The Effect of Uncoupling Agents on Carbon Dioxide Fixation by a *Thiobacillus*

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SUMMARY

A newly isolated strain (c) of *Thiobacillus thio-parus* is described. The organism oxidizes thiosulphate, sulphide, tetrathionate or trithionate to sulphate; carbon dioxide fixation is coupled to the oxidation of each of these compounds. Concentrations of arsenate, 2,4-dinitrophenol, and other inhibitors of oxidative phosphorylation, inhibit carbon dioxide fixation without appreciably affecting oxygen uptake. Carbon dioxide fixation coupled to sulphide oxidation is more sensitive to arsenate and 2,4-dinitrophenol inhibition than that coupled to thiosulphate oxidation. This result is consistent with the suggestion that thiosulphate oxidation is linked to a substrate phosphorylation which is relatively insensitive to inhibition by 2,4-dinitrophenol and arsenate.

INTRODUCTION

Thiobacilli can grow autotrophically, obtaining energy from the oxidation of inorganic sulphur compounds, while carbon dioxide is fixed by the reactions of the Calvin cycle and by the carboxylation of phospho-enolpyruvate (Aubert, Milhaud & Millet, 1957; Suzuki & Werkman, 1958). Carbon dioxide fixation and subsequent biosynthetic processes require ATP and reduced nicotinamide adenine dinucleotide (Trudinger, 1956), which must be generated during sulphur compound oxidation. When thiosulphate is oxidized, NAD is reduced by extracts of *Thiobacillus thio-parus* (Vishniac & Trudinger, 1962), and ATP is formed by whole *T. denitrificans* organisms (Milhaud, Aubert & Millet, 1957). ATP is probably formed at several stages in the oxidation of a substrate such as thiosulphate, and both substrate-level and electron transport phosphorylations may take place. Possibly thiosulphate and sulphide are oxidized through polythionate intermediates with oxidative phosphorylations coupled to the flow of electrons to the oxidant (Vishniac, 1952; Trudinger, 1959; Jones & Happold, 1961). Experiments of Peck (1960, 1962) indicate that at least part of the ATP formed during thiosulphate oxidation arises from substrate-level phosphorylation. In cell-free extracts of *T. thio-parus* Peck demonstrated enzymes which (a) reductively split thiosulphate to sulphide and sulphite; (b) oxidized sulphite in the presence of adenosine monophosphate to form adenosine-5'-phosphosulphate (APS); (c) exchanged the sulphate of APS for phosphate to yield adenosine diphosphate and sulphate. ATP can be formed from ADP by adenylic kinase present in the extracts. This substrate phosphorylation is unaffected by 2,4-dinitrophenol, which uncouples oxidative phosphorylations (Peck &

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Fisher, 1962). We have briefly reported the effect of 2,4-dinitrophenol on CO_2 -fixation by a newly isolated strain of *T. thioparus* and have suggested that both oxidative phosphorylations and the substrate phosphorylation studied by Peck may be coupled to thiosulphate oxidation by this organism (Kelly & Syrett, 1963). In the present paper these observations are extended and the effect of other uncoupling agents on CO_2 -fixation described.

METHODS

The organism. A strain of *Thiobacillus* was isolated from canal water by enrichment culture in the medium for *Thiobacillus thioparus* described by Vishniac & Santer (1957). A pure culture was obtained by subculture of single colonies from medium solidified with 2% agar (Difco). Pure cultures were maintained on slopes of 1% thiosulphate medium, incubated at 30° , and subcultured every other day. The organism was a highly motile Gram-negative rod, about $2\text{--}3\ \mu \times 0\cdot5\ \mu$ in size. Minute clear colonies were formed on agar; these developed an orange centre and deposited sulphur when allowed to age. The organism had a high cytochrome content, with absorption bands at 522 and 551 $m\mu$. Washed organisms oxidized thiosulphate, tetrathionate, trithionate, sulphide and (very slowly) sulphur; sulphate was the end product. Nitrate or ammonium ion could be used for growth, which was the more rapid with ammonium as nitrogen source. The organism did not grow anaerobically with nitrate as oxidant, nor grow on thiocyanate. It did not grow on a wide range of heterotrophic nutrient media. The organism is regarded as a strain of *T. thioparus* (Vishniac & Santer, 1957), and is known as strain c.

