

Effects of Nutritional Characteristics of *Streptococcus agalactiae* on Inhibition of Growth by Lactoperoxidase-Thiocyanate-Hydrogen Peroxide in Chemically Defined Culture Medium

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Five cultures of *Streptococcus agalactiae* have an absolute requirement for L-cystine to grow in a chemically defined medium. The L-cystine could be replaced with cysteine, glutathione, or the disulfide form of glutathione. Dithiothreitol could not substitute for the sulfur-containing amino acids or glutathione; hence, the growth requirement appears to be truly nutritional. Growth was maximum with 4 to 5 μg of L-cystine per ml. If the concentration of L-cystine was no greater than 4 to 5 $\mu\text{g}/\text{ml}$, complete growth inhibition could be obtained by the addition of lactoperoxidase, thiocyanate, and H_2O_2 . The growth inhibition, however, was nullified by additions of L-cystine 10-fold or more in excess of the concentration needed for maximum growth. During the aerobic degradation of glucose by cell suspensions, H_2O_2 accumulation could be shown with cultures 317 and 11-13, the only cultures the growth of which was inhibited without addition of exogenous H_2O_2 . All of the cultures had varying degrees of peroxidase activity. The balance between H_2O_2 generation and peroxidase activity of the culture evidently determined whether growth could be inhibited with lactoperoxidase and thiocyanate without H_2O_2 addition. The growth yields per 0.5 mol of the disulfide forms (cystine and oxidized glutathione) were 1.5 and 1.9 times greater than that per 1 mol of the sulfhydryl forms (cysteine and glutathione).

Jones and Simms (3) have demonstrated a growth inhibitory complex in raw milk that is bactericidal for *Streptococcus pyogenes* but causes only a delay in growth initiation with cultures of *Streptococcus agalactiae*. Wright and Tramer (19) and Portmann and Auclair (11) have established lactoperoxidase (LPO) (EC 1.11.1.7) as one component of the inhibitor complex in milk. Reiter et al. (12) have shown that, in addition to LPO present in the milk and H_2O_2 formed in the metabolism of the streptococci, a thermostable, dialyzable component present in milk is necessary for the growth inhibition of LPO-sensitive organisms. The latter substance was identified as SCN^- . In a chemically defined culture medium, the addition of purified LPO and SCN^- completely inhibits the growth of *S. pyogenes* but causes only a delay in the growth of *S. agalactiae* (5). This difference was attributed to the presence in *S. agalactiae* under aerobic growth conditions of a less peroxidase-sensitive oxidative pathway for supplying growth energy. Additional studies on the oxidative metabolism of *S. agalactiae* have been reported (6-8).

The chemically defined medium (5) may have an excessive concentration of some factor that antagonizes growth inhibition and that is present in milk in a more limited concentration. In studying growth stimulatory properties of milk that antagonize LPO-mediated growth inhibition for *S. agalactiae*, Brown (2) has identified L-cystine to be one of the factors. In the synthetic medium, growth inhibition of *S. pyogenes* can be nullified with glutathione (GSH) or thioglycolic acid (5). Wilson and Rosenblum (18) have showed that cysteine, GSH, and thioglycolate will overcome the inhibition of streptococcal growth by raw milk. The possibility exists that an excessive concentration of cysteine and cysteine in the synthetic medium is the reason for the failure to cause growth inhibition of *S. agalactiae* with LPO and SCN^- (5). The relationship between the sulfhydryl compounds in the medium and growth inhibition of *S. agalactiae* by LPO is the subject of this report.

