# The Inhibition of Streptococci by Lactoperoxidase, Thiocyanate and Hydrogen Peroxide

## THE EFFECT OF THE INHIBITORY SYSTEM ON SUSCEPTIBLE AND RESISTANT STRAINS OF GROUP N STREPTOCOCCI

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1. The growth of the lactoperoxidase-sensitive *Streptococcus cremoris* 972 in a synthetic medium was inhibited by lactoperoxidase and thiocyanate. The glycolysis and oxygen uptake of suspensions of *Strep. cremoris* 972 in glucose or lactose were also inhibited. The lactoperoxidase-resistant *Strep. cremoris* 803 was not inhibited under these conditions but was inhibited in the absence of a source of energy. 2. Lactoperoxidase (EC 1.11.1.7), thiocyanate and hydrogen peroxide completely inhibited the hexokinases of non-metabolizing suspensions of both strains. The inhibition was reversible, hexokinase and glycolytic activities of *Strep. cremoris* 972 being restored by washing the cells free from inhibitor. The aldolase and 6-phosphogluconate-dehydrogenase activities of *Strep. cremoris* 972 were partially inhibited but several other enzymes were unaffected. 3. The resistance of *Strep. cremoris* 803 to inhibition was not due to the lack of hydrogen peroxide formation, to the destruction of peroxide, to the inactivation of lactoperoxidase or to the operation of alternative pathways of carbohydrate metabolism. 4. A 'reversal factor', which was partially purified from extracts of *Strep. cremoris* 803, reversed the inhibition of glycolysis of *Strep. cremoris* 972. The 'reversal factor' also catalysed the oxidation of $NADH_2$ in the presence of an intermediate oxidation product of thiocyanate and was therefore termed the $NADH_2$-oxidizing enzyme. 5. The $NADH_2$-oxidizing enzyme was present in lactoperoxidase-resistant streptococci but was absent from lactoperoxidase-sensitive streptococci.

It is well known that cow's milk inhibits the growth of a number of bacterial species. Its effect on streptococci was extensively studied by Jones and his collaborators (Jones & Little, 1927; Jones, 1928*a*,*b*; Jones & Simms, 1929, 1930), who named the antistreptococcal substance 'lactenin'. Wilson & Rosenblum (1952*a*,*b*,*c*) confirmed and extended this work to most of the then known Lancefield serological groups of streptococci, and Auclair (1953) reported that milk contains two lactenins. Earlier, Hanssen (1924) suggested that the antibacterial action of milk against *Salmonella typhosa* and *Salmonella paratyphosa* was due to oxidative enzymes since the destruction of the inhibitor on heating was accompanied by a loss of oxidase activity. By the same reasoning Wright & Tramer (1958) concluded that one of the lactenins affecting some strains of lactic acid streptococci (serological group N) was LP,* and it was shown by Portmann & Auclair (1959) that horseradish peroxidase could not substitute for LP. Wright & Tramer (1958) proposed that, in the presence of hydrogen peroxide, LP catalysed the formation in milk of inhibitory quinonoid-type compounds, but attempts by Stadhouders & Veringa (1962) and Jago & Morrison (1962) to demonstrate inhibitory substances were unsuccessful. More recently, however, the growth of lactic acid streptococci and the respiration of resting cells were found to be inhibited by LP in the presence of hydrogen peroxide and another factor that was isolated from milk and identified as the $SCN^-$ ion (Reiter, Pickering, Oram & Pope, 1963; Reiter, Pickering & Oram, 1964). The antimicrobial action of some organic thiocyanates (mustard oils) was studied by M. Saarivirta (reported by Virtanen, 1962) and Zeldow (1963) found that thiocyanate was also involved in the inhibition of lactobacilli by a heat-labile non-diffusible factor in saliva. Since saliva contains peroxidase, it was suggested that the same peroxidase–thiocyanate system operates in saliva as in milk (Reiter *et al.* 1963). This was confirmed by Klebanoff & Luebke (1965), who found

* Abbreviation: LP, lactoperoxidase.

that an oral strain of *Lactobacillus acidophilus* was inhibited by lactoperoxidase, thiocyanate and hydrogen peroxide. Lactoperoxidase and thiocyanate also inhibit *Streptococcus pyogenes* (Reiter *et al.* 1964; Professor M. N. Mickelson, U.S.D.A., Ames, Iowa, personal communication), *Streptococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli* (Klebanoff, Luebke & Clem, 1965).

The effect of the inhibitory system LP–thiocyanate–hydrogen peroxide on the metabolism of LP-sensitive lactic acid streptococci and the reasons for the resistance to inhibition of some strains are examined in the present paper. The accompanying paper (Oram & Reiter, 1966) is concerned with the chemical nature of the inhibitory compound(s).

## MATERIALS

*Enzymes and chemicals.* LP was purified from raw skim milk by the method of Morrison & Hultquist (1963). The purity of the preparations varied, possessing $E_{412}/E_{280}$ ratios between 0·6 and 0·91, compared with the most highly purified preparation of Morrison & Hultquist (1963) which had an $E_{412}/E_{280}$ ratio 0·95. Glucose oxidase (EC 1.1.3.4) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Horseradish peroxidase, NAD, NADP, $NADH_2$ and various glycolytic enzymes and phosphorylated carbohydrates were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany. Trypsin and chymotrypsin A were obtained from Seravac Laboratories (Pty) Ltd., Maidenhead, Berks.