When cultivated at 30° in liquid medium containing 1% (w/v) $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and flushed vigorously with air, growth was exponential with a generation time of about 2 hr (Fig. 1). No elementary sulphur was deposited during logarithmic growth; growth ceased when thiosulphate was exhausted. Small quantities of trithionate and tetrathionate sometimes accumulated during growth (see Jones & Happold, 1961).

Growth of cultures. Cultures were grown in medium of initial pH 6.7–7.0, but the growth rate was unchanged as the pH value decreased to about 6 as a result of acid production. Growth ceased in unneutralized cultures when they approached pH 3. A final culture volume of 1 l. was made by adding 200 ml. inoculum to 800 ml. sterile medium. The inoculum was grown in the medium of Vishniac & Santer (1957), containing only 0.2% (w/v) $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, and shaken at 30° for 14–16 hr after inoculation from a 48 hr slope, and was added, aseptically, to 800 ml. of Pyrex glass-distilled water containing 10 g. $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$; 0.8 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.4 g. NH_4Cl ; 3.2 g. K_2HPO_4 ; 3.2 g. KH_2PO_4 ; 8.0 ml. trace element solution (Vishniac & Santer, 1957); 0.8 g. KHCO_3 . The culture was forcibly aerated at 30° in a cylindrical culture vessel with a basal sintered-glass air inlet. After 7–8 hr the pH value began to decrease, and the culture was subsequently kept approximately neutral by adding saturated NaHCO_3 at intervals until all the thiosulphate was exhausted; this took 12–13 hr in all. Aeration for a further 12 hr provided the suspensions of starved organisms used in most experiments. The yield of bacteria was about 4.4 g. dry wt./mole thiosulphate oxidized.

Organisms were harvested by centrifugation, washed, and suspended in 0.1–0.13 M-phosphate buffer (pH 7.0). Suspensions were diluted to give either dilute sus-

pensions (equivalent to about 0.2 mg. dry wt./ml.) or dense suspensions (equiv. about 2.5 mg. dry wt./ml.). Dense suspensions oxidized thiosulphate too rapidly for the rate to be measured accurately by manometry; accurate measurements were possible with more dilute suspensions.

Gas exchange was measured by Warburg manometry at 30°.

Carbon dioxide fixation was estimated by sampling Warburg flasks in which $\text{KH}^{14}\text{CO}_3$ had been added to the suspension of organisms during the experiment. At the end of the experiment, a sample of suspension was pipetted into an equal volume of ethanol containing 5% (v/v) acetic acid. Portions of these mixtures were dried on ground-glass planchets as infinitesimally thin samples and ^{14}C counted with a scintillation counter or thin end-window G/M tube.

Sulphate was determined turbidimetrically as barium sulphate (Gleen & Quastel, 1953) after removal of organisms by centrifugation.

Thiosulphate was determined by volumetric iodine titration.

Chromatography of sulphur compounds. This was based on the methods of Pollard (1954) and Skarżyński & Szczepkowski (1959). With Whatman no. 4 filter paper, descending chromatograms were run at 22° with *n*-propanol + acetone + water (5 + 2 + 3, by vol.) containing 2 g. potassium acetate/100 ml. solvent, or acetone + butanol + water (2 + 2 + 1, by vol.) as solvents. Dried chromatograms were sprayed with 0.5% (w/v) AgNO_3 in ammonia solution (5 vol. aqueous ammonia solution, sp.gr. 0.88, to 95 vol. water), and spots developed by drying and heating the paper to about 100° for a few minutes to decompose the silver compounds to silver sulphide.

Chemicals. Analytical reagent grade sodium thiosulphate and sodium sulphide were used. Sodium sulphide solutions were always freshly prepared and kept in ice to minimize loss of sulphide. Dr F. H. Pollard (Bristol University) kindly gave specimens of potassium trithionate and potassium tetrathionate which were chromatographically pure.

