SYMPOSIUM ON AUTOTROPHY[1]

V. Carbon Dioxide Fixation and Substrate Oxidation in the Chemosynthetic Sulfur and Hydrogen Bacteria

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I. Occurrence of Ribulose 1,5-Diphosphate Carboxylating Reaction

The enzymatic basis for carbon dioxide fixation in the chemosynthetic bacteria is a combination of reactions which are part of widely distributed pathways, and specialized reactions which are found only among the autotrophs. The path of carbon in photosynthesis and chemosynthesis draws upon reactions which are also part of glycolysis, pentose phosphate metabolism, and dicarboxylic acid metabolism. In addition to these widely distributed reactions are two which are peculiar to the autotrophs: the phosphorylation of ribulose 5-phosphate to ribulose 1,5-diphosphate (RDP) and the subsequent carboxylation of RDP with the resulting formation of 3-phosphoglyceric acid. The one reported occurrence of these two reactions in a heterotrophic organism, namely *Escherichia coli*, was not substantiated on reinvestigation (R. C. Fuller, *personal communication*).

The occurrence of CO_2 fixation by the RDP pathway among the chemosynthetic bacteria was found by Santer and Vishniac (18) in *Thiobacillus thioparus* and in several hydrogen bacteria (*unpublished data*). Simultaneously, Trudinger (25) made a similar observation on *Thiobacillus denitrificans*. Subsequent work by Milhaud, Aubert, and Millet (12) showed that the pattern of radioactivity distributed among the metabolites after fixation of $C^{14}O_2$ was similar to the distribution found among photosynthetic organisms by Calvin and his collaborators (1). The similarity in key reaction sequences in CO_2 fixation between the chemosynthetic and photosynthetic organisms was also confirmed by enzymatic analysis of *T. denitrificans* by Trudinger (26), and the study of *Thiobacillus thiooxidans* by Suzuki (23).

If there are similarities in the carbon metabolism of chemosynthetic and photosynthetic organisms, there are also decided differences. Some of these differences reflect the different end products that may be formed. In photosynthetic plants the products of photosynthesis partly accumulate as storage carbohydrates, especially starch and sucrose. The chemosynthetic bacteria do not store these compounds; instead there is an extensive formation of acidic products, some of which may arise by secondary carboxylation reactions. The rapid labeling of dicarboxylic acids has been found both in the sulfur and in the hydrogen bacteria (*unpublished data*), in the latter especially by Koffler (*personal communication*). Fixation of $C^{14}O_2$ by *Hydrogenomonas facilis* led to rapid labeling of formic acid. In addition there was carboxyl-labeled glycine, doubly labeled acetic acid, and doubly labeled glycol. Malic acid was uniformly labeled, suggesting that it arose by a condensation of two-carbon compounds, rather than by the carboxylation of a three-carbon compound.

In pointing out the distribution of C^{14} in the

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sugar phosphates of *T. denitrificans* after $C^{14}O_2$ fixation, the experiments of Milhaud et al. (12) indicate the significance of RDP carboxylase in chemosynthesis. Independent evidence for the role of this reaction can be found in the adaptive changes which the level of RDP carboxylase undergoes in the facultatively autotrophic bacteria. M. Santer and W. Vishniac (*unpublished data*) have examined *Hydrogenomonas ruhlandii* and several unidentified hydrogen bacteria for the occurrence of RDP carboxylase under various conditions. It was found that the highest level of activity occurred in organisms grown under strictly autotrophic conditions, that in the presence of organic matter the level of this enzyme fell off, and that in cultures raised entirely on an organic substrate, such as lactate, the enzyme level had fallen to less than 2% of the autotrophic level. Since all known hydrogen bacteria are facultative autotrophs, such experiments are easily performed in each instance. It is more difficult to obtain similar evidence for the sulfur bacteria, since members of the genus *Thiobacillus* are generally strictly autotrophic. However, in 1935 Starkey (21) described a facultatively autotrophic sulfur bacterium, *Thiobacillus novellus*. R. L. Starkey kindly supplied us with a culture of *T. novellus*, with the apologetic note that the organism had been cultivated on peptone agar for the past twenty-odd years and he was therefore not certain that it would still oxidize thiosulfate. M. Santer, in the course of several transfers, was able to coax this organism back to thiosulfate oxidation, and confirmed entirely the original report of its facultatively autotrophic nature. Here, too, RDP carboxylase followed the conditions of cultivation. On a mineral medium *T. novellus* contained a high level of this enzyme, sufficient in activity to account for all CO_2 fixation necessary to give the organism a generation time of 2.5 hr. In cells grown on organic media the enzyme level fell to less than 2% of the autotrophic level.

