[26] One of these [Perey, M., A. Chevallier, and C. Lausecker, *Compt. Rend. Soc. Biol.*, **146**, 1141 (1952)] is of possible interest. In it were reported experiments showing that tumors induced in rats by 3,4-benzpyrene concentrated Cs^{134} and Fr^{223} but not Rb^{86}.

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*PATH OF SULFUR IN SULFIDE AND THIOSULFATE OXIDATION BY THIOBACILLI**

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Nathansohn, the discoverer of the thiobacilli, initially considered two alternative pathways for thiosulfate oxidation.[1] The first included elemental sulfur as a key product since it was formed in large quantities by *Thiobacillus thioparus* during growth on thiosulfate. He rejected this idea since no rational stoichiometry could be established between thiosulfate disappearance and sulfur and sulfate formation. The second involved polythionates as intermediates and was proposed primarily because tetrathionate could be detected along with sulfate in culture filtrates at the end of growth. In this formulation, sulfur deposition was attributed to an abiological reaction between thiosulfate and tetrathionate.

Despite considerable experimentation over the ensuing years, the opposing viewpoints, which may not be mutually exclusive, still have strong adherents.[2, 3] Additional evidence has accumulated supporting the polythionate hypothesis. It has been shown that tetra- and trithionate are oxidized by resting cell suspensions of thiobacilli.[4, 5] The oxygen uptake during thiosulfate oxidation usually followed a biphasic curve with a distinct decrease in slope at an oxygen consumption corresponding to that required for conversion of added thiosulfate to tetrathionate.[4, 5] Tetrathionate was shown to accumulate during the initial phase and subsequently to disappear. The rate of oxidation of added tetrathionate by such suspensions was essentially identical to the rate of the second phase of thiosulfate oxidation. Further, Trudinger[6] has shown by isotopic and chromatographic techniques the sequential appearance and utilization of tetra- and trithionate during thiosulfate oxidation by suspensions of *T. thioparus*.

Despite the compelling evidence in its support, the polythionate hypothesis has been weakened by the inability of investigators to obtain cell-free systems capable of catalyzing tetra- and trithionate oxidation. In numerous attempts, only the enzymatic conversion of thiosulfate to tetrathionate could be demonstrated.[7, 8] It was probably this negative result which led Vishniac[8] to postulate an intermediate organic carrier through which tetrathionate enters the pathway of sulfide and thiosulfate oxidation.

The hypothesis that sulfur is a key product in thiosulfate oxidation has been supported by two main lines of evidence: the deposition of sulfur during growth of *T. thioparus* on thiosulfate,[9] and the ability of thiobacilli to oxidize elemental sulfur. It was greatly strengthened by Peck's[10] demonstration of a thiosulfate reductase system in *T. thioparus* and *T. thiooxidans* which in the presence of reduced gluta-

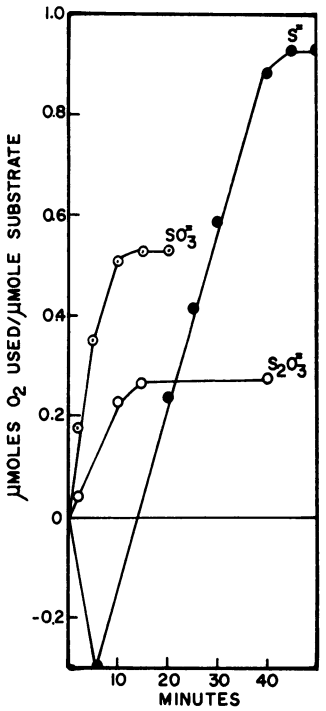

FIG. 1.—The oxidation of reduced sulfur compounds by Hughes press extract of *T. thioparus*. Reaction mixtures: 25 mg extract protein; 45 μmoles phosphate buffer, pH 6.9; 10 μmoles Na_2S, Na_2SO_3, or $Na_2S_2O_3$; 0.1 ml 20% KOH in center well except in sulfide flask; total volume 2.1 ml.

