The latter ether extract was washed with water, dried over calcium chloride, filtered and distilled to dryness through a column. The residual crude benzoic acid, 0.89 g. (88%), was recrystallized twice from water, then vacuum sublimed prior to radioactivity assay, m.p. 122–122.5°. The radioactivity assays of the benzoic acid samples arising from each of the 2-phenylbutane samples of Table II are given in Table I.

2-(2,4-Diacetylaminophenyl)-butane-3-C^{14}.—3-Phenyl-1-butene-2-C^{14} (2.30 g.) was hydrogenated with the use of platinum oxide catalyst and hydrogen as described, yielding 1.91 g. (82%) of labeled 2-phenylbutane. This was nitrated at room temperature using 20 ml. of a 2:1 sulfuric–nitric acid mixture. The dinitro product was isolated and reduced, and the resulting diamino product was acetylated as described above for the preparation of the corresponding derivative of ethylbenzene. The crude 2-(2,4-diacetylaminophenyl)-butane-3-C^{14}, 0.79 g. (22%), m.p. 183–185°, was recrystallized thrice from 80% ethanol, when it had a constant m.p. of 189–190°, unchanged after vacuum sublimation. The m.p. of this derivative is given as 191.5°[30] and 193°[26]; radioactivity assay: 0.0888, 0.0884 mc./mole.

(30) T. E. Zalesskaya, *J. Gen. Chem. U.S.S.R.*, **17**, 489 (1947); *C. A.*, **42**, 844 (1948).

Radioactivity assays were accomplished by wet combustion of the labeled samples to carbon dioxide,[31] followed by counting[32] the latter in an ionization chamber with the aid of a Cary model 31 vibrating reed electrometer. Due to the extremely low levels of radioactivity in several of the samples assayed it was necessary to make corrections for background radiation and electrometer drift in order to minimize errors from these sources. In general this was accomplished by (a) correcting the slow observed rate of drift curves for "instantaneous plateaus" due to bursts of background radiation and (b) correcting the slow ion-current rate of drift for the slower rate of drift due to ion chamber charge leakage, the latter itself separately corrected for "instantaneous plateaus" caused by background radiations. Such corrections have been applied in the calculations of all of the radioactivity assays in Table I. The assays of the benzoic acid samples in Table I, while significantly above background level, are nevertheless so low that we are unable to estimate their absolute experimental validity at the present time.

(31) O. K. Neville, THIS JOURNAL, **70**, 3501 (1948).

(32) V. A. Raaen and G. A. Ropp, *Anal. Chem.*, **25**, 174 (1953).

STANFORD, CALIF.

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF THE JOHNS HOPKINS UNIVERSITY]

Properties of Proto- and Mesoheme Imidazole Complexes[1]

BY ALSOPH H. CORWIN AND STEPHEN D. BRUCK

RECEIVED APRIL 19, 1958

An improved method for the preparation of proto- and mesohemes is presented. In the crystalline state, both imidazole proto- and mesohemochromes have the ability to combine reversibly with molecular oxygen. Like hemoglobin itself, the iron of the hemochrome upon oxygenation remains in the ferrous condition, although as a result of repeated cycling, it slowly oxidizes. The quantity of oxygen bound to the complexes approaches one mole per atom of iron, deviations probably being due to impurities. The work has been extended to a liquid solution of imidazole in pyridine. Under these conditions, both imidazole proto- and mesohemochromes indicate spectroscopically their combination with molecular oxygen.

Hemoglobin possesses the property of combining reversibly with molecular oxygen without oxidation of its iron from the ferrous to the ferric state. The complexity of the protein portion of this molecule has rendered studies on the relationship between structure and properties very difficult. However, simplified synthetic models can be used to throw light upon this relationship. Corwin and Erdman[2] observed that passivity with respect to oxidation by atmospheric oxygen can be secured by the exclusion of coördinating substances, such as water, which provide an electron transfer mechanism. Corwin and Reyes[1] found that crystalline di-imidazole protohemochrome combines reversibly with molecular oxygen in the absence of water, thus establishing the double linkage to imidazole as a sufficient condition for oxygenation in the crystalline state. This work was very laborious, however, and an improved method of preparation was indicated. The object of the present undertaking was to extend this work to other iron–porphyrin complexes and to liquid solutions.