*Bacteria.* The following strains of group N streptococci were used (National Collection of Dairy Organisms catalogue numbers are given in parentheses): *Streptococcus lactis* C10 (509), and *Streptococcus cremoris* 803 (1240), 972 (1241), E8 (1196). The cultures were subcultured daily in autoclaved litmus milk at 22°. Washed suspensions were prepared from cocci cultured in glucose–Lemco broth (GL broth) or in lactose–Lemco broth (LL broth) consisting of glucose or lactose, 10g./l.; Oxoid Lab Lemco, 10g./l.; Evans peptone, 10g./l.; NaCl, 5g./l.; pH 7·2. After incubation for 16hr. at 30° the cocci were washed twice with a quarter-strength Ringer phosphate solution (Ringer phosphate consisting of: NaCl, 25g./l.; KCl, 1·0g./l.; $CaCl_2$, 0·75g./l.; $KH_2PO_4$, 1·36g./l.; $K_2HPO_4$, 1·74g./l.; pH 6·8) and stored at 4°.

## METHODS

*Bacterial growth.* This was measured turbidimetrically by following the increase of $E_{580}$; for cultures in the exponential phase of growth, an $E^{1cm.}_{580}$ value of 1 corresponded to about $9 \times 10^8$ cocci/ml.

*Enzyme assays.* LP was measured by following the oxidation of *o*-dianisidine by $H_2O_2$ at 37°. A solution (1·0ml.) containing $H_2O_2$ (1·0$\mu$mole) and *o*-dianisidine (100$\mu$g.) was added to 4·0ml. of 0·1M-sodium acetate buffer, pH 5·7, containing the enzyme and $E_{520}$ measured after 5min. One unit of activity was defined as the amount of LP required to give an increase in $E^{1cm.}_{520}$ of 1·0 under these conditions. Hexokinase (EC 2.7.1.1) was measured by the method of Crane & Sols (1955), phosphohexokinase (EC 2.7.1.11) by that of Ling, Byrne & Lardy (1955), fructose diphosphate aldolase (EC 4.1.2.13) by that of Taylor (1955) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) by that of Velick (1955). Phosphohexose isomerase (EC 5.3.1.9) was measured by following the isomerization of glucose 6-phosphate (10$\mu$moles) in 0·5ml. of 0·1M-potassium phosphate buffer, pH 7·0. The amount of fructose 6-phosphate formed in 5min. was measured by the method of Roe (1934) by using the factor given by Umbreit, Burris & Stauffer (1959) to convert the assay values from fructose into fructose 6-phosphate. Glucose 6-phosphate-dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate-dehydrogenase (EC 1.1.1.43) activities were assayed spectrophotometrically at 340m$\mu$ by following the reduction of 1·0$\mu$mole of NADP in the presence of 10$\mu$moles of the substrate and 100$\mu$moles of tris–citrate buffer, pH 7·5, in a volume of 3·0ml. Lactate dehydrogenase (EC 1.1.1.27) was measured by the method of Kornberg (1955) in the presence of 1mM-fructose 1,6-diphosphate, which acts as an activator of the enzyme (Oram, 1965). $NADH_2$-oxidase (EC 1.6.99.3), $NADH_2$-peroxidase (EC 1.11.1.1) and $NADPH_2$-oxidase (EC 1.6.99.2) activities were measured in 0·1M-potassium phosphate buffer, pH 7·0, at room temperature, by using a quartz cuvette fitted with a Thunberg-type side bulb as described by Dolin (1955). In each case 0·5$\mu$mole of $NADH_2$ or $NADPH_2$ was oxidized after the admission of air or the addition of 5$\mu$moles of $H_2O_2$, as appropriate.

$NADH_2$-oxidizing enzyme (see the Results section) was measured as follows. This enzyme catalysed the oxidation of $NADH_2$ by an intermediate oxidation product of the LP-catalysed oxidation of thiocyanate by $H_2O_2$. Since peroxide/thiocyanate ratios above 0·5 also favoured the formation of another intermediate that acted as an electron acceptor in the LP-catalysed oxidation of $NADH_2$ (Oram & Reiter, 1966) it was essential to employ low concentrations of $H_2O_2$ in the measurement of $NADH_2$-oxidizing enzyme. The oxidation of 0·6$\mu$mole of $NADH_2$ was measured spectrophotometrically at 340m$\mu$ on the addition of 0·4$\mu$mole of $H_2O_2$ to a solution containing an extract of *Strep. cremoris* 803, LP (0·9unit), KSCN (1·0$\mu$mole) and sodium acetate buffer, pH 5·8 (100$\mu$moles), in a volume of 3·0ml. at room temperature. One unit of $NADH_2$-oxidizing enzyme was defined as the amount of enzyme that catalysed the oxidation of 1·0$\mu$mole of $NADH_2$/min. under these conditions. The rate of oxidation of $NADH_2$ in the range 0·01–0·08unit of enzyme was proportional to the amount of enzyme present and was linear for the oxidation of approx. 0·4$\mu$mole of $NADH_2$ (e.g. for about 5min. in the presence of 0·08unit of enzyme).