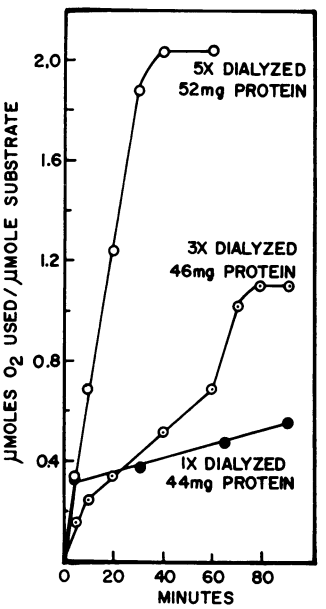

FIG. 2.—Oxidation of thiosulfate by dialyzed *T. thioparus* extracts. Reaction mixtures: extract protein as indicated; 10 μmoles $Na_2S_2O_3$; 0.1 ml 20% KOH in center well; water to 2.1 ml.

thione cleaves thiosulfate to sulfite and sulfide, the latter being oxidized to S°. The objection remains, nevertheless, that no rational stoichiometry has been shown for sulfur production from thiosulfate.[1, 8] Further, cultures with low thiosulfate concentrations or maintained near neutrality form little or no sulfur.[8, 11]

Data presented in this paper provide enzymatic evidence implicating tetra- and trithionate as intermediary products in sulfide and thiosulfate oxidation, and explain, at least in part, past failures to demonstrate enzymatic oxidation of the polythionates. The significance of thiosulfate reductase and sulfur formation in the oxidations will be considered elsewhere.

Methodology.—The strains of *T. thioparus* and *T. thiooxidans* used were obtained by enrichment from natural sources[12, 13] and possess the characteristic properties that define these species. Cultures were grown in Baalsruds' medium[8] supplemented with 20 mg $FeCl_3$ per liter.

Cells were harvested from 40 liters of culture incubated for 48 hr at 30°C with vigorous aeration and continuous automatic regulation of pH between 6.0 and 6.2 or 4.5 and 4.7 for *T. thioparus* or *T. thiooxidans*, respectively. The cell paste, 15–20 gm wet weight, was washed twice in 0.05 *M* potassium phosphate buffer, pH 6.9, and resuspended in 40–60 ml of the same buffer. The suspension was placed in the cup of a 10-kc Raytheon sonic oscillator, made anaerobic by flushing with a stream of nitrogen for 10 min, and treated at full power for 20 min, main-

taining a temperature of 6–8°C. The unbroken cells and debris were removed by centrifugation at 10,000 × *g* for 45 min. Hughes press extracts were prepared by placing 5–8 gm of cells into a frozen block, holding overnight at −12°C and processing. The cell mass was then extracted with 0.05 *M* phosphate buffer, pH 6.9, for 30 min at 10°C and centrifuged as above.

Some extracts were dialyzed against 30 per cent polyethylene glycol (Carbowax 6000) to a 50–60 per cent decrease in extract volume. When an extract was dialyzed more than once, the residue at each step was restored to its initial volume by the addition of water and centrifuged to remove precipitated protein. Carbon-treated extracts were obtained by mixing 5–10 mg of acid-washed Norit A with 20 ml cold extract and removing the carbon 5 min later by centrifugation.

Tetra- and trithionate were synthesized by the method of Stamm *et al.*;[14] dithionate by the method of Pfanstiel.[15] Thiosulfate, tetra- and trithionate were determined iodometrically[16] and chromatographically.[6, 17] Protein was determined by the biuret reaction; sulfate, turbidimetrically as $BaSO_4$. Radioautographs were prepared from paper chromatograms spotted with heat-deproteinized and clarified reaction mixtures containing sufficient S^{35} to give approximately 1000 cpm per spot (Nuclear-Chicago gas flow counter). Conventional manometric procedures were employed using an air atmosphere and 30°C in all experiments. All manometric data are corrected for oxygen consumption of extracts in absence of substrate.

Results.—Growth and resting cell studies: The *T. thioparus* and *T. thiooxidans* strains grew well with thiosulfate or tetrathionate as the energy source. Large amounts of tetrathionate and sulfur accumulated in unneutralized thiosulfate-grown cultures of *T. thioparus*. Comparable cultures of *T. thiooxidans* contained only small amounts of tetrathionate and no sulfur at the cessation of growth. Little or no tetrathionate or sulfur accumulated in neutralized *T. thioparus* cultures.

