Heme Sulfuric Anhydrides. I. Synthesis and Reactions of Mesoheme Sulfuric Anhydride*

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ABSTRACT: Mesoheme sulfuric anhydrides are activated derivatives of mesoheme, in which one or both of the propionic acid carboxyl groups has been converted into a sulfuric anhydride. These anhydride derivatives react readily with nucleophiles such as amino groups of amino acids to form amide bonds. The synthesis of mesoheme sulfuric anhydrides and their reactions with histidine and methionine methyl ester are described. The monohistidine, bishistidine, monomethionine, bismethionine, and histidine–methionine derivatives of mesoheme can be purified by chromatography on silicic acid. The covalently bound amino acids can serve as ligands to the fifth and sixth coordination positions of the heme. These derivatives may be useful models for physical studies on heme proteins.

We have recognized the need for well-defined model compounds to aid in the interpretation of the spectral, electrochemical, and optical rotatory properties of heme proteins. The most useful type of model compound would be one in which ligands are covalently bound to the heme molecule in a manner allowing coordination to the heme iron. Covalent binding of the ligands to the heme molecule should maximize the probability of coordination of the ligand, thus avoiding the medium effects which undoubtedly result when high concentrations of noncovalently bound ligands are added to achieve the same objective. With covalent ligands, it is also possible to study the properties of mixed-ligand complexes. This is not generally possible by addition of a mixture of noncovalently bound ligands, since one type of ligand or the other is usually preferentially coordinated.

One route to the synthesis of model heme derivatives with covalently bound ligands is to attach cysteine to the vinyl side chains of protoheme *via* thioether linkages, as in cytochrome *c*. This method has been used by Lautsch *et al.* (1958) and by Sano *et al.* (1964). The carboxyl or amino groups of the cysteine can then serve as a nucleus for addition of potential liganding groups. Another possible route is to react the liganding group with the propionic acid side chains of the heme molecule. We have followed the latter route, using the sulfuric anhydride derivative of mesoheme (Figure 1) as an activated intermediate. This water-soluble heme derivative is capable of reaction with α- and ϵ-amino groups of amino acids and proteins to form amide bonds. The sulfuric anhydride method for carboxyl group activation was introduced by Kenner and Stedman (1952) for use in peptide synthesis. Aside from the high yields of peptides attainable by this method, the principal advantage is that the peptide bond can be formed in aqueous solution. This property is important for a derivative which is designed to react with proteins as well as amino acids. Lautsch and coworkers (1957) have used other peptide synthesis methods including the azide, acid chloride, isocyanate, and phosphazo methods for synthesis of heme peptides with amino acids covalently bound at the propionic acid side chains of heme. However, these methods are not well suited to reaction with proteins, since the activated compounds are either insoluble in water or hydrolyze rapidly.

Since mesoheme has two propionic acid side chains, both monoanhydride and bisanhydride derivatives are possible. In this paper, we describe the preparation of mesoheme mono- and bissulfuric anhydrides, their reaction with histidine and methionine, and the purification of the various histidine and methionine derivatives of mesoheme. Histidine-containing derivatives of mesoheme have previously been synthesized by the phosphazo method (Lautsch *et al.*, 1958).

Experimental Section

Preparation of SO_3–Dimethylformamide Complex. The complex of sulfur trioxide with dimethylformamide was prepared by a modification of the method of Clayton *et al.* (1959). Instead of repeated distillations of SO_3 from 60% oleum as used by these workers, a stabilized liquid form of SO_3, Sulfan, was distilled directly into dimethylformamide. All operations with SO_3 and the SO_3–dimethylformamide complex were performed under dry nitrogen to exclude moisture. The Sulfan (5.5 ml) was distilled (45°) over a 1–2-hr period into 50 ml of anhydrous dimethylformamide in a cooled (−10°) receiver flask fitted with a stirrer and a P_2O_5 guard tube. The distillation was stopped when about 80% of the SO_3 had distilled over and then the solution was diluted with anhydrous dimethylformamide (about 50 ml) to give an estimated concentration of 1 M SO_3–dimethylformamide complex in dimethylformamide. The SO_3–dimethylformamide solution was stable for several months when stored over P_2O_5 at −20°.

