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Role of Keto Acids and Reduced-Oxygen-Scavenging Enzymes in the Growth of *Legionella* Species

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Keto acids and reduced-oxygen-scavenging enzymes were examined for their roles in supporting the growth of *Legionella* species and for their potential reactions between the chemical components of the media. When grown in an experimental ACES {2-[(2-amino-2-oxoethyl)-amino] ethanesulfonic acid}-buffered chemically defined (ABCD) broth, the presence of keto acids shortened the lag periods, increased the rates of growth, and gave maximum cell yields. In addition, keto acids affected the specific activities of reduced-oxygen-scavenging enzymes determined during growth. The specific activities of superoxide dismutase of *Legionella pneumophila* (Knoxville) and *L. dumoffii* (TEX-KL) were increased three- to eightfold, while that of *L. bozemanii* (WIGA) was not affected. All strains appeared to be equally sensitive to the effects of superoxide anion (O_2^-) generated by light-activated riboflavin, and all were equally protected by the presence of keto acids in the ABCD broth. Production of trace amounts of acetate and succinate in pyruvate- and alpha-ketoglutarate-containing media exposed to light suggested that hydrogen peroxide was formed. Pyruvate and alpha-ketoglutarate were products of growth on amino acids, and there was no quantitative evidence that these keto acids were metabolized when they were added to the medium. The rate of cysteine oxidation in ABCD broth was increased by the presence of ferric ion or by exposure to light or by both, and keto acids reduced the rate of this oxidation. ACES buffer was a substrate for the production of O_2^- in the presence of light, and the combined addition of Fe^{2+} ions, cysteine, and either keto acid to the medium strongly inhibited the production of O_2^-. Thus, keto acids inhibited the rate of cysteine oxidation, they stimulated rapid growth by an unknown process, and, in combination with added Fe^{2+} ions and cysteine, they reversed the toxic effects of light by inhibiting O_2^- production.

Satisfactory growth of legionellae is obtained with only a few complex organic media. Iron and cysteine are requirements for the growth of this strictly aerobic organism (9, 41), but addition of these components to common laboratory media does not promote growth. However, the addition of charcoal to broth or agar strongly stimulates growth in yeast extract media containing Fe^{3+} and cysteine (40, 47). Of the compounds tested for their effect on growth of *Legionella pneumophila* in semisynthetic media, pyruvate and alpha-keto-glutaric acid when added to agar media strongly stimulated rates of growth and cell yields at both pH 6.5 and 7.2 (41). However, *L. pneumophila* (Philadelphia 1) showed little rate response to these keto acids in broth media and gave little increase in cell yields. But when charcoal was added to these semisynthetic media, the growth of dilute inocula was more rapid and more CFU were observed (L. Pine and G. B. Malcolm, unpublished data). It was found that exposure of yeast extract broth to fluorescent light generated O_2^- and hydrogen peroxide to potentially toxic levels and that charcoal rapidly decomposed these products (28, 52).

To determine those factors which might inhibit growth of this organism in common laboratory media, strong consideration was given to the apparent conflict between the oxygen demands of *Legionella* spp. and its requirement for cysteine and Fe since Fe^{3+} catalyzes the rapid oxidation of cysteine by oxygen (9, 40, 46). These considerations included the experimental results of other workers describing the toxic effects of hyperbaric oxygen on the bacterial growth (6, 15, 20, 27, 44), the generation of hydrogen peroxide or O_2^- or both in the presence of cysteine and metals (7, 36, 37), the reaction of alpha-hydroxycarbonyl compounds, such as glucose and glyceraldehyde, with phosphate to form toxic concentrations of hydrogen peroxide and O_2^- (5), and the formation of toxic compounds by the action of light on conventional or riboflavin-containing media (12, 16, 54, 55). None of these studies involved legionellae. Closely allied with the reported toxicity of hydrogen peroxide or O_2^- for several microorganisms are the positive effects on growth reported for sulfite, pyruvate, alpha-ketoglutarate, catalase, and superoxide dismutase (SOD) which are known to scavenge these reduced-oxygen compounds (5, 7, 10, 17, 26, 27, 31, 36).

Recently, we observed that catalase, peroxidase, and SOD were not uniformly present in all *Legionella* species (43). *L. pneumophila* had only peroxidase, *L. bozemanii* had only catalase, and *L. dumoffii* had both peroxidase and catalase. We have asked what is the nature of the relationships of these reduced-oxygen-scavenging enzymes to the growth of each of these three enzymatic groups of *Legionella* species and whether there was any growth advantage given by the presence of one or more of these enzymes. In addition, the positive or negative roles of keto acids added to chemically defined medium supporting growth of *Legionella* species were examined in the context of the presence or absence of these enzymes.

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MATERIALS AND METHODS

Bacterial strains, media, and cultural conditions. All bacterial strains were from stock cultures identified at the Center for Infectious Diseases, Centers for Disease Control, and were described earlier (43). The *Legionella* sp. strains used were *L. pneumophila* (Knoxville 1), *L. dumoffii* (TEX-KL), and *L. bozemanii* (WIGA). The legionellae were cultured on 2-[(2-amino-2-oxoethyl)-amino] ethanesulfonic acid (ACES)-buffered yeast extract agar slants (BCYE) (38) for 48 to 72 h and stored at 5°C; transfers were made at 6-week intervals, and fresh working cultures from frozen stock cultures were prepared every 6 to 8 months. Fresh inocula were prepared from 2- to 3-day-old inoculated BCYE slants; the cells were washed from the slant surface with 2 ml of distilled water, the suspension was diluted to 5 to 10 ml, a sample was taken to determine the cell absorbance in test tubes (20 by 150 mm) at 660 nm, and sufficient cell suspension was added to freshly prepared liquid media to give an initial absorbance of 0.05 to 0.10. In general, cultures had 10 ml of media in 50-ml side-arm flasks or 25 ml in 125-ml side-arm flasks. These were shaken at 75 and 100 rpm, respectively, at 35°C in the absence of light. When cultures were incubated in the light, they were incubated 12 in. (30.48 cm) beneath two 15-W, 16-in. (40.64-cm) fluorescent bulbs giving approximately 25 ft- (269 lx)/cm^2 total at the base of the flasks. Control flasks were wrapped in aluminum foil.