RESULTS

Thiosulphate. This was rapidly and completely oxidized to sulphate by suspensions of washed organisms, usually at a constant rate. The QO_2 was commonly 1600 $\mu\text{l. O}_2/\text{hr/mg. dry wt.}$, and values up to 4000 were observed. With relatively dilute suspensions (equiv. 0.10–0.45 mg. dry wt. organism/flask), the rate of O_2 uptake was proportional to the mass of organisms, but with more dilute suspensions (equiv. 0.05–0.10 mg. dry wt. organism/flask) the rate of oxidation sometimes slowed down after 40–50 min. and the thiosulphate was incompletely oxidized (compare Vishniac & Trudinger, 1962). Up to the time at which the change in rate took place, chromatography showed that thiosulphate and trithionate were both present in the flasks; after the change to a slower rate of oxidation only trithionate was found. During thiosulphate oxidation, $^{14}\text{CO}_2$ was fixed. Fixation ceased when the thiosulphate had been completely oxidized, and there was no $^{14}\text{CO}_2$ fixation in the absence of thiosulphate (Fig. 2*a*). The amount of $^{14}\text{CO}_2$ fixed was proportional to the quantity of thiosulphate oxidized (Fig. 2*b*). Dense suspensions sometimes fixed a little more CO_2 /unit thiosulphate than very dilute ones. Maximally, in these experiments, 7 mole CO_2 were fixed/100 mole thiosulphate oxidized.

Sulphide. Sulphide below 0.3 mM was oxidized rapidly by dilute suspensions, but higher concentrations were inhibitory. When an inhibitory amount of sulphide

was added to a dilute suspension, oxidation proceeded slowly until the sulphide concentration was sufficiently lowered; the remaining sulphide was then oxidized rapidly. The organisms did not adapt to sulphide since, after the oxidation of an initial quantity of sulphide, additional sulphide was still inhibitory and the kinetics of its oxidation were identical with those of the initial quantity. When dense suspensions of organisms were used, the decrease in sulphide concentration proceeded

Fig. 1. Growth of *Thiobacillus C.* Optical density E (—●—●—) and $\log_{10}$ optical density (—●—●—) plotted against time. Note coincidence of growth and thiosulphate oxidation (—○—○—). Temperature, 30° . Initially at pH 7.0.

more rapidly; 1.8 mM-sulphide was oxidized rapidly and completely to the theoretical quantity of sulphate. Sulphide oxidation was also coupled to CO_2 -fixation and, with dense bacterial suspensions, $^{14}\text{CO}_2$ fixation was proportional to the quantity of sulphide oxidized (Fig. 2*b*). From eleven experiments, the quantity of CO_2 fixed during the oxidation of one mole of sulphide was $66.8 \pm 8.6\%$ of that fixed when one mole of thiosulphate was oxidized.

Polythionates. Tetrathionate and trithionate were oxidized quantitatively to sulphate, but the course of oxidation was variable, particularly with trithionate. In some experiments, the oxidation proceeded at a constant rate to completion, but, in others, the rate increased during the experiment. The oxidation of trithionate was slower in the presence of 2,4-dinitrophenol (Fig. 3) so the entry of trithionate into the cells may be an energy-requiring process. $^{14}\text{CO}_2$ was fixed during polythionate

oxidation. With dense bacterial suspensions, $^{14}\text{CO}_2$ fixed per sulphur atom of each compound was estimated from three experiments to be: thiosulphate 100%, tetrathionate $98.8 \pm 6.8\%$, trithionate $79.5 \pm 4.6\%$. Fixation, per molecule oxidized, was thus, thiosulphate 100, tetrathionate 198, trithionate 119.

Sulphite and dithionate were apparently not oxidized by *Thiobacillus* strain c.

Fig. 2. (a) Oxygen uptake (—x—x—) and $^{14}\text{CO}_2$ -fixation (—o—o—) accompanying thiosulphate oxidation. Results from replicate flasks are given. Warburg flasks contained in a final 2.0 ml.: 1.0 ml. 0.15 M-sodium phosphate (pH 7.25); 18 μmole $\text{KH}^{14}\text{CO}_3$ ($0.88 \mu\text{C}$); 15 μmole $\text{Na}_2\text{S}_2\text{O}_3$; equiv. 0.48 mg. dry wt. *Thiobacillus* strain c. Sampling flasks sealed with vaccine stoppers contained five times those quantities. $\text{KH}^{14}\text{CO}_3$ and $\text{Na}_2\text{S}_2\text{O}_3$ were tipped into the Warburg flasks, or injected into the sampling flasks at zero time. Samples were withdrawn at intervals from the latter, by using 1.0 ml. syringes, and the Warburg flasks sampled at the end of the experiment (185 min.). Temperature, 30° . Oxygen uptake for complete oxidation of added thiosulphate = $672 \mu\text{l.}$ 10^6 counts/100 sec. $\equiv 1 \mu\text{C}$. In the absence of thiosulphate there was no oxygen uptake and no $^{14}\text{CO}_2$ -fixation.