Results.—From Table I it can be seen that *both* imidazole protohemochrome and imidazole mesohemochrome combined reversibly with molecular oxygen when subjected to oxygenation in the crystalline state. The amount of oxygen bound by these compounds approached one mole per mole of the heme. The purity of the compounds had a marked effect on their ability to combine reversibly with oxygen. Compounds 1, 2, 5 and 6 were less pure, containing excess imidazole as shown by carbon and hydrogen determinations. The maximum uptake by these less pure compounds did not exceed 0.88 mole per mole of heme. Those compounds which were of higher purity combined with a maximum of 0.96 mole of oxygen per mole of heme.

TABLE I

OXYGEN UPTAKE PER MOLE[a]

No.	Hemochrome	Cycle I		II		III	
		%	Mole	%	Mole	%	Mole
1	Meso[b]	3.45	0.85	3.28	0.81	2.15	0.53
2	Meso[b]	3.53	.87	3.16	.78	2.51	.62
3	Meso[c]	3.63	.89	3.44	.84	2.27	.55
4	Meso[c]	3.73	.92	3.08	.76	2.50	.64
5	Proto[b]	3.59	.88	1.83	.45	..	..
6	Proto[b]	3.02	.74	2.08	.51	0.73	0.18
7	Proto[c]	3.70	.91	3.18	.78	1.18	.29
8	Proto[c]	3.90	.96	3.38	.83	1.92	.47

[a] Based on one mole of O_2 per atom Fe. Calcd. for mesohemochrome, 4.06%; calcd. for protohemochrome, 4.08%. [b] Oxygenation, dry air, 8 hours, room temperature; desorption at 60–65°, 5 mm., 6 hours, under nitrogen. [c] Oxygenation, dry air, 8 hours, room temperature; desorption, 55–60°, 5 mm., 6 hours, under nitrogen.

It was also possible to follow the changes due to oxygenation spectroscopically. Table II summarizes these results.

(1) Porphyrin Studies. XV. Paper XIV, A. H. Corwin and Z. Reyes, THIS JOURNAL, **78**, 2437 (1956).

(2) A. H. Corwin and J. G. Erdman, *ibid.*, **68**, 2473 (1946).

TABLE II

SPECTRA OF IMIDAZOLE IRON–PORPHYRIN COMPLEXES IN PYRIDINE[a]

Compound	mμ	Order of intensity
Oxygenated mesohemochrome[b]	I, 548; II, 518	I = II
Mesohemochrome	I, 545; II, 517	I >> II
Mesohemichrome	I, 545; II, 518	II > I
Oxygenated protohemochrome[b]	I, 556; II, 523	I = II
Protohemochrome	I, 553; II, 521	I >> II
Protohemichrome	I, 553; II, 521	II > I

[a] Hartridge reversion spectroscope, measured immediately. [b] Oxygenated in the crystalline state. Spectrum represents the first cycle of oxygenation. On long standing in pyridine, it showed the spectrum of the hemichrome.

Tables I and II show that the results on oxygen uptake and on spectral shifts with chemical changes were very similar with the meso and proto compounds with the exception that the stability of the proto derivative to recycling was less.

In the course of qualitative experiments, it was observed that treatment of either proto- or mesohemochrome with an imidazole-saturated pyridine solution in air changed the intense hemochrome spectrum almost instantaneously into that of the corresponding hemochrome which had been oxygenated in the crystalline state. No such change was observed when pyridine without dissolved imidazole was used. To ascertain whether or not these observations were the result of oxygenation, experiments were conducted in the absence of oxygen and with deoxygenated pyridine present. A summary of the observations is given in Table III.

TABLE III[c]

Hemochrome Meso[b]	O_2[a] → Oxygenated hemochrome	$Fe(CN)_6^{-3}$[b] → Hemichrome
I, 545 mμ	I, 548 mμ	I, 545 mμ
II, 517 mμ	II, 518 mμ	II, 518 mμ
I >> II	I = II	II > I
Proto		
I, 553 mμ	I, 556 mμ	I, 553 mμ
II, 519 mμ	II, 521 mμ	II, 519 mμ
I >> II	I = II	II > I

[a] In an imidazole-saturated pyridine solution. [b] Two drops of 5% aqueous solution of potassium ferricyanide. [c] Spectra measured by the Hartridge reversion spectroscope.

These results in an imidazole-saturated pyridine solution are qualitatively identical with those obtained with crystalline hemochromes (Table II). We thus have experimental evidence that both the proto- and the mesohemochrome can combine with molecular oxygen when in an imidazole saturated pyridine solution but that this combination does not take place in excess pyridine.

