THE SIGNIFICANCE OF FAT IN SULFUR OXIDATION BY THIOBACILLUS THIOOXIDANS

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The autotrophic sulfur-oxidizing bacterium, *Thiobacillus thiooxidans*, appears to be the principle exception to the otherwise applicable generalization that a cell can derive energy only from oxidations carried out at or within its boundaries. Insoluble food materials are rendered soluble by hydrolytic processes involving little energy change and the soluble products are oxidised within the cell where the energy released can be 'coupled' to the needs of the cell. Yet sulfur, besides being virtually insoluble in the medium employed for growth, cannot be hydrolysed to a soluble product. Therefore the manner in which it could be oxidised by this cell has been a matter of considerable speculation. In a previous paper (Vogler and Umbreit, 1941) it was shown that the cell of *Thiobacillus thiooxidans* must be in direct contact with the sulfur particle before the oxidation of sulfur can take place. This phenomenon was regarded as a reflection of one of at least two possibilities; either that sulfur was oxidised at the surface of the cell, or that sulfur was dissolved in some component of the cell wall.

The purpose of this paper is to demonstrate that the sulfur is dissolved in a component of the cell before oxidation. This component is a fat-like "globule" located in the majority of cases at the ends of the cell. The organism when growing on sulfur

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places the fat globule in contact with the sulfur particle, dissolves the sulfur in the fat, and is thus able to bring the insoluble sulfur into the cell for controlled oxidation. To our knowledge this is the first time fat has been implicated in such a function and the first case of a material insoluble in the medium being oxidised by means of its solubility in the organism attacking it.

THE SIGNIFICANCE OF DIPOLAR STAINING

The first published photograph of this organism (Waksman and Joffe, 1922) shows darkly staining bodies at either end of the cell (figure 3, page 252) which give to the organism a decidedly "dipolar" appearance. These bodies are not always apparent and their detection depends upon the staining technique employed. They are particularly noticeable in stains employing crystal violet and iodine, especially if the iodine is applied before the crystal violet. In wet mounts under a magnification of 1200 diameters they appear as refractile bodies, but are not generally noticeable under the usual oil immersion lens. Nile blue sulfate (wet mount) stains the entire cell a deep blue under acid conditions and a deep red under alkaline conditions. However, a combination of malachite green and alkaline nile blue sulfate stained these bodies red and the rest of the protoplasm green. The iodine fuchsin method of Eisenberg (1908) stained the entire organism yellow and Sudan III gave no detectable coloration. In the Sudan Black B stain of Hartman (1940) these bodies apparently took the stain but because of their refractile nature and their small size it was difficult to be entirely sure of the result. These studies led to the tentative conclusion that the 'dipolar' staining was due to fat-like bodies.

RELATIONSHIP OF THE "FAT BODY" TO SULFUR OXIDATION

There are several observations which associate these globules with sulfur oxidation. Microscopic observations show an orientation of the bacteria around the sulfur particle with the terminal end of the cells in contact with the sulfur. Since the globules are located at these ends, they might logically be considered part of the sulfur-utilizing mechanism. These suppositions were con-

firmed by the following simple experiment. Sulfur was sublimed onto a cover slip which was then sealed to a "hanging drop slide" so that it covered about three-fourths of the area of the depression. By filling the depression with inoculated base media and placing the slide in a horizontal microscope, the course of attachment of the bacteria could be followed. At first, of course, there were too few organisms to be readily discernible but by the 3rd to 8th hour (depending upon the size of the inoculum) there were sufficient to observe in detail. At this period virtually all of the organisms were attached to sulfur particles although the sulfur droplets were not entirely surrounded. At from 8–15 hours the droplets were completely covered with bacteria in a "uni-cellular layer" aligned with one of the ends of the cell in contact with the sulfur. When some of these organisms divided, the cells a row away from the sulfur might remain there for some time as if they too were receiving nourishment from the sulfur. Frequently, however, they would move rapidly away through the medium until they struck some solid object, to which they would immediately attach themselves. If this object were a sulfur particle no further motility was observed, but if, as frequently happened, they collided with the cover glass, they would remain for a few moments and then, apparently "disappointed" by the lack of nutrient, would again move away until a sulfur particle was reached. All of these contacts with solid objects were made with the ends of the cell, at the point where the staining body is located, so that it seemed entirely valid to associate this body with the oxidation of sulfur.