MATERIALS AND METHODS

Cultures and culture medium. Five cultures of *S. agalactiae* were used. *S. agalactiae* 50 was from our

laboratory collection and was also used in the work reported earlier (5). Cultures no. 44, 84, and 11-13 were obtained from Allen Packer, Department of Veterinary Bacteriology, Iowa State University. Culture 317 was obtained by J. McDonald of this laboratory from T. W. Huber, University of Texas Medical School, San Antonio, Tex., where it was isolated from a human infection. The cultures were maintained by transfer every 2 weeks in a liquid medium composed of 1% yeast extract (Difco), 0.5% Casitone (Difco), 0.5% glucose, and 0.1 M phosphate, pH 7. The cystine-limited, chemically defined medium used was a modification of that of Willett and Morse (16). Cystine was added separately to the autoclaved medium from an aqueous solution containing 100 mg of L-cystine per ml sterilized by filtration through an ultrafine sintered-glass filter. The composition of the medium was: (amino acids) L-arginine, 200 µg/ml of final volume; L-histidine, 200 µg; L-glutamic acid, 500 µg; L-isoleucine, L-leucine, L-valine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tyrosine, L-tryptophan, L-proline, and L-aspartic acid, each 100 µg; glycine, 200 µg; and L-alanine, 500 µg; (purine and pyrimidine bases) adenine, guanine, and uracil, each 10 µg/ml of final volume; (B vitamins) thiamine, 0.5 µg/ml of final volume; riboflavin, 0.5 µg; pantothenic acid, 0.5 µg; nicotinic acid, 1.0 µg; pyridoxal, 1.0 µg; and biotin, 0.0025 µg; (salts and glucose) MgSO₄·7H₂O, 0.20 mg/ml of final volume; FeSO₄·7H₂O, 0.01 mg; MnSO₄·4H₂O, 0.01 mg; and NaCl, 0.01 mg. Sodium acetate (NaC₂H₃O₂·3H₂O) and glucose were each at 10 mg/ml of final volume.

The medium was made up to double strength, dispensed in 2-ml portions per culture tube, and made up to 3.6 ml with distilled water and sample. After sterilization for 10 min at 121°C and then cooling, 0.4 ml of sterile 1 M phosphate (pH 7.0) was added.

For studies in the cystine-limited medium, the inoculum was grown in the synthetic medium containing 3 µg of L-cystine per ml. A 4-ml portion was inoculated with 0.1 ml of the stock culture and incubated for 3.5 to 4 h at 37°C. The cells were sedimented by centrifugation and suspended in sterile distilled water to give an optical density of about 0.50 at 610 nm. A 0.1-ml sample of suspension, equal to 0.1 mg of dry cell weight, was used to inoculate each 4 ml of medium. Screw-capped culture tubes (13 by 100 mm) were used, and growth was measured by reading culture densities in a Bausch and Lomb Spectronic 20 colorimeter at 610 nm. The growth values are reported as optical density at 610 nm. The yield of dry cells was estimated from a standard curve prepared from suspensions on which optical density readings were plotted against dry cell weights. Incubation temperature was 37°C.

LPO preparation and assay. LPO was prepared from fresh skim milk by the procedure of Morrison and Hultquist (9). The ratio of absorption of 412 nm to absorption at 280 nm of the 0.5 M sodium acetate eluate from the second resin column was 0.86. This purified LPO was used in all experiments on growth inhibition. The eluate was distributed in 1-ml amounts into 5-dram (ca. 6 g), screw-capped vials

and held at -60°C until used. Each 1-ml portion of the concentrated eluate contained 225 to 250 U of LPO activity as measured by the procedure of Oram and Reiter (10). LPO, diluted to 20 U/ml before use, was pasteurized at 60°C for 30 min and added before inoculation to give 0.7 U of activity per ml of medium.

Cystine, cysteine, glutathione, and DTT. Reduced GSH (Mann Research Laboratories, Inc.), oxidized glutathione (GSSG) (Sigma Chemical Co.), L-cystine, and L-cysteine (Calbiochem) solutions were made in 100-µg/ml concentrations, filtered through an ultrafine sintered-glass filter, and added to the sterilized medium just before inoculation. Catalase and dithiothreitol (DTT) (P-L Biochemicals) and SCN⁻ solutions were sterilized by filtration through a membrane filter disk (0.45-µm pore size; Millipore Corp.).

