(7.2)	(15.9)	(129)

[a] Charge transfer band. [b] From Margoliash *et al.* (1959). These data were collected from the spectra in Figures 6, 8, 10, and 11.

spectrum of apomyoglobin plus mesoheme. The shapes of the curves are very similar and the positions of the maxima are nearly identical in the oxidized forms, although the extinction coefficients differ. In the reduced forms, greater differences can be seen, possibly because of incomplete coordination of the histidine of monohistidine mesoheme in the reduced form. Incomplete coordination would also explain the broadness of the reduced monohistidine mesoheme peaks. The spectrum of bishistidine mesoheme is compared with the spectrum of cytochrome *c* in Figure 11. Here, the similarity is less striking. In the Soret region, the oxidized maxima are separated by 4 mμ and there is a 27% difference

between their extinction coefficients. In the reduced form, the maxima are separated by 2 mμ. Further differences can be seen in the α–β region, where the extinction coefficients of the α and β bands are higher in cytochrome *c* by 37% and 23%, respectively. These extinction coefficient differences apparently stem largely from a general sharpening of the bands of cytochrome *c*. The shallow minimum between the α and β bands of all of the synthetic heme derivatives is another manifestation of the broadness of their absorption bands. The wavelengths and extinction coefficients of the absorption bands of all of these heme compounds are summarized in Table II.

Redox Potentials. Some preliminary measurements of the redox potentials of the heme derivatives have been made in collaboration with Dr. G. S. Wilson. The heme derivatives are well-behaved electrochemically and polarographic measurements were made in dioxane–buffer mixtures. Results of the preliminary studies are summarized in Table III. From these values it is clear that increasing the methionine content increases the redox potential. Results of further studies including the pH dependence of the redox potential and properties of heme derivative electron transfer processes will be published subsequently (G. S. Wilson, 1969, unpublished results).

TABLE III: Redox Potentials of Heme–Amino Acid Derivatives.

Derivative	E_0, pH 7.0, [a] V
Bismethionine mesoheme	+0.02
Monomethionine mesoheme	−0.004
Histidine–methionine mesoheme	−0.11
Monohistidine mesoheme	−0.21
Bishistidine mesoheme	−0.22

[a] Formal potentials as calculated from polarographic half-wave potentials. Measurements were made at an "apparent" pH 7.0 in 0.1 M phosphate buffer, 30% (v/v) dioxane–water. Approximate concentrations were 2×10^{-4} M.

Discussion

A major advantage of studying the covalent heme–amino acid derivatives over the study of mesoheme plus extraneous ligands is that the spectra of pure monoligand complexes are accessible. For example, the closeness of the first and second dissociation constants determined by titration of mesoheme with imidazole dictates that at no point during the titration is the concentration of the monoimidazole mesoheme appreciable. Several lines of evidence indicate that the histidine of monohistidine mesoheme is strongly complexed with the heme iron. First of all, titration of monohistidine mesoheme with imidazole gives constant isosbestic points and conforms to the theoretical curve for dissociation of a single ligand, both suggesting that the single covalently bound histidine is strongly coordinated. Another indication of this fact is that the concentration of imidazole required for half-titration of monohistidine mesoheme is only about one-tenth as high as that required for half-titration of mesoheme. The isosbestic points for titration of mesoheme with imidazole differ significantly from those for the monohistidine mesoheme titration. Furthermore, monohistidine mesoheme has a spectrum which is distinctly different from mesoheme but is very similar to the spectrum of the peptic-tryptic heme octapeptide from cytochrome *c*. Harbury and Loach (1960a,b) have presented evidence that this heme peptide has a single histidine ligand. The last argument is that the high Soret band extinction coefficient of monohistidine mesoheme suggests a single spectral form, since partial dissociation of the histidine should cause the spectrum to partially revert to that of mesoheme, thus broadening the absorption band.

The similarity between the spectrum of mesoheme plus excess imidazole and that of bishistidine mesoheme leaves little doubt that both histidines are coordinated in the latter derivative. The possibility of intermolecular coordination of a histidine from one molecule to the heme iron of another molecule seems to be ruled out by the aggregation studies. Intermolecular interactions should be broken down by either dioxane or dilution, thus causing a shift to the spectrum of monohistidine mesoheme. No evidence of such a shift was observed even at the highest dilution or dioxane concentration. Intermolecular coordination would be more likely for the monohistidine derivative, which would offer no steric hindrance to the approach of a second molecule. Some indications of intermolecular coordination in monohistidine mesoheme have in fact been observed, but only in samples diluted from concentrated stock solutions which had been stored at 0° for more than a day. Such samples gave lower extinction coefficients, took a longer time to achieve the maximum extinction coefficient in dioxane–buffer mixtures, and exhibited a stronger shoulder at about 520 mμ. This effect was at least partially irreversible in dioxane–buffer solution and probably indicates the presence of some of the intermolecular bishistidine form.