Cells of both organisms harvested from periodically neutralized thiosulfate-grown cultures oxidized sulfide, thiosulfate, and tetrathionate to sulfate at a high and constant rate. Neither trithionate nor dithionate was oxidized by such suspensions. However, trithionate was oxidized by suspensions of *T. thioparus* prepared from unneutralized cultures.

Oxidations by cell-free extracts of T. thioparus: Hughes press extracts catalyzed complete oxidation of sulfite to sulfate and partial oxidations of sulfide and thiosulfate (Fig. 1). The partial oxidations ceased at uptakes of 0.9–1.2 μmole and 0.25–0.27 μmole oxygen per μmole of these substrates, respectively. The stoichiometries in both instances correspond to the oxygen requirements for quantitative production of tetrathionate which was found by chromatographic analyses. The initial increase in pressure (calculated as O_2) seen in the sulfide oxidation curves (Figs. 1, 3) is due to liberation of some hydrogen sulfide into the gas atmosphere of the Warburg vessel when sodium sulfide solution was tipped from the side arm into the main compartment.

Untreated extracts prepared by ultrasonic disruption behaved as did the Hughes press extracts, except that there was a slow but definite oxygen consumption after the tetrathionate stage was reached in sulfide and thiosulfate oxidation. Repeated dialysis against polyethylene glycol markedly altered the properties of such

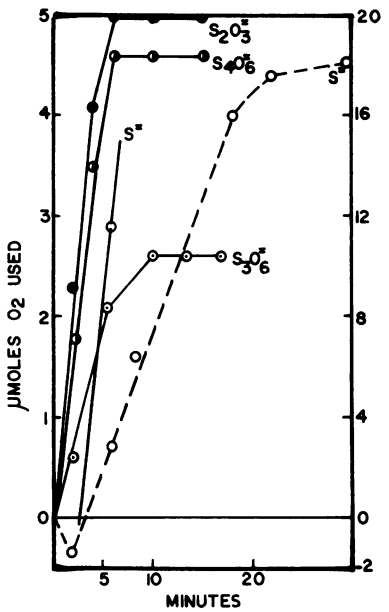

FIG. 3.—Oxidation of reduced sulfur compounds by a 5 × dialyzed, Norit A-treated extract of *T. thioparus*. Reaction mixture: 90 mg protein; 10 μmoles Na_2S, 2.5 μmoles $Na_2S_2O_3$, 1.25 μmoles $K_2S_4O_6$, or 1.25 μmoles $K_2S_3O_6$; 0.1 ml 20% KOH; water to 2.1 ml. - - -, $S^=$ oxidation (scale on right).

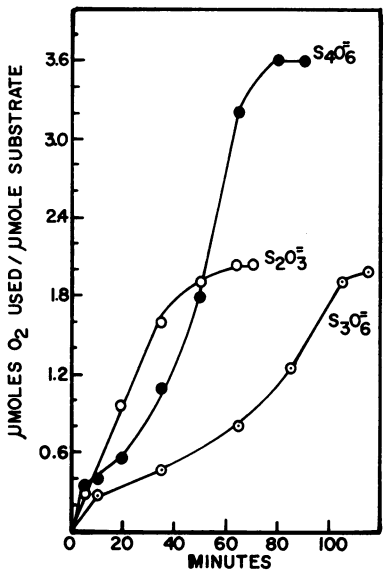

FIG. 4.—Oxidation of reduced sulfur compounds by *T. thiooxidans* extract. Reaction mixtures: 70 mg extract protein; 5 μmoles $Na_2S_2O_3$, 2.5 μmoles $K_2S_4O_6$, or 5 μmoles $K_2S_3O_6$; 0.1 ml 20% KOH in center well; water to 2.1 ml.

extracts. In the example shown (Fig. 2) the oxidation by a once dialyzed extract proceeded rapidly until 0.3 μmole oxygen per μmole thiosulfate was consumed, and very slowly thereafter. The thrice dialyzed extract showed a rapid initial oxygen uptake (0.25 μmole/μmole thiosulfate) followed by a slower one, involving an additional 0.45 μmole oxygen per μmole substrate; the rate then increased, and the reaction stopped with an oxygen consumption of only about 50 per cent of that required for the quantitative production of sulfate. The positions of the first and second rate change varied from 0.25 to 0.46 and 0.70 to 1.1 μmoles oxygen per μmole thiosulfate, respectively, with other batches of extract. The data imply the temporary and sequential accumulation of thiosulfate and tetrathionate.

