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X-Ray Diffraction Studies of the Sulfur Globules Accumulated by *Chromatium* Species

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Isolated wet and dried sulfur globules, obtained by osmotic lysis of lysozyme-ethylenediaminetetraacetic acid prepared spheroplasts of *Chromatium okenii*, *C. weissei*, and *C. warmingii*, were studied by polarizing microscopy and X-ray diffraction. When viewed through crossed Nicol prisms, the sulfur globules, whether in the cell or isolated in a pure, wet state, had a characteristic maltese cross appearance. The observation that rotation of the mount did not change the orientation of the arms suggested a symmetrical radial arrangement of the birefringent units. X-ray diffraction patterns of freshly isolated, wet sulfur globules gave two broad and diffuse diffraction rings with maxima at 0.36 and 0.52 nm. This pattern closely resembled the diffraction pattern of liquid sulfur. When allowed to stand in the wet state, the sulfur globules eventually converted into crystalline orthorhombic sulfur after passing through an unstable crystalline phase not previously described by X-ray diffraction. Vacuum drying of the sulfur globules accelerated the change into crystalline orthorhombic sulfur.

The prominent morphological feature of the *Thiorhodaceae* and the large apochlorotic sulfur bacteria is the numerous, highly refractile sulfur globules seen within cells grown in the presence of sulfide. Although the chemical nature of these globules has been known for sometime (14), only recently have studies been undertaken to determine the allotropic form of the sulfur. La Riviére (6) demonstrated by X-ray diffraction that the sulfur isolated from the colorless sulfur bacterium *Thiovulum majus* was of the orthorhombic type. Subsequent observations by Trüper and Hathaway (13) revealed that the orthorhombic modification was also the principal allotrope of the sulfur accumulated by marine photosynthetic bacteria. In both studies, the sulfur, isolated from the cells by osmotic shock, was examined by X-ray diffraction after drying. Since the orthorhombic allotrope is the expected form of sulfur at room temperature (7), the question arises as to whether this allotrope may have been preceded by an unstable precursor, a transformation known to occur in sulfur chemistry (7). The morphological evidence suggests that this may be the case, because the spherical form of sulfur accumulated by these microorganisms is not a typical habit of orthorhombic sulfur (4, 7). Therefore, the present study was undertaken to determine whether drying of the isolated sulfur globules resulted in a transformation from an unstable allotropic form of sulfur to the orthorhombic modification.

MATERIALS AND METHODS

Chromatium okenii Perty strain Melbourne and *C. weissei* Perty strain SCNP, derived from Pfennig's strain 6211, were grown in screw-capped bottles in the medium employed by Pfennig (9). The cultures were incubated at 25 to 27 C with continuous illumination from a bank of 100-w tungsten lamps placed at such a distance from the cultures as to give illumination of 300 ft-c. *C. warmingii*, obtained from Pfennig, was grown in the same manner, but at an illumination of 50 ft-c in a 16-hr light cycle. The sulfide concentration was replenished every 3 to 4 days during incubation, depending on cell density.

Cells were harvested by centrifugation for 15 min at 10,000 × *g* 4 hr after addition of fresh sulfide solution to the bottles. The organisms were washed twice with 0.01 M phosphate buffer (*p*H 7.0), flushed with oxygen-free nitrogen, and resuspended in the same buffer. Spheroplasts were formed by the addition of 100 μg of lysozyme per ml and 1.28×10^{-3} M ethylenediaminetetraacetic acid (EDTA) to the phosphate buffer.

Sulfur globules were isolated from osmotically lysed cells by centrifugation at 500 × *g* for 10 min in screw-capped conical centrifuge tubes which had been flushed with high-purity nitrogen and washed twice with nitrogen-flushed distilled water. No change in the macro- or microscopic appearance of the sulfur globules was observed when boiled distilled water was substituted for the nitrogen-flushed water used in the washing process.