II. Oxidation of Sulfur Compounds

For the biosynthesis of organic matter the autotrophic bacteria require a continuous supply of adenosine triphosphate (ATP) and reduced pyridine nucleotides. Unlike the photosynthetic organisms in which the photochemical apparatus produces these components of assimilatory power, the chemosynthetic bacteria are dependent upon respiratory processes for their generation. Under favorable growth conditions thiobacilli quantitatively oxidize sulfide or thiosulfate to sulfate, so that eight electrons are transferred for every atom of sulfur which is oxidized from sulfide to sulfate. Although it is not yet known which of these electron transport steps is linked to pyridine nucleotide reduction, we know that such a reduction can occur. When extracts of acetone-dried cells of *T. thioparus* are mixed with a few micromoles of thiosulfate, there is at first a so far unexplained decrease in optical density over a wide spectral range. The subsequent addition of diphosphopyridine nucleotide (DPN) leads to an increase in optical density at 340 mμ. The individual steps of thiosulfate oxidation are little known, although a coherent picture of the nature of this pathway is beginning to emerge.

The end products of thiosulfate metabolism by growing cultures of thiobacilli show considerable variation and different workers have interpreted their results in terms of the following equations:[4]

$$3\ S_2O_3^{=} + 2\ O_2 \rightarrow S_4O_6^{=} + SO_4^{=} \quad (1)$$

$$S_2O_3^{=} + \tfrac{1}{2}\ O_2 \rightarrow SO_4^{=} + S \quad (2)$$

$$5\ S_2O_3^{=} + 4\ O_2 + H_2O \rightarrow 6\ SO_4^{=} + 4\ S + 2\ H^{+} \quad (3)$$

Moreover, Parker and Prisk (15) reported that, whereas *T. thioparus* metabolized thiosulfate according to equation 3 without the formation of tetrathionate, the closely related *Thiobacillus X* gave rise to somewhat different products, including a large amount of tetrathionate. This early work was carried out with unneutralized cultures and some of the differences can no doubt be ascribed to variable inactivation of the cells as the culture becomes acid. Nevertheless, Skarzynski, Ostrowski, and Krawczyk (19) and Peck (16) have reported that neutralized cultures of *T. thioparus* carried out an incomplete oxidation of thiosulfate with the formation of large amounts of elementary sulfur, but no detectable quantities of polythionates. In our experience, carefully neutralized and aerated cultures of *T. thioparus*, *Thiobacillus X*, and *T. novellus* oxidize thiosulfate rapidly and completely to sulfate with little or no sulfur deposition. Under these conditions, tetrathionate is invariably found in significant amounts during the early stages of growth.

The divergent results of growth experiments

[4] Equation 1 (13); equation 2 (3); equation 3 (15, 22, 24).

have led to contrasting views on the mechanism of thiosulfate oxidation. Tamiya, Haga, and Huzisige (24) and later Vishniac and Santer (30) proposed that the sulfur atoms of thiosulfate are transformed to sulfate through a series of polythionates. The transient but extensive formation of polythionates during the oxidation of thiosulfate by *T. thioparus* (29) and by *Thiobacillus X* (27) supported this hypothesis, which was further strengthened by the isolation of a soluble enzyme system catalyzing the quantitative conversion of thiosulfate to tetrathionate. This enzyme has been found in *Thiobacillus X* (28), in *T. thioparus*, and in autotrophically grown *T. novellus* (M. Santer, *personal communication*). The thiosulfate-oxidizing enzyme is absent, or at least drastically reduced, in *T. novellus* grown on organic media (W. P. Hempfling, *personal communication*).