Assay of SO_3–Dimethylformamide Complex. The total sulfur content of the SO_3–dimethylformamide solution was determined as sulfuric acid by diluting a measured volume into a nearly equivalent amount of standard base and titrating

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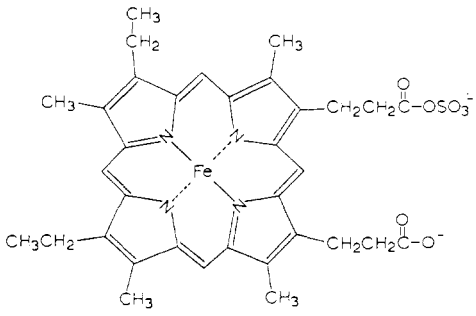

FIGURE 1: Structure of mesoheme monosulfuric anhydride.

to the phenolphthalein end point. The SO_3–dimethylformamide complex could be conveniently assayed by reacting it with acetate to form acetylsulfuric anhydride according to eq 1. The product, acetylsulfuric anhydride, was used to

$$SO_3\text{–}HCN(CH_3)_2 + CH_3CO^- \longrightarrow CH_3COSO_3^- + HCN(CH_3)_2 \quad (1)$$

$$CH_3COSO_3^- + NH_2CH_2COCH_2CH_3 \longrightarrow CH_3CNHCH_2COCH_2CH_3 + HSO_4^- \quad (2)$$

acetylate glycine ethyl ester (see eq 2). The extent of glycine acetylation was determined by the loss of ninhydrin-positive material. To ensure virtually complete reaction of the SO_3–dimethylformamide with acetate to form acetylsulfuric anhydride, excess acetic acid was used in the activation reaction. In the acetylation step, excess glycine ethyl ester was used to drive the reaction to completion. The assay conditions were as follows: 0.20 ml of glacial acetic acid (3.50 mmoles) and 0.80 ml of triethylamine (5.72 mmoles) were mixed in 1.5 ml of anhydrous dimethylformamide and cooled to 0°. An aliquot of this solution (50 μl, 7.0×10^{-2} mmole) was added to 50 μl of the standardized SO_3–dimethylformamide solution ($\sim 5 \times 10^{-2}$ mmole) at 0° under N_2 and allowed to react for 30 min at 0°. Glycine ethyl ester hydrochloride (50 mg, 0.36 mmole) was dissolved in 1.0 ml of anhydrous dimethylformamide with gentle warming and 0.167 ml (1.19 mmoles) triethylamine was added. After shaking for 10 min at room temperature, triethylamine hydrochloride was removed by centrifugation and 0.20 ml of the supernatant was added at 0° to the acetylsulfuric anhydride solution prepared above. After reaction for 1 hr at room temperature, colorimetric ninhydrin analyses were performed on the glycine ethyl ester solution before and after reaction with acetylsulfuric anhydride. Using this method, a 91–93% yield of *N*-acetylglycine ethyl ester (based on the total amount of sulfur present) was obtained with the SO_3–dimethylformamide preparation used in these studies.

Preparation of Mesoheme Lithium Chloride Salt. Mesoheme was prepared by hydrogenation of protohemin on PtO_2 as described by Baker and Corwin (1966). When prepared in this way, the mesoheme was contaminated with by-products arising from over-reduction of protoheme. These by-products were detected by thin-layer chromatography on silicic acid in a butanol–pyridine–water (1:1:1) system. Mesoheme had an R_F value of 0.65 in this solvent. Several by-products could be observed at R_F values of 0.0, 0.3, and 0.45. To purify this preparation, the crude dry mesoheme (0.8 g) was ground in a mortar to a fine powder and extracted repeatedly with 100-ml portions of chloroform on a filter funnel fitted with Whatman No. 1 paper. The extraction on the filter was continued until the filtrate was only slightly colored (about 2 l. of $CHCl_3$). The combined filtrates were run through a 3×5 in column of Whatman No. 1 cellulose powder, standard grade (equilibrated with $CHCl_3$), at a flow rate of about 500 ml/hr. The column was washed with $CHCl_3$ until the effluent was light brown in color and the combined eluates (total volume about 2.5 l.) were evaporated to dryness. The mesoheme residue was dissolved in 6 ml of 1 M LiOH, diluted with 300 ml of water, and then quantitatively precipitated by addition of 6 ml of 1 M HCl. The mesoheme lithium chloride salt was collected by centrifugation and the pellet was suspended in 200 ml of water, recentrifuged, and dried *in vacuo*, then over P_2O_5. This method gave a 55% yield of a product which produced a single spot (R_F 0.65) in the butanol–pyridine–water (1:1:1) thin-layer system. The pyridine hemochrome spectrum (2.1 M pyridine, 0.075 M NaOH, Thunberg cuvet) given by the purified mesoheme differed significantly from that of the crude mesoheme but agreed closely with the literature value (Falk, 1964).