(b) Proportionality of $^{14}\text{CO}_2$ -fixation to quantity of thiosulphate (—o—o— and —x—x—) or sulphide (—●—●—) oxidized. The results of two separate experiments with thiosulphate are shown. ^{14}C -fixation was determined after added quantities of substrate had been completely oxidized. pH 7.0. Temperature, 30° .

The effect of uncoupling agents on $^{14}\text{CO}_2$ fixation

2,4-Dinitrophenol. CO_2 -fixation was inhibited by 2,4-dinitrophenol when any one of the above sulphur compounds was oxidized. Thiosulphate-coupled fixation was inhibited by concentrations of 2,4-dinitrophenol which stimulated or did not affect the rate of oxidation (Table 4; also Kelley & Syrett, 1963). CO_2 -fixation coupled to sulphide oxidation by dense bacterial suspensions was inhibited by a similar range of 2,4-dinitrophenol concentrations (Fig. 4) and the degree of inhibition was independent of the initial sulphide concentration (Table 1). However, sulphide-linked fixation was always inhibited more than that coupled to thiosulphate oxidation, whatever the dinitrophenol concentration (Fig. 4).

Fixation of $^{14}\text{CO}_2$, with tetrathionate as substrate, appeared to be about as sensitive to uncoupling by 2,4-dinitrophenol as that with thiosulphate as substrate (Table 2). The effect of dinitrophenol on the trithionate-linked fixation was somewhat inconsistent; this is possibly related to the variability of the time course of oxidation of this compound. Table 3 shows the results of an experiment in which trithionate was oxidized at a constant rate with a Q_{O_2} of 180. $^{14}\text{CO}_2$ -fixation coupled to its

Fig. 3

Fig. 4

Fig. 3. Inhibition of trithionate oxidation by 2,4-dinitrophenol. Equiv. 4.6 mg. dry wt. *Thiobacillus* strain c were incubated in 2.5 ml. phosphate buffer, containing 2,4-dinitrophenol, for 30 min.; 8 μmole potassium trithionate and 9 μmole KHCO_3 then added (zero time on graph). Temperature 30° ; pH 7.0. 2,4-dinitrophenol concentrations: 0 ($\times-\times$), 10^{-5}M ($\text{O}-\text{O}$), $5 \times 10^{-5}\text{M}$ ($\bullet-\bullet$), 10^{-4}M ($+-+$).

Fig. 4. Inhibition by 2,4-dinitrophenol of $^{14}\text{CO}_2$ -fixation coupled to sulphide or thiosulphate oxidation. The results of two experiments are shown. Equiv. 6.8 mg. dry wt. *Thiobacillus* strain c in 2.5 ml. phosphate buffer (pH 7.0), oxidized 4.0 μmole Na_2S or $\text{Na}_2\text{S}_2\text{O}_3$ in the presence of 9 μmole $\text{KH}^{14}\text{CO}_3$ ($1.5 \mu\text{C}$). Sulphate recovery in experiments 1 and 2 was 3.1 and 4.5 μmole from sulphide, and 8.5 and 8.2 from thiosulphate, respectively. $^{14}\text{CO}_2$ -fixation, as % of controls without 2,4-dinitrophenol, is shown for sulphide (Expt. 1, $\text{O}-\text{O}-\text{O}$; Expt. 2, $\bullet-\bullet-\bullet$) and thiosulphate (Expt. 1, $-+-+$; Expt. 2, $-x-x-x$).

Table 1. Relationship between quantity of sulphide oxidized and the effect of 2:4-dinitrophenol on coupled $^{14}\text{CO}_2$ -fixation by *Thiobacillus* strain c

The equivalent of 5.6 mg. dry wt. *Thiobacillus* strain c in 2.5 ml. phosphate buffer (pH 7.0) oxidized Na_2S in the presence of 9 μmole $\text{KH}^{14}\text{CO}_3$ ($3 \mu\text{C}$). $^{14}\text{CO}_2$ -fixation was estimated when oxidation ceased. Temperature 30° .