Skarzynski et al. (19), on the basis of growth experiments with S^{35}-labeled thiosulfate, have concluded that thiosulfate is first cleaved at the cell membrane to sulfate and a S_1-compound, the latter being the only part of the molecule to enter the cell and be metabolized. A similar idea has been proposed by Peck (16) who studied thiosulfate oxidation by extracts of *T. thioparus*. Peck suggested that the initial reaction is the reduction of thiosulfate to sulfide and sulfite, and that these products are then oxidized to sulfur and sulfate. All enzymes necessary for such a metabolic sequence were demonstrated in the extracts.

We have attempted to resolve some of these discrepancies by studing the influence of experimental conditions on the oxidation of thiosulfate by washed suspensions of thiobacilli. These experiments have been carried out primarily with *Thiobacillus X*, but qualitatively similar results have been obtained with *T. novellus* (autotrophic) and *T. thioparus*.[5] Reaction mixtures were analyzed at various stages in the oxidation of thiosulfate, while the concentrations of cells, substrate, and oxygen were varied. Sufficient buffer was present to maintain the pH near 7. The only compounds found in significant amounts during thiosulfate oxidation were tetrathionate, elementary sulfur, and sulfate. Traces of trithionate, pentathionate, and an unknown material (possibly a polysulfide) were also routinely detected, but it is not yet clear whether they arose as the result of biological or purely chemical reactions. The relative amounts of products formed during thiosulfate oxidation are determined by the combined effects of variations in cell density, cell condition (age and storage), aeration rate, and oxygen concentration. It is simplest to discuss these changes as a function of cell density, and for this purpose we wish to describe three types of experimental circumstances or "conditions."

In condition 1, the rate of oxygen uptake is almost directly proportional to cell density. This nearly linear relationship holds from very low concentrations up to the point at which the rate of oxygen consumption is 50 to 60% of the maximum which can be obtained by using excessive cell densities (without varying the shaking rate). In condition 3, with very high cell densities, rate of oxygen uptake has reached a maximum and is no longer influenced by cell density. Condition 2 describes the transition from condition 1 to condition 3 in which the linear relationship between cell density and rate of oxygen consumption is lost, and increases in cell density result in progressively smaller increments in rate of oxygen consumption, until the latter becomes independent of the former. The numerical values which describe these conditions depend on species, age at harvesting, and storage of cells, whereas the maximal value of oxygen consumption is influenced by rate of shaking and partial pressure of oxygen.

Under condition 1, tetrathionate was formed in large amounts and the oxidation of thiosulfate was incomplete. This effect became progressively more pronounced as the cell concentration was lowered until in extreme cases thiosulfate was almost quantitatively converted to tetrathionate when oxidation ceased. Little or no elementary sulfur was formed. When the cell concentration was raised until the rate of oxygen uptake was above 50 to 60% of the maximal attainable (conditions 2 and 3), the formation of tetrathionate was much reduced and thiosulfate was oxidized rapidly to completion.

Extensive formation of elementary sulfur (greater than 1 to 2 μatoms per 100 μmoles of substrate) has been observed only under condition 3. However, sulfur formation is not an invariable phenomenon, and we have prepared

[5] Part of this work was carried out in the Biology Department, Haverford College, Haverford, Pa.

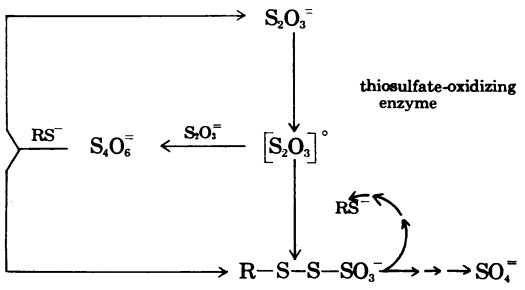

FIG. 1. *Relation between thiosulfate and tetrathionate metabolism.*

many batches of cells which produced insignificant amounts of sulfur even though the cell concentrations exceeded 3 or 4 times those required for maximal oxygen consumption. The reason for this variability at present eludes us. When sulfur was formed it was derived solely from the outer sulfur atom of thiosulfate, and was subsequently oxidized during later stages of incubation. Sulfur production also increased with increasing substrate concentration as has been shown previously by Vishniac (29) and Lange-Posdeeva (9). Under conditions 2 and 3, thiosulfate concentration has little effect on the proportion of thiosulfate converted to tetrathionate and sulfate. However, under condition 1, the amount of thiosulfate converted to sulfate increases as the substrate concentration is lowered.