In contrast to the partial oxidations observed with the once and thrice dialyzed extracts, a complete oxidation of thiosulfate to sulfate at a uniform and high rate was catalyzed by a five times dialyzed preparation (Fig. 2). Although repeated dialyses always increased the extent of thiosulfate oxidation, its complete conversion to sulfate was only infrequently observed. However, if dialysis was preceded by a Norit A treatment, the extracts consistently induced essentially complete oxidations of sulfide, thiosulfate, tetra-, and trithionate to sulfate (Fig. 3) at uniform and similar rates. Dithionate was not oxidized.

Analyses were made of replicate reaction mixtures of a three times dialyzed extract oxidizing thiosulfate, halting the reactions just after the first change in rate, just before the second rate change, and about midway in the third phase of oxida-

tion. The data show (Table 1) that all thiosulfate had disappeared by the first rate change. Large amounts of polythionate were present by this time and throughout the second stage of oxidation. Qualitatively, the results support the interpretation made of the oxygen uptake data.

TABLE 1

DETERMINATION OF REACTANTS AND PRODUCTS DURING THE COURSE OF THIOSULFATE OXIDATION BY A 3 × DIALYZED EXTRACT OF *Thiobacillus thioparus**

O_2 used (μmoles)	Residual $S_2O_3^-$ (μmoles)	SO_4^- formed (μmoles)	Polythionate formed† (μmoles)
2.6	0	0.4	3.3(6.6)
6.3	0	2.0	2.9(5.8)
10.2	0	4.0	0

* Initial substrate concentration, 8 μmoles; conditions otherwise as in Fig. 2.
† The analytical procedure[16] results in the reduction of 1 $I_2/S_4O_6^-$ and $1/2$ $I_2/S_3O_6^-$; the data are calculated as $S_4O_6^-$ or $S_3O_6^-$ (in parentheses)

Oxidations by cell-free extracts of T. thiooxidans: Sonically prepared extracts catalyzed rapid oxidations of sulfide, thiosulfate, tetra-, and trithionate to sulfate without prior dialysis or Norit A treatment (Fig. 4). Thiosulfate oxidation proceeded at a constant rate from zero time while polythionate oxidations were usually preceded by a lag which could be decreased in extent or eliminated by decreasing substrate and increasing extract protein concentrations. As with undialyzed and dialyzed *T. thioparus* extracts, sulfite was rapidly oxidized and dithionate was not attacked.

The shape of the curve suggests that intermediate products did not accumulate in appreciable quantities during the course of thiosulfate oxidation. Indeed, periodic analyses of reaction mixtures showed that thiosulfate disappearance roughly paralleled oxygen consumption and that considerable substrate remained late in the oxidation (Table 2). Polythionates were not detected and sulfate formation was more rapid than in oxidations mediated by *T. thioparus*.

TABLE 2

DETERMINATIONS OF REACTANT AND PRODUCTS DURING THE COURSE OF THIOSULFATE OXIDATION BY AN EXTRACT OF *Thiobacillus thiooxidans**

O_2 used (μmoles)	Residual $S_2O_3^-$ (μmoles)	SO_4^- formed (μmoles)	Polythionate formed (μmoles)
4.1	6.6	1.7	0
8.9	5.0	11.4	0
13.0	3.6	15.4	0

* Initial substrate 10 μmoles; conditions otherwise as in Fig. 4.

