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Reduction of Sulphite to Sulphide Catalysed by Desulfoviridin from *Desulfovibrio gigas*

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Abstract

Desulfoviridin from *D. gigas* was partially purified by column chromatography and further purified by gel electrophoresis into a major band preparation and a minor band preparation. All partially purified and electrophoretically purified preparations catalysed the methylviologen-linked reduction of sulphite to sulphide, but stoichiometric reduction to sulphide was not demonstrated with the major band preparation. Desulfoviridin did not catalyse the reduction of thiosulphate or trithionate.

Introduction

Desulfoviridin is a green pigment characteristic of *Desulfovibrio* species (Postgate 1956). Suh and Akagi (1969) showed that this pigment from *D. vulgaris* catalysed the reduction of sulphite to thiosulphate, while Lee and Peck (1971) found that purified desulfoviridin from *D. gigas* catalysed the reduction of sulphite to trithionate. However, Skyring and Trudinger (1972) showed by disc electrophoresis that the site of reduction of sulphite to sulphide in crude extracts of *D. gigas* was coincident with desulfoviridin and that the desulfoviridin had no thiosulphate reductase activity. In these latter experiments it was not certain that the reduction of sulphite to sulphide was catalysed by desulfoviridin or by other proteins with similar electrophoretic properties.

In this paper it is shown that partially purified and electrophoretically purified preparations of desulfoviridin from *D. gigas* catalyse the reduction of sulphite, at least partially to sulphide. On completion of this work Kobayashi *et al.* (1972) reported that a purified desulfoviridin preparation from *D. vulgaris* also catalysed the reduction of sulphite to sulphide, but with trithionate and thiosulphate as major and minor products.

Methods

Growth Conditions

The growth medium consisted of $(NH_4)_2SO_4$, 5 g; NaCl, 10 g; $MgSO_4$, 0·5 g; $CaCl_2$, 0·1 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; sodium lactate, 3·5 g; Difco yeast extract, 1 g; Difco vitamin-free casamino acids, 4 mg; cysteine hydrochloride, 1 mg; *i*-inositol, 2 mg; thiamine hydrochloride, 400 μg; biotin, 10 μg; *p*-aminobenzoic acid, 200 μg; vitamin B_{12}, 2 μg; calcium pantothenate, 800 μg; pyridoxine hydrochloride, 400 μg; riboflavin, 200 μg; H_3BO_3, 200 μg; $CuSO_4$, 40 μg; KI, 100 μg; $FeCl_3$, 200 μg; $MnSO_4$, 400 μg; Na_2MoO_4, 200 μg; $ZnSO_4$, 400 μg; $CoCl_2$, 200 μg; 2-mercaptoethanol, 0·01% (v/v); distilled water, 1 litre; pH 6·8. In preparing the medium aqueous K_2HPO_4 was added last, and the medium was sterilized by filtration through a Millipore GS filter. It was incubated for 15–17 h at 35°C before inoculation.

Using a 10% (v/v) inoculum, *D. gigas* (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; strain number 9332) was grown for 3–4 days at 35°C in completely filled 20-litre containers.

Preparation of Cell Extract

Organisms were harvested by centrifugation at 4°C, washed once in 0·1M KCl–1 mM EDTA–0·01M tris–HCl buffer, pH 7·8, and stored at −20°C for a maximum of 4 weeks.

Cells from 360 litres of growth medium (28 g dry wt) were suspended in the 0·1M KCl–1 mM EDTA–0·01M tris–HCl buffer, pH 7·8, containing $MgCl_2$ (0·01M) and deoxyribonuclease (0·005% w/v), and passed twice through a French pressure cell at 20000 lb in^{-2} ($\sim 1{\cdot}4 \times 10^8$ N m^{-2}). The resultant preparation was centrifuged twice at 10000 *g* for 10 min. The supernatant was further centrifuged at 198000 *g* for 1 h then at 105000 *g* for 1 h and concentrated from 120 ml to 40 ml by means of an XM50 Diaflo membrane (Amicon Corp., Mass.) under argon at 50 lb in^{-2} ($\sim 3{\cdot}4 \times 10^5$ N m^{-2}).