*Protein.* This was measured by the biuret reaction (Dittebrandt, 1948).

*Thiocyanate.* This was measured spectrophotometrically as $Fe(SCN)_3$ (Crosby & Sumner, 1945). Chromogen formation was inhibited in a number of common buffer solutions (including phosphate) but acetate at concentrations up to 0·1M did not interfere.

*Hydrogen peroxide.* This was measured enzymically with horseradish peroxidase and *o*-dianisidine at 37°. Horseradish peroxidase (1mg.) and *o*-dianisidine (10mg.) were dissolved in 100ml. of 0·1M-potassium phosphate buffer, pH 6·8, and 3·0ml. of this reagent was added to 2·0ml. of the test solution. The increase in $E^{1cm.}_{520}$ was measured after 5min. and was proportional to peroxide concentrations in the range 0·02–0·2mM in the test solution.

*Preparation of cell-free extracts.* Washed streptococci were suspended in one-hundredth of the culture volume of Ringer phosphate and either broken at 4° in an extrusion press (Milner, Lawrence & French, 1950) or lysed by the addition of sufficient phage lysin (Oram & Reiter, 1965) to lyse 80–90% of the cells within 1 hr. at 37°. Unbroken or unlysed cells and cell debris were removed by centrifuging at 10000*g* for 30 min. in a refrigerated centrifuge.

*Measurement of lactic acid formation by suspensions of streptococci.* A suspension of about $2 \times 10^{10}$ cocci in 20 ml. of 0·1 M-glucose– or 0·1 M-lactose–10 mM-potassium phosphate buffer, pH 6·8, was stirred at room temperature in a pH-stat (Pye Ltd., Cambridge) and the amount of lactic acid produced determined from the uptake of 0·05 N-or 0·1 N-NaOH.

## RESULTS

*Effect of LP and thiocyanate on growth, lactic acid formation and oxygen uptake.* The effect of LP and thiocyanate on streptococci was highly strain-specific. Whereas the growth of *Strep. cremoris* 972 in the synthetic medium of Reiter & Oram (1962), modified by the omission of cysteine and ascorbic acid, was completely inhibited in the presence of LP and about 2 μM-potassium thiocyanate (Fig. 1), that of *Strep. cremoris* 803 was unaffected even in the presence of 1 mM-potassium thiocyanate. However, both strains were inhibited at higher concentrations (10 mM) of thiocyanate even in the absence of LP. In addition, the oxygen uptake and lactic acid formation of suspensions of strain 972 in 0·1 M-glucose–10 mM-potassium phosphate buffer, pH 6·8, were also inhibited by LP and thiocyanate, although strain 803 was not inhibited under these conditions. These results indicated that the inhibitory system acted on the energy-generating processes of LP-sensitive (strain 972) but not of LP-resistant streptococci (strain 803). Since $NADH_2$-oxidase, $NADH_2$-peroxidase and $NADPH_2$-oxidase activities were found in cell-free extracts of strain 972, it seemed probable that the inhibition of oxygen uptake was due to an effect either on the formation of $NADH_2$ or $NADPH_2$ (e.g. on glycolytic or on pentose phosphate-cycle enzymes) or on the $NADH_2$-oxidase, $NADPH_2$-oxidase or $NADH_2$-peroxidase systems. Consequently, the effect of the inhibitory system on the activities of some of the enzymes catalysing those steps was investigated.

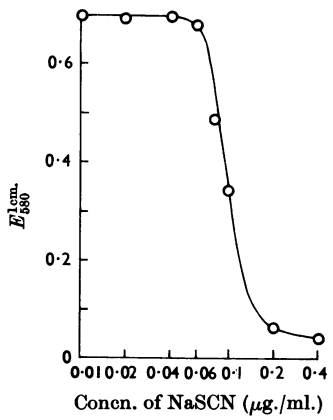


Fig. 1. Effect of LP and thiocyanate on the growth of *Strep. cremoris* 972 in a synthetic medium. Approx. $10^3$ colony-forming chains were added to 10 ml. of the medium of Reiter & Oram (1962), from which cysteine and ascorbic acid were omitted, containing 10 units of LP/ml. and NaSCN as indicated in 150 ml. conical flasks. The $E^{1cm.}_{580}$ was measured after 24 hr. at 30°.

*Effect of LP, thiocyanate and hydrogen peroxide on some streptococcal enzymes.* The streptococci obtained from 1 l. of GL broth were suspended in 25 ml. of Ringer phosphate containing LP (50 units) and potassium thiocyanate (5 μmoles) and were shaken gently at 30°. Hydrogen peroxide (20 μ-moles) was added from a dialysis sac to give an 'inhibited' suspension and water was added to a second 'control' suspension. After 30 min. each suspension was washed twice with Ringer phosphate and tested for its ability to reduce 0·1 mM-2,6-dichlorophenol-indophenol in the presence of 0·1 M-glucose. Only the 'control' suspension was able to reduce the dye. The 'inhibited' and 'control' streptococci were then broken in the extrusion press.