These data indicate some reasons for the variations in the products of thiosulfate metabolism by growing cultures. Such variations depend not only upon the composition of the growth medium and degree of aeration, but also upon the different stages of growth as the concentration of substrate and the relationship of cell concentration to oxygen supply change with increasing cell density. We have in fact obtained a variety of balance sheets, some of which approximate those described by equations 1 to 3.

The following additional observations are pertinent to the formulation of a reaction mechanism to explain the results already described:

a) The change from condition 1 to condition 2 is accompanied by a marked increase in the ratio of reduced to oxidized cytochrome, indicating a drop in the redox potential of the cell.

b) The initial rate of thiosulfate oxidation was increased by 100% oxygen over that in air, whereas tetrathionate oxidation was inhibited strongly by 100% oxygen.

c) Tetrathionate, but not thiosulfate, was metabolized anaerobically, the products being sulfate, derived almost entirely from the inner (—SO_3) sulfur, thiosulfate, other polythionates, and a compound with the properties of a polysulfide. Little or no sulfur was formed anaerobically.

d) Tetrathionate metabolism was inhibited by very low concentrations (10^{-7} M) of *p*-chloromercuribenzoic acid (pCMB). Higher concentrations (10^{-4} M) were required to inhibit thiosulfate oxidation, but thiosulfate was still being oxidized as far as tetrathionate. The soluble thiosulfate oxidizing enzyme was insensitive to pCMB.

The observations on the effect of oxygen and the oxidation state of the cytochromes suggest that the metabolism of thiosulfate and polythionates is directed by the redox potential within the cell. The effect of pCMB suggests further that this control is expressed through a thiol-disulfide system. Lees (10) has also recently implicated such a system in thiosulfate metabolism, but his reaction sequence failed to account for the presence of a soluble pCMB-insensitive thiosulfate oxidizing enzyme. We have envisaged the events depicted in Fig. 1 to account for our observations.

The mechanism of action of the thiosulfate-oxidizing enzyme is unknown, but a reasonable hypothesis is that a two step electron removal from the thiosulfate results in an enzyme-bound uncharged radical which is then accepted by an appropriate anion. In cell-free extracts, thiosulfate is the only anion available and tetrathionate is formed. In the cell, however, other anions (e.g., RS^-) might be acceptors, as shown in Fig. 1. In this scheme an efficient conversion of thiosulfate and tetrathionate to sulfate is conditional upon the supply of RS^-. At high redox potentials RS^- would be low, tetrathionate oxidation suppressed, and thiosulfate would tend to be oxidized only as far as tetrathionate. Anaerobically thiosulfate could not enter the sequence owing to the lack of an electron acceptor for the thiosulfate-oxidizing enzyme, but tetrathionate could.

The further metabolism of the sulfenyl thiosulfate is assumed to be a hydrolysis to sulfate and an organic polysulfide, since sulfate and not sulfite is formed anaerobically from tetrathionate (equation 4).

$$R—S—S—SO_3^- \xrightarrow{OH^-} R—S—S^- + HSO_4^- \qquad (4)$$

Should an organic polysulfide be formed in this way it must at some stage be oxidized further to sulfate. Such oxidation could occur directly (equation 5) or after prior formation of elementary sulfur or sulfide by reactions 6 and 7, for both of which chemical precedents exist.

$$R{-}S{-}S^- \xrightarrow{3O} R{-}S{-}SO_3^- \xrightarrow{OH^-} RS^- + HSO_4^- \quad (5)$$

$$RS{-}S^- \longrightarrow RS^- + S \quad (6)$$

$$RS{-}S^- + RS^- \longrightarrow RSSR + S^= \quad (7)$$

Some preliminary evidence obtained in this laboratory suggests that at least some of the outer sulfur of thiosulfate is oxidated without the intermediate formation of S or $S^=$.

Anaerobically, or under conditions of oxygen deprivation, reactions 6 and 7 could account for the formation of "polysulfide" and sulfur.