Measurement of respiration, peroxide formation, and peroxide destruction. H₂O₂ accumulation during respiration on glucose, H₂O₂ destruction by peroxidase activity during respiration, and the rate of respiration were measured with freshly prepared cell suspensions of each of the five cultures. Cell suspensions were prepared from cultures grown for 10 h on a shaker (120 oscillations/min) at 37°C in 50 ml of 1% yeast extract-0.5% Casitone-0.5% glucose-0.1 M phosphate (pH 7) in 125-ml, loosely capped Erlenmeyer flasks. Experiments were done manometrically at 37°C with 2.0-ml volumes of 0.02 M glucose-H₂O₂ (2 µmol/ml)-0.1 M phosphate, pH 7, and the equivalent of 4 to 5 mg of dry bacteria per Warburg flask. When cyanide was used as a respiratory inhibitor, 0.2 ml of 1 M KCN rather than KOH was used in the alkali well of the respirometer flasks. H₂O₂ was omitted from flasks in which the respiration rate was determined.

Experiments were terminated by adding 0.5 ml of 6 N H₂SO₄ to the reactants from the side arm. Contents of the flasks were transferred to a 15-ml centrifuge tube with successive washings of five 1-ml portions of distilled water. H₂O₂ was estimated titrimetrically as previously described (5). A flask, the contents of which were acidified before the cell suspension was added, was used to estimate initial peroxide concentration.

Comparison of thiocyanate, cystine, cysteine, and GSH as reducing agents for H₂O₂ in the presence and absence of LPO. Because an excess of cystine nullified the inhibition of growth by LPO, SCN⁻, and H₂O₂, SCN⁻, cystine, cysteine, and GSH were compared as electron donors for the LPO-H₂O₂ oxidizing system. The *O*-dianisidine color reaction was used to measure unused H₂O₂. A reaction volume of 4.4 ml containing 2.0 ml of 0.2 M sodium acetate (pH 5.7) and 0.20 U of LPO was allowed to react with varying amounts of H₂O₂ ranging from 0.15 to 0.55 µmol for 10 min at room temperature, after which 0.1 ml of 0.1% *O*-dianisidine in 95% ethanol was added. Color development was allowed to proceed for 10 min at room temperature, the reaction was stopped with 0.5 ml of 4 N HCl, and the color was read in a Klett-Summerson colorimeter with a no. 42 filter. Under these conditions, color development was maximum with 0.35 µmol of H₂O₂

(0.08 $\mu\text{mol/ml}$). The value obtained was used as a reference point in determining the amount of unreacted H_2O_2 after incubation with SCN^- , cystine, cysteine, and oxidized and reduced GSH. The reaction was repeated with 0.35 μmol of H_2O_2 and the following quantities of the reducing agents: sodium thiocyanate, 1.0 μmol ; cystine, 0.5 μmol ; cysteine, 1.0 μmol ; GSH, 1.0 μmol ; or GSSG, 0.5 μmol . After a 10-min reaction period, *O*-dianisidine was added to measure the amount of unreacted H_2O_2 . The reaction was also run with only H_2O_2 present, followed by the addition of LPO and *O*-dianisidine to measure the nonenzymatic reduction of H_2O_2 with each of the compounds.

RESULTS

Growth with the five cultures of *S. agalactiae* was maximum with 4 to 5 μg of cystine per ml (Table 1).

TABLE 1. Cystine requirement of five cultures of *S. agalactiae* in a chemically defined medium^a

L-Cys- tine ($\mu\text{g/ml}$)	Cystine requirement (OD_{610}) ^b of culture:				
	50	44	84	317	11-13
0	0.018	0.009	0.023	0.023	0.043
2	0.360	0.658	0.569	0.516	0.272
4	0.620	0.850	0.850	0.810	0.634
6	0.700	0.917	0.877	0.890	0.741
8	0.740	0.917	0.908		0.757

^a Incubation was for 18 h at 37°C.

^b OD_{610} , Optical density at 610 nm.