Synthesis of Mesoheme Sulfuric Anhydride. The lithium chloride salt of mesoheme as prepared above was converted into its sulfuric anhydride derivative by dissolving 50 mg (0.075 mmole) in 1.0 ml of 1 M SO_3–dimethylformamide solution in dimethylformamide. After the heme had completely dissolved, triethylamine (0.05–0.15 ml, 0.35–1.10 mmoles) was added and the anhydride formation was allowed to proceed for 30 min at room temperature. All of the above operations were performed under an atmosphere of dry nitrogen to exclude moisture. If anhydrous conditions are maintained, the mesoheme anhydride solution can be stored for up to 1 month at −20° without significant change. Since mesoheme has two propionic acid side chains available for anhydride formation, the reaction mixture generally contains both monoanhydride and bisanhydride, as well as small amounts of unreacted mesoheme. The extent of anhydride formation can be controlled roughly by varying the amount of triethylamine within the range indicated above. At the lower triethylamine concentration, predominantly monoanhydride is formed, while at the high triethylamine concentration, the major product is the bisanhydride. A qualitative indication of the extent of anhydride formation was routinely obtained by reacting the anhydride mixture with histidine and separating the products by thin-layer chromatography. For this purpose, a 5-μl sample (0.38 μmole) of the anhydride solution as prepared above was mixed with 0.1 ml of 0.33 M L-histidine buffer, pH 9.0 (0°), and reacted for 2 hr at room temperature. A small aliquot was spotted on an Eastman Chromagram silicic acid plate and ascending chromatography was carried out in butanol–acetic acid–water (3:1:1). In this solvent system, the product of the reaction of the bisanhydride with histidine has an R_F value of 0.35, while the product of the reaction of the monoanhydride with histidine has an R_F value of 0.60. Unreacted mesoheme has an R_F value of 0.80

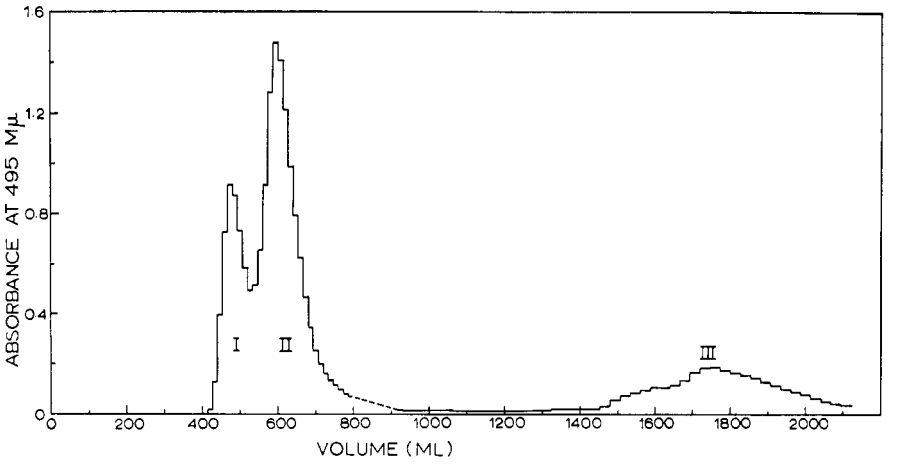

FIGURE 2: Elution profile for silicic acid chromatography of monohistidine and bishistidine mesoheme: fractions I, II, and III contain mesoheme (22%), monohistidine mesoheme (54%), and bishistidine mesoheme (24%), respectively. The column was eluted with butanol–acetic acid–water (4:1:1).

in this system. In order to avoid interference by excess histidine on the thin-layer plate, the test samples were in some cases diluted to 1.0 ml and adjusted to pH 5 with 1 M HCl. The precipitated heme peptides were collected by centrifugation and the supernatant, containing most of the excess histidine, was decanted. The heme peptides were then dissolved in acetic acid and chromatographed as above.

