The Inhibition of Streptococci by Lactoperoxidase, Thiocyanate and Hydrogen Peroxide

THE OXIDATION OF THIOCYANATE AND THE NATURE OF THE INHIBITORY COMPOUND

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1. The products of the lactoperoxidase-catalysed oxidation of thiocyanate by hydrogen peroxide were sulphate, carbon dioxide and ammonia. Cyanate, sulphite and a compound showing increased extinction at 235 m μ (the '235 compound') were intermediate oxidation products. 2. Two of the intermediates acted as electron acceptors in the oxidation of NADH₂. Thus NADH₂ was oxidized by sulphite in the presence of lactoperoxidase (EC 1.11.1.7) and Mn²⁺ and by the '235 compound' in the presence of an enzyme, the NADH₂-oxidizing enzyme, present in extracts of lactoperoxidase-resistant streptococci. Sulphur dicyanide also acted as an electron acceptor in the latter reaction. The '235 compound' was also reduced non-enzymically by sulphite. 3. The glycolysis of lactoperoxidase-sensitive streptococci suspended in glucose solution was not inhibited by sulphite, cyanate, cyanide or the '235 compound' but was inhibited by sulphur dicyanide. The inhibition by 0.1 mM-sulphur dicyanide could be reversed, as could that caused by lactoperoxidase, thiocyanate and hydrogen peroxide, by washing the cells or by the addition of a cell-free extract of a lactoperoxidase-resistant streptococcus. 4. The effects of 0.1 mM-sulphur dicyanide on catabolic enzymes of resting streptococci were very similar to those of the lactoperoxidase-thiocyanate-hydrogen peroxide system. Thus hexokinase was completely inhibited, glucose 6-phosphate dehydrogenase and aldolase were partially inhibited and phosphohexokinase was little affected in both cases.

The inhibition of some strains of lactic acid streptococci in raw milk is due to the action of a short-lived intermediate in the LP*-catalysed oxidation of thiocyanate by hydrogen peroxide (Reiter, Pickering & Oram, 1964). The only previous investigation of the enzymic oxidation of thiocyanate by hydrogen peroxide appears to be that of Sörbo & Ljunggren (1958), who reported its oxidation by myeloperoxidase (from leucocytes) to cyanide and sulphate with the formation of an intermediate oxidation product that showed increased extinction at 235 m μ . Portmann & Auclair (1959) found that horseradish peroxidase had no effect on milk cultures of streptococci and Reiter *et al.* (1964) suggested that this was due to its inability to catalyse the oxidation of thiocyanate. The effect of the inhibitory system LP-thiocyanate-hydrogen peroxide on streptococci was described in the preceding paper (Oram & Reiter, 1966). The chemical nature of the products of the LP-catalysed oxidation of thiocyanate and their effects on the

growth and metabolism of some strains of streptococci are examined in the present paper.

MATERIALS

Enzymes and chemicals. KS¹⁴CN (4.4 μ C/ μ mole) was obtained from The Radiochemical Centre, Amersham, Bucks. S(CN)₂ was prepared by treating AgCN with SCl₂ in CS₂ (Beilstein III, p. 180). After recrystallization from CS₂, it was sublimed *in vacuo* at 30°, stored at -20° and resublimed just before use. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), NADP and NADH₂ were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany. LP and streptococcal NADH₂-oxidizing enzyme were prepared as described by Oram & Reiter (1966).

Bacteria. Streptococci from the following serological groups were used: group A: *Streptococcus pyogenes* Pope, Richardson, J/5 and J/17; group K streptococcus, Fry charwoman; group L streptococcus, P34; group N: *Streptococcus cremoris* 803 and 972. They were cultured in skim milk or in glucose-Lemco broth (GL broth) as described by Oram & Reiter (1966), or in the synthetic medium of Mickelson (1964). Milk and broth cultures of the group N

* Abbreviation: LP, lactoperoxidase.

streptococci were incubated at 22° or 30° respectively and cultures of other streptococci were incubated at 37°.