Purification of Desulfoviridin

Cell extract (36 ml; 165 mg protein/ml) was applied to a column (45 by 2·5 cm) of DE52 resin (Whatman) in 0·1M KCl–1 mM EDTA–0·01M tris–HCl buffer, pH 7·8. Elution was with successive 600-ml amounts of 0·2 and 0·3M KCl in 1 mM EDTA–0·01M tris–HCl buffer, pH 7·8, and 13-ml fractions were collected. The fractions containing desulfoviridin were eluted by 0·3M KCl; they were green and fluoresced red in light of 365 nm wavelength after addition of one drop of 1N NaOH to one drop of the fraction (Postgate 1959). The main desulfoviridin fractions were pooled (130 ml) and concentrated to 10 ml by use of an XM50 Diaflo membrane to constitute the DE52 preparation. This was diluted 1 : 10 with 0·1M tris–HCl buffer, pH 7, before use in reductase reactions.

Two lots of 3 ml (65 mg protein/ml) of the DE52 preparation were chromatographed separately on a Sephadex G200 column (45 by 2·5 cm) with 0·1M tris–HCl buffer, pH 7·0, as the eluant. Fractions of 3 ml were collected and the main desulfoviridin fractions were again pooled (47 ml), concentrated by membrane filtration to 2 ml and applied to a similar Sephadex G200 column. Fractions were collected in 1·6-ml aliquots and the main desulfoviridin fractions were pooled (25 ml) to constitute the G200 preparation which was stored at −20°C in 0·5-ml amounts.

A quantity of the G200 preparation (128 μg; 3·4 mg protein/ml) was subjected to electrophoresis in 7% polyacrylamide gels by methods previously described (Skyring and Trudinger 1972). Desulfoviridin in the gels was recognized by its green colour and by the fluorescent reaction. After electrophoresis two adjacent desulfoviridin bands were clearly visible, the leading band being the major one. These were sliced separately from 121 gels, gently macerated, and the desulfoviridin eluted by shaking slowly overnight at 4°C in 0·5M tris–HCl buffer, pH 7·0. After centrifugation the supernatants were kept at 4°C before concentration by membrane filtration; the 0·5M buffer was replaced with 0·1M tris–HCl, pH 7·0, and the two desulfoviridin preparations were stored at 4°C. These are referred to as major band (1·8 ml, 1·3 mg protein/ml) and minor band (0·6 ml, 1·3 mg protein/ml; see Table 1) preparations.

Reductase Reactions

Reductase reactions were performed in polyacrylamide gels, in which the formation of black FeS was used as an indicator of sulphide production (Skyring and Trudinger 1972), or in a 3·8-ml capacity Thunberg-type cuvette of 1 cm path length. The cuvette contained 5 μmol of substrate in 3 ml of 0·01M EDTA–0·1M tris–HCl buffer, pH 7. The desulfoviridin preparation (50 or 100 μl) was placed in the bulb and, after stoppering the cuvette with the bulb, deoxygenated nitrogen [obtained by passage through reduced methylviologen (MVH)] was bubbled through the contents for 10 min via the side-arm of the cuvette. MVH (0·3 ml of a 0·3% w/v solution) in 0·01M EDTA–0·1M tris–HCl buffer, pH 7, was then added through the side-arm and deoxygenated nitrogen bubbled through for a further 10 min. (MVH was obtained by shaking the methylviologen solution for 8–10 min in a 1-ml capacity syringe containing a few pieces of acid-washed granulated zinc.) The cuvette was placed in a water-bath at 37°C for 2 min and then transferred to a compartment at 37°C in a Cary model 14 spectrophotometer equipped with neutral density filters in the reference compartment.

The initial extinctions at 600 nm wavelength were in the range 3–4. After recording the extinction for an equilibration period of 20 min the desulfoviridin preparation was added to form the complete system by mixing the bulb and cuvette contents. Reduction of the substrate was followed by recording the decrease in extinction at 600 nm due to oxidation of MVH. The reference cuvette contained the same system as the sample cuvette but was aerobic so that the MVH became oxidized before the extinction was recorded.

A molar absorption coefficient of 13100 per centimetre for MVH in 0·01M EDTA–0·1M tris–HCl buffer, pH 7, at 37°C was obtained by addition of known amounts of $FeCl_3$ from the bulb of the cuvette (method 1 as described by Trudinger 1970*a*). A lower value, equivalent to that reported by Trudinger (1970*a*), was obtained at room temperature.