The activities of some glygolytic and pentose phosphate-cycle enzymes of cell-free extracts of 'inhibited' and 'control' streptocci of strains 972 and 803 are given in Table 1. The activities of some of the enzymes, e.g. phosphohexokinase (strain 972), aldolase (strain 803) and glucose 6-phosphate dehydrogenase (both strains), were more or less equal in extracts of 'inhibited' and 'control' cocci. In other experiments it was found that the 'inhibited' cocci also possessed normal lactate-dehydrogenase, $NADH_2$-oxidase and $NADH_2$-peroxidase activities. The activities of phosphohexose isomerase (strain 972), phosphohexokinase (strain 803) and glyceraldehyde 3-phosphate dehydrogenase (both strains) were somewhat higher in the 'inhibited' than in the 'control' extracts. Increased phosphohexose-isomerase activities were consistently observed in extracts of inhibited cocci of strain 972. Two enzymes, aldolase and 6-phosphogluconate dehydrogenase, were partially inhibited in strain 972 but were little affected in strain 803. The most marked effect of the inhibitory system on the resting cells of both strains, however, was on hexokinase. No hexokinase activity could be detected in the extracts of 'inhibited' strain 972 and the enzyme

Table 1. *Effect of LP, thiocyanate and hydrogen peroxide on some glycolytic and pentose phosphate-cycle enzymes of* Strep. cremoris 972 *and* Strep. cremoris 803

Resting cell suspensions of strains 972 and 803 were inhibited with LP, KSCN and $H_2O_2$ as described in the text, peroxide being omitted from 'control' suspensions. Values refer to specific activities, i.e. μmoles of substrate changed/mg. of protein/min. at room temperature.

| | *Strep. cremoris* 972 | | | *Strep. cremoris* 803 | | |
|---|---|---|---|---|---|---|
| Enzyme | Control | Inhibited | Inhibited as % of control | Control | Inhibited | Inhibited as % of control |
| Hexokinase | 0·147 | 0 | 0 | 0·186 | 0·0517 | 27·8 |
| Phosphohexose isomerase | 1·33 | 1·90 | 143 | 2·95 | 3·43 | 117 |
| Phosphohexokinase | 0·00545 | 0·00583 | 107 | 0·0129 | 0·0170 | 132 |
| Aldolase | 1·48 | 0·79 | 54 | 1·45 | 1·48 | 102 |
| Glyceraldehyde 3-phosphate dehydrogenase | 0·0186 | 0·0276 | 148 | 0·0234 | 0·0462 | 198 |
| Glucose 6-phosphate dehydrogenase | 0·0468 | 0·0400 | 86 | 0·0767 | 0·0896 | 117 |
| 6-Phosphogluconate dehydrogenase | 0·0152 | 0·0093 | 61 | 0·0198 | 0·0177 | 89·5 |

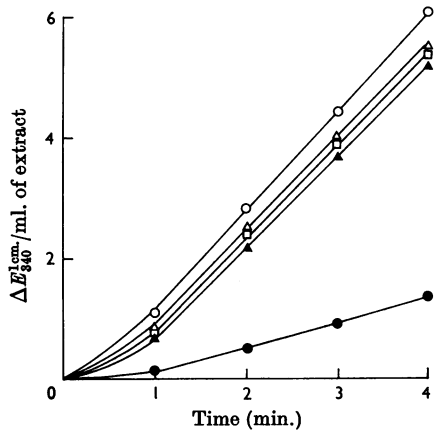


Fig. 2. Effect of LP, thiocyanate and $H_2O_2$ on streptococcal hexokinases. Hexokinase activities were measured 10min. after the addition of LP (3·4units), KSCN (0·5μmole) and $H_2O_2$ (1·0μmole) to extracts of *Strep. cremoris* 972 in 1·0ml. of 0·1M-sodium acetate buffer, pH5·8, at room temperature. ○, Control (no additions); □, LP and KSCN; △, KSCN and $H_2O_2$; ▲, LP and $H_2O_2$; ●, LP, KSCN and $H_2O_2$.

was 78% inhibited in those of strain 803. In a second experiment the hexokinase activity of resting suspensions of strain 803 was also completely inhibited by LP–thiocyanate–hydrogen peroxide. The hexokinase activities of cell-free extracts of both strains 972 and 803 were also inhibited by the addition of LP, thiocyanate and hydrogen peroxide to the cell-free extracts (Fig. 2). It was not possible to restore the activities of extracts of inhibited cocci by the addition of reducing agents such as cysteine or by dialysis against Ringer phosphate at 4°. However, glycolytic activity could be restored by washing the inhibited cells with Ringer phosphate, and extracts prepared from these cells possessed normal hexokinase activities.

The inhibition of *Staph. aureus* by organic thiocyanates (in the absence of peroxidase) was observed by M. Saarivirta (Virtanen, 1962). Since high concentrations of thiocyanate also inhibit *Strep. cremoris* 972 and 803 in the absence of LP the effect of thiocyanate alone on hexokinase was examined by using a cell-free extract of strain 972. In the absence of thiocyanate, 0·5ml. of extract catalysed the formation of 0·176μmole of glucose 6-phosphate/min. at room temperature. The addition of thiocyanate (3·3mM) caused only a slight inhibition of hexokinase but the enzyme was inhibited by 27·5% at 33mM-thiocyanate and by 77% at 167mM-thiocyanate. The concentration of thiocyanate required to inhibit streptococcal hexokinase is considerably higher than that (10mM) needed to inhibit the growth of *Strep. cremoris* in the absence of LP.

