BBA 45897

PRODUCTS OF SULPHIDE OXIDATION IN EXTRACTS OF *THIOBACILLUS CONCRETIVORUS*

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(Received September 30th, 1969)

SUMMARY

1. An early intermediate of sulphide oxidation in cell-free extracts of *Thiobacillus concretivorus* is bound to a membrane fraction.

2. The compound is probably at the oxidation level of elemental sulphur, and its properties suggest that it is in a linear polymeric form rather than the more stable S_8 ring structure.

3. Dialysed extracts oxidize sulphide as well as the bound sulphur intermediate completely to sulphate, without forming thiosulphate or polythionates. In undialysed preparations however inorganic sulphur compounds accumulate, and sulphate formation from sulphide is considerably less.

4. A mechanism is proposed for the enzymic oxidation of sulphide to sulphate *via* a bound sulphur polymer and sulphite.

INTRODUCTION

The thiobacilli, a group of chemoautotrophic bacteria, obtain energy for growth by oxidising reduced inorganic sulphur compounds. Sulphide oxidation has received less attention by previous workers than has the oxidation of sulphur, thiosulphate and sulphite. Most thiobacilli oxidize sulphide to sulphate but the mechanisms involved are not well defined. Elemental sulphur[1,2], thiosulphate and polythionates[2,3] have all been proposed as intermediates in the process.

An enzymic mechanism for sulphide oxidation, involving an electron transfer chain, has been reported in *Thiobacillus concretivorus*[4]. Polysulphides were tentatively identified as early stable intermediates. The products of sulphide oxidation in this bacterium have now been investigated in more detail, and the results reported in this paper.

METHODS AND MATERIALS

Organism

T. concretivorus (N.C.I.B. 9514) was grown and harvested as described previously[4].

Preparation of extracts

Cells suspended in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM EDTA (sodium salt) (1 g wet cells/4 vol. buffer) were broken by a French pressure cell at 20000 lb/inch^2 at 4°. The crude homogenate was centrifuged at 20000 $\times$ *g* for 40 min. The supernatant fraction was used as the crude extract, and unless stated otherwise, it was dialysed overnight against the same buffer. Protein was determined by the method of ITZHAKI AND GILL[5] using bovine serum albumin as a standard. Extraction of the crude extract with cold acetone was carried out as described previously[4].

Separation and identification of sulphur compounds

Sulphur compounds were separated by electrophoresis on DEAE-cellulose paper (Whatman DE81) in 0.3 M sodium citrate buffer (pH 5.0) at 1500 V for 75 min or at 2500 V for 45 min. The apparatus used was that described by TATE[6] for routine electrophoresis. In each run, thiosulphate was used as a marker compound. Polythionates, thiosulphate and sulphide were detected by dipping the electrophoretograms in a solution of $AgNO_3$ (8 g) in water (10 ml) and acetone (90 ml); sulphite was detected by basic fuchsin (1 %, w/v) in water (10 ml) and acetone (90 ml); and thiocyanate by $Fe(NO_3)_3$ (10 g) in 2 M HNO_3 (10 ml) and acetone (90 ml). The mobility of sulphate was determined with $^{35}SO_4^{2-}$.

Radioactivity measurements

Radioactive areas on the electrophoretograms were detected by cutting the paper into small parts (1 cm $\times$ 2 cm) and counting in a toluene solution of 2,5-diphenyloxazole (PPO) (3.0 g/l) and 1,4-bis-(5-phenyloxazolyl-2)benzene (POPOP) (0.2 g/l) in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375). A convenient check on the identity of radioactive areas, provided not all anions are present at the same time, can be made by oxidation of the sample with I_2 (0.1 M in 0.1 M KI), prior to running in the electrophoresis system. Total activity in solutions was measured either by spotting 0.02-ml portions of the reaction mixture onto paper, drying, and counting as above, or by counting 0.2 ml of the sample in 10 ml dioxan containing PPO (10 g/l) and POPOP (250 mg/l) and naphthalene (100 g/l). Corrections when necessary, were made for quenching.

Assay of O_2 uptake and sulphide utilization

Sulphide uptake and O_2 uptake were determined polarographically as described previously[4]. The absolute amount of O_2 in the reaction vessel was determined by measuring the O_2 reduced by 0.5 μmole of NADH in the presence of NADH oxidase. At equilibrium, the displacement of the recorder pen thus corresponded to 0.25 μmole O_2, permitting calibration of the oxygen electrode.