TABLE 2. Growth response of *S. agalactiae* culture 84 to cysteine, cystine, GSH, GSSG, and DTT in a chemically defined medium

Concn ($\mu\text{g/ml}$)	Growth response (OD_{610}) ^a to:										
	L-Cysteine		L-Cystine		GSH			GSSG			DTT
	15 h	20 h	15 h	20 h	20 h	40 h	48 h	20 h	40 h	48 h	48 h
0.0	0.009	0.013	0.009	0.013	0.013	0.018	0.018	0.013	0.018	0.018	0.018
1.5	0.268	0.523	0.598	0.792	0.037	0.057	0.089	0.023	0.028	0.197	
3.0	0.654	0.846	0.560	0.877	0.032	0.072	0.157	0.032	0.228	0.629	
4.5	0.565	0.846	0.616	0.882	0.052	0.394	0.598	0.052	0.634	0.654	
6.0	0.456	0.886	0.611	0.912	0.067	0.519	0.598	0.047	0.669	0.669	0.028

^a OD_{610} , Optical density at 610 nm.

TABLE 3. Comparative growth of *S. agalactiae* cultures with L-cysteine, L-cystine, GSH, and GSSG in a chemically defined medium

Culture	Dry cells (mg/ml) of culture				Cell yield ratio	
	L-Cysteine ^a	L-Cystine	GSH	GSSG	0.5 mol of cystine/1.0 mol of cysteine	0.5 mol of GSSG/1.0 mol of GSH
50	0.24	0.42	0.38	0.63	1.75	1.66
317	0.45	0.83			1.85	
11-13	0.54	0.75	0.26	0.49	1.40	1.88
44	0.52	0.78	0.21	0.37	1.50	1.76
84	0.56	0.85	0.21	0.30	1.50	1.44

^a L-Cysteine, L-cystine, GSH, and GSSG were at concentrations of 1.5 $\mu\text{g/ml}$.

The growth response of *S. agalactiae* culture 84 to cysteine, cystine, GSH, GSSG, and DTT is shown in Table 2. The latter was included to determine whether the growth stimulation was due to a lowering of the oxidation potential with a reducing agent or to fulfilling of an amino acid nutritional requirement. There was no growth response to DTT with culture 50 over the concentration range of 1.5 to 6 $\mu\text{g/ml}$; the other cultures were tested at only the 6- $\mu\text{g/ml}$ concentration.

When cysteine or cystine were autoclaved in the medium, the growth response was 25 to 35% less than when they were sterilized separately by filtration and then added to the medium. Cysteine, GSH, and GSSG can replace cystine in the medium for each of the cultures tested except 317. Although cystine or cysteine are readily used and yielded maximum growth in 20 h or less, incubation for up to 48 h was required for maximum growth with the two forms of glutathione. An example of the difference in growth rates with cystine, cysteine, and the glutathiones is shown in Table 2.

With the concentrations of the sulfur-containing compound that gave about one-half maximal growth, the cellular growth yield per mole of compound was considerably greater with cystine or GSSG than with cysteine or GSH (Table 3).

Experiments were carried out to determine

whether growth inhibition of several cultures of *S. agalactiae* could be shown in a chemically defined medium by LPO and SCN⁻ if the concentration of cystine used was not in excess of that required for maximal growth. Cystine was used in a concentration of 5 µg/ml, along with various combinations of LPO, SCN⁻, H₂O₂, and catalase. The results obtained with five cultures of *S. agalactiae* are shown in Table 4. Growth inhibition was complete in the presence of LPO and SCN⁻ consistently only when H₂O₂ was added. When catalase was added before H₂O₂ was added, the growth inhibition was nullified. LPO and H₂O₂, SCN⁻ and H₂O₂, or H₂O₂ alone did not inhibit growth.

In addition to catalase, excess amounts of cystine also completely nullified growth inhibition with LPO, SCN⁻, and H₂O₂ (Table 5). In some experiments, growth inhibition was partial to complete with only LPO and SCN⁻ present, particularly with cultures 317 and 11-13 (line 3, Table 5).