The impossibly high concentration of *N*-acetylmethioninamide which would be required to achieve a low-spin bismethionine complex with oxidized mesoheme indicates that either methionine is a very weak ligand or that a bismethionine spectrum is actually of the high-spin type and is quite similar to that of mesoheme. The former interpretation is favored in view of the ease of displacement by added imidazole. Harbury *et al.* (1965) found that addition of 2 M *N*-acetylmethionine to the heme octapeptide from cytochrome *c* produced a complex with a low-spin-type spectrum, but there is good reason to believe that this heme peptide already has a histidine ligand, which probably makes coordination of a second ligand, even a weak ligand like methionine, a more favorable process. Since complete complexation in this favorable case requires such a high concentration of methionine, the prospect of forming a bismethionine complex appears dim. The spectrum of histidine–methionine mesoheme in the oxidized form (Figure 8) appears to be a cross between the spectrum of bishistidine mesoheme and monohistidine mesoheme (Figure 6). This would suggest that the methionine is partially coordinated in this derivative, which would again be favored by prior coordination of the histidine. Of course, in both of these cases, the presence of a histidine ligand could be the decisive factor for the spin type.

One possible reason for the lack of complete coordination of the ligands in the synthetic heme derivatives is that in some of the molecules, both propionic acid side chains are directed

toward the same side of the heme plane. The molecular model studies discussed previously (Warme and Hager, 1970) indicated that rotation around the heme to β-carbon bond of the propionate groups is sterically hindered by the neighboring methyl group side chains of the heme. However, the X-ray structure of myoglobin by Kendrew (1961) shows the propionic acid side chains extending toward opposite sides of the heme, while the X-ray analysis of cytochrome *c* by Dickerson *et al.* (1968) indicates that both propionate groups are on the same side of the heme plane. Thus, the question of propionate isomers of the heme–amino acid derivatives remains open. Because of the tightly restricted geometry in the heme peptides, an intermolecular bisligand complex would be sterically hindered if the propionate groups are directed toward opposite sides of the heme plane.

The value of these heme peptides as models for interpretation of hemeprotein spectra is best exemplified by comparison of the monohistidine mesoheme spectrum with that of mesoheme myoglobin, in which the normal protoheme prosthetic group is replaced by mesoheme (Figure 10). Myoglobin has been shown to have a single histidine ligand by the X-ray diffraction analysis of Kendrew (1961), and the same conclusion may be drawn from the similarity of its spectrum to that of the model compound. The spectrum of mesoheme myoglobin possesses a general similarity to the normal protoheme myoglobin in both the oxidized and reduced forms, although the bands are all displaced by 10 to 15 mμ to lower wavelengths.

The possibility that cytochrome *c* has two histidine ligands was first suggested by Theorell (1941) and Theorell and Akesson (1941). Since then, this hypothesis has been extended by a variety of lines of experimental evidence. The correspondence of the spectrum of bishistidine mesoheme with that of cytochrome *c* is not very close, although there are definite similarities. In the α–β region, the principal differences are in the extinction coefficients. These spectral similarities point out potential dangers in extrapolating the results of heme–amino acid studies to cytochromes, in view of the more recent reports that methionine is very likely one of the ligands in cytochrome *c*, at least in the reduced form (Hettinger *et al.*, 1966). However, the spectrum of histidine–methionine mesoheme is even more different from the spectrum of cytochrome *c* than is bishistidine mesoheme, probably because the methionine is only partially coordinated, at least in the oxidized form.

Preliminary experiments on the redox potential of the heme derivatives corroborate the conclusions drawn from spectral data. If the redox potential is viewed as a reflection of the relative strengths of the oxidized and reduced complexes, these potentials are seen to be in agreement with the spectrophotometric studies on the heme peptides. The methionine ligand is weak in the oxidized form but is strong in the reduced form, whereas the opposite is true of the histidine ligand. The redox potential of bishistidine agrees closely with the potential of the heme octapeptide of cytochrome *c* measured in the presence of added histidine (Harbury and Loach, 1960a). The potential of histidine–methionine mesoheme (-0.11) compares with a value of -0.05 for the heme octapeptide of cytochrome *c* in the presence of added methionine (Harbury *et al.*, 1965). Both values are far below the redox potential of cytochrome *c* ($+0.26$ V). This could be a result of the very weak and incomplete coordination of methionine in the oxidized heme derivatives which was indicated by the spectrophotometric studies. It is likely that histidine–methionine mesoheme has one histidine ligand and one water ligand in the oxidized form. If all of the forces of the protein are brought to bear on the methionine in cytochrome *c* to cause complete coordination even in the oxidized form, the potential might be altered considerably. This appears to be the case, since the spectrum of cytochrome *c* is definitely of the low-spin type, which indicates strong coordination with two ligands.

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