Estimation of Sulphide

Sulphide formed in the cuvette during sulphite reduction was estimated by the *N*-ethylmaleimide reaction (Trudinger 1970*b*). The contents of the reference cuvette served for the blank estimate. Sulphide was also detected by the formation of methylene blue in the presence of *p*-aminodimethylaniline and Fe^{3+} using the method of Gustafsson (1960) modified by the substitution of 9M H_2SO_4 for 3·5M H_2SO_4.

Spectra

Spectra of desulfoviridin preparations were recorded using cuvettes of 1 cm path length and a Cary model 14 spectrophotometer.

Estimation of Molecular Weight

The molecular weight of desulfoviridin was estimated on a Sephadex G200 column (45 by 2·5 cm) in 0·1M sodium phosphate buffer, pH 7. The column was calibrated with human γ-globulin, bovine serum albumin, ovalbumin, equine heart myoglobin and equine heart cytochrome *c*. Molecular weights of these proteins were those quoted by Andrews (1965).

Protein Estimations

Protein concentrations were estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as the reference protein.

Results

Purification of Desulfoviridin

The characteristic absorption peak of desulfoviridin is at 627 nm (Fig. 2). Thus the extent of purification at each stage of the procedure is indicated by the ratio

$$(E_{627}-E_{700})/[\mathrm{P}],$$

where $(E_{627}-E_{700})$ is the difference between the extinction at 627 nm and that at 700 nm, and [P] is the protein concentration (mg/ml). For the cell extract, DE52, G200 and major band preparations these ratios were 0·03, 0·12, 0·24 and 0·31 respectively. After elution from the polyacrylamide slices the green colour of the minor band preparation decreased considerably before concentration and this was accompanied by a decrease in the extinction at 627 nm (see Table 1) and by the appearance of red fluorescence on illumination of the membrane filter effluent with light of 365 nm wavelength.

The major proteins present in gels of the cell extract, DE52 and G200 preparations were coincident with those of desulfoviridin. Protein staining of the G200 preparation in 7% gels showed that in addition to the two densely staining desulfoviridin bands (major and minor, $m=0·46$ and 0·41 respectively) there were nine

weakly staining bands (Fig. 1*a*), four of which were recognized as weak desulfoviridin bands by their fluorescence in the presence of NaOH at 365 nm (m=0·20, 0·27, 0·52 and 0·58 respectively). Some of the faintly staining bands in these and other gels were impossible to resolve photographically. When the major desulfoviridin band from the G200 preparation was sliced from 7% gels and re-run separately on 5, 7 and 10% gels with an initial pH of 9·5 and on a 7% gel with an initial pH of 7·0 only protein bands recognizable as desulfoviridin were present. When the minor band was re-run on a 5% gel a similar result was obtained.

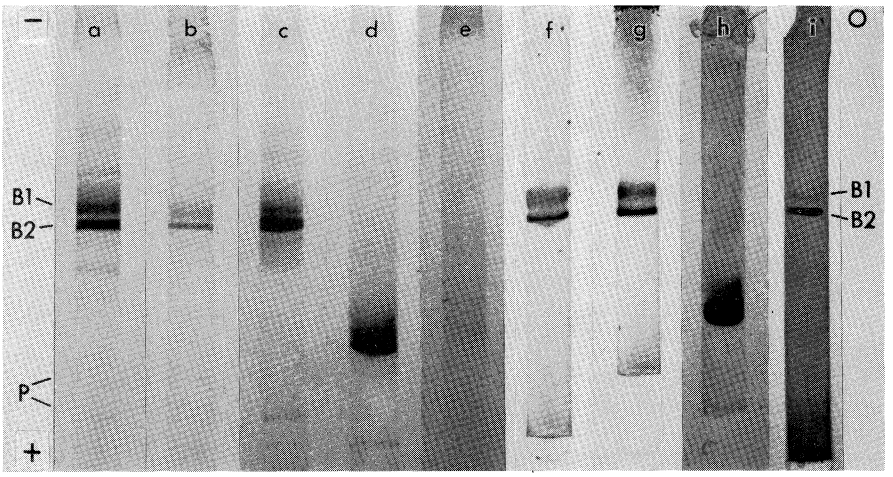

Fig. 1. Polyacrylamide gel disc electrophoresis of desulfoviridin preparations showing protein bands stained with aniline blue black (CANALCO) (*a–e*) or sites of sulphite reductase activity (*f–i*; bands are ferrous sulphide). *O*, origin; *B1*, *B2*, minor and major bands respectively; *P*, marker protein.

a, *b* G200 preparation in 7% gels containing 64·0 and 12·8 μg protein per gel respectively.

c, *d* Major band preparation in 7 and 5% gels respectively, containing 184 μg protein per gel. All protein bands were coincident with desulfoviridin bands.

e Minor band preparation in 5% gel containing 39 μg protein per gel (see Table 1). The fastest band was weakly fluorescent.

f DE52 preparation in 7% gel.

g, *h* G200 preparation in 7 and 5% gels respectively.

i Major band preparation in 7% gel.