In manometric experiments, the relationship between the respiration rate, the accumulation of H₂O₂, and the rate of peroxide decomposition

during aerobic degradation of glucose was determined. Data with cell suspensions of five cultures of *S. agalactiae* are shown in Table 6. Only with cultures 11-13 and 317 was there detectable accumulation of H₂O₂. Culture 50 had a respiration rate six to seven times greater than that of the other cultures and also had four to seven times greater peroxidase activity. Peroxidase activity was inhibited 95 to 97%, and respiration rate was inhibited 75 to 82% by 0.001 M cyanide. In the four other cultures, the lower respiration rates were inhibited 60 to 70% by cyanide, but their low peroxidase activity was not seriously altered.

In Table 5, there is evidence that with cultures 11-13 and 317, growth was completely inhibited for at least 10 h by LPO and SCN⁻, and growth was not yet maximum after 20 h of incubation.

The data in Table 7 show a comparison of cystine, cysteine, GSSG, GSH, and SCN⁻ as reducing agents for H₂O₂ in the presence and absence of LPO. Only thiocyanate, GSH, and cysteine were oxidized under the conditions used.

TABLE 4. Growth inhibition of *S. agalactiae* cultures by LPO and SCN⁻ in a chemically defined medium^a

Medium	Growth inhibition (OD ₆₁₀) in culture:				
	50	44	84	317	11-13
1. Basal medium, without cystine	0.018	0.000	0.000	0.023	0.022
2. L-Cystine (5 µg/ml)	0.480	0.841	0.810	0.865	0.650
3. (2) + LPO + SCN ⁻	0.678	0.841	0.815	0.868	0.018
4. (2) + LPO + SCN ⁻ + H ₂ O ₂	0.000	0.027	0.000	0.005	0.000
5. (2) + LPO + H ₂ O ₂	0.428	0.820	0.792	0.868	0.611
6. (2) + SCN ⁻ + H ₂ O ₂	0.482	0.796	0.783	0.859	0.654
7. (2) + H ₂ O ₂	0.394	0.534	0.824	0.823	0.634
8. (2) + LPO + SCN ⁻ + H ₂ O ₂ + catalase	0.602	0.757	0.732	0.850	0.602

^a Concentrations used: LPO, 0.7 U/ml; H₂O₂, 0.25 µmol/ml; NaSCN, 0.7 µmol/ml; catalase, 100 µg (Pabst beef liver, 3,000 U/mg)/ml. Incubation was for 24 h at 37°C. OD₆₁₀, Optical density at 610 nm.

TABLE 5. Nullification of growth inhibition of *S. agalactiae* cultures by LPO-SCN⁻-H₂O₂ with high concentrations of cystine in a chemically defined medium^a

Medium	Growth inhibition (OD ₆₁₀) in culture:									
	50		44		84		317		11-13	
	10 h	20 h	10 h	20 h	10 h	20 h	10 h	20 h	10 h	20 h
Basal medium without cystine	0.034	0.034	0.012	0.019	0.029	0.039	0.029	0.059	0.014	0.028
Cystine	0.800	0.850	0.320	0.800	0.212	0.890	0.234	0.700	0.140	0.830
Cystine + LPO + SCN ⁻	0.802	0.859	0.169	0.854	0.418	0.978	0.009	0.491	0.047	0.460
Cystine + LPO + SCN ⁻ + H ₂ O ₂	0.000	0.000	0.005	0.010	0.005	0.005	0.000	0.000	0.014	0.014
LPO + SCN ⁻ + H ₂ O ₂										
+ Cystine (50 µg/ml)	0.000	0.818	0.059	0.823	0.125	0.605	0.589	0.837	0.067	0.820
+ Cystine (100 µg/ml)	0.015	0.959	0.699	0.937	0.246	0.973	0.607	0.837	0.102	0.850

^a Concentrations used: cystine, 5 µg/ml, except where high levels are indicated; LPO, 0.75 U/ml; NaSCN, 0.74 µmol/ml; H₂O₂, 0.25 µmol/ml. OD₆₁₀, Optical density at 610 nm.