The NADH oxidase was prepared as a crude extract from bakers' yeast, using similar methods to those described for the extracts of *T. concretivorus*. Sulphide was determined by titration with a standard $AgNO_3$ solution.

Mass spectrometry

Samples for mass spectrometry were frozen in liquid N_2 immediately after the enzyme reaction was completed and then they were freeze-dried. These samples were

analysed in a Hitachi Perkin Elmer RMU-6D double-focus mass spectrometer using the heated inlet system. Spectra were run after heating the samples at 250° for 5 min.

Spectrophotometry

Absorption spectra were obtained with the Unicam SP 800 fitted with a scale expansion unit as described previously[4].

Chemicals

Standard A.R. grade chemicals were made up in double glass-distilled water. Sodium sulphide solution was freshly prepared each day from washed crystals of $Na_2S \cdot 9H_2O$. Bovine serum albumin and NADH were obtained from Sigma Chemical Co., U.S.A.; sodium tetrathionate was made by the method of TRUDINGER[12]; sodium trithionate was kindly supplied by Dr. D. P. Kelly, Baas Becking Geobiological Laboratory, Canberra, Australia. Radioactive chemicals were purchased from the Radiochemical Centre, Amersham, England.

RESULTS

Formation of bound intermediate

95 % of the $^{35}S^{2-}$ incubated with crude extract for 2 min, was excluded with the protein fraction from a Sephadex G-50 column (Table I). The remaining 5 % was composed of low-molecular-weight compounds, which occur as impurities in the radioactive sulphide. This method was used for routine collection of protein-bound ^{35}S material used in subsequent experiments. Under identical conditions in a control experiment with extracts of *Nitrosomonas europaea* only 5 % of the ^{35}S was associated with the protein fraction.

The results of equilibrium dialysis (Table II), indicate that boiled extracts were ineffective in binding ^{35}S. When the crude extract was incubated with $^{35}S^{2-}$ (as in Table I), and centrifuged at 224000 × *g* for 30 min, about 70 % of the ^{35}S was recovered in the pellet, indicating an association with the lipoprotein membrane fraction.

TABLE I

BINDING OF $^{35}S^{2-}$ BY CELL FRACTIONS OF *T. concretivorus*

Each fraction (containing 2.5 mg protein) was incubated with $Na_2{}^{35}S$ (0.3 μC) and 1 μmole Na_2S carrier in 0.5 ml 50 mM phosphate buffer (pH 7.0) containing 0.2 mM EDTA (sodium salt) for 2 min at 25°. The reaction mixture was then applied to a Sephadex G-50 column (10 cm × 1.5 cm) previously equilibrated with the buffer at 4°. The column was eluted with the same buffer. An extract of *Nitrosomonas europaea*, prepared in the same way as the *T. concretivorus* crude extract, was used in a control experiment.

Fraction	*Protein-bound ^{35}S excluded by Sephadex G-50* (%)
Crude extract	95.0
Pellet obtained after centrifuging crude extract at 224000 × *g* for 1 h	95.0
Supernatant left after centrifuging crude extract at 224000 × *g* for 1 h	5.0
Nitrosomonas europaea crude extract	5.0

TABLE II

MEMBRANE-BOUND ^{35}S INTERMEDIATE, A PRODUCT OF THE ENZYMIC OXIDATION OF $^{35}S^{2-}$

Incubations were carried out as described in Table I, except that 5 mg protein in 2 ml buffer was used. After incubation the extracts were dialysed against 10 mM borate buffer (pH 9.0) for 6 h at 4°.

Fraction	^{35}S *retained with dialysate* (%)
Crude extract	80
Crude extract (boiled 2 min)	10

Characterization of ^{35}S membrane-bound intermediate

Stoichiometry of sulphide oxidation. The ratio of O_2 uptake to sulphide consumption in the crude extract was found to be 1 mole O_2 per 2.2 $\pm$ 0.1 moles of S^{2-}.

Mass spectrometry. A primarily hydrocarbon background spectrum of crude extracts was consistently reproducible. In crude extracts which had been incubated with sodium sulphide for 10 min, a typical S_8 sulphur spectrum was superimposed on this background at mass numbers 32, 64, 96, 128, 160, 192, 224, and 256. In addition, smaller but significant changes were noted with the ^{34}S isotope at the appropriate mass numbers. Part of this spectrum is shown in Fig. 1. The masses of the S_6 fragments of the S_8 molecule at 192, 194 and 196 are sufficiently less than the corresponding background, to enable an unambiguous analysis of the isotopic abundance to be made. The same applied for the S_7 and S_8 species, although for convenience they are not shown in Fig. 1. This confirms that these are fragments of sulphur.