Preparation of Hemes.—The equation for the introduction of iron into porphyrins may be represented as

$$\text{Prophyrin(HH)} + Fe^{++} \rightleftharpoons \text{Porphyrin(Fe)} + 2H^+ \rightleftharpoons \text{Porphyrin(Fe)}^+ + e^-$$

As ordinarily performed, advantage is taken of the oxidation to the ferric state to drive the reaction to completion. Vestling[3] recommended the use of a sodium acetate buffer to control the acidity and thus to favor metal introduction. We find that this measure is not sufficient to permit complete formation of the heme when oxygen is rigidly excluded from the system by the use of all-glass apparatus and by its removal from all solvents. Under these conditions some porphyrin was always in equilibrium with the heme in acetic acid solution. Rough values for the equilibrium constant can be calculated from the isolated yields. Best yields were obtained by the use of a suspension of the porphyrin, probably due to a favorable solubility differential between the porphyrin and the heme. Results are summarized in Table IV.

TABLE IV

PREPARATION OF PROTOHEME IN THE ABSENCE OF OXYGEN

Expt.[a]	Glac. acetic, cc.	$Fe(Ac)_2$,[c] cc.	Molarity of NaAc in glac. acetic	Reflux time, min.	Yield, %	$\frac{100K[PH_2][Fe^{++}]}{[PFe]}$
1	20	10	0.10	2	45.0	4.2
2	20	10	.10	5	41.5	
3	20	10	.10	15	36.5	
4	15	10	.10	2	48.5	4.4
5	20	10	.25	2	50.2	3.8
6	10	10	.25	2	62.4	3.0
7	10	15	.50	2	68.2	2.9
8	10[b]	20	.50	2	79.3	
9(a)	10[b]	20	.50	5	86.3	
9(b)	10[b]	20	.50	5	87.5	

[a] In each experiment 50 mg. of protoporphyrin hydrochloride was taken. [b] In these experiments the porphyrin was suspended without the preliminary heating required to bring it into solution. [c] Containing 6 mg. of iron per ml. in acetic acid.

Analysis of Table IV reveals that the addition of sodium acetate produces little, if any, improvement in the yield of the heme, the critical variables being, instead, concentration of porphyrin and of ferrous acetate. A close approximation to equilibrium is reached in two minutes, since equilibrium constants calculated from these data differ only by a factor of 1.5.

Discussion

Wang, Nakahara and Fleischer[4] recently have concluded that protection of hemes from water by hydrophobic groups in hemoglobin is responsible for its observed passivity. Their view is that the oxidation process requires a separation of ions which does not proceed rapidly in a medium of low dielectric constant. Thus they argue that the critical factor in passivity is the dielectric constant of the medium immediately surrounding the iron.

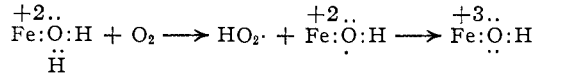
In 1925, Hill[5] reported that short exposure of pyridine hemochromogen to oxygen in dry pyridine failed to oxidize it. Corwin and Erdman[2] took advantage of the greater stability of mesoporphyrin derivatives to show essentially indefinite passivity of the ferrous iron in dry pyridine. They interpreted this as due to the lack of an electron transfer mechanism in this solvent. Stoichiometrically, water or a similar coördinating substance is necessary to balance the equation for the oxidation of

(3) C. S. Vestling, *J. Biol. Chem.*, **135**, 623 (1940).

(4) J. H. Wang, A. Nakahara and E. B. Fleischer, THIS JOURNAL, **80**, 1112 (1958).

(5) R. Hill, *Biochem. J.*, **19**, 341 (1925).

iron by oxygen. Mechanistically, the electron transfer can be represented as



According to this view, a mechanism must be provided for the transfer of an iron electron from a non-bonding position to a bonding position in order for the oxidation to proceed. In the case of a coordinated hydrate, this mechanism is provided through the loss of a hydrogen atom from one side of the oxygen and a gain of a bonding electron from the non-bonding position in the iron on the other side. Passivity should, thus, be dependent upon the specific chemical properties of available coordinating substances.

Our current finding that heme will not oxygenate in pyridine alone but will oxygenate in pyridine containing sufficient imidazole argues for a specific chemical effect of the imidazole on the ability of the iron to undergo oxygenation. These two conditions, then, give partial specifications for the structure that should combine passivity to oxidation with ease of oxygenation.