TABLE 6. Respiration rate, accumulation of H_2O_2 , and rate of H_2O_2 decomposition by cell suspensions of *S. agalactiae*^a

Culture	Reaction time (min)	O ₂ consumed (μ mol)/mg of cells/h	Cyanide (0.001 M)	H ₂ O ₂ accumulated (μ mol)/mg of cells/h	H ₂ O ₂ decomposed (μ mol)/mg of cells/h
50	90	7.30	-	0	0.540
		1.30	+	0	0.012
	90	7.24	-	0	0.465
		1.85	+	0	0.021
317	90	0.94	-	0.153	0.068
		0.21	+	0	0.081
	105	0.76	-	0.119	0.077
		0.27	+	0.141	0.086
11-13	90	1.31	-	0.055	0.091
		0.41	+	0.142	0.057
	90	1.17	-	0	0.111
		0.39	+	0.126	0.147
44	105	1.04	-	0	0.149
		0.28	+	0	0.094
84	105	0.91	-	0	0.084
		0.36	+	0	0.043

^a Concentrations used: glucose, 0.02 M; H_2O_2 , 2 μ mol/ml; phosphate (pH 7), 0.1 M; cells, 4 to 5 mg (dry weight) per 2 ml of reaction volume. H_2O_2 was omitted from flasks in which oxygen consumption was measured.

TABLE 7. Comparison of SCN^- , cystine, cysteine, GSH, and GSSG as electron donors to LPO- H_2O_2 and H_2O_2 ^a

Electron donor	% H_2O_2 ^b	
	LPO- H_2O_2	H_2O_2
NaSCN ⁻ (1.0 μ mol)	0	3.1
Cystine (0.5 μ mol)	100	100.0
Cysteine (1.0 μ mol)	67	92.0
GSH (1.0 μ mol)	0	0.57
GSSG (0.5 μ mol)	93	98.0

^a The color developed in a reaction mixture of 0.22 U of LPO and 0.35 μ mol of H_2O_2 was used as the standard for comparison.

^b Percentage of H_2O_2 remaining after reaction for 10 min.

DISCUSSION

When cystine was omitted from the chemically defined medium, there was no growth with any of the five *S. agalactiae* cultures that were tested. The fact that there was no growth with DTT indicates that the effect of cysteine, cystine, GSH, and GSSG was to satisfy a nutritional need for the amino acid cystine and was not the result of lowering the oxidation-reduction potential of the medium. Growth was generally slower with the glutathiones than with cysteine or cystine, except with culture 50. Culture 50 grew more rapidly than the other four cultures, and growth on cysteine, cystine, GSH, and GSSG reached a maximum in about 20 h,

whereas growth of the other cultures on the glutathiones usually did not reach a maximum until 24 to 48 h of incubation. An example is shown with culture 84 in Table 2. The delay in growth initiation may be related to the synthesis of the enzymes for cleavage of the tripeptide. Culture 317 would not grow with either of the glutathiones. Evidently, this organism was unable to hydrolyze the tripeptide.

Especially interesting were the increased cell yields obtained from growth on cystine and GSSG as compared with yields on cysteine and GSH (Table 3). Brown (2) observed a greater stimulation of acid formation with *S. agalactiae* from cystine than from cysteine when the two amino acids were added to a medium composed of pasteurized skim milk (to provide LPO) diluted in steamed skim milk (LPO destroyed). The magnitude of the increase in acid formation from cystine over cysteine was in the same range as that of the increase in growth yields herein reported. In the chemically defined medium, with concentrations of cysteine and cystine of 1.5 μ g/ml, which limited growth to about one-half the maximum, the yield of dry cells per ml from 0.5 mol of L-cystine ranged from 1.4 to 1.85 times greater than from 1 mol of L-cysteine for all cultures. Likewise, at growth-limiting concentrations of the glutathiones, 1.5 and 3.0 μ g/ml, the yield of dry cells per ml was 1.45 to 1.9 times greater per 0.5 mol of GSSG