METHODS

Sulphate. This was measured by the method of Belcher, Bhasin, Shah & West (1958).

Ammonia. This was estimated by nesslerization with the reagent described by Umbreit, Burris & Stauffer (1959).

Carbon dioxide. This was measured by the indirect method of Dixon (Umbreit, Burris & Stauffer, 1959).

Cyanate. This was measured as ammonia after the removal of interfering cations by ion-exchange chromatography (Shaw & Bordeaux, 1955).

Cyanide. This was estimated by the modified Leibig method of Archer (1958).

Protein, thiocyanate and hydrogen peroxide. These were estimated by the methods described by Oram & Reiter (1966).

Enzyme assays. LP, streptococcal hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were measured by the methods described by Oram & Reiter (1966), and fructose diphosphate aldolase (EC 4.1.2.13) and phosphohexokinase (EC 2.7.1.11) by the methods of Wu & Racker (1959).

Radioautography of oxidized ^{14}C -labelled thiocyanate. Samples containing about 50 μC of KS^{14}CN or its oxidation products were chromatographed in the ethanol-pyridine-water-aq. ammonia (sp.gr. 0.88) (15:5:4:1, by vol.) system of Elbeigh & Abo-Elnaga (1960), in butanol-ethanol-water (1:1:1, by vol.) or in 0.1M-ammonium acetate. The separation time was limited to 1-3 hr. and the drying period to about 1 hr. at room temperature to minimize the extent of breakdown of unstable oxidation products.

Effects of oxidation products of thiocyanate on streptococci.

(a) Growth. Solutions were sterilized by filtration through Oxoid membrane filters, and added to 10 ml. quantities of Mickelson's medium or skim milk heated at 70° for 30 min. to destroy LP. The media were inoculated with about 10^3 washed cocci obtained from a 16 hr. GL broth culture and growth followed either by making viable counts by the method of Miles & Misra (1938) or by turbidity measurements at 580 μm .

(b) Lactic acid formation of resting cell suspensions. This was measured as described by Oram & Reiter (1966).

(c) Oxygen uptake. Approx. 2×10^{10} cocci were suspended in 5 ml. of 1% (w/v) glucose-0.1M-potassium phosphate buffer, pH 6.8, and the uptake of oxygen was measured at room temperature with an oxygen electrode (Radiometer Ltd., Copenhagen, Denmark). The suspension was mixed gently for a few seconds with a magnetic stirrer after each addition of known or suspected oxidation products of thiocyanate.

RESULTS

LP-catalysed oxidation of thiocyanate. The complete oxidation of thiocyanate in 33mm-sodium acetate buffer, pH 5.8, required 3.0-3.5 equiv. of hydrogen peroxide. There was no simple linear proportionality between the amounts of added hydrogen peroxide and oxidized thiocyanate (Fig.

Fig. 1. Extent of oxidation of thiocyanate by LP at various molar ratios of H_2O_2 . The residual thiocyanate was determined 10 min. after the oxidation of 0.96 μmole of KSCN by 70 units of LP in 3.0 ml. of 33 mm-sodium acetate buffer, pH 5.8, at room temperature.

1); the oxidation appeared to proceed in three stages corresponding, very approximately, to the addition of 1, 2 and 3 equiv. of hydrogen peroxide. The inflexion in Fig. 1, which resulted from the low $\Delta[\text{SCN}^-]/\Delta[\text{H}_2\text{O}_2]$ ratios at intermediate peroxide concentrations, was also obtained when thiocyanate at several other concentrations was oxidized. These inflexions corresponded to the oxidation of approximately half of the thiocyanate by 1.7-2.25 equiv. of hydrogen peroxide. However, experiments with ^{14}C -labelled thiocyanate (see below) showed that the E_{460} values (Fig. 1) were probably given by residual thiocyanate rather than by an intermediate oxidation product giving a red chromogen with the ferric nitrate reagent.