Synthesis and Purification of Bishistidine and Monohistidine Mesoheme Derivatives. For large-scale synthesis of the histidine derivatives of mesoheme, 0.40 ml (0.03 mmole) of the mesoheme anhydride solution prepared above was mixed with 4 ml of 0.32 M L-histidine buffer, pH 9.0, at 0° and reacted for 2 hr at room temperature. The deep red solution was then mixed with 4 ml of glacial acetic acid and 16 ml of 1-butanol and charged on a 6 × 25 cm column of Unisil silicic acid (200–325 mesh). The silicic acid was prepared by equilibrating and deaerating 320 g of Unisil in 700 ml of butanol–acetic acid–water (4:1:1) prior to pouring the column. The column was developed with the same solvent at maximal flow rate (~250 ml/hr) and 13.3-ml fractions were collected. The elution profile (Figure 2) shows resolution of the three expected products, mesoheme, monohistidine mesoheme, and bishistidine mesoheme in order of elution. The unreacted histidine was eluted after bishistidine mesoheme. Since monohistidine mesoheme was incompletely resolved on this column, it was necessary to repeat the chromatography step to obtain pure monohistidine mesoheme. After evaporating the monohistidine mesoheme and bishistidine mesoheme fractions to dryness, the residues were dissolved in a minimal amount of 1 M KOH, diluted to 10 ml with water, and precipitated by addition of 1 M HCl to pH 5. The precipitate was collected by centrifugation, redissolved in KOH, and reprecipitated with HCl. The resulting precipitate was centrifuged down, suspended thoroughly in 10 ml of distilled water, recentrifuged, dried *in vacuo*, and then over P_2O_5. The yield of bishistidine mesoheme was about 4 mg (20% based on the amount of mesoheme used) and the yield of monohistidine mesoheme was about 10 mg (50%). The yield of bishistidine mesoheme could be improved at the expense of monohistidine mesoheme by doubling the amount of histidine buffer used in the coupling reaction and maintaining pH 8.7 throughout the reaction.

Synthesis and Purification of Bismethionine, Monomethionine, and Histidine–Methionine Mesoheme Derivatives. A mixture of mesoheme mono- and bissulfuric anhydrides was prepared from 100 mg of mesoheme (LiCl salt) as described above. A solution of 1.54 mmoles of L-histidine monohydrochloride (0.295 g) in 5 ml of 1 M KOH was added to 6.2 mmoles of L-methionine methyl ester hydrochloride (1.24 g) in 15 ml of dimethylformamide. The pH was adjusted to 9.0 with 1 M KOH and the slightly turbid solution was cooled to 0°. The mesoheme anhydride solution was added dropwise with stirring at 0° and then the pH was readjusted to 8.8 with 1 M KOH. The mixture was warmed to room temperature and reacted for 2 hr at room temperature while maintaining the pH between 8.5 and 8.8. A red precipitate formed during the reaction. After the 2-hr reaction period, the mixture was adjusted to pH 4 and diluted with 55 ml of water to complete the precipitation. After standing overnight at 0°, the precipitate was collected by centrifugation and then resuspended twice in 40 ml of water and centrifuged to remove the residual unreacted amino acids.

A silicic acid column was prepared from 250 g of Unisil silicic acid suspended in 1.25 l. of butanol–ammonia buffer (1-butanol–0.1 M NH_3 (9:1) adjusted to pH 10 with acetic acid). The gel was deaerated under gentle vacuum before packing a 6 × 23 cm column bed. The heme peptide precipitate above was dissolved in 15 ml of butanol–ammonia buffer, centrifuged to remove a slight precipitate, and the supernatant was placed immediately on the silicic acid column. The column was developed with the same butanol–NH_3 buffer, giving the elution diagram in Figure 3. The major peaks I, II, III, and IV were evaporated to dryness on a rotary evaporator (40–50°). Thin-layer chromatography (Eastman Chromagram silicic acid plates) in the same solvent as used for the column gave major spots at R_F 0.67, 0.24, 0.18, and 0.08 for fractions I through IV, respectively (Figure 4). These spots were subsequently identified as the bismethionine methyl ester (I), monomethionine methyl ester (II), histidine–methionine methyl ester (III), and bishistidine (IV) derivatives

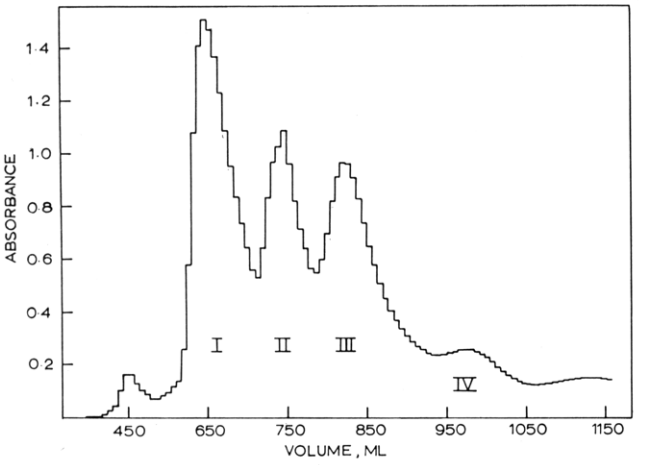

FIGURE 3: Elution profile for silicic acid chromatography on methionine heme derivatives: fractions I, II, III, and IV contain bismethionine mesoheme, monomethionine mesoheme, histidine–methionine mesoheme, and bishistidine mesoheme, respectively. The column was eluted with butanol–0.1 M ammonium acetate, pH 10 (9:1).