*Mechanisms of resistance to inhibition.* In marked contrast with the LP-sensitive streptococci, many strains of lactic acid streptococci grow as well in raw or pasteurized milk as in milk heated to destroy LP (Wright & Tramer, 1958; Portmann & Auclair, 1959). However, resting cell suspensions of the LP-resistant strain *Strep. cremoris* 803 were resistant only in the presence of a source of energy, indicating that resistance was an energy-requiring process, and several possible reasons for resistance were investigated.

*Lack of hydrogen peroxide formation.* Dolin (1955) has shown that *Strep. faecalis* possesses both $NADH_2$ oxidase and $NADH_2$ peroxidase, and similar enzymes are present in both LP-sensitive and LP-resistant streptococci (Oram, 1965). Hydrogen peroxide would be expected to accumulate therefore only when the activity of $NADH_2$ oxidase exceeded that of $NADH_2$ peroxidase. Suspensions of both sensitive (strain 972) and resistant streptococci (strain 803) produced hydrogen peroxide from glucose under aerobic conditions although variable amounts were formed. The inhibition of strain 972 by LP and thiocyanate was also rather irregular, and to ensure inhibition either hydrogen peroxide or a hydrogen peroxide-generating system (glucose plus glucose oxidase) was added to the suspensions. More peroxide was produced by cells grown in a lactose-containing medium (LL broth) and resuspended in lactose than by glucose-grown cells resuspended in glucose. Hydrogen peroxide formation was enhanced considerably by the addition of ascorbic acid (10mM), thus allowing inhibition to occur in experiments where the addition of hydrogen peroxide was undesirable. Unlike those of strain 972, suspensions of strain 803 were not inhibited by LP and thiocyanate, even after the addition of hydrogen peroxide (Fig. 3), and, as it was found that hydrogen peroxide was not destroyed under these conditions, it is apparent that resistance to inhibition did not result from the non-availability of hydrogen peroxide.

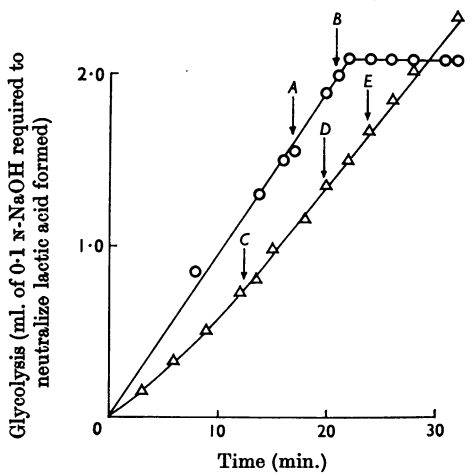


Fig. 3. Effect of LP, thiocyanate and $H_2O_2$ on the glycolysis of suspensions of *Strep. cremoris* 972 (○) and *Strep. cremoris* 803 (△) in 0·1M-glucose–10mM-potassium phosphate buffer, pH 6·8. Additions were: *A*, LP (142 units), and *B*, KSCN (2·5 μmoles), to strain 972; *C*, LP (142 units), *D*, KSCN (5 μmoles), and *E*, $H_2O_2$ (10 μmoles), to strain 803.

*Inactivation of LP.* Stadhouders & Veringa (1962) reported a correlation between the ability of cultures of lactic acid streptococci to inactivate LP and their resistance to inhibition, but this was not confirmed with suspensions of strain 803. Thus when 142 units of LP and 1·0 μmole of potassium thiocyanate were added to suspensions of strain 972 or 803 in 20ml. of 0·1M-glucose–10mM-potassium phosphate buffer, pH 6·8, the suspension of inhibited strain 972 contained 66% and the suspension of non-inhibited strain 803 contained 79% of the original LP activity after 30 min.

*Operation of alternative pathways of carbohydrate metabolism.* The aerobic carbohydrate metabolism of both strains 972 and 803 proceeds largely homofermentatively with a 75–80% conversion of glucose into lactic acid (Oram, 1965). The addition of the inhibitory system did not appear to affect the glucose metabolism of suspensions of strain 803. Thus the rate of lactic acid formation was unaltered when LP, thiocyanate and hydrogen peroxide were added to suspensions of strain 803 in glucose solution. Further, there were no significant differences in the amounts of carbon dioxide, ethanol or acetic acid produced by strain 803 in the presence and absence of the inhibitory system. It therefore seems improbable that the resistance of strain 803 was due to its ability to by-pass LP-sensitive pathways of energy production.