The end products of the LP-catalysed oxidation of thiocyanate under the above conditions included sulphate, ammonia and carbon dioxide, but not cyanide (Table 1). The concentrations of ammonia and carbon dioxide in the reaction products increased over a period of several hours, indicating that they arose from the breakdown of some intermediate oxidation product. This intermediate could be adsorbed to anionic but not cationic exchange resins, was hydrolysed by 2N-sulphuric acid and could be measured as cyanate by the method of Shaw & Bordeaux (1955).

Oxidation of reduced coenzymes by intermediate oxidation products of thiocyanate. The reduced coenzymes NADH_2 and NADPH_2 are oxidized by an intermediate in the LP-catalysed oxidation of thiocyanate (Reiter *et al.* 1964). By studying the oxidation of NADH_2 by LP, thiocyanate and

Table 1. *Products of the LP-catalysed oxidation of thiocyanate by hydrogen peroxide*

Assays were made 10 min. after the addition of H_2O_2 to 20 μmoles of KSCN and 12 units of LP dissolved in 3.0 ml. of 33 mM-sodium acetate buffer, pH 5.8, at room temperature.

Amount of added H_2O_2 (μmoles)	Product (μmoles)				
	Thiocyanate	Sulphate	Cyanide	Cyanate	Ammonia
0	20	0	0	0	0
10	14.9	5.3	0	2.5	1.0
20	10.1	9.9	0	4.2	1.3
30	6.0	12.0	0	7.2	1.4
40	3.8	11.5	0	9.0	1.8

Fig. 2. Effect of the NADH_2 -oxidizing enzyme on the extent of the oxidation of thiocyanate by LP at various molar ratios of H_2O_2 . Reaction mixtures contained sodium acetate buffer, pH 5.8 (100 μmoles), LP (0.9 unit), NADH_2 (0.6 μmole), KSCN (1.0 μmole) and H_2O_2 as indicated. ○, With 0.1 ml. of an extract of *Strep. cremoris* 803; ●, without the extract.

hydrogen peroxide in the presence or absence of the NADH_2 -oxidizing enzyme (Oram & Reiter, 1966) evidence has now been obtained for the formation of two electron acceptors. Thus the oxidation of NADH_2 required about 1.7 equiv. of hydrogen peroxide in the presence but about 4.0 equiv. in the absence of the NADH_2 -oxidizing enzyme. Whereas in the presence of this enzyme there was a direct proportionality between the amounts of added peroxide and of NADH_2 oxidized, in its absence there was a threshold concentration of hydrogen peroxide below which no oxidation occurred (Fig. 2).

Nature of the electron acceptors. (a) Oxidation catalysed by LP. The intermediate involved in the oxidation of NADH_2 in the absence of the NADH_2 -oxidizing enzyme was stable for less than 1 min. at room temperature. Klebanoff (1960) observed that the oxidation of NADH_2 by either horseradish

Fig. 3. Oxidation of NADH_2 by LP, sulphite and Mn^{2+} . The complete reaction mixture (○) contained potassium phosphate buffer, pH 6.8 (100 μmoles), LP (1.0 unit), NADH_2 (0.5 μmole), Na_2SO_3 (0.5 μmole) and MnSO_4 (0.1 μmole) in 3.0 ml. at room temperature. Δ, Without Na_2SO_3 ; □, without MnSO_4 ; ●, without LP.

peroxidase or myeloperoxidase and catalytic amounts of Mn^{2+} required either sulphite or bisulphite. We have found that in the presence of Mn^{2+} , but not in its absence, either compound could replace thiocyanate and hydrogen peroxide in the LP-catalysed oxidation of NADH_2 (Fig. 3). Sulphate, cyanate, cyanide and thiosulphate were unable to act as electron acceptors in this reaction. It therefore seems probable that sulphite or bisulphite is formed in the oxidation of thiocyanate by LP and hydrogen peroxide.