S. D. B. wishes to acknowledge a grant-in-aid from the Hynson, Westcott and Dunning Research Fund.

Experimental

Hemin.—The procedure was a modification of that of Fischer[6] and was identical to that described by Corwin and Erdman[2]; yield 84%.

Protoporphyrin IX, Dihydrochloride.—Protoporphyrin dimethyl ester was first prepared essentially by the method of Grinstein.[7] It was then hydrolyzed by the following procedure.

Two grams of the dimethyl ester was dissolved in a 25% solution of hydrochloric acid and allowed to stand overnight in a refrigerator. The product was neutralized with aqueous KOH and the precipitate filtered and washed with distilled water. The crystals were treated with a 5.5% solution of hot hydrochloric acid and filtered quickly. After cooling, the dihydrochloride of the porphyrin separated as fine needles. The product was collected on a sintered glass filter, washed three times with small amounts of cold 5.5% hydrochloric acid and dried in a vacuum desiccator over sodium hydroxide for 24 hours; yield 1.8 g. or 84.7%.

Mesoporphyrin IX, Dihydrochloride.—The palladium oxide catalyst was prepared according to Starr and Hixon.[8] The procedure was essentially that of Corwin and Erdman; yield 1.5 g. or 78.9%.

Mesohemin Chloride.—Introduction of the iron was essentially by the method of Fischer, Treibs and Zeile[9] as modified by Corwin and Erdman.[2]

Imidazole–Ferrimesoporphyrin.—The method was essentially that used by Corwin and Reyes for the corresponding protoporphyrin derivative.

Anal. Calcd. for $C_{40}H_{45}O_5N_8Fe$: C, 60.98; H, 5.16. Found: C, 61.21; H, 5.25.

The Preparation of Imidazole Meso- and Protohemochromes by the Direct Introduction of Fe^{+2} into the Porphyrin in the Absence of Oxygen. A. The Apparatus.—The apparatus was so designed as to eliminate all rubber connections in favor of glass construction and to allow all operations to be carried out in the complete absence of oxygen and metallic impurities. The all-glass apparatus consisted of three general areas: 1, nitrogen purifier; 2, solvent deoxygenating area; 3, reaction area.

1. **The Nitrogen Purifier.**—Commercial pre-purified grade nitrogen was used which was led through a column of ascarite and a column of anhydrone and then to a tube filled with fine copper turnings. This latter tube was heated to 400° by an electric furnace which in turn was controlled by a thermocouple. The pure nitrogen then passed through a small column of silver and gold turnings to remove any mercury vapors which may have been carried over from the trap through which the nitrogen first entered.

The purity of the nitrogen could be tested by sealing a tungsten wire (0.20 mm. in diameter) in a 10-mm. glass tube and sealing this into the system in the path of the purified nitrogen. The tungsten wire was electrically heated to reduce it. In the presence of traces of oxygen, the wire discolors. The test is claimed to be sensitive to 0.0004% of oxygen by Heyne.[10]

2. **The Solvent Purifier.**—All solvents were deoxygenated by means of Wood metal alloy. The method was originally applied only for the complete deoxygenation of water by Patrick and Wagner[11] and was successfully extended by us by deoxygenate benzene, absolute ethyl alcohol and glacial acetic acid. Wood metal alloy consists of 50% bismuth, 25% lead, 12.5% tin and 12.5% cadmium. The particular sample used in these experiments melted at 70° Since the solvents selected all had higher boiling points than 70°, the Wood metal alloy could melt and deoxygenate the solvents. It was possible to simultaneously deoxygenate up to five different solvents by means of five similar deoxygenating vessels. Each solvent was refluxed with finely ground Wood metal alloy (approx. 200 g. per 500 cc. of solvent) for three hours. The deoxygenated solvents were then distilled directly into the reaction chamber through airtight glass connections.

3. **The Reaction Area.**—Due to the intricacy of the equipment and the numerous steps required for the process, only the fundamental principles will be described below. The reaction area consisted, essentially, of three parts. Chamber 1 served for the preparation of the ferrous acetate, chamber 2 stored the imidazole–benzene solution, and compartment 3 enabled the introduction of Fe^{+2} into the porphyrin and the subsequent coördination with imidazole.