For X-ray diffraction examination, the washed sulfur globules were concentrated into pellets at the closed ends of 0.7- or 0.5-mm diameter, thin-walled, glass capillaries by centrifugation at 1,500 × *g*. Excess water was removed from the capillary by siphoning; only a small layer of water immediately above the pellet was kept to maintain the sulfur in an aqueous milieu. The capillaries were then fully sealed to prevent moisture loss and were mounted on brass rods. The X-ray diffraction diagrams were recorded on film with 57.3- and 114.6-mm diameter Debye-Scherrer powder cameras. Nickle-filtered copper radiation ($\lambda = 0.1542$ nm) was employed as the X-ray source. All recordings were made at room temperature with exposure times of 0.5 to 3.0 hr. After initial X-ray exposure, the capillaries were stored with the ends containing the collected sulfur in a downward position to minimize turbulent dispersion of the pellets. Reexamination of the specimens by X rays was carried out at irregular intervals up to 6 weeks.

Microscopic examination of sulfur globules during both isolation and X-ray examination was performed with a Leitz Dialux-Pol polarizing microscope.

RESULTS AND DISCUSSION

The first observable stage in the photooxidation of sulfide to sulfur was the appearance of small, phase-dark areas within the living cell. Polarizing microscopy revealed these areas to be birefringent, but their minute size prevented observation of a discernible birefringent pattern. As these areas increased in size, they became highly refractile, spherical globules that seemed to occupy most of the cell volume. The appearance of refractility was accompanied by a birefringence having a characteristic maltese cross appearance when the sulfur globules were viewed through crossed Nicol prisms (Fig. 1). The fact that rotation of the mount did not change the orientation of the arms of the cross suggested a radial, symmetrical arrangement of the birefringent units. Liberation of the sulfur globules by osmotic lysis of lysozyme-EDTA prepared spheroplasts, and the subsequent washing and collection procedures necessary for X-ray diffraction studies, did not alter the microscopic characteristics of these globules provided they were kept wet (Fig. 2). Upon drying, however, the globules converted into birefringent crystals.

The X-ray diffraction diagrams of the freshly isolated wet sulfur globules obtained from all strains of *Chromatium* tested gave two broad and diffuse diffraction rings with maxima at 0.36 and 0.52 nm (Fig. 3a). The 0.36-nm diffraction ring in these diagrams was always more prominent, being about twice as intense as the 0.52-nm ring. It is apparent that the aqueous environment enveloping the specimen does not interfere with or weaken the sulfur pattern, because the 0.215- and 0.32-nm diffraction maxima of water, shown for comparison in Fig. 3b, were not evident in the diffraction diagrams of the sulfur.

The amorphous X-ray pattern obtained from freshly isolated sulfur globules more closely resembles the diffraction pattern of liquid sulfur than that of plastic sulfur. Liquid sulfur has two maxima at 0.35 and 0.5 nm, with the inner maximum about one-half as intense (2, 5). Fresh