(b) Oxidation catalysed by the NADH_2 -oxidizing enzyme. In the presence of an extract of the LP-

Table 2. Comparison of the stabilities of two oxidation products of thiocyanate acting as electron acceptors in the oxidation of NADH₂ by LP

KSCN (0.5 μmole) was oxidized by 0.7 unit of LP and 0.5 μmole of H₂O₂ in the presence or absence of the NADH₂-oxidizing enzyme (as 0.1 ml. of an extract of *Strep. cremoris* 803; see the text) in 3.0 ml. of 0.1 M-potassium phosphate buffer, pH 6.5, at room temperature. NADH₂ (0.5 μmole) was added before or after the addition of H₂O₂ to the reaction mixture and the fall in $E_{340}^{1\text{cm.}}$ was measured.

Delay in the addition of NADH ₂ to oxidized KSCN (min.)	$\Delta E_{340}^{1\text{cm.}}$	
	NADH ₂ -oxidizing enzyme present	NADH ₂ -oxidizing enzyme absent
0	0.717	0.325
2	—	0.055
10	0.521	0.009
53	0.225	—
83	0.105	—

resistant strain *Strep. cremoris* 803, a much more stable intermediate acted as an electron acceptor. Thus 70% of its original concentration was present when NADH₂ was added 10 min. after the oxidation of thiocyanate (Table 2). Several compounds, including sulphite, bisulphite, sulphate, thio-sulphate and cyanate, were unable to act as electron acceptors in this reaction and the NADH₂-oxidizing enzyme was inhibited by 1 mM-cyanide. The peroxidase system could be replaced, however, by sulphur dicyanide, which was found by Wilson & Harris (1961) to be formed in the non-enzymic oxidation of thiocyanate by hydrogen peroxide. The oxidation of NADH₂ by sulphur dicyanide (Fig. 4) occurred only when sulphur dicyanide was added last to the otherwise complete system and even then only about 75% of the stoicheiometric amount of NADH₂ was oxidized. Although solutions of sulphur dicyanide in water lost very little electron-acceptor activity after several hours at room temperature, a total loss of activity occurred within a few minutes, regardless of pH, in a variety of commonly used buffer solutions. The loss in electron-acceptor activity was accompanied by the formation of thiocyanate and cyanide. Since cyanide inhibited the NADH₂-oxidizing enzyme, its formation from sulphur dicyanide under these conditions could explain the absence of NADH₂ oxidation when sulphur dicyanide was added before NADH₂ to buffered solutions of the NADH₂-oxidizing enzyme.

The '235 compound' also acted as an electron acceptor in the oxidation of NADH₂ catalysed by the NADH₂-oxidizing enzyme and was much more stable than sulphur dicyanide in solutions of

Fig. 4. Oxidation of NADH₂ by S(CN)₂ in the presence of the NADH₂-oxidizing enzyme. S(CN)₂ (O, nil; Δ, 0.3 μmole; □, 0.6 μmole; ●, 0.9 μmole) was added last to 0.6 μmole of NADH₂ and 0.1 ml. of an extract of *Strep. cremoris* 803 in 3.0 ml. of 33 mM-sodium acetate buffer, pH 5.8, at room temperature.

Fig. 5. Effect of NADPH₂ and the NADH₂-oxidizing enzyme on the '235 compound'. The oxidation of 2.0 μmoles of KSCN by 0.3 μmole of H₂O₂ was catalysed by 0.27 unit of LP in 0.33 M-potassium phosphate buffer, pH 7.0, at room temperature. After 7 min., 10 μmoles of glucose 6-phosphate were added to the complete system (O), which also contained 0.025 μmole of NADP, 10 μg. of glucose 6-phosphate dehydrogenase and 0.08 unit of NADH₂-oxidizing enzyme. Δ, Without glucose 6-phosphate dehydrogenase; □, without NADP; ●, without NADH₂-oxidizing enzyme.

electrolytes. The disappearance of the '235 compound' was measured in the presence of the NADH₂-oxidizing enzyme and NADPH₂ formed by the enzymic reduction of NADP (which was added in low concentration, since it also absorbs ultra-violet light strongly at 235 mμ) with glucose

6-phosphate dehydrogenase and glucose 6-phosphate. No decrease in E_{235} occurred in the absence of either the NADH_2 -oxidizing enzyme or of any component of the NADPH_2 -regenerating system (Fig. 5). The amounts of reduced coenzymes oxidized by the NADH_2 -oxidizing enzyme were proportional to the concentration of the '235 compound', which, as shown in Fig. 6, was highest at about 0.35–0.4 equiv. hydrogen peroxide. At higher concentrations of hydrogen peroxide, the E_{235} rose initially but then fell rapidly with concomitant losses in electron-acceptor activities. The oxidation of NADH_2 by the NADH_2 -oxidizing enzyme and the '235 compound' was accompanied by an increase in the thiocyanate concentration to that observed before the addition of peroxide.