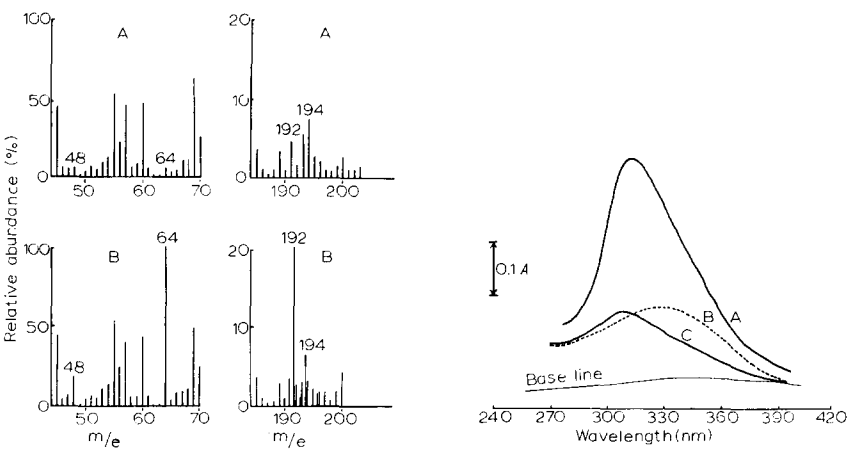

Fig. 1. Mass spectra of membrane-bound sulphur compounds. A. Membrane fraction prepared by passing crude extract only, through a Sephadex G-50 column as described in Table I. B. Membrane fraction prepared as above, but using 1 ml crude extract (10 mg protein) in 50 mM phosphate buffer (pH 7.0) and 0.2 mM EDTA (sodium salt), incubated with 5 mM Na_2S for 10 min at 25°.

Fig. 2. Effect of cyanide and sulphite on ultraviolet difference spectra of products of sulphide oxidation. Sample and reference cuvettes contained 2.5 ml crude extract (20 mg protein) in 50 mM phosphate buffer (pH 7.0) and 0.2 mM EDTA (sodium salt). A. 2 min after adding 10 μmoles Na_2S to sample cuvette. B. 1 min after adding a few crystals of Na_2SO_3 to A. C. 15 min after adding a few crystals of KCN to A.

A small increase in the peak at mass 48 suggests that some SO may also be present. If so, it is likely that SO_2 as well as S_2 contribute to the mass 64 peak.

In crude extracts incubated with sulphide for 1 h the peaks at masses 48 and 64 were more prominent, indicating the presence of a compound which fragments to SO_2 in the mass spectrometer.

Membrane-bound ^{35}S compounds were precipitated in the 0–40 % $(NH_4)_2SO_4$ fraction. The relative abundance of SO_2 was about 5 times greater, and SO about 10 times greater, than that in the mass spectrum shown in Fig. 1. This indicates a

TABLE III

ELECTROPHORESIS OF SOME INORGANIC SULPHUR COMPOUNDS AND THE MEMBRANE-BOUND ^{35}S FRACTION

The membrane-bound ^{35}S fraction was obtained by filtering the incubated reaction mixture through Sephadex G-50 as described in Table I. Where stated, I_2 (as 0.1 M solution in 0.1 M KI) was applied to the paper prior to the addition of the membrane-bound ^{35}S. Details of electrophoresis are given in METHODS AND MATERIALS.

Compounds	*Mobility relative to thiosulphate*	*^{35}S in sulphur compounds (%)*	
		Membrane-bound ^{35}S	*I_2 + membrane-bound ^{35}S*
Elemental S or membrane-bound S compounds	0	100	90
Tetrathionate	0.40		
Sulphide	0.45		
Trithionate	0.54		
Thiocyanate	0.70		
Sulphite	0.85		
Thiosulphate	1.00		
Sulphate	1.12		10

TABLE IV

CHEMICAL REACTIONS OF THE MEMBRANE-BOUND ^{35}S

Crude extract (20 mg protein) in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM EDTA (sodium salt), $Na_2{}^{35}S$ (0.5 μC) and 2 μmoles Na_2S carrier in a total volume of 2 ml, was incubated at 25° in a reciprocating water bath. After 10 and 60 min, respectively, 1 ml was passed through a Sephadex G-50 column as described in Table I. Portions of the Sephadex-filtered membrane fraction from the 10-min incubation were then treated with each of the reagents at the final concentrations shown, for 15 min at 25°. In addition, a portion of the Sephadex-filtered membrane fraction from the 60-min incubation was treated with KCN. Sulphur compounds in 20-μl samples were separated by electrophoresis (see METHODS AND MATERIALS).