Sulphide oxidized (μmole)	$^{14}\text{CO}_2$ fixed (counts/100 sec./flask)		Inhibition of CO_2 -fixation (%)
	Control	$+ 5 \times 10^{-5}\text{M-DNP}^*$	
1.0	11,000	5,900	46
2.0	33,450	20,150	40
4.0	68,050	40,300	41

* DNP = 2,4-dinitrophenol.

oxidation was inhibited by dinitrophenol and, in this experiment, the inhibition was less than when thiosulphate was the substrate.

Other substituted phenols. Several aromatic compounds were tested for the ability to uncouple thiosulphate-dependent CO₂-fixation (Table 4). Phenol was ineffective; *o*-nitrophenol was ineffective as an uncoupling agent, and is known to have little

Table 2. *Effect of 2,4-dinitrophenol on ¹⁴CO₂-fixation coupled to thiosulphate and tetrathionate oxidation*

Dense suspensions of *Thiobacillus* strain c (equiv. 2.5 mg. dry wt./ml.) oxidized thiosulphate, or tetrathionate, completely to sulphate. ¹⁴CO₂-fixation and sulphate production were then measured. Sulphate recoveries in experiments 3 and 4, as % of expected amounts, were 107.5 and 115 for thiosulphate, and 109 and 108 for tetrathionate. Oxygen consumption always approximated to the theoretical values. Temperature 30°. pH 7.0.

Experiment	2,4-Dinitrophenol concentration (M)	Oxidation of	
		S ₂ O ₃ ²⁻	S ₄ O ₆ ²⁻
		Inhibition of CO ₂ -fixation (%)	
1	2.5 × 10 ⁻⁴	69	80
2	2.5 × 10 ⁻⁴	76.5	71.5
3	2 × 10 ⁻⁴	77.5	88
4	1 × 10 ⁻⁵	9	2
	5 × 10 ⁻⁵	32	36
	1 × 10 ⁻⁴	44	55

Table 3. *Fixation of ¹⁴CO₂ during thiosulphate or trithionate oxidation by *Thiobacillus* sp. strain c and the effect of 2,4-dinitrophenol*

Thiobacillus c equiv. 6.3 mg. dry wt. oxidized Na₂S₂O₃ (9 μmole) or K₂S₃O₆ (6 μmole) in the presence of KH¹⁴CO₃. The 2,4-dinitrophenol concentration was 5 × 10⁻⁵ M; pH 7.0; temperature, 30°.

Treatment	¹⁴ CO ₂ fixed (counts/100 sec.)	¹⁴ CO ₂ fixed (%/μatom sulphur)	Inhibition of fixation by 2,4- dinitrophenol (%)
Na ₂ S ₂ O ₃	7.12 × 10 ⁴	100	—
K ₂ S ₃ O ₆	5.10 × 10 ⁴	72	—
Na ₂ S ₂ O ₃ + 2,4-dinitrophenol	4.08 × 10 ⁴	100	42.5
K ₂ S ₃ O ₆ + 2,4-dinitrophenol	3.66 × 10 ⁴	89.5	28.5

effect on oxidative phosphorylation in other systems (Cross *et al.* 1949; Clowes *et al.* 1950). Picric acid inhibited fixation at high concentrations but its effect was probably complex; in other systems it is a poor uncoupling agent. Dichloro- and *p*-nitrophenol are well established as inhibitors of oxidative phosphorylation (Hackett, 1960; Clowes *et al.* 1950) and effectively uncoupled CO₂-fixation by our *Thiobacillus*. Low concentrations of 2,6-dichloro-4-nitrophenol probably have a similar effect (Krahl & Clowes, 1936) but high concentrations inhibit the oxidation of thiosulphate. 2,4-Dinitrophenol (2 mM) depressed the rate of oxygen uptake by more than 80 %.