B. The Method of Preparation.—To initiate the reaction, 120 mg. of iron wire (J. T. Baker, analyzed purity) was placed into compartment 1 and held on the inside wall of the chamber by means of a magnet. This device enabled one to raise any excess (undissolved) iron above the surface of the ferrous acetate solution without the opening of the system. One hundred milligrams of mesoporphyrin dihydrochloride (or protoporphyrin dihydrochloride), 410 mg. of sodium acetate and a small magnetic bead were placed on the sintered glass filter of compartment 3. Next, 414 mg. of imidazole was placed in compartment 2. All chambers were sealed. By the careful manipulation of nitrogen pressure and vacuum, deoxygenated glacial acetic acid was transferred into chamber 1 and the ferrous acetate solution was prepared.

The ferrous acetate solution was then similarly transferred to the chamber which contained the porphyrin. After a few minutes of refluxing, the iron–porphyrin complex was filtered off by means of the built-in sintered glass filter. It is noticed that the Fe^{+2} was introduced in the porphyrin while the latter was held in *suspension* in glacial acetic acid. The iron–porphyrin complex was dried for several hours by passing through it pure nitrogen and heating it gently with a heat lamp.

In the meantime, deoxygenated benzene was transferred in chamber 2 containing the imidazole. By gently heating this chamber (approx. 50°), all the imidazole dissolved. The imidazole–benzene solution was then transferred by means of vacuum to the chamber holding the *thoroughly dried* iron–porphyrin complex. Complexing was accomplished again in the crystalline state by stirring the iron–porphyrin in the benzene–imidazole solution. Usually 30 minutes was sufficient to achieve coördination. The product was filtered through the built-in sintered glass filter and washed twice with small portions of deoxygenated benzene at 50°, twice with deoxygenated absolute ethyl alcohol. The compound finally was dried by passing pure, dry nitrogen through it for several hours and by applying gentle heat (approx. 50°) with a heat lamp. The product was then further dried under a vacuum of 5–6 mm. for three hours and heated at 50°. The compound was kept in the chamber until further use.

(6) H. Fischer, *Org. Syntheses*, **16**, 77 (1936).
(7) M. Grinstein, *J. Biol. Chem.*, **167**, 515 (1947).
(8) D. Starr and R. M. Hixon, *Org. Syntheses*, **16**, 77 (1936).
(9) H. Fischer, A. Treibs and K. Zeile, *Z. physiol. Chem.*, **195**, 1 (1931).
(10) G. Heyne, *Z. angew. Chem.*, **38**, 1099 (1925).
(11) W. A. Patrick and H. A. Wagner, *Anal. Chem.*, **21**, 752 (1941).

Oxygenation Experiments. General Procedure.—To carry out these experiments, a 5-mm. Pyrex sample tube was prepared which was long enough to fit inside a 10-mm. Pyrex glass tube. This latter one resembled an absorption tube used ordinarily in carbon and hydrogen determinations, but was smaller in size. The sample tube was constricted at one end and a small glass wool plug was inserted inside this part. The outer tube, which was equipped with glass valves at each end, was just long enough to accommodate the sample tube. The volume of the outside tube was, therefore, decreased to a minimum.

Both the sample tube and the absorption tube were properly tared by means of an identical set. Taring was accomplished after both tubes had been swept with pure nitrogen and the glass valves at each end of the tubes had been closed.

A small portion (approx. *10 mg.*) of the imidazole hemochrome was then introduced into the sample tube with a micro-spatula. The sample tube was fitted inside the absorption tube and was then placed inside a small, electrically heated furnace. The tube was evacuated to a mercury pressure of 5 mm., heated and kept at 60–65° for two hours. At the end of this period, pure nitrogen was allowed to pass through the tube for two hours, while the temperature was kept at 60–65°. This operation was necessary to remove any oxygen which may have been taken up by the sample. The tube was then weighed. Next, dry air was passed through the sample for eight hours, at room temperature, at a rate of 20 cc. per minute, after which the tube was swept with pure nitrogen for one minute and again weighed. Desorption of the oxygen was carried out by heating the sample in the tube to 60–65° and passing pure nitrogen through it for six hours while the 60–65° temperature was maintained. The sample then was weighed. A second and a third cycling were carried out as described above.

BALTIMORE 18, MD.

COMMUNICATIONS TO THE EDITOR

THE CRYSTAL STRUCTURE OF TOSYL-L-PROLYL-L-HYDROXYPROLINE MONOHYDRATE

Sir:

Hydrolysis studies[1,2] have shown that the sequence -gly-pro-hypro- is a common one in collagen and gelatin and this evidence has been used as a restrictive factor in building models of collagen.[3,4] Hence the structure analysis of a peptide involving the sequence -pro-hypro- would be of interest. The compound tosyl-L-prolyl-L-hydroxyproline was available to us[5] and additional interest lay in testing the usefulness of a "marker group" (in this case *p*-toluenesulfonyl) in the X-ray determination of relatively complex structures.