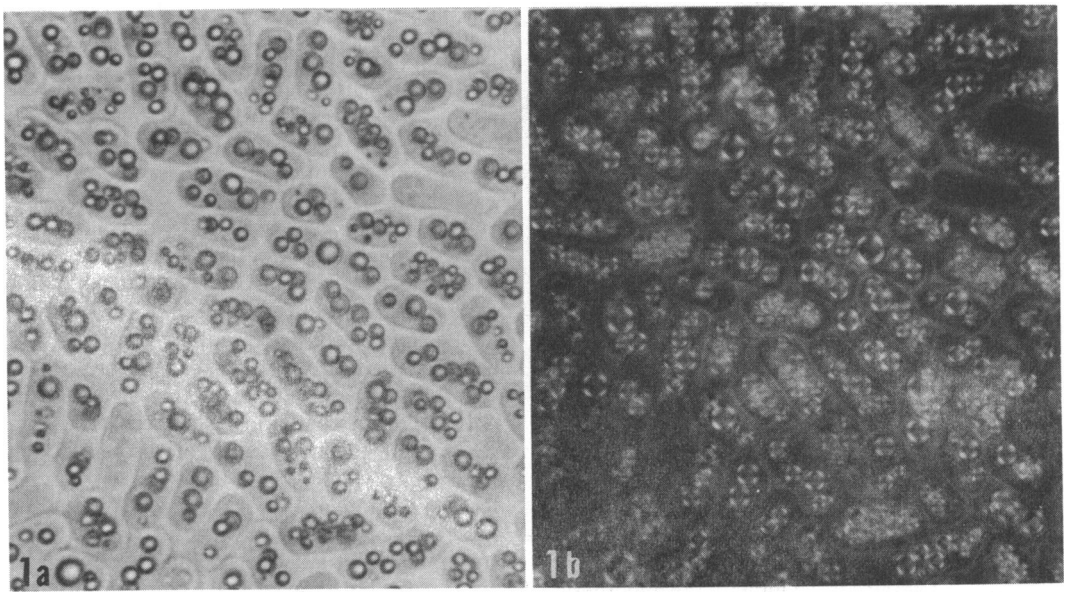

FIG. 1. *Photomicrographs of Chromatium okenii containing sulfur globules.* × *1,500. (a) Bright-field microscopy. (b) Polarizing microscopy.*

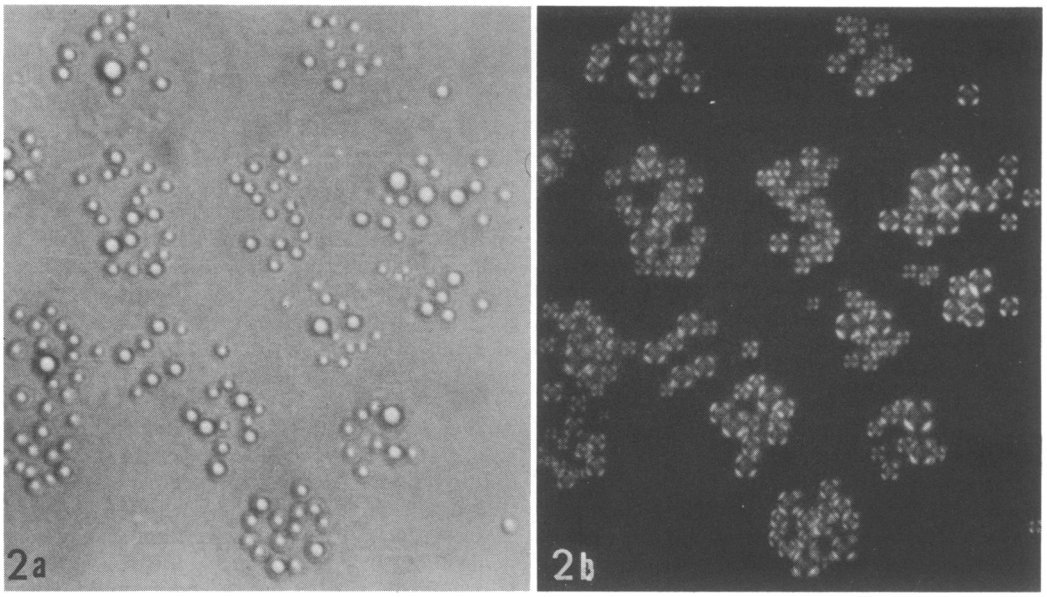

FIG. 2. *Photomicrographs of isolated wet sulfur globules.* × *1,700.* (*a*) *Bright-field microscopy.* (*b*) *Polarizing microscopy.*

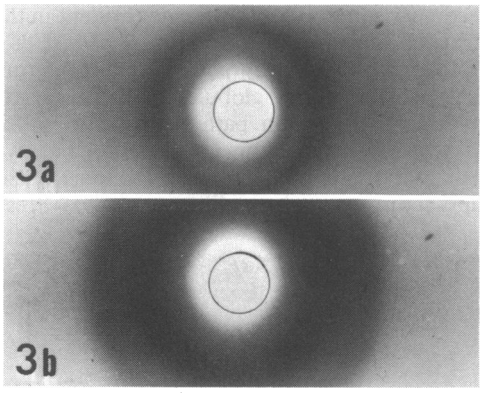

FIG. 3. *X-ray diffraction patterns of* (*a*) *wet sulfur globules taken immediately after isolation and* (*b*) *water.*

plastic sulfur gives only one diffuse band occurring at 0.35 nm (2, 5). Although liquid sulfur normally is stable only at temperatures above 113 C, it has been reported (3) that liquid sulfur can be kept for as long as 3 days in a supercooled state at room temperature if prepared in the form of small spherules, a shape similar to the globules produced from the photooxidation of sulfide by *Chromatium*.