*Removal of the inhibitor.* Although none of the components of the inhibitory system appeared to be affected by resistant streptococci, resistance might have been due to a removal or inactivation of the inhibitory intermediate oxidation product of thiocyanate. Cell-free extracts of strain 803 were examined for their ability to reverse the inhibition of suspensions of strain 972 in 0·1M-glucose–10mM-potassium phosphate buffer, pH 6·8. The addition of LP to a suspension of strain 972 in glucose plus thiocyanate led to the oxidation of about 10% of the thiocyanate, by metabolically produced hydrogen peroxide, and the inhibition of glycolysis (Fig. 4). On adding 1·0ml. of an extract of strain 803 there was an immediate reversal of the inhibition of glycolysis and a steady fall in the concentration of thiocyanate, which had remained almost constant during the period of inhibition. The renewed acid formation did not result from the glycolytic activity of the extract of strain 803, which did not produce acid from glucose. Extracts of strain 972 failed to reverse the inhibition. The factor responsible for the reversal of the inhibition of strain 972 was termed the 'reversal factor'.

*Characterization of the 'reversal factor'.* The 'reversal factor' was non-diffusible, was inactivated by heating at 80° for 5min. and lost activity when kept at 4° overnight or on freezing and thawing. It was not destroyed, however, by incubation at

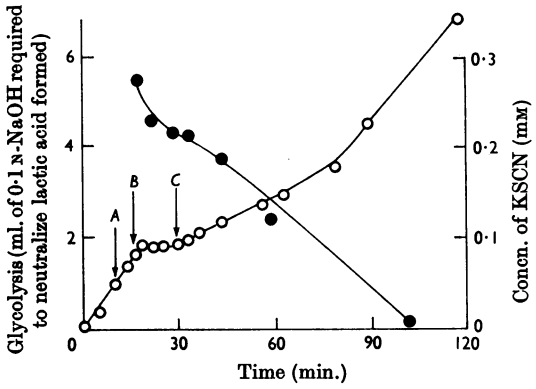


Fig. 4. Inhibition of the glycolysis of *Strep. cremoris* 972 by LP and KSCN and its reversal by an extract of *Strep. cremoris* 803. KSCN (5 $\mu$moles) (*A*) and LP (70 units) (*B*) were added to a suspension of strain 972 in 0·1 M-glucose–10 mM-potassium phosphate buffer, pH 6·8, and extract of strain 803 (1·0 ml.) (*C*) was added later. ○, Glycolysis; ●, concn. of residual KSCN, corrected for volume changes.

pH 8·0 with either trypsin or chymotrypsin (0·5 mg./ml.) at 37° for 3·5 hr. It therefore seemed probable that the 'reversal factor' was an enzyme that affected the course of the LP-catalysed oxidation of thiocyanate by hydrogen peroxide, so preventing any accumulation of the inhibitory oxidation product(s). This type of activity would be expected to alter the concentrations of intermediate oxidation products, including, perhaps, the intermediate found by Reiter *et al.* (1964) to act as an electron acceptor in the LP-catalysed oxidation of $NADH_2$ by hydrogen peroxide. The rate and extent of the oxidation of $NADH_2$ was in fact found to be greatly increased in the presence of extracts of strain 803 (Oram & Reiter, 1966).

The streptococcal enzyme catalysing the oxidation of $NADH_2$ and $NADPH_2$ in the presence of an oxidation intermediate of thiocyanate was termed the $NADH_2$-oxidizing enzyme. Its activity was very much greater than those of the $NADH_2$ oxidase and $NADPH_2$ oxidase that were also present in the unfractionated extracts. When the properties of the $NADH_2$-oxidizing enzyme were compared with those of the 'reversal factor' it was found that both were present in strain 803 but not in strain 972, were destroyed by heating at 80° for 5 min. but were little or unaffected by incubation with trypsin or chymotrypsin. The chemical nature of the intermediates acting as electron acceptors for the $NADH_2$-oxidizing enzyme are considered in the next paper (Oram & Reiter, 1966).

*Distribution of the $NADH_2$-oxidizing enzyme in LP-resistant and LP-sensitive streptococci.* A number of strains of lactic acid streptococci were examined for their sensitivity to LP, thiocyanate and hydrogen peroxide and for $NADH_2$-oxidizing enzyme activity. The enzyme was present in all the LP-resistant strains but absent from those that were sensitive to LP. Whereas strain 803, which possessed the $NADH_2$-oxidizing enzyme, was unaffected by LP, thiocyanate and hydrogen peroxide, the fermentation of glucose was inhibited in strains 972 and E 8, from which the enzyme was absent (Fig. 5). An LP-resistant culture of *Strep. lactis* C 10, from which Auclair & Vassal (1963) obtained LP-sensitive variants, was streaked on to GL agar, and well-isolated colonies were cultured in GL broth and examined for sensitivity to LP and possession of the $NADH_2$-oxidizing enzyme. There was a gradation in sensitivity to inhibition among different strains that was paralleled by a similar range in $NADH_2$-oxidizing enzyme activities. Thus the specific activities ($\mu$moles of $NADH_2$ oxidized/mg. of protein/min.) of extracts of three sub-strains of strain C 10 were 0·64, 0·122 and 0·06 respectively. The first of these sub-strains was not inhibited by LP, thiocyanate and hydrogen peroxide, the second was temporarily inhibited and the third was completely inhibited.