than per 1 mol of GSH for all the cultures except 317. Culture 317 would not grow when either GSH or GSSG was substituted for cystine. The reason for the increased growth response to the disulfide as compared with that of the sulfhydryl forms of the compounds is not known. We are tempted to suggest that the disulfide bond facilitates transport across the cell membrane. An energy-dependent transport for GSSG, but only in the outward direction, has been reported for human erythrocytes (13). Transport into the erythrocytes could not be shown, and GSH, found only inside the cell, was not transported outward. Similar outward transport of GSSG has been observed in lens and rat liver tissue, and the process is believed to be a means of accounting for the turnover of glutathione in the cell (4). When the γ -glutamyl cycle and the role of glutathione in the transport of amino acids were considered, Thompson and Meister (14) reported that L-cystine was considerably more active than L-cysteine as an acceptor for the γ -glutamyl moiety. The data referred to from mammalian cell studies may indicate that the disulfide forms of the amino acid and glutathione may be the transport form. No reports have been found relating to similar work with microorganisms.

The LPO-SCN⁻-H₂O₂ complex is an effective growth inhibitor for *S. agalactiae* in a chemically defined growth medium if the concentration of cystine is not greatly in excess of the amount necessary for maximum growth. However, a 10-fold excess of cystine nullified the growth inhibition. Earlier unsuccessful attempts (5) to inhibit growth in a chemically defined medium were no doubt influenced by the presence of a 10- to 20-fold excess of cystine in the medium. The complex is also effective as a growth inhibitor only when there is H₂O₂ present, either added as a reagent or formed by the bacteria during growth. H₂O₂ formation during aerobic degradation of glucose by cell suspensions was noted only with culture 317 and 11-13 (Table 6), both of which were inhibited in their growth for a limited period of time with only LPO and SCN⁻ added to the medium. All of the five cultures had peroxidase activity and in variable amounts. Evidently, the balance between H₂O₂ generation and H₂O₂ utilization by peroxidase activity in each culture determines whether growth will be inhibited when only LPO and SCN⁻ are added to the medium. Nullification of the growth inhibition by catalase, when H₂O₂ was added to the medium, was undoubtedly due to destruction of the H₂O₂.

Cyanide was a strong inhibitor of peroxidase activity in culture 50 (Table 6). The cyanide-

sensitive respiration regularly observed in this organism (5-7), and thought to be an inhibition of some component in the electron transport chain, may, in fact, be due to inhibition of the peroxidase-catalyzed destruction of H₂O₂, which was generated during respiration.

Possibly, when excessive amounts of cystine were present, the nullification of growth inhibition by the LPO-SCN⁻-H₂O₂ complex was caused by competition with thiocyanate as a reducing agent for the LPO-H₂O₂ oxidizing system. The data in Table 7 indicate that under the conditions used, thiocyanate and GSH caused complete reduction of the H₂O₂ present, and cysteine reduced about one-third of the H₂O₂. Cystine or GSSG did not reduce the H₂O₂. Evidently cystine is unable to reduce H₂O₂ in the presence of LPO unless the cystine is first reduced to cysteine inside the bacterial cell. Little or no cystine is present in living cells (15), and the cystine in proteins is formed by oxidation of cysteine residues after their incorporation into peptide chains. The fact that cysteine, cystine, and the two glutathiones all can fulfill the nutritional need for cystine would suggest that in growing cultures, the compounds are all convertible to cysteine. With cystine present 10-fold or more in excess of that amount needed for maximal growth, it may, during rapid growth of the culture, be reduced to cysteine rapidly enough to compete with thiocyanate as an electron donor and thus prevent growth inhibition. An unstable intermediate of thiocyanate oxidation is involved as the cause of growth inhibition (1).

Oram and Reiter (10) reported that *S. cremoris* 972 was inhibited by LPO and SCN⁻ from growing in a chemically defined medium from which they omitted the cysteine and ascorbic acid; evidently, in their studies, these reducing compounds interfered also with the functioning of thiocyanate in growth inhibition.

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