Radial distribution analyses of liquid sulfur patterns by Gingrich (5) have shown that each sulfur atom has only two permanent nearest neighbors, a finding consistent with the hypothesis that the molecules in liquid sulfur at a temperature immediately above the melting point (113 C) are mostly in the form of a closed ring of eight atoms (5, 12). This cyclooctasulfur form is also the only stable molecular species at room temperature (7). These data on liquid sulfur, together with the close analogy between the X-ray diffraction pattern and the observed pattern of birefringence seen by polarizing microscopy, suggest that the sulfur globules isolated from *Chromatium* species are spherically symmetrical aggregates of radially arranged arrays of S^8 molecules. A somewhat analogous situation exists with polymers that crystallize in a spherical form. Known as spherulites, they exhibit the maltese cross optical effect between crossed Nicol prisms, but the birefringent units, in this case, are the fiberlike crystals comprizing the spherulites (8). The lack of discrete Bragg maxima in the X-ray diffraction pattern of the sulfur globules is evidence, however, that the birefringent units here are not sulfur crystals but the sulfur molecules themselves. When such molecules are radially arranged into spherelike aggregates, Bragg maxima are not to be expected. Though ordered, this arrangement is not rectilinearly periodic, a structural condition necessary for the observance of discrete Bragg maxima.

Reexamination of the isolated wet sulfur globules revealed that they slowly crystallized. At room temperature, a crystalline pattern began to appear in the X-ray diffraction diagram after 24 hr and was fully developed by 4 days (Fig. 4a).

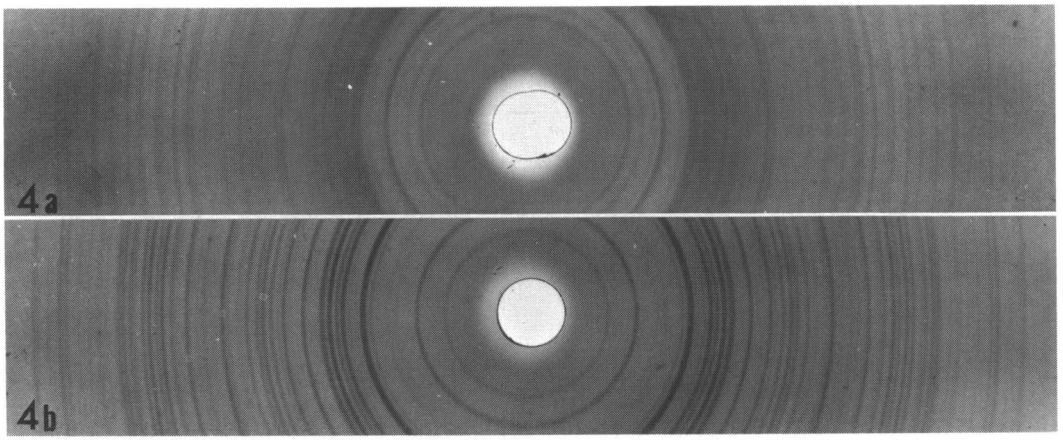

FIG. 4. *X-ray diffraction patterns of (a) wet sulfur taken 4 days after isolation and (b) 6-day-old sulfur globules after vacuum drying.*

The angular positions, converted into the corresponding interplanar *d* spacings, and the relative intensities of the diffraction lines in this pattern, are given in Table 1. However, a positive identification of the crystalline phase from the X-ray pattern was not possible. This phase does not appear to be a common allotrope of sulfur. Its X-ray pattern does not match those reported for orthorhombic (α) sulfur (American Society for Testing and Materials Powder Diffraction File, card 8-247), monoclinic (β) sulfur (11), rhombohedral sulfur (1), or insoluble (μ) sulfur (10). Nor do the observed *d* spacings compare with the *d* spacings calculated from published unit-cell data for monoclinic (γ) sulfur (4), hexagonal (w) sulfur (12), or fibrous sulfur (4).