Non-enzymic reduction of the '235 compound'. The '235 compound' was unstable above the minimum concentration of peroxide required for the formation of the short-lived electron acceptor for NADH_2 (Fig. 2). The addition of 0.2 μmole of sodium sulphite after the oxidation of 2 μmoles of potassium thiocyanate by 0.6 μmole of hydrogen peroxide resulted in an immediate fall in both E_{235} and electron-acceptor activity in the oxidation catalysed by the NADH_2 -oxidizing enzyme. It therefore seems probable that the instability of the '235 compound' in the presence of LP and excess of hydrogen peroxide resulted from its reaction with sulphite or some similar reducing agent.

Fig. 6. Relationship between the concentration of the '235 compound' and the extent of oxidation of NADH_2 by the NADH_2 -oxidizing enzyme. KSCN (2.0 μmoles) was oxidized by 5 units of LP and various concentrations of H_2O_2 in 2.8 ml. of 36 mM-potassium phosphate buffer, pH 7.0, at room temperature. The increase in E_{235} was measured after 10 min. (○), and the oxidation of 0.5 μmole of NADH_2 by 0.1 ml. of an extract of *Strep. cremoris* 803 was followed at 340 $\mu\mu$ (Δ).

Oxidation of ^{14}C -labelled thiocyanate. No radioactive compound other than thiocyanate was detected on radioautographs of KS^{14}CN oxidized by LP and various molar ratios of hydrogen peroxide in several buffer solutions in the range pH 5.8–7.5. Similar results were obtained with radioautographs of the '235 compound' and the products of both its non-enzymic reduction by sulphite or its enzymic reduction with NADH_2 and the NADH_2 -oxidizing enzyme.

Effects of oxidation products of thiocyanate on streptococci. (a) Growth. Sulphite, cyanate, cyanide, sulphur dicyanide and thiosulphate added singly or in combination at concentrations between 0.1 and 10 mM were without effect on the growth of streptococci in the synthetic medium of Mickelson (1964) or in skim milk the LP of which had been inactivated by heating at 70° for 30 min. The inability of sulphur dicyanide to inhibit the growth of streptococci was not unexpected in view of its very limited stability in ionic environments.

(b) Lactic acid formation and oxygen uptake. Several oxidation products of thiocyanate were, with some related compounds, examined for their effects on the acid production and oxygen uptake of LP-sensitive and LP-resistant strains of streptococci. Sulphite, sulphate, cyanate, cyanide, thiosulphate and ammonium ions were all inactive even at a concentration of 10 mM. However, 0.2 mM-sulphur dicyanide inhibited the lactic acid formation of *Strep. cremoris* 972 for 20–30 min. but was without effect on that of the LP-resistant strain *Strep. cremoris* 803. The effect of sulphur dicyanide on strain 972 was similar to that of LP-thiocyanate-hydrogen peroxide (Oram & Reiter, 1966) in that it could be reversed by the addition of a cell-free extract of strain 803 but not by an extract of strain 972. Sulphur dicyanide also inhibited the oxygen uptake of several LP-sensitive strains of streptococci but caused only a slight check to that of the LP-resistant strain 803 (Fig. 7). The '235 compound' was without effect on the lactic acid formation of suspensions of strain 972 in 0.1 M-glucose–10 mM-potassium phosphate buffer, pH 6.8. Thus, although the cocci were inhibited by the oxidation *in situ* of 0.3 μmole of potassium thiocyanate/ml. by 0.4 equiv. of hydrogen peroxide, no inhibition occurred when the reaction products were added to the cocci 2 min. after the oxidation of thiocyanate. Since the concentrations of '235 compound' decreased by only about 20% in an hour under these conditions, the inhibitor appeared to be much less stable than the '235 compound'. Since sulphur dicyanide appeared to simulate the effects of LP, thiocyanate and hydrogen peroxide on suspensions of streptococci, we examined its effect on some enzymes of resting streptococci.