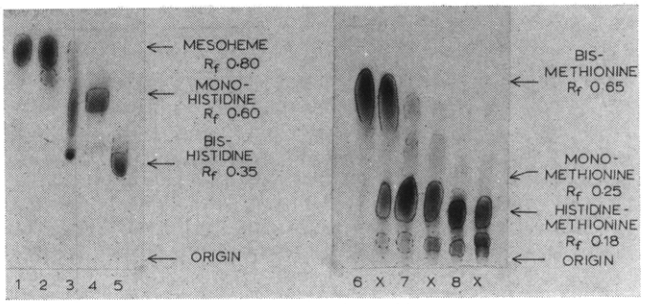

FIGURE 4: Thin-layer chromatography on heme derivatives. Mesoheme (spot 1), base-hydrolyzed mesoheme sulfuric anhydride (spot 2), histidine test sample (spot 3), monohistidine mesoheme (spot 4), bishistidine mesoheme (spot 5), bismethionine mesoheme (spot 6), monomethionine mesoheme (spot 7), and histidine–methionine mesoheme (spot 8) were chromatographed according to the procedures described in the text. The spots, marked by ×'s indicate the chromatographic behavior of fractions recovered between the major peaks on the column.

of mesoheme. The minor peak eluted before fraction I has not been identified.

Since some intercontamination of fractions II and III was indicated by the thin-layer results, these fractions were further purified by rechromatography in the manner described above. Fraction III was still contaminated with some heme component having an R_F value of 0.05 on the thin-layer plate. This contaminant might well result from partial hydrolysis of the methyl ester of the main component under the mildly basic conditions of the chromatographic separation. Only the peak tubes containing about 75% of the heme from each fraction were taken through the remaining steps of the procedure. Fractions I, II, and III were evaporated to dryness on a rotary evaporator (40–50°) and the residues were dissolved in 3.5 ml of acetone plus 0.8 ml of water.

The methyl ester groups were hydrolyzed by the addition of 0.2 ml of 1 M KOH. After 30 min at room temperature, most of the acetone was evaporated by blowing nitrogen over the surface of the samples while keeping them at 30° in a water bath (about 30 min). Each sample was then diluted to 20 ml with water and precipitated by the addition of 0.2 ml of 1 M HCl. The precipitates were collected by centrifugation, redissolved in 0.1 ml of 1 M KOH, diluted to 20 ml with water, and reprecipitated with 0.1 ml of 1 M HCl. After centrifugation, the precipitates were suspended thoroughly in 20 ml of water to dissolve salts and any residual amino acids and then recentrifuged. The precipitates were dried *in vacuo* and then over P_2O_5. The yields of the various fractions were: bismethionine mesoheme (I), 26 mg; monomethionine mesoheme (II), 9 mg; and histidinemethionine mesoheme (III), 19 mg. The combined yield, corrected to the molecular weight of mesoheme, was 38%.

Conversion of the Methionine-Containing Heme Derivatives into Porphyrins. This procedure was patterned after the method of Lemberg *et al.* (1955). About 0.1–0.2 mg of each of the monomethionine, histidine–methionine, and bismethionine mesoheme derivatives was dissolved in 1.0 ml of glacial acetic acid and the tubes were flushed with nitrogen. A solution of 40 mg of ferrous sulfate was prepared in 0.4 ml of 12 N HCl and 40 μl was added to the above heme peptide solutions under nitrogen. The formation of the porphyrin was indicated by the appearance of a fluorescent violet color. After 5–10 min, the solutions were adjusted to pH 4 by addition of 1.0 ml of 3 N NaOH and the porphyrins were extracted into about 2 ml of ethyl acetate. The purplish organic layer was washed twice with 2-ml portions of water to remove traces of iron. Some of the histidine–methionine mesoporphyrin was extracted into the aqueous layer of the second wash, so in this case the aqueous layer was back-extracted with 1.0 ml of ethyl acetate. The ethyl acetate extracts were evaporated first under a stream of nitrogen and finally *in vacuo* to remove the residual acetic acid completely. The residues were dissolved in 0.1 ml of 0.025 M NaOH. The spectra of the porphyrins in pyridine had peaks at 621, 568, 532, 501, and 401 mμ. These peak positions correspond closely to the literature values for mesoporphyrin dimethyl ester, so the extinction coefficient for this compound at 401 mμ (160) was used for evaluation of the porphyrin derivative concentrations (Falk, 1964).