*Purification of the $NADH_2$-oxidizing enzyme and its relationship to the 'reversal factor'.* The $NADH_2$-oxidizing enzyme was purified from extracts of strain 803 by gel filtration with Sephadex G-200 equilibrated with 0·1 M-potassium chloride–1 mM–potassium phosphate buffer, pH 6·8, at 4°. In one experiment 3·0 ml. of streptococcal extract, containing 29·5 mg. of protein/ml. and 43 units of $NADH_2$-oxidizing enzyme activity/ml., was applied to a 50 cm. × 2·3 cm. column. The flow rate was adjusted to 20 ml./hr. and 3·0 ml. fractions were collected. The specific activity ($\mu$moles of $NADH_2$ oxidized/mg. of protein/min.) of the fraction with the highest $NADH_2$-oxidizing enzyme activity was 43 times that of the crude extract and an overall recovery of about 90% was obtained (Fig. 6). At lower ionic strengths considerable losses in activity occurred.

Washed cocci of strain 972 from 500 ml. of an LL broth culture were suspended in 30 ml. of 0·1 M-lactose–10 mM-potassium phosphate buffer, pH 6·8, containing ascorbic acid (10 mM) and were inhibited by the addition of LP (70 units) and potassium thiocyanate (2·5 $\mu$moles). At 15 min. after the onset of inhibition, 0·5 ml. samples of fractions from the Sephadex G-200 column were added to 2·0 ml. of the inhibited suspensions in the presence or absence of 0·5 $\mu$mole of $NADH_2$. There was a resumption of lactic acid production in those suspensions that received both the $NADH_2$-oxidizing enzyme and $NADH_2$ (Fig. 6) but the inhibition was not reversed by fractions without $NADH_2$-oxidizing enzyme activity. None of the fractions was able to

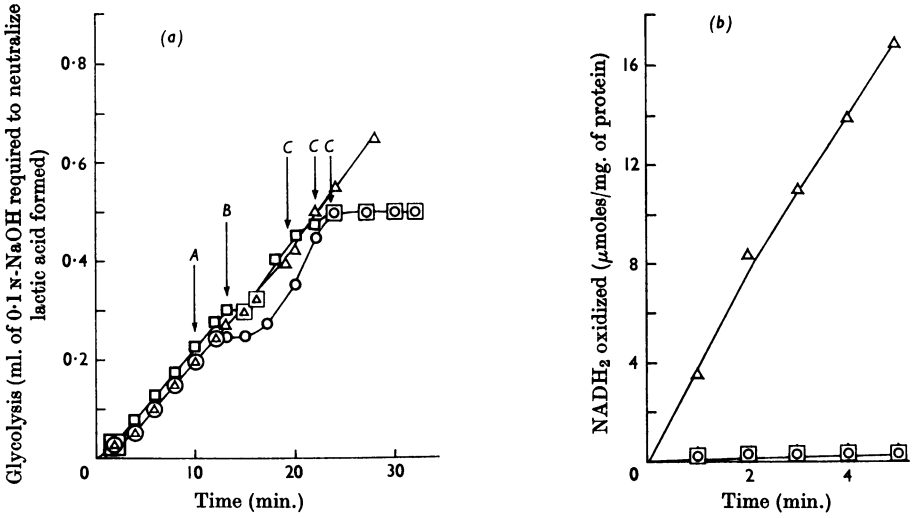


Fig. 5. Relationship between the effect of LP and thiocyanate on streptococci and their possession of the $NADH_2$-oxidizing enzyme. (*a*) Effect of LP (70 units) (*A*), KSCN, (10 μmoles) (*B*) and glucose oxidase (100 μg.) (*C*) on the glycolysis of suspensions of streptococci in 0·1 M-glucose–10 mM-potassium phosphate buffer, pH 6·8. (*b*) $NADH_2$-oxidizing enzyme activities of extracts of streptococci. ○, *Strep. cremoris* 972; △, *Strep. cremoris* 803; □, *Strep. cremoris* E8.

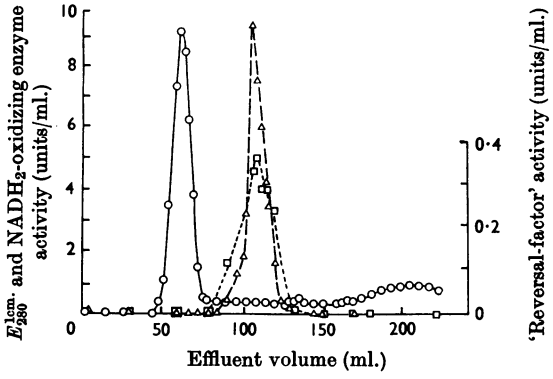


Fig. 6. Relationship between the $NADH_2$-oxidizing enzyme and the 'reversal-factor' activities of *Strep. cremoris* 803. An extract (3·0 ml.) of *Strep. cremoris* 803 was passed through a column of Sephadex G-200 as described in the text; 3·0 ml. fractions were collected and examined for the activities of $NADH_2$-oxidizing enzyme (△) and 'reversal factor' (□). One unit of 'reversal-factor' activity was defined as the amount of factor that permitted the formation of 1·0 μmole of lactic acid/hr. by 2·0 ml. of a suspension of *Strep. cremoris* 972 that had previously been inhibited by LP and KSCN as described in the text. ○, $E_{280}^{1cm.}$.

reverse the inhibition in the absence of $NADH_2$, showing that both $NADH_2$-oxidizing enzyme and $NADH_2$ were required for the 'reversal-factor' activity of the extracts of strain 803.