Incubation time before separation of membrane fraction (min)	*Treatment*		*^{35}S in various sulphur compounds (%)*						
			Elemental S or membrane-bound S compounds	*SH^-*	*$S_3O_6{}^{2-}$*	*CNS^-*	*$HSO_3{}^-$*	*$S_2O_3{}^{2-}$*	*$SO_4{}^{2-}$*
10	NaOH	(0.5 M)	30	0	0	0	45	8	17
10	Na_2SO_3	(0.01 M)	25	0	25	0	5	40	5
10	KCN	(0.01 M)	26	0	0	73	0	0	1
60	KCN	(0.01 M)	10	0	0	86	0	0	4

chemical reaction of the sulphur compounds with $(NH_4)_2SO_4$, so that care is necessary when interpreting the results of enzymic oxidation of sulphur.

Absorption spectra. Early products of sulphide oxidation by crude extracts have absorption bands in the region 300–400 nm (ref. 4) (Fig. 2A). Within about 1 min of adding sulphite to an extract containing these products, the absorbance of the 300–400-nm band decreased markedly (Fig. 2B). Cyanide also caused a reduction in the 300–400-nm absorption band but more slowly than sulphite (Fig. 2C). Thus the decrease in absorbance over the range 300–400 nm suggests that sulphite and cynanide react with the compounds which absorb strongly in this region.

Chemical reactions. The mobilities of some inorganic sulphur compounds separated by electrophoresis are given in Table III. Elemental S and any membrane-bound S remained at the origin. Most sulphide applied to the paper, at pH 5.0, was usually lost as H_2S but some remained, presumably as SH^-. Sulphide was estimated after oxidation to sulphur by prior application of I_2 at the origin. No free anions were detected with the membrane-bound ^{35}S but some sulphate was formed on treating it with I_2 (Table III).

The chemical reactions of the membrane-bound ^{35}S, shown in Table IV, are typical of nucleophilic displacement from a polymeric sulphur compound[11]. The ^{35}S which remained on the origin during electrophoresis, suggests that not every ^{35}S atom was susceptible to nucleophilic substitution.

When the membrane-bound ^{35}S was treated with HCl (1.0 M), up to 10% of the activity was often lost, presumably as H_2S. Variable quantities (up to 10%) of SH^- and SO_4^{2-} were detected by electrophoresis. This suggests that under some conditions, polysulphides and sulphur–oxygen compounds may be formed.

On shaking the membrane-bound ^{35}S with carbon disulphide or benzene, between 5 and 10% of the ^{35}S was extracted into these solvents indicating only small amounts of free sulphur.

Complete oxidation of sulphide

The dialysed crude extract completely oxidised sulphide to sulphate (Table V) and no thiosulphate or polythionates were detected. In undialysed crude extracts

TABLE V

COMPLETE OXIDATION OF SULPHIDE BY CRUDE EXTRACTS DIALYSED FOR 12 h AGAINST 50 mM PHOSPHATE BUFFER (pH 7.0) CONTAINING 0.2 mM EDTA (SODIUM SALT)

Reaction mixture as in Table IV. 20-μl fractions were removed at 1 min and 2 h from the reaction mixture for electrophoresis.

Sulphur compound in reaction mixture	*^{35}S compounds (%)*			
	Undialysed crude extract		*Dialysed crude extract*	
	1 min	*2 h*	*1 min*	*2 h*
Elemental S or membrane-bound S	70	20	70	10
Tetrathionate	0	5	0	0
Sulphide	25	0	25	0
Trithionate	5	20	5	0
Thiosulphate	0	5	0	0
Sulphate	0	50	0	90

however, polythionates and thiosulphate accumulated and sulphate formation was much reduced. In boiled preparations about 75 % of the $^{35}S^{2-}$ added was lost as H_2S and the remainder gradually formed a mixture of sulphur, thiosulphate and polythionates.