Many other sulfur allotropes have been reported to exist (7), but X-ray diffraction data on these modifications are not available from the literature. These latter allotropes have the common property of being unstable and ultimately converting into one of the more common forms for which X-ray data are available, most notably orthorhombic (α) sulfur. The crystalline sulfur allotrope which formed after 24 hr shared this property of limited stability. At room temperature, it slowly transformed into orthorhombic (α) sulfur. Orthorhombic diffraction lines were seen in X-ray patterns of 2-week-old wet preparations, and after 6 weeks these lines indicated that orthorhombic sulfur had become the dominant phase. Drying the preparation accelerated the change into orthorhombic sulfur. A 6-day-old preparation showing no signs of orthorhombic sulfur converted almost completely into this form when dried for 1 hr in air or in vacuo (Fig. 4b). The *d* spacings and relative intensities for the observed allotropes, as well as the reference standard for orthorhombic sulfur, are shown in Table 1. There is good agreement between the corresponding *d* values of the final crystalline phase and the orthorhombic standard. The position of the two additional lines observed in the final crystalline phase (0.537 and 0.453 nm) suggests that a small amount of the intermediate phase remains in the dried sample.

Orthorhombic (α) sulfur is the stable allotrope of elemental sulfur at room temperature. For this reason, the sulfur preparations were not examined beyond the point where this allotrope became the principal phase.

The physical transition into orthorhombic (α) sulfur demonstrates that the intermediate crystalline phase seen in sulfur preparations from *Chromatium* species is a solid allotrope of elemental sulfur, even though this is not a commonly occurring modification. Further, the crystallization into orthorhombic sulfur shows that the molecular species in this intermediate crystalline allotrope is cyclooctasulfur, since this species is also the molecular unit in orthorhombic sulfur. This finding is consistent with the deduction that cyclooctasulfur is the dominant molecular species in the liquidlike sulfur globules.

Thus, the elemental sulfur isolated from *Chromatium* is characterized by the presence of three allotropes: one in a liquidlike state, and two in solid form. They are, in the order of appearance, (i) the liquidlike globule formed within the cell by the rapid photooxidation of sulfide, (ii) an intermediate crystalline phase that has not been previously described by X-ray diffraction and is, like the globules, of limited stability, and (iii) stable orthorhombic (α) sulfur.

Although this complex behavior was unexpected, it is not without precedent. It has been reported that unstable allotropes have been prepared in the precipitation of sulfur from solutions

TABLE 1. *X-ray powder diffraction spacing data of sulfur isolated from Chromatium okenii*

Liquidlike phase[a]		Intermediate crystaline phase[b]		Final crystalline phase[c]		Orthorhombic sulfur ASTM 8-247	
d	*I*[d]	*d*	*I*[d]	*d*	*I*[d]	*d*	*I*[d]
nm		*nm*		*nm*		*nm*	
				0.774	w	0.769	6
		0.650	vw				
		0.604	mw				
				0.575	m	0.576	14
						0.568	5
		0.538	w	0.537	vw		
0.52	m (broad)						
		0.484	vw	0.482	vw	0.480	2
		0.457	m	0.453	vw		
		0.435	vw				
						0.419	2
		0.402	w	0.405	vw	0.406	11
						0.391	12
				0.385	s	0.385	100
		0.374	m (broad)				
0.36	s (broad)	0.366	ms				
				0.355	vw	0.357	8
		0.351	mw				
		0.342	m	0.345	ms	0.344	40
				0.336	w	0.338	3
		0.332	mw	0.330	w	0.333	25
		0.327	m				
				0.322	ms	0.321	60
		0.314	m				
						0.311	25
				0.3095	m	0.308	17
		0.305	w			0.306	1
		0.285	w	0.285	mw	0.284	18
		0.262	mw	0.262	w	0.2621	13
		0.255	w				