## DISCUSSION

It is apparent that the inhibition of streptococci by LP results from the interaction of several highly specific complex biochemical processes. The inhibitory system, which affects only certain strains of streptococci, is specific for both LP and thiocyanate. Reiter *et al.* (1964) suggested that the inability of horseradish peroxidase to inhibit milk cultures of LP-sensitive streptococci (Portmann & Auclair, 1959) was related to its inability to oxidize thiocyanate (Sörbo & Ljunggren, 1958). Wright & Tramer (1958) considered that LP catalysed the formation of an inhibitory oxidation product of a quinonoid nature, but, although quinones do inhibit lactic acid streptococci, the inhibition does not show the same strain-specificity as LP and thiocyanate (B. Reiter & J. D. Oram, unpublished work). In the presence of dialysed saliva (Zeldow, 1963) or of LP (Klebanoff & Luebke, 1965) *L. acidophilus* is inhibited by very low concentrations of thiocyanate, which can be replaced by much higher, non-physiological, concentrations of iodide. However, the inhibition of streptococci by LP and iodide or other oxidizable substances in milk, e.g. indican (indoxyl sulphuric acid), is also non-specific (Reiter *et al.* 1964).

The LP-catalysed oxidation of thiocyanate depends on the formation of hydrogen peroxide in the aerobic metabolism of the streptococci. Consequently, factors influencing the formation of

hydrogen peroxide by LP-sensitive streptococci must also affect the oxidation of thiocyanate to inhibitory compounds that, by their action on streptococcal metabolism, affect in turn the formation of peroxide. The factors affecting hydrogen peroxide formation are at present poorly understood. Thus the uptake of oxygen by suspensions of strain 972 did not always lead to the accumulation of sufficient hydrogen peroxide for the inhibition of strain 972 by LP and thiocyanate. The stimulation of hydrogen peroxide formation of suspensions of strain 972 by ascorbic acid (which is present in milk) serves to underline the complexity of the factors involved in peroxide formation.

The inhibition of streptococcal hexokinase by the inhibitory system would appear to explain the inhibition of the growth, oxygen uptake and lactic acid formation in LP-sensitive lactic acid streptococci. The effect of the inhibitory system on hexokinase might also account for the inhibition of glutamic acid uptake in *L. acidophilus* (Clem & Klebanoff, 1965), since the incorporation of amino acids into bacteria is well known to require a supply of energy. Owing to the extreme lability of the inhibitor(s) (Oram & Reiter, 1966), it has not been possible to study its mode of action on hexokinase.

Since reducing agents, in particular cysteine, were known to reverse the inhibition of the growth of streptococci in milk (Wilson & Rosenblum, 1952*a*,*b*,*c*; Auclair, 1953; Wright & Tramer, 1958), it was proposed by Stadhouders & Veringa (1962) that inhibition was due to the LP-catalysed oxidation of cysteine, which was held to be an essential growth factor for lactic acid streptococci. This explanation seems improbable, however, as cysteine is not required for the growth of these streptococci (Reiter & Oram, 1962). Stadhouders & Veringa (1962) also reported a parallel between variations in sensitivity to inhibition and differences in the ability of the streptococci to inactivate LP. A strain would also be expected to be resistant to inhibition if it failed to produce hydrogen peroxide (Wright & Tramer, 1958). Our own work has shown that the resistance of *Strep. cremoris* 803 to inhibition by LP and thiocyanate was not due to the non-availability of hydrogen peroxide, to the destruction of LP, to the oxidation of an essential nutrient (since inhibition was immediate and occurred in the absence of growth), or to the operation of an alternative pathway of carbohydrate metabolism. Resistance appeared to be associated, however, with the presence of an enzyme that, in the presence of $NADH_2$, reduced an intermediate oxidation product of thiocyanate. The role of this enzyme in resistance is discussed further in the accompanying paper (Oram & Reiter, 1966). The distribution of the $NADH_2$-oxidizing enzyme is at present being examined in a wider range of bacteria, since streptococci of other serological groups and some other bacterial species such as *E. coli* and *Staph. aureus* (Klebanoff *et al.* 1965) are also inhibited by LP and thiocyanate.

Since peroxidase and thiocyanate are both widely distributed in animal tissues this inhibitory system may be of a wider biological significance. Conditions during phagocytosis would appear to be particularly favourable for the formation of antibacterial substances, since thiocyanate is oxidized by leucocyte peroxidase (Sörbo & Ljunggren, 1958) and the amount of hydrogen peroxide produced by polymorphonuclear leucocytes is greatly enhanced during phagocytosis (Iyer, Islam & Quastel, 1961). It seems possible that many bacteria that are unaffected by LP and thiocyanate because they fail to produce hydrogen peroxide may be inhibited in the presence of an exogenous supply of hydrogen peroxide, which would be available, for example, in the phagocytosing leucocyte.

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