It should be noted that in the proposed reaction sequence free tetrathionate is not an intermediate, but is formed as a consequence of thiosulfate entering the anion competition for the reactive uncharged radical derived from thiosulfate. Whether tetrathionate or sulfur accumulates during growth would depend upon the relative contribution of all the reactions described, which in turn would depend upon experimental conditions and possibly also upon the physiological state of the cells. The latter is largely an unknown factor and may well vary from experiment to experiment when the usual methods of cultivation with intermittent neutralization are employed. A pH-Stat is being used in this laboratory to study the effects of, for instance, pH of the culture medium and to obtain cultures grown under accurately reproducible conditions.

Sulfenyl derivatives have been suggested to be intermediates in reactions catalyzed by other enzymes acting on sulfur compounds, notably rhodanase (20), sulfite oxidase (6), and thiosulfate reductase (8). Peck has described sulfite oxidation by thiobacilli, and we have found that this oxidation is strongly inhibited by reduced glutathione. Such inhibition is consistent with a mechanism involving a reaction between SO_3^- and R—S—S—R as proposed by Fridovich and Handler (6).

Thus there may be a basic similarity in the mode of action of all these diverse enzymes, the essential substrate being a sulfenyl derivative of the general formula $RS(S)_xSO_3^-$ which can react further in the following ways:

$$R{-}S{-}(S)_xSO_3^- \begin{cases} \xrightarrow{OH^-\,(AMP)?} SO_4^= \\ \xrightarrow[RSH]{} S^- + SO_4^= \\ \xrightarrow{CN^-} SCN^- + SO_4^= \end{cases} \quad (8)$$

The above sequence of reactions is merely intended to provide a working hypothesis for the pathway of sulfur during thiosulfate and polythionate oxidation. Many variations can be imagined (e.g., in regard to the sulfur chain length of the intermediates) without altering the over-all interpretation.

The experiments of Peck (16) suggest that a large part of the ATP requirement of the sulfur bacteria may be satisfied by the oxidation of sulfite to sulfate. It is indeed conceivable that all necessary ATP can be supplied by sulfite oxidation and there is as yet no evidence for electron-transport phosphorylation in these organisms. The medium which we commonly employ for *T. thioparus* contains 1% sodium thiosulfate, or a 4×10^{-2} M solution. The formation of sulfate could therefore give rise to 8×10^{-2} moles of ATP. According to Bauchop and Elsden (2), the yield of microorganisms grown on a carbohydrate substrate is 10 g dry weight per mole of available ATP. Since chemosynthetic organisms require additional ATP to reduce CO_2 to the carbohydrate level (3 moles of ATP per CO_2), the yield of a chemosynthetic organism should be 2.5 g dry weight per mole of ATP. The formation of 8×10^{-2} moles of ATP in a liter of medium should therefore give rise to 0.2 g dry weight of thiobacilli, or roughly 2 g wet weight. This figure exceeds our best observed yield of 1.5 g wet weight of cells per liter of medium, and although we have undoubtedly not yet obtained optimal yields, the figures are certainly comparable.

III. Oxidation of Hydrogen

In the hydrogen bacteria we have at least information on the origin of reduced DPN. The activity described as "hydrogenase" includes many different enzymes, among which especially those of the clostridia and desulfovibrios have been studied (7, 10, 17). We have worked with the hydrogenase activity in the autotrophic hydrogen bacteria, especially *Hydrogenomonas*

ruhlandii. We have found two types of enzymes, a soluble enzyme (14) and an activity associated with particles (5). The soluble enzyme has been purified some 80-fold and it appears to be a single enzyme which catalyzes reversibly the reaction:

$$H_2 + DPN^+ \leftrightarrow DPNH + H^+ \qquad (9)$$

The enzyme is colorless and an absorption spectrum shows no peak other than the protein absorption around 280 mμ. There are no soluble, easily dialyzable cofactors and the reaction is specific for DPN. The reversibility of the reaction can be demonstrated either by deuterium exchange, or by coupling the enzyme to the DPN-linked glucose 6-phosphate dehydrogenase of *Leuconostoc mesenteroides*. In the presence of glucose 6-phosphate and a catalytic amount of DPN, a steady evolution of hydrogen takes place. On purification the enzyme loses the ability to reduce hydrogen acceptors other than DPN; purification by adsorption, either by treatment with alumina gel or with charcoal, removes bound DPN and the enzyme will no longer catalyze a reduction of methylene blue, while its ability to reduce benzyl viologen is drastically reduced. Upon the addition of a catalytic amount of DPN, reduction of these dyes with hydrogen is restored.