At a concentration of 184 μg desulfoviridin per gel, the protein bands of the major band preparation in 7% gels were coincident with all the desulfoviridin bands in the G200 preparation (Fig. 1*c*). When the major band preparation was run on a 5% gel, there were four strongly fluorescent bands (m 0·75, 0·73, 0·71 and 0·68) and three weakly fluorescent bands (m 0·47, 0·53 and 0·81); no other proteins were present. On a 5% gel, two proteins were detected (m 0·72, 0·65) in the minor band preparation (Fig. 1*e*). The slower band did not fluoresce at 365 nm in the presence of NaOH. Both bands were coincident within experimental error (Skyring and Trudinger 1972) with desulfoviridin bands (m 0·71, 0·68) in a 5% gel of the major band preparation. It is possible that the slower band which did not fluoresce in the minor band preparation was a degradation product of desulfoviridin resulting from loss of chromophore during preparation (see above).

From the ratio $(E_{627}-E_{700})$: [P] for the major band preparation, desulfoviridin constitutes about 10% of the cell-extract proteins and about 3% of the cell dry weight.

Spectra

Absolute spectra of all preparations were similar and resembled the spectrum of desulfoviridin (Postgate 1956). Peaks at 627, 588 and 409 nm, shoulders at 390 and 378 nm and an almost imperceptible broad peak around 490 nm were present (Fig. 2.) With spectra of all preparations, on reduction with sodium dithionite the extinctions at 409 and 588 nm decreased slightly, but that at 627 nm remained unchanged (Fig. 2). The peak at 588 nm reappeared after oxidation with potassium ferricyanide.

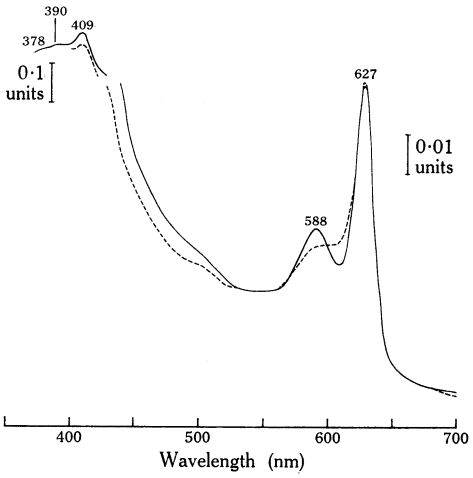

Fig. 2. Absolute spectra of G200 desulfoviridin preparation (0·34 mg protein/ml) before reduction (——) and after reduction with sodium dithionite (- - -). The vertical scales indicate extinction units.

Molecular Weight

The molecular weight of desulfoviridin in the cell extract was approximately 200000, which is identical with the result reported by Lee and Peck (1971).

Reductase Reactions

After electrophoresis of the cell extract, DE52, G200 and major band preparations reduction of sulphite to sulphide was demonstrated only at the positions of the major and minor desulfoviridin bands (Figs 1*f*–1*i*). Thiosulphate reductases were detected only in the cell extract and DE52 preparations. In the cell extract one broad band with $m=0\cdot18$ was found. In the DE52 preparation this was resolved into two bands ($m=0\cdot14$ and $0\cdot18$); on continued incubation the two bands fused into one broad band similar to that in the cell extract. These thiosulphate reductases were well separated from the major and minor desulfoviridin bands ($m=0\cdot46$ and $0\cdot43$).

In cuvette experiments with the DE52, G200, major band and minor band preparations, oxidation of MVH in the presence of sulphite was accompanied by formation of sulphide. Furthermore, if deoxygenated nitrogen was blown through the cuvette for 40 min after the reaction had ceased, sulphide was not detected. The DE52 preparation oxidized MVH non-specifically at a slower rate than when sulphite was present (Table 1). This reaction may have resulted from the presence of reducible substrates or from hydrogenase activity. The DE52 preparation reaction and the enzymic activity of all preparations was destroyed by boiling for 30 min.