Comparison of the effects of LP-thiocyanate-

hydrogen peroxide and sulphur dicyanide on streptococcal enzymes. Cells of strain 972 were harvested from 1.5l. of GL broth, washed twice with 500ml. of water and resuspended in 30ml. of water to give approx. 2×10^{10} cells/ml. Then 200 units of LP and 10 μ moles of potassium thiocyanate were added to 10ml. of the suspension, which also received 20 μ moles of hydrogen peroxide in a dialysis sac. One μ mole of sulphur dicyanide was added to another 10ml. of the suspension and a third portion served as a control. The suspensions were shaken gently at 30° for 30 min. after which only the control cells were able to ferment glucose. Each suspension was

centrifuged and the cells were washed twice with 10ml. of water at 4°, suspended in 10ml. of Ringer phosphate at 37° and lysed by the addition of 10mg. of partially purified phage lysin as described by Oram & Reiter (1966). Measurements of the hexokinase, phosphohexokinase, aldolase and glucose 6-phosphate-dehydrogenase activities of the extracts showed that sulphur dicyanide and the LP-thiocyanate-hydrogen peroxide system had very similar effects (Table 3). Thus hexokinase was completely inhibited, phosphohexokinase was little affected, and aldolase and glucose 6-phosphate dehydrogenase were partially inhibited in both cases. Since washing the cells inhibited by either sulphur dicyanide or LP-thiocyanate-hydrogen peroxide led to a restoration of their glycolytic activity, the effect of either inhibitor was reversible. Increasing the sulphur dicyanide concentration to 1 mM, however, resulted in an irreversible inhibition of glycolysis and the complete inhibition of aldolase and glucose 6-phosphate dehydrogenase as well as hexokinase. Phosphohexokinase, which was little affected by either 0.1 mM-sulphur dicyanide or LP-thiocyanate-hydrogen peroxide, was inhibited by about 85% by 1 mM-sulphur dicyanide.

Fig. 7. Effect of $S(CN)_2$ on the oxygen uptake of streptococci. Sulphur dicyanide (1.0 μ mole) was added, as indicated by the arrows, to suspensions in 5ml. of 1% (w/v) glucose-0.1 M-potassium phosphate buffer, pH 6.8, of the following strains of streptococci: 1, group L streptococcus, P34; 2, *Strep. cremoris* 972; 3, *Strep. cremoris* 803; 4, *Strep. pyogenes* J/5; 5, group K streptococcus, Fry charwoman; 6, *Strep. pyogenes* J/17.

DISCUSSION

The LP-catalysed oxidation of thiocyanate required 3–3.5 mol.prop. of hydrogen peroxide compared with the non-catalysed oxidation, which requires 4 mol.prop. of hydrogen peroxide at neutral pH (Wilson & Harris, 1960) and 3 mol.prop. in acid solutions (Wilson & Harris, 1961). The major end products in the LP-catalysed oxidation, namely sulphate, carbon dioxide and ammonia, were the same as those found by Wilson & Harris (1960) for the non-enzymic oxidation at neutral pH. However, cyanide, which is formed in the oxidations of thiocyanate catalysed by acid (Wilson & Harris, 1961) or myeloperoxidase (Sörbo & Ljunggren, 1958), was not detected by chemical or radiochemical methods in the LP-catalysed reaction. Ammonia and

Table 3. Comparison of the effects of LP-thiocyanate-hydrogen peroxide with those of sulphur dicyanide on enzymes of *Strep. cremoris* 972

Resting cell suspensions were inhibited by LP-KSCN- H_2O_2 or by 0.1 mM- $S(CN)_2$ as described in the text. Values refer to specific activities, i.e. μ moles of substrate changed/mg. of protein/min. at room temperature.