Detection of intermediate products: Cell-free extracts of *T. thioparus* (Norit A-treated and 5 × dialyzed) and *T. thiooxidans* which catalyzed complete oxidations of sulfide and thiosulfate, as illustrated in Figures 3 and 4, were used for the oxidation of Na_2S^{35} and $S^{35}{\cdot}SO_3Na_2$. Reaction mixtures were chromatographed at intervals during the oxidations and radioautographs prepared. These showed the formation of thiosulfate, tetra-, and trithionate during sulfide oxidation, and tetra- and trithionate during thiosulfate oxidation by extracts of both organisms (Fig. 5). In sulfide oxidation by *T. thiooxidans* extracts, the three intermediates were present at the earliest time sampled and persisted throughout the experiment. Thiosulfate was predominant; the polythionates were much less abundant. Similarly, the two polythionates were present at all stages of thiosulfate oxidation tested,

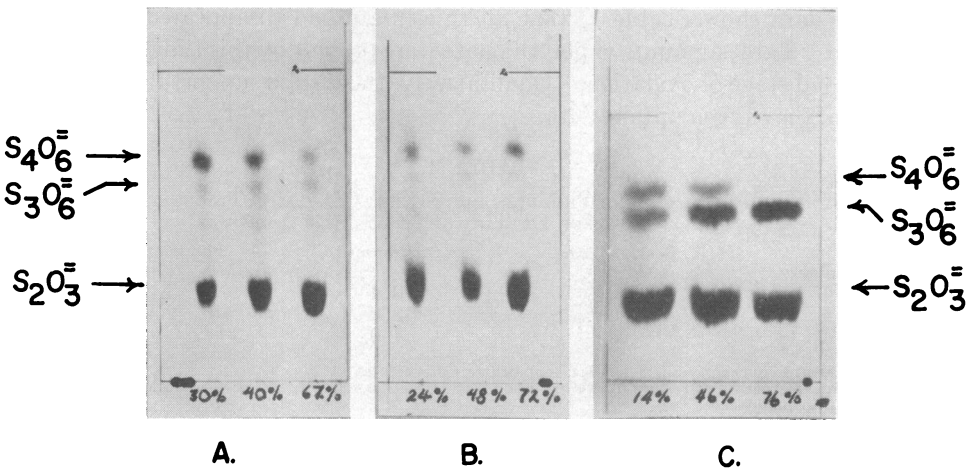

FIG. 5.—Reproduction of radioautographs. (*A*) and (*B*), sulfide and thiosulfate oxidation by *T. thiooxidans* extract; (*C*), thiosulfate oxidation by a 5 × dialyzed, Norit A-treated *T. thioparus* extract. Oxygen consumed at time of sampling is shown (below origin) as % of that required for complete oxidation to sulfate. Solvent system: pyridine; acetone; water (3:5:5).[6]

with tetrathionate in greater concentration. The quantity of polythionates present was small, which explains our failure to detect them iodometrically (Table 2). The faint spots on the radioautographs below trithionate probably are polythionic acids (see Pollard *et al.*[17]).

Tetra- and trithionate were also formed early in thiosulfate oxidation by *T. thioparus* extracts. In contrast to the results with *T. thiooxidans*, tetrathionate could not be detected late in the oxidation, and the relative concentration of trithionate increased with time. The findings suggest that the rate-limiting step was the oxidation of trithionate. This is in accord with the somewhat lower rate of trithionate oxidation observed in manometric experiments (Fig. 3).

Discussion.—The data presented show unequivocally that extracts of *T. thioparus* and *T. thiooxidans* contain the enzymes required for the complete oxidation of sulfide, thiosulfate, tetra- and trithionate, and sulfite. The addition of cofactors, primers, or reducing agents is not required for these oxidations. Further, the stoichiometries at the rate changes in multirate oxidations, the analyses of products present at these points, and the radioautographs prepared during the course of complete oxidations, show the sequential intermediate formation of thiosulfate, tetra-, and trithionate during sulfide oxidation, and tetra- and trithionate during thiosulfate oxidation by extracts of both organisms. These results, along with the demonstration by us and others[4–6, 13] that cell suspensions and growing cultures oxidize tetrathionate and—under certain conditions—trithionate, and produce these compounds during thiosulfate oxidation, leave little doubt that the oxidation of sulfide and thiosulfate by thiobacilli proceeds by way of the lower polythionates. The path of sulfur in these oxidations can be formulated schematically as follows:

$$4S^= \rightarrow 2\ S_2O_3^= \rightarrow S_4O_6^= \rightarrow SO_3^= + S_3O_6^= \rightarrow 4\ SO_3^= \rightarrow 4\ SO_4^=.$$

It is improbable that the conversions of sulfide to thiosulfate, or tetrathionate to trithionate are single-step reactions and much remains to be learned about the

details of these oxidations. Also, little can be said concerning the steps between trithionate and sulfate except that dithionate, whose oxidation could not be demonstrated, is probably not involved.