Apart from the non-specific action of the DE52 preparation, both sulphite and enzyme were required for MVH oxidation and for sulphide formation. When thio-

sulphate and trithionate were used as substrates for the G200 preparation, MVH oxidation and sulphide formation did not occur.

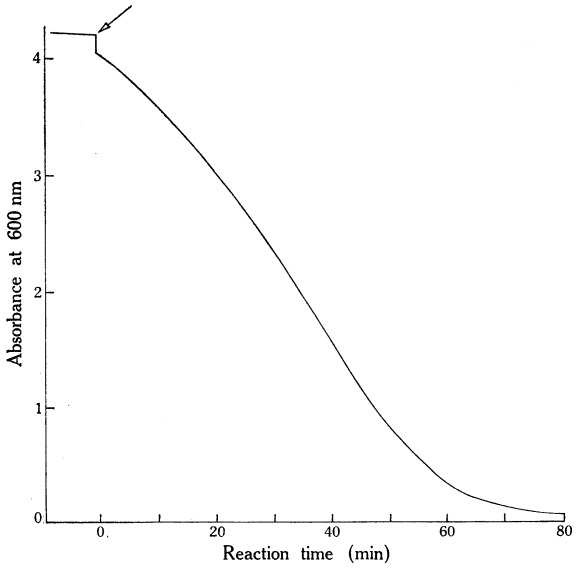

Fig. 3. Oxidation of reduced methylviologen in the presence of sulphite by G200 desulfoviridin preparation (0·34 mg protein). The complete incubation system is described in the Methods section. Arrow indicates addition of the G200 preparation.

Fig. 3 shows oxidation of MVH during reduction of sulphite to sulphide by the G200 preparation. The rate increases as the extinction falls to 2·1, then becomes approximately linear down to 1·2 and finally decreases at the lower extinction values. This pattern was similar for all preparations, and rates of reactions were therefore compared over extinctions of 1·2–2·1 (Table 1).

Table 1. Sulphite reduction catalysed by desulfoviridin preparations

The complete incubation system is described in the Methods section

Preparation	ΔE[A]	Protein (mg/ml)	Protein used in cuvette (mg)	Incubation system	Incubation time (min)	Initial MVH (μmol)[B]	MVH oxidized (μmol) (M)	Oxidation rate (nmol MVH/min)[C]	Sulphide produced (μmol) (S)	Ratio M : S
DE52	0·8	6·5	0·65	Complete	80	0·954	0·935	23·5	0·16	5·85
DE52	0·8	6·5	0·65	No SO_3^{2-}	80	0·783	0·441	4·2	0·01	44·1
DE52	0·8	6·5	0·65	No MVH	80				0	
G200	0·8	3·4	0·34	Complete	80	1·034	1·009	18·6	0·182	5·5
G200	0·8	3·4	0·34	Complete	80	1·05	1·029	20·8	0·179	5·76
G200	0·8	3·4	0·17	Complete	120	0·98	0·946	11·7	0·154	6·15
G200	0·8	3·4	0·17	Complete	120	0·817	0·794	10·6	0·114	6·96
Major band	0·4	1·3	0·13	Complete	100	0·8	0·782	14·2	0·061	12·8
Minor band	0·24	(1·3)[D]	(0·13)[D]	Complete	160	0·927	0·836	7·12	0·122	6·85
—				No enzyme	80	1·16	0·067		0·016	

[A] Extinction at 627 nm minus that at 700 nm.
[B] Determined from the extinction at 600 nm immediately after addition of enzyme.
[C] Rates determined at extinction values between 1·2 and 2·1 (see text).
[D] Based on an extinction at 280 nm equivalent to that of the major band preparation.

The ratios of amount of MVH oxidized to amount of sulphide produced are given in Table 1. Values in the region of 6 : 1, indicating stoichiometric reduction of sulphite to sulphide, occurred with the DE52, G200 and minor band preparations

but the major band preparation gave values around 12 : 1. The value given for the DE52 preparation (5·85 : 1) would be lower if the non-specific MVH oxidation was taken into account. However, since it should not be possible to have less than 6 μmol of MVH oxidized per μmol of sulphide produced, this indicates that in the presence of sulphite the non-specific MVH oxidation was markedly reduced.