Enzyme	Addition to cell suspensions	Specific activity		
		None (control)	LP-KSCN- H_2O_2	0.1 mM- $S(CN)_2$
Hexokinase		0.32	0.008	0
Phosphohexokinase		0.116	0.120	0.105
Aldolase		0.392	0.136	0.232
Glucose 6-phosphate dehydrogenase		0.228	0.202	0.175

carbon dioxide appear to be formed by the hydrolysis of cyanate, but the steps between thiocyanate and cyanate are as yet unknown. The lack of labelled products on radioautographs of oxidized KS^{14}CN indicates that the intermediate oxidation products are unstable, with possibly all the isotope being lost as $^{14}\text{CO}_2$.

Wilson & Harris (1961) reported that sulphur dicyanide was produced in the perchloric acid-catalysed oxidation of thiocyanate by hydrogen peroxide. We have not detected this compound in the LP-catalysed oxidation, although this may have been due to its instability under these conditions. The relationship between the '235 compound' and sulphur dicyanide is at present obscure. Sulphur dicyanide showed little absorption at $235\text{m}\mu$ and was much less stable than the '235 compound' in solutions of electrolytes. However, both compounds acted as electron acceptors for the NADH_2 -oxidizing enzyme, which would therefore appear to act as an NADH_2 -sulphur dicyanide oxidoreductase. Since the enzyme was without activity at the low ionic strengths at which sulphur dicyanide is stable it has not been possible to make a detailed study of this reaction. However, it appears that the NADH_2 -oxidizing enzyme catalyses the reduction of the '235 compound' to thiocyanate. The '235 compound' was also reduced non-enzymically by sulphite, which was found by Goehring (1943) to react with sulphur dicyanide giving sulphate, thiocyanate and cyanide. Although we were unable to find cyanide after the reduction of the '235 compound' by either sulphite or NADH_2 it seems possible that the '235 compound' and sulphur dicyanide may be structurally related.

The following criteria must be satisfied before an oxidation product of thiocyanate can be identified as an inhibitor. It must affect LP-sensitive but not LP-resistant organisms, inhibiting the same metabolic processes or individual enzymes as the LP-thiocyanate-hydrogen peroxide system. Further, inhibition should be reversed by NADH_2 and the NADH_2 -oxidizing enzyme (Oram & Reiter, 1966). None of the identified oxidation products of thiocyanate or the '235 compound' inhibited LP-sensitive streptococci. Although not shown to be an intermediate, sulphur dicyanide appeared to possess the predicted properties of the inhibitor. Thus it inhibited the lactic acid formation and oxygen uptake of metabolizing suspensions of LP-sensitive but not LP-resistant streptococci. Moreover, the effects of 0.1mM -sulphur dicyanide on resting cells of strain 972 were very similar to those of LP-thiocyanate-hydrogen peroxide, both inhibitors affecting various enzymes to a similar

degree. Further, the inhibition of the glycolysis of strain 972 by sulphur dicyanide was reversed by cell-free extracts of the resistant strain, *Strep. cremoris* 803. In view of these properties it therefore appears that sulphur dicyanide is closely related to, if not identical with, the inhibitor produced in the oxidation of thiocyanate by LP and hydrogen peroxide. The reversal of the inhibitory activity of LP-thiocyanate-hydrogen peroxide and sulphur dicyanide by extracts of strain 803 appears to be related to the reduction of the '235 compound' or sulphur dicyanide. It seems probable, moreover, that the resistance of certain strains of streptococci to inhibition by LP-thiocyanate-hydrogen peroxide is related to their ability to reduce the '235 compound' or compounds such as sulphur dicyanide. Alternatively, if the inhibitor were in chemical equilibrium with the '235 compound', then the reduction of the latter might also lead to the removal of the inhibitor. Although it did not inhibit streptococci, the '235 compound' may, in the absence of NADH_2 -oxidizing enzyme activity, be further oxidized to an inhibitory compound.

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