Pyridine Hemochrome Spectra. The conditions for pyridine hemochrome determinations were those suggested by Paul *et al.* (1953). The heme sample was diluted into a basic pyridine solution in a Thunberg cuvet to give a final concentration of 2.1 M pyridine–0.075 M KOH. A small amount (less than 1 mg) of sodium hydrosulfite was placed in the side arm and the cuvet was shaken vigorously while evacuating and flushing with nitrogen for three cycles before reduction.

Amino acid analyses were performed on a Beckman Model 120C amino acid analyzer. Before analysis, the heme peptides were hydrolyzed in 6 N HCl for 21 hr at 110° in evacuated, sealed ampoules.

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

Materials. Sulfan was obtained from Allied Chemical Company. Protoheme IX (hemin) was purchased from Pierce Chemical Co. Spectrograde dimethylformamide was purchased from Matheson Coleman and Bell. Unisil silicic acid was purchased from Clarkson Chemical Co. Eastman Chro-

TABLE I: Amino Acid Compositions of the Synthetic Heme Peptides.

Sample		Residues per Heme		Oxidized Methionine	Total Methionine
		Histidine	Methionine		
Monohistidine mesoheme		1.10			
Bishistidine mesoheme		1.94			
Monomethionine mesoheme	1	0.06	0.37	0.71	1.08
	2	0.05	0.63	1.16	1.79
Histidine–methionine mesoheme	1	0.79	0.0	0.71	0.71
	2	1.07	0.35	0.82	1.17
Bismethionine mesoheme	1	0.0	0.0	1.25	1.25
	2	0.0	0.67	1.50	2.17

TABLE II: Pyridine Hemochrome Wavelengths and Ratios of Mesoheme Derivatives.

Sample	Wavelength (mμ)				Ratio		
	α	min	β	Soret	α:β	α:min	Soret:α
Crude mesoheme	546	531	516	408	1.65	3.00	4.20
Purified mesoheme	546	531	516	407	1.70	3.38	4.11
Mesoheme (Falk, 1964)	547	531	518	407			4.23
Mesoheme anhydride mixture							
(1) 0.1 M KOH, pH 10.3	545	530	515	392	1.80	3.33	8.9
(2) 0.1 M KOAc, pH 5.3	546	531	516	393	1.68	3.16	8.9
Monohistidine mesoheme	546	530	516	407	1.69	3.37	4.29
Bishistidine mesoheme	546	531	516	408	1.71	3.50	4.36
Histidine–methionine mesoheme	546	530	516	407	1.77	3.58	4.19
Monomethionine mesoheme	547	531	516	408	1.77	3.32	4.14
Bismethionine mesoheme	547	531	517	408	1.78	3.59	4.02

magram Sheets are a product of Eastman Kodak Co. CPK Atomic Models were purchased from the Ealing Corporation. L-Histidine monohydrochloride was a product of Nutritional Biochemicals Corp. L-Methionine methyl ester was obtained from Cyclo Chemical Corp.

Results

Identification of Heme Derivatives. A thin-layer chromatogram (butanol–acetic acid–water, 3:1:1) of selected heme compounds is shown in Figure 4 (left). Purified mesoheme (spot 1) gives a single spot having an R_F value of 0.80. Sample 2 was prepared by diluting the mesoheme sulfuric anhydride reaction mixture into 0.1 M KOH in order to hydrolyze the anhydride. In addition to the mesoheme spot (R_F 0.8), this sample had a small amount (10–15%) of a component having an R_F value of 0.7 which is apparently a by-product of the anhydride-forming reaction. The identity of this by-product has not been determined. Sample 3 is a histidine test to measure the extent of anhydride formation, performed as described under Methods. For comparison, samples of purified monohistidine mesoheme (spot 4) and bishistidine mesoheme (spot 5) were included on the plate. In the test sample (spot 3), the presence of unreacted histidine alters the R_F values of the products to some extent, compressing the bishistidine spot and spreading the monohistidine spot. This histidine test shows that the particular anhydride solution used in this experiment contained about equal amounts of monoanhydride and bisanhydride, along with a trace of mesoheme. Removing the excess histidine from the test sample before chromatography, as described in Methods, gave R_F values and shapes for the heme derivative spots which compared closely to those of the purified derivatives.