Discussion

Kobayashi *et al.* (1969) proposed that in *Desulfovibrio* species sulphite is reduced in three steps, sulphite → trithionate → thiosulphate → sulphide, each mediated by a separate enzyme system. However, results presented here indicate that desulfoviridin from *D. gigas* can catalyse the formation of sulphide from sulphite. The following points support this conclusion.

1. When replicates of the major desulfoviridin band from the G200 preparation were excised and re-run on four different gel systems only protein bands recognizable as desulfoviridin were detected. When the minor band was re-run on a 5% gel a similar result was obtained.

2. With all desulfoviridin preparations ferrous sulphide bands were coincident only with desulfoviridin bands in polyacrylamide gels. The stoichiometric relationship between MVH oxidized and sulphide produced indicates that sulphite was reduced solely to sulphide with all except the major band preparation. Unfortunately, because of insufficient major band preparation, assays for the unidentified products were impossible.

3. Only the DE52 preparation showed thiosulphate reductase activity in polyacrylamide gels, but the site of this reaction was not coincident with desulfoviridin proteins. Neither the electrophoretically purified nor the G200 preparations in the present investigations contained detectable thiosulphate or trithionate reductases. Therefore the detection of sulphide in the reductase assays of these preparations cannot be attributed to contamination by these enzymes. These results suggest either that thiosulphate and trithionate are not intermediate products in reduction of sulphite to sulphide by desulfoviridin, or that the enzyme must be "primed" by reaction with earlier products or sulphite in order to reduce thiosulphate or trithionate.

At a concentration of 18 μg per gel, the major band preparation was electrophoretically comparable with the purified preparation of Lee and Peck (1971) and also the hydroxyapatite fraction of Kobayashi *et al.* (1972). The latter authors apparently considered the minor band a contaminant. However, at higher concentrations (184 μg per gel) of the major band preparation on both 5 and 7% gels other weak desulfoviridin bands were detected (Figs 1*c*, 1*d*). Reasons for this multiplicity of bands are not clear. Some molecular changes appear to occur during or before electrolysis since both the major band and G200 preparations have similar desulfoviridin patterns in 7% gels (pH 9·5).

Suh and Akagi (1969) showed that in the hydrogenase-linked reduction of sulphite catalysed by desulfoviridin, 72% of the hydrogen consumed was accounted for by thiosulphate formation. Another protein was required for complete conversion of sulphite to thiosulphate. Using a similar manometric system and ^{35}S-labelled sulphite it was shown by Lee and Peck (1971) that of the ^{35}S recovered 74% occurred as trithionate and the rest as sulphate. Neither detected sulphide as an end product. Kobayashi *et al.* (1972) apparently used a major band preparation of desulfoviridin

and reported sulphide, thiosulphate and trithionate as the products of hydrogenase-linked sulphite reduction in mole ratios of 0·53 : 0·3 : 0·8 respectively.

The variability of end products is difficult to explain. One possibility is that differences between the results of Suh and Akagi (1969), Lee and Peck (1971) and Kobayashi *et al.* (1972) are due to variation between strains of the different species of *Desulfovibrio* used as sources of desulfoviridin. However, this possibility does not seem to apply to the present results since the strain of *D. gigas* used was the same as that reported by Lee and Peck (1971). Whether differences in procedures for isolating desulfoviridin affect its enzymic action remains to be clarified. As regards assay methods, the G200 preparation also catalysed the production of sulphide from sulphite in phosphate buffer at pH 6·0 (Suh and Akagi 1969; Lee and Peck 1971; Kobayashi *et al.* 1972). The influence of other differences in assay methods is being investigated.

The possibility that thiosulphate or trithionate formed from sulphite by enzymic action could be converted to sulphide non-enzymically under the conditions described here was negated by experiments in which thiosulphate and trithionate were added to the complete system minus desulfoviridin in the gels and the cuvette.

Since the major and minor band preparations catalysed the production of different amounts of sulphide from sulphite it could be interpreted that different electrophoretic forms of desulfoviridin may vary in their enzymic action.

Reduction of sulphite to sulphide also occurred at visible desulfoviridin bands after gel electrophoresis of crude extracts of other *Desulfovibrio* species (Skyring and Trudinger 1973). In addition, G200 desulfoviridin preparations from *D. gigas* and *D. africanus* grown in a modified Starkey's medium (medium B as described by Jones 1971) also showed sulphite to sulphide reductase activities. It thus appears that sulphide is at least a partial end-product of sulphite reduction by desulfoviridin.

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