[a] Diagram of wet sulfur taken immediately after isolation.
[b] Diagram of wet sulfur taken 4 days after isolation.
[c] Diagram of 6-day-old wet sulfur after vacuum drying.
[d] The intensity (*I*) of the lines corresponding to the interplanar spacing (*d*) was read as follows: vw (very weak), w (weak), mw (medium weak), m (medium), ms (medium strong), s (strong).

containing organic solvents (7). These unstable intermediates formed whenever the precipitation rate exceeded the crystallization rate for orthorhombic sulfur. When the differences between the two became sufficiently large, the sulfur precipitated in an amorphous form. The lifetime of the unstable species varied, but they eventually converted into orthorhombic sulfur. Even though the conditions employed in these synthetic experiments were highly nonphysiological, the results suggest that the events observed with the sulfur isolated from *C. okenii*, *C. weissei*, and *C. warmingii* are kinetically controlled processes taking place in a highly unequilibrated situation, and reflect the extreme rapidity at which elemental sulfur is generated from the photooxidation of sulfide by these microorganisms.

Although the X-ray diffraction studies reported here have been limited to the sulfur globules isolated from three species of *Chromatium*, other representatives of the *Thiorhodaceae* (*Thiospirillum jenense*, *Chromatium* strain D) and *Chlorobium thiosulfatophilum* were examined by polarizing microscopy. The sulfur globules formed by these microorganisms also showed the characteristic maltese cross pattern. Whether this similarity represents the same allotropic form of sulfur as that accumulated by the organisms studied cannot be ascertained at this time without further X-ray diffraction data.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Caron, A., and J. Donohue. 1960. The x-ray powder pattern of rhombohedral sulfur. J. Phys. Chem. **64**:1767–1768.
2. Das, S. R. 1938. Study of sulfur allotropes by the x-ray diffraction method. Indian J. Phys. **12**:163–181.
3. Das, S. R., and K. Das Gupta. 1939. X-ray diffraction by super-cooled liquid sulphur. Nature **143**:332.
4. Donohue, J., and B. Meyer. 1965. The naming of sulfur allotropes, p. 1–11. *In* B. Meyer (ed.), Elemental sulfur. Interscience Publishers, Inc., New York.
5. Gingrich, N. S. 1965. X-ray and neutron diffraction studies of liquid structure, p. 172–200. *In* J. F. Hughel (ed.), Liquids: structure, properties, solid interactions. Elsevier, Amsterdam.
6. La Rivière, J. W. M. 1963. Cultivation and properties of *Thiovulum majus* Hinze, p. 61–72. *In* C. H. Oppenheimer (ed.), Marine microbiology. Charles C Thomas, Publisher, Springfield.
7. Meyer, B. 1964. Solid allotropes of sulfur. Chem. Rev. **64**: 429–451.
8. Morse, H. W., C. H. Warren, and J. D. H. Donnay. 1932. Artificial spherulites and related aggregates. Amer. J. Sci. **23**:421–439.
9. Pfennig, N. 1965. Anreicherungskulturen für rote und grüne Schwefelbakterien. Zentralbl. Bakteriol. Parasitenk. Infehtionskr. Hyg. Abt. I Orig. Suppl. 1, p. 179–189, 503–504.
10. Pinkus, A. G., J. S. Kim, J. L. McAtee, Jr., and C. B. Concilio. 1957. New lines in the x-ray diffraction pattern of orthorhombic sulfur. "Amorphous" (μ) sulfur. J. Amer. Chem. Soc. **79**:4566–4567.
11. Pinkus, A. G., J. S. Kim, J. L. McAtee, Jr., and C. B. Concilio. 1959. X-ray diffraction pattern for monoclinic sulfur. J. Amer. Chem. Soc. **81**:2652–2654.
12. Schmidt, M. 1962. Sulfur polymers, p. 98–158. *In* F. G. A. Stone and W. A. G. Graham (ed.), Inorganic polymers. Academic Press Inc., New York.
13. Trüper, H. G., and J. C. Hathaway. 1967. Orthorhombic sulfur formed by photosynthetic sulfur bacteria. Nature **215**:435–436.
14. Winogradsky, S. N. 1887. Über Schwefelbakterien. Bot. Ztg. **45**:489–508.