It thus seems probable that the turbidity noted in cultures of *Thiobacillus thiooxidans* is due, not to growth throughout the medium, but to the presence of organisms moving through the medium in search of sulfur particles. This would imply that while oxidising sulfur the organism builds up a supply of reserve nutrients which enables it to live for long periods in the absence of sulfur. Such actually seems to be the case for distilled water suspensions of *Thiobacillus thiooxidans* cells survive for periods as long as three months without detectable loss in respiratory activity. The nature of these reserve products and the means

by which they supply energy are not yet known in detail, but there is little doubt that they exist.

CHEMICAL STUDIES

The preceding sections have shown that the material responsible for 'dipolar' staining is associated with the oxidation of sulfur and that it appears to be fat-like in nature. To confirm this latter conclusion chemical analysis of the cells was necessary. If the "globule" were indeed fat, this should be reflected in an 'abnormal' fat content. Staining reactions, especially those with iodine, implied that the fat would also be highly unsaturated. To test these possibilities large quantities of cells of *Thiobacillus thiooxidans* were necessary. Since the growth of an autotrophic bacterium is hardly vigorous, it was necessary to employ large cultures. 100 liters of the base medium previously described (Vogler and Umbreit, 1941) were distributed in half-liter quantities in 2-liter flint glass bottles, which were plugged with cotton and sterilized for one hour at 15 pounds. After cooling, the bottles were slanted to provide maximum surface and sterile sulfur (sterilized by steaming for 8 hours on two successive days) was blown over the surface with sterile air. Each bottle was then inoculated with 1 ml. of a pure culture of *Thiobacillus thiooxidans*. After 10 to 12 days, when most of the sulfur had disappeared, the cultures were filtered through cotton (to remove any sulfur particles remaining) and the cells removed by means of a steam-driven Sharples Centrifuge at 30,000 r.p.m. The cells thus obtained were suspended in distilled water and recentrifuged in an ordinary centrifuge. Under these conditions we cannot claim absolute purity for the cells obtained, since under such large scale conditions a certain amount of contamination undoubtedly occurred. However, with the medium at the start at pH 4.5 and at harvest at pH 1.0 to 1.2; with no energy supply except sulfur; and with no organic matter except the cells of *Thiobacillus thiooxidans*, few contaminants could grow. Such suspensions showed no increase in oxygen uptake in the presence of glucose, and hence, for purposes of chemical analysis, they were pure. Another difficulty was the presence of ash. In some

samples this constituted as much as 30 per cent of the dry weight while in others it could be reduced to 4 to 6 per cent. The ash material undoubtedly arose from the presence of finely divided inorganic materials (usually calcium sulfate) which centrifuged with the cells. It was particularly evident when the cultures were neutralized before centrifuging, and could be eliminated to some extent by centrifuging at pH 1.0 and thorough washing with distilled water.

Three samples of cells so prepared have been analysed for fat. The first required over a week to reach constant weight at 100°C. The cells turned black and ether extracts yielded only 6 per cent

TABLE 1

SOLVENT TREATMENT	EXTRACTED		IODINE NUMBER	NATURE OF MATERIAL
	Per cent dry weight	Per cent ash free		
Acetone	8.7	9.06	Very high (212)	Fat-like, soft light gold, semi-liquid
Ether	0.8	.83		
Alcohol-ether 2 per cent HCl followed by chloroform	1.9	1.98	Very low (below 10)	Wax-like, hard brittle, black traces of decomposition due to treatment
Alcohol-ether 2 per cent NaOH followed by chloroform	0.95	0.99		
Total	12.35	12.86		

of the dry weight as fat. It was concluded that considerable decomposition had occurred and that at least some of the fat was lost by volatilization. The second sample was dried in a vacuum oven at 65°C., reached constant weight in one day, and yielded considerable fat on extraction. However, the ash content of this sample was 32 per cent of the dry weight. If 10 per cent of the ash found is assumed to represent that actually in the cells, and 22 per cent to represent material precipitated from the medium, the fat content of the cells was calculated at 12 per cent. The third sample, comprising 6 grams of dry matter, is reported in detail. It was dried at 65°C. at a pressure of 10

mm. mercury and reached constant weight in 12 hours. It was first exhaustively extracted with acetone, and then with ether. At this point microscopic examination of the cells showed that the dipolar appearance of the cells was almost entirely absent although the rest of the cell structure seemed intact. The sample was then treated with 2 per cent HCl in alcohol-ether (1 to 1) followed by 2 per cent NaOH in alcohol-ether, each treatment being extracted with chloroform. This treatment removes the "bound" lipides. The data obtained are listed in table 1.