Arsenate. The effect of a range of arsenate concentrations on ¹⁴CO₂-fixation by

dense bacterial suspensions oxidizing thiosulphate or sulphide was measured (Table 5); in these experiments, the phosphate concentration remained constant. $^{14}\text{CO}_2$ -fixation coupled to oxidation of thiosulphate or sulphide was inhibited by arsenate:phosphate ratios of the order of unity, but the fixation coupled to sulphide

Table 4. *Effect of substituted aromatic compounds on thiosulphate-linked $^{14}\text{CO}_2$ -fixation by Thiobacillus strain c*

Compound	Concentration (M)	Inhibition of $^{14}\text{CO}_2$ -fixation (% of control)	Initial oxidation rate (% difference from controls)
Experiment 1			
Phenol		0	.
<i>o</i> -Nitrophenol		8.5	.
<i>p</i> -Nitrophenol		34	.
2,4-Dinitrophenol		48.5	+15
2,4,6-Trinitrophenol	2×10^{-4}	10.5	.
<i>p</i> -Nitroaniline		14	.
<i>p</i> -Nitroanisole		0	.
<i>p</i> -Nitrobenzylalcohol		1	.
<i>o</i> -Nitrophenol	10^{-4}	14.5	-10
	5×10^{-4}	16	-13
<i>p</i> -Nitrophenol	10^{-5}	0	.
	5×10^{-5}	4	.
	10^{-4}	12	.
	5×10^{-4}	91	+20
Experiment 2			
2,4-Dinitrophenol	10^{-5}	3.5	.
	5×10^{-5}	14	.
	10^{-4}	49.5	.
	5×10^{-4}	74.5	.
2,4,6-Trinitrophenol	10^{-4}	7.5	-7
	5×10^{-4}	38.5	-15
2,4-Dichlorophenol	10^{-5}	0	.
	5×10^{-5}	12.5	.
	10^{-4}	47	-21
	5×10^{-4}	100	-48
2,6-Dichloro-4-nitrophenol	10^{-5}	0	.
	5×10^{-5}	27	.
	10^{-4}	33	.
	5×10^{-4}	99	-50*

* 5×10^{-4} M 2,6-dichloro-4-nitrophenol blocked the oxidation after only 3.8 μ mole of the theoretical 20 μ mole of oxygen had been consumed.

oxidation was the more sensitive to the inhibitor. The rate of thiosulphate oxidation by dilute bacterial suspensions was unaffected by these arsenate:phosphate ratios.

Azide. Sodium azide inhibited both thiosulphate oxidation and $^{14}\text{CO}_2$ fixation. Both reactions were inhibited by 50 % by 10^{-4} M-azide and completely inhibited by 5×10^{-4} M. Azide, therefore, has no specific uncoupling effect on this system. Iwatsuka, Kuno & Maruyama (1962) obtained a similar result with *Thiobacillus thiooxidans*.

Table 5. *Inhibition of ¹⁴CO₂-fixation in Thiobacillus strain c by arsenate*

Dense suspensions of *Thiobacillus* strain c in 0.003M-phosphate buffer (pH 7) were added to phosphate+arsenate buffer mixtures in Warburg flasks and incubated at 30°, pH 7.0, for 30–40 min. KH¹⁴CO₃ and Na₂S or Na₂S₂O₃ were added. ¹⁴CO₂-fixation was measured when oxidation was complete. The final phosphate concentration was 0.05M in each experiment.

Arsenate concentration (M)	Arsenate:phosphate ratio	Oxidation of	
		S ₂ O ₃ ²⁻ - ¹⁴ CO ₂ fixed (%)	S ²⁻ - ¹⁴ CO ₂ fixed (%)
Experiment 1			
0	0	100	100
0.02	0.4	115	77
0.05	1.0	102	61
Experiment 2			
0	0	100	100
0.001	0.02	105	96
0.01	0.2	80	89
0.05	1.0	81	33
0.1	2.0	80	22
Experiment 3			
0	0	100	100
0.01	0.2	112	87
0.025	0.5	102	82
0.05	1.0	72	56
0.1	2.0	21	17

DISCUSSION

CO₂-fixation in *Thiobacillus* takes place chiefly by the Calvin cycle (Aubert *et al.* 1957) and presumably these reactions require a supply of reduced NAD or NADP and ATP (Racker, 1955). Since, in our experiments, CO₂-fixation is inhibited by low concentrations of 2,4-dinitrophenol, which uncouples phosphorylation in other systems, one can assume that the amount of CO₂-fixation indicates the quantity of high-energy phosphate available to the organisms. Taking the value with thio-sulphate as substrate as 100, CO₂-fixation/mole substrate oxidized was 119 units with trithionate, 198 with tetrathionate and 67 with sulphide. Since, however, these compounds contain different numbers of sulphur atoms, a better comparison is CO₂-fixation/sulphur atom oxidized. The figures then are, with thiosulphate as 100, trithionate 80, tetrathionate 99 and sulphide 134.