A preliminary identification of the methionine-containing heme derivatives was made on the basis of their behavior on thin-layer chromatography plates using a 2,6-lutidine–water (3:2) system in an atmosphere of ammonia. The R_F value in this solvent decreases with an increasing number of free carboxyl groups (Falk, 1964). The major components in fractions I through IV had R_F values of 0.94, 0.84, 0.82, and 0.65, respectively. After basic hydrolysis of the methyl esters, all of the samples had an R_F value of 0.65 in the same solvent system. Thus, fraction I was tentatively identified as bismethionine methyl ester mesoheme. Judging from their lower mobilities, fractions II and III should contain a heme derivative having a single methionine methyl ester group. A bishistidine mesoheme structure was assigned to fraction IV, since its chromatographic behavior indicates that it has no

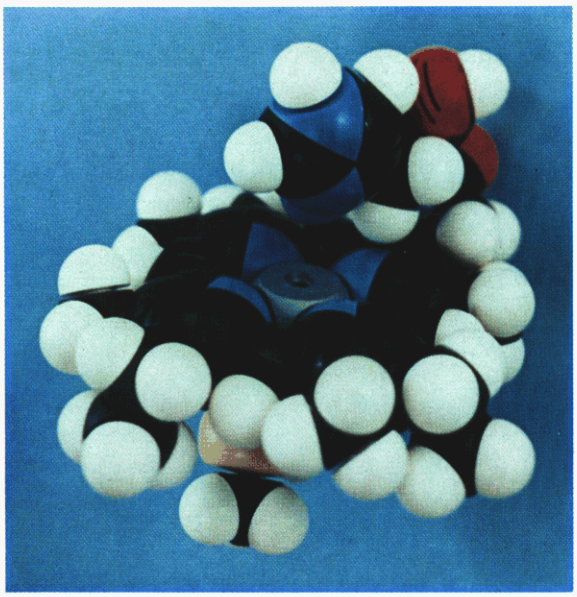

FIGURE 5: Model of histidine–methionine mesoheme: view 1.

FIGURE 6: Model of histidine–methionine mesoheme: view 2.

methyl ester groups. Since polar groups generally decrease the R_F value in silicic acid chromatography, fraction III was tentatively identified as the histidine–methionine methyl ester derivative in view of its lower R_F value. The thin-layer plate shown on the right in Figure 4 was developed in butanol–0.1 M ammonia, pH 10 (9:1). Samples 5, 6, and 7 are the purified fractions of bismethionine, monomethionine, and histidine–methionine mesoheme, respectively. The samples indicated by x's were obtained from the areas between the major peaks on the silicic acid column (Figure 3). These samples were not carried through the final purification steps.

Characterization of Heme Derivatives. Amino acid analyses of acid-hydrolyzed samples of the histidine derivatives of mesoheme gave the results shown in Table I. The heme concentrations were determined by the pyridine hemochrome method (Paul *et al.*, 1953). Amino acid analyses on the bismethionine, monomethionine, and histidine–methionine derivatives were complicated by oxidation of methionine during acid hydrolysis, as shown by the results listed for analysis 1 in Table I. The total methionine values are also low for these analyses. Acid hydrolysis of a methionine standard in the presence of iron gave both oxidation and destruction and several unidentified by-products were detected. Thus, it is likely that the heme iron plays a role in the destruction of methionine. The iron was removed from each of the methionine-containing heme derivatives as described in Methods and acid hydrolysates of these samples gave the results listed under 2 in Table I. The methionine contents of the porphyrins are close to the expected values except for the monomethionine derivative, which is apparently heavily contaminated with bismethionine mesoheme.

Pyridine Hemochrome Parameters. The pyridine hemochrome parameters for the various mesoheme compounds mentioned above are summarized in Table II. A significant difference between the extinction ratios of the crude mesoheme and purified mesoheme was noted. The parameters for the purified mesoheme agree closely with the literature values of Falk (1964). Samples of the mesoheme sulfuric anhydride reaction mixture gave strange pyridine hemochrome results for aliquots diluted into aqueous solutions. The Soret band extinction coefficient before reduction increased slowly with time, while the Soret band in the reduced from decreased with time and was in a different position (391 mμ) than that of mesoheme under the same conditions (407 mμ). The Soret band: α band ratios were extremely high (8.9) in comparison with the normal ratio for mesoheme (4.2). The reason for these anomalous pyridine hemochrome results with crude mesoheme sulfuric anhydride is not presently known in detail, but apparently this has no effect on the reaction of mesoheme anhydride with amino acids or proteins. The pyridine hemochrome parameters of the synthetic heme derivatives are similar enough to those of mesoheme to allow the assumption that the extinction coefficients are the same. Thus concentrations of the heme derivatives have been determined by using an extinction coefficient of 140.4 at the Soret band of the reduced pyridine hemochrome spectrum, as reported for mesoheme. Before reduction of their basic pyridine solutions, the Soret maxima of monohistidine and bishistidine mesoheme are at 401 and 407 mμ, respectively, in contrast to mesoheme, which has a Soret maximum at 392 mμ. These differences indicate that before reduction, the heme peptides retain their histidine ligands, in spite of the high pyridine concentration. However, pyridine is a stronger ligand than histidine in the reduced form and probably displaces the histidine ligands to give a bispyridine complex after reduction.