This particular sample contained 4.0 per cent ash. The actual fat content found is reasonably higher than most bacteria and in fact closely approaches that of the acid-fasts. The fat can be extracted with acetone and ether and such treatment removes "dipolar" staining. The fat so obtained has an exceedingly high iodine number (the highest of any naturally occurring fat known). There is, therefore, no doubt but what the material causing "di-polar" staining is a highly unsaturated fat. There has not been enough material available to make further studies of the chemistry of this fat and its exact nature is not known.

DISCUSSION

The data presented in this paper permit the following conclusions. At the ends of the cells of *Thiobacillus thiooxidans* there exists a body which gives to the cells, under certain staining procedures, a 'dipolar' appearance. This body is definitely concerned in sulfur oxidation and is placed in contact with the sulfur whenever sulfur oxidation occurs. This body is composed of a fat which has an iodine number higher than any known natural fat. Since sulfur is soluble in fat in proportion to the unsaturation of the fat, this "globule" is eminently suited for its solution. It is therefore concluded that the sulfur is dissolved in the fat globule and is thereby taken within the cell where it can be oxidised under controlled conditions. To our knowledge this is the first time that fat has been implicated in this function. However, it is desirable to point out that this conclusion is entirely in accord with several observations in the literature and that it offers a reasonable explanation of many phenomena that were formerly obscure.

One of the most interesting of these is the early finding that the organism was gram-positive (Waksman and Joffe, 1922) and the later studies which have shown it to be gram-negative (Starkey, 1935). Both observations are undoubtedly correct. Inasmuch as the early stains were not well buffered, and since the organism grows in such an acid medium, this result is surprising only in that the usual transformation of gram-positive to gram-negative reaction under acid conditions is here reversed. However, the information now at hand enables one to interpret these reactions. Under acid conditions the iodine combines with the unsaturated fat and in the presence of even small amounts of crystal violet tends to mordant these into the fat globule, yielding a "gram-positive" dipolar stain. Under alkaline conditions, due to buffered stain, the iodine-fat reaction is much slower, the mordanting does not show up under the conditions used, and the organism appears as a short gram-negative rod.

In the larger sulfur bacteria, globules of 'sulfur' have been frequently noted. These globules have been thought to be "amorphous" sulfur. We may now suggest that they are possibly fat containing large quantities of dissolved sulfur. Their chemical reactions as reviewed by Starkey (1937) are compatible with this viewpoint.

It has been noted that in old cultures of *Thiobacillus thiooxidans* streamers of opalescent material frequently appear. These have been thought to be calcium sulfate (Waksman, 1932, page 87) but at least some of this material is fat, presumably originating from disintegrated cells. We have been able to obtain as much as 20 per cent of the dry weight of these opalescent streamers in ether extracts of the separated material.

Finally, we cannot help but remark on the possible correlation between these studies and the mechanism of acid resistance in other organisms. *Thiobacillus thiooxidans* is noted for its ability to withstand, and even to grow, in media that are exceedingly acid. It is relatively high in fat. The acid-resisting *Azotobacter* (Starkey and De, 1939) also contains a fat globule, and there is evidence that in the fungi the lower the pH the greater the amount of fat in the mycelium (Wenck, Peterson, and Fred, 1935). Might not the mechanism of acid resistance in many

organisms be related to a high fat content? It would seem that a study of this relationship should prove profitable.

CONCLUSIONS

Upon the basis of the data contained in this paper it is concluded that *Thiobacillus thiooxidans*, an obligate autotrophic sulfur-oxidising bacterium, oxidises insoluble sulfur by dissolving it in a fat globule located at the ends of the cell. This fat is highly unsaturated, having an iodine number greater than any known natural fat, and may, under given staining procedures, give the organism a "dipolar" appearance. During sulfur oxidation this fat globule is placed in contact with the sulfur particle, in such a manner that sulfur dissolves in it and is taken into the cell for oxidation. While it is true that only soluble food materials can be oxidised in a bacterial cell, these materials need not be soluble in the medium employed for growth but may, as demonstrated here, be soluble in some constituent of the cell itself.

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