The question arises as to why previous investigators did not detect cell-free oxidation of the polythionates. The answer, in large part, stems from the happenstance that *T. thioparus* was thought to be somewhat more convenient to grow in quantity than *T. thiooxidans*, and past investigations almost exclusively used extracts of the former organism or closely related strains. As was shown, such untreated extracts catalyze only an incomplete oxidation of thiosulfate, or sulfide, to tetrathionate. Yet the same extracts after extensive dialysis oxidize these substrates in a manner identical to that observed with intact resting cells or with continuously neutralized growing cultures—that is, at a uniform rate, to completion, and without appreciable accumulation of intermediate or side products. One can only conclude that past inabilities to demonstrate enzymatic oxidation of the polythionates resulted from the procedures used and not from any inherent characteristic of the particular strains of thiobacilli investigated.

Dialysis of extracts against polyethylene glycol has two obvious consequences: the concentration of extract protein by water loss and the removal of diffusible molecules, both of which probably play a role in the observed changes in oxidative properties. Hughes press extracts which were relatively low in protein content never catalyzed thiosulfate oxidation beyond tetrathionate while undialyzed sonically prepared extracts of much higher protein content usually showed a very slow but definite oxidation beyond the tetrathionate stage. In this connection it is important to mention that by appropriately diluting a five times dialyzed extract, the sequence of changes shown in Figure 3 could be reversed and multirate curves and partial oxidation obtained. This not only indicates the influence of protein concentration, but also shows that dialysis does not substitute one oxidation system for another but merely permits expression to different degrees of a single system.

Since extracts of high protein content can be prepared which do not yield complete oxidations and since Norit A treatment facilitates the preparation of highly active extracts, it is apparent that the removal of some inhibitor from the system is necessary to obtain complete activity. Because repeated dialyses are required, the compound(s) involved is probably bound, at least in part. The nature of the compound(s) will be dealt with elsewhere.

Summary.—It has been shown that cell-free extracts of *T. thioparus* and *T. thiooxidans* catalyze the complete oxidation of sulfide, thiosulfate, tetra- and trithionate to sulfate. The formation of thiosulfate, tetra- and trithionate during sulfide oxidation and tetra- and trithionate during thiosulfate oxidation was demonstrated. These and other data indicate that sulfide and thiosulfate are oxidized by way of tetra- and trithionate in the thiobacilli.

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*ON THE ORIGIN OF THE OPTICAL ACTIVITY IN THE UROBILINS**

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Some of the more fascinating problems involving the bile pigments are associated with that group of compounds referred to collectively as the urobilins.[1-3] Although the molecular structure of all the various urobilins has not been established unequivocally,[1] the commonly accepted structures for the three urobilins which shall concern us here are those given in Figure 1. Gray and Nicholson,[4] in particular, have marshaled important evidence for the structures shown for *l*-stercobilin (I) and *d*-urobilin (II). Also, in collaboration with Klyne and Jones,[5] they measured the optical rotatory dispersion (ORD) curves for the hydrochlorides of these substances, and noted the large amplitudes of rotation associated with the intense dipyrrylmethene absorption in the vicinity of 490 mμ. In the present note we should like to report some further ORD measurements of these intensely rotatory urobilins. These data shed light on the origin of the optical activity in these compounds, on their conformations in solution, and on the relative configurations at the asymmetric centers labeled *a* and *a'* in Figure 1.

Chromophoric Dissymmetry and Optical Activity.—Recent theoretical studies[6, 7] directed toward relating molecular geometry to the orders of magnitude encountered in measurements of optical activity have demonstrated the advantage of classifying certain optically active chromophores as "inherently dissymmetric," and others as "inherently symmetric, but asymmetrically (more generally, dissymmetrically) perturbed."[8, 9] In the latter case, the intrinsic geometry of the isolated chromophore has sufficiently high symmetry that optically active chromophoric transitions would be precluded if the local symmetry of the chromophore were the sole consideration. Only after account is taken of a dissymmetric molecular environment provided for the chromophore by the rest of the molecule, would optical activity be expected to manifest itself. For example, the electronic transitions of the