When the bound ^{35}S fraction was incubated with fresh, dialysed crude extracts, sulphate was rapidly produced (and polythionates and thiosulphate were not detected), which supports the hypothesis that this bound sulphur compound is a true intermediate (Table VI).

TABLE VI

SULPHATE FORMATION FROM MEMBRANE-BOUND ^{35}S

0.5 ml membrane-bound ^{35}S (5 mg protein), obtained as described in Table I, was mixed with 0.5 ml of low-molecular-weight fractions from the same Sephadex G-50 column, or with 0.5 ml fresh dialysed crude extract (5 mg protein) and incubated at 25°. 50-μl fractions were removed at various times for separation of sulphur compounds by electrophoresis (see METHODS AND MATERIALS).

Compounds	^{35}S *compounds* (%)					
	Membrane-bound ^{35}S		*Membrane-bound* ^{35}S + *low-mol.-wt. fraction*		*Membrane-bound* ^{35}S + *crude extract*	
	1 min	*2 h*	*1 min*	*2 h*	*1 min*	*2 h*
Elemental S or membrane-bound S	100	95	75	68	100	5
Trithionate	—	—	18	18	—	—
Sulphate	—	5	7	14	—	95

Effect of DEAE-cellulose on sulphur

When elemental ^{35}S in benzene was applied to DEAE-cellulose paper, and run in the standard electrophoresis system, considerable degradation to polythionates occurred. Thus the DEAE-cellulose promoted chemical oxidation of free sulphur, but it had no effect on the membrane-bound sulphur compounds. This suggests that free sulphur was not present with the membrane-bound material.

DISCUSSION

It has already been shown[4] that sulphide is oxidised by a rapid enzymic process in extracts of *T. concretivorus*, and that the first stable products have distinctive spectra, similar to those of polysulphides. The protein-bound ^{35}S intermediate is a product of this sulphide oxidation because only enzymically active cell extracts formed this complex. Chemical reactions which might account for the binding of sulphide to proteins occurred to a much lesser extent as shown by the control experiments. Much of the membrane-bound ^{35}S intermediate reacts with cyanide and sulphite, giving products expected from reactions between these reagents and a polymeric sulphur compound. The decrease in absorbance which occurred in the ultraviolet region when cyanide and sulphite reacted with the oxidation products of sulphide suggest that the distinctive spectra obtained previously[4] are due to the same polymeric sulphur compounds.

Because the molar ratio for sulphide oxidised to O_2 uptake was a little greater than 2, it is likely that although most atoms in the polymeric sulphur compounds are at the oxidation level of elemental sulphur, some would be in a more reduced state. Thus the stoichiometry for this step in sulphide oxidation is consistent with the formation of polysulphides.

Under the conditions used to obtain the mass spectrum, if sulphur were not already in its most stable form (*viz.* S_8) it would rapidly revert to it. Thus the mass spectra support the conclusion that some atoms of the bound intermediate are at the oxidation level of sulphur, but its precise structure cannot be deduced from these results.

An upper limit for free elemental sulphur in the complex is suggested by the amount extracted into carbon disulphide or benzene (5–10 %).

Scission of the S_8 ring in aqueous solution does not readily occur at room temperature with the nucleophilic reagents used here. Since the membrane-bound ^{35}S intermediate formed during the enzymic oxidation of sulphide is very reactive, it is likely to be a linear form of polymeric sulphur, rather than an S_8 ring (for review, see ref. 7). This conclusion must be regarded as tentative since the modifying effects of the lipoprotein environment on reaction rates are unknown.

After reaction of the membrane-bound ^{35}S with KCN, much of the ^{35}S which did not form thiocyanate, remained at the origin during electrophoresis. This suggests that the reduced sulphur atoms at the ends of the polymeric chains are bound to the lipoprotein fraction, because they would otherwise have been released as free anions.

The sulphate formed on treating the membrane-bound ^{35}S with cyanide may result from an inorganic oxidation of the sulphite formed as shown in Eqn. 1.

$$R\text{–}S\text{–}S\text{–}S\text{–}SO_3^- + CN^- \rightarrow R\text{–}SSSCN + SO_3^{2-} \qquad (1)$$

The SO and SO_2 detected by the mass spectrometer, and the sulphate formed from membrane-bound ^{35}S by treatment with I_2, may be derived from a sulphonate group.