The enzyme is sensitive to pCMB but can be reactivated with cysteine or 2,3-dimercapto-1-propanol (Bal). Preincubation with DPN protects the enzyme to a certain extent against pCMB. We have found similar enzymes in other strains of the hydrogen bacteria, including *Pseudomonas saccharophila*. This enzyme is lost or at least becomes negligible when the hydrogen bacteria are cultivated on organic media, but reappears under autotrophic conditions. Since this enzyme carries out a reversible reduction of the pyridine nucleotide, since DPN appears to be normally bound to it, and since sulfhydryl groups appear to play a role in the function of the enzyme, we have proposed the name "hydrogen dehydrogenase" for this particular enzyme (4).

In addition there appears to be a particle-bound, non-DPN-linked activity which also increases and decreases adaptively, depending upon whether the organism is grown on mineral or organic media. We have at present no indication concerning the role of this other hydrogenase.

The hydrogen bacteria carry out a respiratory electron-transport phosphorylation as judged by the incorporation of $P^{32}O_4^{\equiv}$ into ATP when cell-free extracts are incubated under a mixture of hydrogen and oxygen.[6] In addition there seems to be some phosphorylation associated with the reduction of DPN by hydrogen. The only evidence for this is the incorporation of radioactivity into ATP when extracts of *H. ruhlandii* are incubated under hydrogen with catalytic amounts of DPN, pyruvate, and lactic dehydrogenase. No net formation of ATP or, under suitable conditions, glucose 6-phosphate has been observed, but the incorporation of P^{32} into ATP depends strictly upon the uptake of hydrogen when pyruvate is the terminal electron acceptor. Under helium and in other controls only very low levels of P^{32} are incorporated.

IV. Obligate Autotrophy

A discussion of the metabolism of the autotrophic bacteria inevitably leads to a consideration of the most puzzling feature which they present, namely, the obligate autotrophy which one finds among the thiobacilli. Although the hydrogen bacteria are all able to live on either mineral or organic media, *T. novellus* is the only one of the sulfur bacteria that is similarly facultatively autotrophic. We may have some comprehension of the way in which the other thiobacilli are able to make use of sulfur oxidation to carry out CO_2 fixation, but we still have no understanding of their inability to utilize organic matter directly. This problem is made more puzzling by the increasing evidence for the permeability of the thiobacilli toward organic compounds. Thus J. Postgate (*personal communication*) has been able to demonstrate that *T. denitrificans* is freely permeable toward glycerol in an experiment based on Mager's procedure (11). C. B. van Niel and G. Cohen (*personal communication*) found that *T. denitrificans* would concentrate labeled valine from the medium several hundredfold within a few minutes. We have performed experiments with the autotrophic strain of *T. novellus* and found that labeled leucine was concentrated about 5-fold within 90 sec. It is therefore impossible to maintain that the thiobacilli are impermeable with respect to organic compounds. Their inability to make use of them therefore becomes so much more difficult to understand.

The hydrogen and sulfur bacteria are both

[6] Experiments performed by S. Bernstein.

members of the suborder *Pseudomonadineae*, to which the nitrifying bacteria also belong. We feel confident that other autotrophic bacteria must exist and that the resurgence of interest in autotrophic life will lead to the study of the less well known autotrophs also.

V. Acknowledgment

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VI. Addendum

S. R. Elsden has pointed out to one of us that the ATP requirement for the growth of *Thiobacillus thioparus* is likely to be much greater than 4 moles of ATP per 10 g dry weight of cells. The yield described by Bauchop and Elsden (2) applies to cultures in which bacterial protein is synthesized from amino acids provided in the medium. Since *T. thioparus* derives all organic matter from CO_2, the additional biosynthetic reactions raise the ATP requirement by an estimated factor of 3 or 4. This consideration lowers the expected growth yield to 0.6 to 0.8 g dry weight per mole of ATP. Based on substrate level phosphorylation alone our cultures should therefore yield 0.04 to 0.06 g dry weight, or roughly 0.4 to 0.6 g wet weight per liter. Our observed yield of 1.5 g wet weight strongly suggests the occurrence of electron transport phosphorylation.

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