Discussion and Conclusions. Mesoheme rather than protoheme was selected for the initial studies reported in this paper because preliminary experiments indicated that a side reaction which involved the vinyl side chains of protoheme occurred in the activation step. Since most apoheme proteins have a high affinity for mesoheme as well as protoheme, it was felt desirable to eliminate the side reaction by using mesoheme. The sulfuric anhydrides of protoheme can be prepared by the methods outlined here for mesoheme, although further purification is necessary to remove by-products.

The use of mesoheme sulfuric anhydride for synthesis of heme peptides has several advantages over other methods. The high water solubility and low rate of hydrolysis of this activated derivative allows reaction with free amino acids in aqueous solution, whereas other methods require reaction in organic solvents and carboxyl-protected amino acids must be used. Heme peptides in which the amino acids are attached to the propionic acid side chains have a higher effective concentration of the ligand at the heme iron than heme peptides in which the amino acid is attached to the cysteine of heme C, where the ligand has more translational freedom. Furthermore, the ease of synthesis of the heme peptides by the sulfuric anhydride method recommends its use.

Except for a slight (less than 10%) contamination of the monohistidine mesoheme with bishistidine mesoheme, the histidine heme peptides are chromatographically pure. The amino acid analyses on the histidine–methionine and bismethionine derivatives indicate that these heme peptides are also essentially pure. However, the methionine content of the of the monomethionine derivative indicates greater than 50% contamination with bismethionine mesoheme. The column purification and the R_F values of these derivatives on thin-layer chromatograms depend almost entirely on the number of polar groups, so if one of the methyl ester groups of bismethionine methyl ester mesoheme is hydrolyzed, the product will behave like monomethionine methyl ester mesoheme. Thus, it is likely that the mildly basic conditions used during the preparation and purification of these methionine heme peptides cause partial hydrolysis of the methyl ester groups. No means has yet been found for separation of the bismethionine and monomethionine derivatives after hydrolysis of the methyl ester, although several methods have been tried. These efforts led to the conclusion that in the absence of a charge difference, the chromatographic properties are determined largely by the comparatively large heme group, which overshadows the effects of the amino acid side chains.

A space-filling molecular model of histidine–methionine mesoheme was constructed with Corey–Pauling–Koltun atomic models (Figures 5 and 6). View 1 (Figure 5) shows the extreme steric constraints placed on the histidine side chain when the imidazole group is coordinated to the heme iron. In fact, the conformation shown, with the 1-nitrogen coordinated, is probably the only one in which the nitrogen is both close enough to the iron and is at a favorable distance and angle for coordination. View 2 shows another orientation where the heme plane is perpendicular to the plane of the paper. This view shows that the methionine can also be placed in a configuration which places the sulfur very near the heme iron. Because of the steric constraints placed on the positions of the nitrogen and sulfur atoms in this model, the question may be raised whether this is the optimum geometry for coordination. Almost certainly it is not, and this will probably be reflected in the physical properties of these derivatives. On the other hand, it may be just this factor which makes these heme peptides most valuable as model compounds, since their structure can be accurately defined. From the model of bishistidine mesoheme it was found that the imidazole 1-nitrogen is confined within a hemisphere of radius about 8 Å. This leads to a figure of 1.5 M for the effective concentration of imidazole in the heme peptides, which is far in excess of the concentration required for complete complexation. The simple structure of these heme peptides eliminates many of the uncertainties involved in studying the more complex heme peptides which have been obtained from enzymatic hydrolylates of cytochrome *c* (Margoliash *et al.*, 1959; Harbury *et al.*, 1965).

The next paper in this series describes the spectral properties of the various histidine and methionine mesoheme derivatives and demonstrates that the covalently bound ligands are capable of coordination to the heme iron.

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