The complete oxidation to sulphate of one molecule of sulphide or thiosulphate requires two molecules of oxygen. Nevertheless, only about two-thirds as much CO₂-fixation is coupled to the oxidation of sulphide. If the amount of CO₂ fixed does indicate the quantity of high-energy phosphate made available during oxidation, this difference suggests that the oxidation of a molecule of thiosulphate is linked to more phosphorylating steps than that of one of sulphide. An additional phosphorylation would occur during thiosulphate oxidation if the substrate-level phosphorylation studied by Peck (1962) in cell-free extracts also takes place in intact organisms.

The inhibition of CO₂-fixation by 2,4-dinitrophenol, and other phenolic compounds

which are known to uncouple oxidative phosphorylations in mitochondrial systems, indicates that oxidative phosphorylation plays a part in the coupling between substrate oxidation and CO_2 -fixation in *Thiobacillus*. However, CO_2 -fixation coupled to sulphide oxidation is always more sensitive to 2,4-dinitrophenol inhibition than is the CO_2 -fixation coupled to thiosulphate oxidation. Such a difference in sensitivity would be expected if the substrate phosphorylation, studied by Peck, is relatively more important in supplying energy for CO_2 -fixation during the oxidation of thiosulphate than it is when sulphide is oxidized. This view is supported by the observation that the ATP content of intact *Thiobacillus* organisms increases rapidly after the addition of thiosulphate or sulphide (Kelly & Syrett, unpublished); but, whereas, when thiosulphate is added, this increase is unaffected by 2,4-dinitrophenol, it is strongly inhibited when sulphide is the substrate. If the only phosphorylations coupled to sulphide oxidation are oxidative ones, sensitive to 2,4-dinitrophenol, then our data indicate that two-thirds of the phosphorylations coupled to thiosulphate oxidation are of this type and one-third are unaffected by 2,4-dinitrophenol (Kelly & Syrett, 1963). As yet, the pathway of sulphide oxidation is uncertain and the possibility of some substrate phosphorylation during sulphide oxidation cannot be excluded.

Arsenate can replace phosphate in many enzyme reactions and, with it, ADP-sulphurylase catalyses the arsenolysis of adenosine-5'-phosphosulphate (Robbins & Lipmann, 1958). Both this enzyme and adenosine phosphosulphate are reactants in the phosphorylation system studied by Peck & Fisher (1962) and, in their cell-free extracts, arsenate could replace for phosphate in catalysing the oxidation of thiosulphate to sulphate. However, when phosphate and arsenate were both present, in equimolar quantities, phosphate esterification was unaffected by arsenate. Thus arsenate competes poorly with phosphate in the reaction with adenosine phosphosulphate. On the other hand, an arsenate:phosphate ratio of unity uncouples oxidative phosphorylation in a mitochondrial system (Crane & Lipmann, 1953). Our results with whole organisms of *Thiobacillus* show that arsenate resembles 2,4-dinitrophenol in that a given concentration inhibits $^{14}\text{CO}_2$ -fixation more when sulphide is the substrate than with thiosulphate. The difference in sensitivity again suggests that thiosulphate oxidation is linked to a type of phosphorylation which is relatively less important in the oxidation of sulphide.

When tetrathionate is added, CO_2 -fixation/sulphur atom oxidized is very similar to fixation with thiosulphate as substrate and the sensitivity to 2,4-dinitrophenol inhibition is much the same. Such similarity is to be expected if the first step in the metabolism of a molecule of tetrathionate is its conversion to two molecules of thiosulphate (Peck & Fisher, 1962). The present results with trithionate suggest less CO_2 -fixation/sulphur atom oxidized than with the other substrates and a lower sensitivity to 2,4-dinitrophenol. It is attractive to think that the trithionate ion, $\text{S}_3\text{O}_6^{2-}$, might be reduced to two sulphite ions and one of sulphide. If this sulphite were then oxidized with a coupled substrate phosphorylation as suggested by Peck, phosphorylations insensitive to 2,4-dinitrophenol might be quantitatively more important in the oxidation of trithionate in *Thiobacillus* than in the oxidation of thiosulphate, $\text{S}_2\text{O}_3^{2-}$, where only one sulphite ion, together with one of sulphide, is formed on reduction.

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