Sulphate production from sulphide by dialysed crude extracts proceeds without formation of detectable polythionates or thiosulphate as intermediates. The only intermediates are the membrane-bound ^{35}S compounds, as detected by electrophoresis and radioassay. In undialysed extracts, or in aged preparations, the production of sulphate from sulphide is slow.

TAYLOR[10] has shown that GSH, cysteine and β-mercaptoethanol inhibit sulphur oxidation by *Thiobacillus neapolitanus*. This observation might explain why dialysis of the crude extract promoted complete oxidation of sulphide to sulphate. The reading of a sulphide electrode in the crude extract was considerably less after dialysis, showing that free thiols and sulphide had been removed. Thus for effective enzymic oxidation it seems that the polymeric sulphur compound should be bound to the membrane fraction, and not occur as a free polysulphide.

Sulphide oxidation proceeds in two stages. The first stage (with the stoichiometry of 2.2 ± 0.1 moles S^{2-} to 1 mole O_2) is more rapid than the second, and is not affected by CO (ref. 4). In the first stage, sulphide may lose two electrons. Polymerisation of the resulting sulphur atoms would commence, but, free sulphide which is present until the end of the first stage may react with the developing polymeric sulphur chains, to form polysulphides. This would explain why the stoichiometry for sulphide oxidation is variable and greater than 2:1. The second stage of sulphide oxidation

could be the subsequent oxidation of short-chain polysulphides to longer membrane-bound polymeric sulphur compounds. Since CO inhibits O_2 uptake during this stage[4], a different enzymic system may be involved.

These tentative reactions are illustrated in Eqns. 2–6 where X represents the group linking the polysulphide chain to the lipoprotein membrane fraction.

$$SH^- \xrightarrow[\text{oxidase}]{\text{sulphide}} [S] + H^+ + 2e^- \quad (2)$$

$$2[S] \longrightarrow [S\text{–}S] \quad (3)$$

$$[S\text{–}S] + SH^- \longrightarrow {}^-S\text{–}S\text{–}SH \quad (4)$$

$${}^-S\text{–}S\text{–}SH + X^+ \longrightarrow X\text{–}S\text{–}S\text{–}SH \quad (5)$$

$$2[X\text{–}S\text{–}S\text{–}SH] \xrightarrow[\text{oxidase}]{\text{polysulphide}} X\text{–}S_6\text{–}X \quad (6)$$

Subsequent oxidation may proceed by a mechanism similar to that suggested by SUZUKI[8] and SUZUKI AND SILVER[9]. The terminal sulphur atom in the chain would be oxygenated to form a sulphonate and then released as sulphite. Sulphite would be oxidised to sulphate by a sulphite oxidase, which is present in *T. concretivorus*[14].

Mass spectra provide no evidence for the accumulation of large amounts of sulphur oxides. It is likely then that only a small proportion of the sulphur atoms present are oxygenated at any given time.

ACKNOWLEDGEMENTS

We are grateful to Dr. J. H. Bowie and Mr. D.B. Cobb of the Organic Chemistry Department, University of Adelaide, for the mass spectrometry.

REFERENCES

1 C. D. PARKER AND J. PRISK, *J. Gen. Microbiol.*, 8 (1953) 344.
2 W. VISHNIAC AND M. SANTER, *Bacteriol. Rev.*, 21 (1957) 195.
3 J. LONDON AND S. C. RITTENBERG, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 1183.
4 D. J. W. MORIARTY AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 184 (1969) 114.
5 R. F. ITZHAKI AND D. M. GILL, *Anal. Biochem.*, 9 (1964) 401.
6 M. E. TATE, *Anal. Biochem.*, 23 (1968) 141.
7 R. E. DAVIS, in A. F. SCOTT, *Survey of Progress of Chemistry*, Vol. II, Academic Press, New York, 1964, p. 189.
8 I. SUZUKI, *Biochim. Biophys. Acta*, 104 (1965) 359.
9 I. SUZUKI AND M. SILVER, *Biochim. Biophys. Acta*, 122 (1966) 22.
10 B. F. TAYLOR, *Biochim. Biophys. Acta*, 170 (1968) 112.
11 W. A. PRYOR, *Mechanisms of Sulphur Reactions*, McGraw-Hill, New York, 1962.
12 P. A. TRUDINGER, *Biochem. J.*, 78 (1961) 680.
13 J. E. BAER AND M. CARMACK, *J. Am. Chem. Soc.*, 71 (1949) 1215.
14 D. J. W. MORIARTY, Ph. D. thesis, University of Adelaide, 1969.