Tosyl-L-prolyl-L-hydroxyproline monohydrate,[6] M = 400.44, crystallizes from water as plates, frequently twinned. The crystal system is monoclinic, a = 6.291, b = 7.689, c = 19.640 Å., β = 99°27.5′. The space group is P2, with Z = 2, the density calculated being 1.419, measured 1.415.

With information derived from partial three-dimensional data, image-seeking processes[8] were applied to the sharpened-up zero-layer Patterson function $P_0(u, w)$. Guided by the use of models of the component molecular units, tosyl,[9] prolyl[10] and hydroxyproline,[11] a reasonable disposition in the *ac* projection area was achieved. The reliability index, ($R = \Sigma\,|F_0 - F_c|/\Sigma|F_0|$), initially 0.52, has been reduced to 0.18 for the observed *h0l* structure amplitudes.[12] The corresponding electron-density distribution is shown in Fig. 1 with

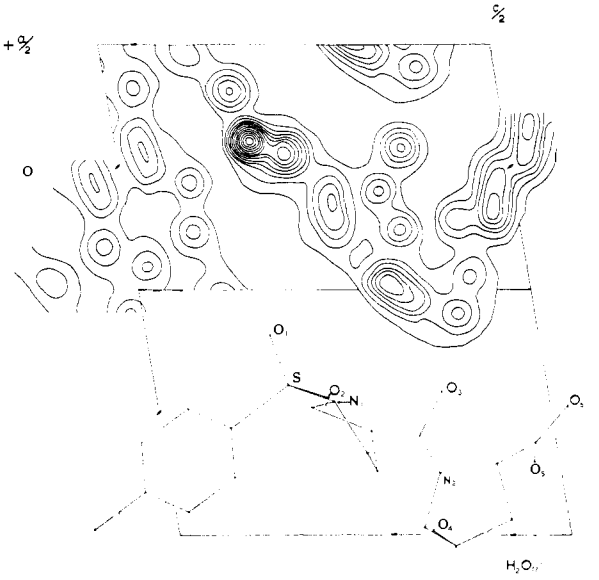


Fig. 1.—Crystal structure of tosyl-L-polyl-L-hydroxyprolin monohydrate.

the molecular interpretation adjacent.[13] The y parameters are being determined from the three-dimensional data.

(1) W. A. Schroeder, L. M. Kay, J. LeGette, L. Honnen and F. C. Green, THIS JOURNAL, **76**, 355b (1954).

(2) T. D. Kroner, W. Tabroff and J. J. McGarr, *ibid.*, **77**, 335 (1955).

(3) A. Rich and F. H. Crick, *Nature*, **176**, 915 (1955).

(4) P. M. Cowan and S. McGavin, *ibid.*, **176**, 1062 (1955).

(5) A. F. Beecham, THIS JOURNAL, **79**, 3262 (1957).

(6) Difference maps[7] revealed the presence of an atom additional to those in the molecular formula.[5] Assumed to be a water molecule, independent physical measurements confirmed this. *Anal.* Calcd. for $C_{17}H_{22}N_2O_6S\cdot H_2O$: C, 50.99; H, 6.04; N, 7.00; O, 27.97; S, 8.01. Found: C, 50.93; H, 6.12; N, 6.48; O, 27.80; S, 7.94.

(7) W. Cochran, *Acta Cryst.*, **4**, 81 (1951).

(8) M. J. Buerger, *ibid.*, **4**, 531 (1951).

(9) A. McL. Mathieson and J. M. Robertson, *J. Chem. Soc.*, 724 (1949).

(10) A. McL. Mathieson and H. K. Welsh, *Acta Cryst.*, **5**, 599 (1952).

(11) (a) J. Zussman, *ibid.*, **4**, 72, 493 (1951); (b) J. Donohue and K. Trueblood, *ibid.*, **5**, 419 (1952).

(12) Y. C. Leung and R. E. Marsh, *ibid.*, **10**, 815 (1957); **11**, 17 (1958).

(13) A table of provisional x, z parameters of the 27 atoms has been deposited as Document Number 5599 with the ADI Auxiliary Publications Project, Photoduplications Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document Number and by remitting $1.25 for photoprints, or $1.25 for 35 mm. microfilm in advance by check or money order payable to: Chief, Photoduplication Service, Library of Congress.