Preparation of ABCD broth (with keto acids). An experimental ACES-buffered chemically defined (ABCD) broth was prepared, using the following stock solutions in deionized water, unless stated otherwise. Solution 1 had 20 g of ACES buffer, 0.44 g of KH_2PO_4, and 0.30 g of Na_2SO_4 per liter. Solution 2 had 55.5 mg of $CaCl_2$, 21.5 g of $MgSO_4$, 117 mg of NH_4VO_3, and 2.875 g of $ZnSO_4 \cdot 7H_2O$ per liter. Solution 3 had 47.6 mg of $CoCl_2 \cdot 6H_2O$, 2.5 mg of $CuSO_4 \cdot 5H_2O$, 2.0 mg of $MnCl_2 \cdot 4H_2O$, 121.0 mg of $NaMO_4 \cdot 2H_2O$, and 52.6 mg of $NiSO_4 \cdot 6H_2O$ per liter of 0.05% HCl. Solution 4 had 4.0 g of $FeSO_4 \cdot 7H_2O$ per liter in 0.05% HCl. Solution 5 had 20 g of serine and 1.0 g each of the following amino acids per liter: L-alanine, L-arginine, L-asparagine · H_2O, L-aspartic acid, L-glutamine, L-glutamic acid, glycine, L-histidine HCl, L-isoleucine, L-leucine, L-lysine HCl, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, and L-valine. Solution 6 was 10% sodium pyruvate. Solution 7 was 10% alpha-ketoglutaric acid. Solution 8 had 200 mg of *i*-inositol, 200 mg of thiamine HCl, 200 mg of calcium pantothenate, 100 mg of nicotinamide, and 10 mg of biotin. Solution 9 had 10 mg of DL-thioctic acid per 10 ml of 95% ethanol, solution 10 had 10 mg of coenzyme A per 10 ml of deionized water, and solution 11 had 2 mg of hemin per ml of dilute NH_4OH. To prepare 1 liter of ABCD broth the following constituents were added in the following sequence and mixed well: 500 ml of solution 1, 10 ml of solution 2, 10 ml of solution 3, 100 ml of solution 5, 10 ml of solution 6, 10 ml of solution 7, 10 ml of solution 8, 0.1 ml of solution 9, 0.1 ml of solution 10, 500 mg of cysteine HCl, 500 mg of glutathione (reduced), 50 mg of L-tyrosine, and 10 ml of solution 4. The solution was adjusted to pH 6.5 with 20% KOH, 1 ml of solution 11 was added, and the final volume was filtered for sterilization. The above medium without keto acids is referred as the "basal ABCD" broth.

Chemical and enzymatic analyses. Using the modifications and procedures described previously for whole cells and cell-free extracts (42, 43), we determined catalase by the Beers-Sizer procedure (3), peroxidase with *o*-dianisidine (58), and SOD with pyrogallol (33) without chromatographic separation of the individual enzymes. We determined cysteine and cystine by the procedure of Gaitonde (13). Hydrogen peroxide was determined with peroxidase and *o*-dianisidine (58); with this test, standard curves obtained in the presence and absence of basal ABCD broth were linear in the range of 0.25 to 3 μg of added hydrogen peroxide per ml. Acetic, pyruvic, lactic, succinic, and alpha-ketoglutaric acids were determined qualitatively and quantitatively by gas-liquid chromatography (32). Volatile acids were determined directly by acid extraction into ether; nonvolatile acids were converted to methyl esters and extracted into chloroform. Phenylacetic acid was used as an internal standard for the quantitative determination of nonvolatile acids; volatile and nonvolatile acid standards were prepared as described previously (32). A Perkin-Elmer 3920 gas chromatograph having a flame detector was used with a 10% SP 1000–1% H_3PO_4 100/120 Chromosorb W/AW (Supelco) column (3 mm inside diameter by 157.5 cm). Volatile acids were run at a column temperature of 140°C; the injection port and detector were at 200°C. For the methyl esters of lactic, pyruvic, and succinic acids, the temperature was maintained at 140°C for 32 min followed by a 2°C/min rise to a final temperature of 200°C to determine alpha-ketoglutarate methyl ester. The injection port and detector temperatures were 225°C; the carrier gas was helium at 60 ml/min.

Effect of medium components on generation of O_2^-. O_2^- was measured with EDTA, riboflavin, and fluorescent light, using the nitroblue-tetrazolium (NBT) assay of Winterbourn et al. (57) except NaCN was not used. Starch, at a final concentration of 0.1%, was added to prevent the precipitation of reduced NBT (formazan); sensitivity of the reaction was increased twofold with the use of starch. When the reactants had EDTA, the following reagents were added to test tubes (13 by 100 mm): 1.5 ml of potassium phosphate (0.067 M; pH 7.8), 0.15 ml of 3% soluble potato starch, 0.2 ml of sodium EDTA (0.1 M; pH 7.8), 0.1 ml of NBT (1.5 mM), and 0.05 ml of riboflavin (4.5 mg/100 ml). To this, 1.5 ml of sample was added. When EDTA was not used as a reductant for light-activated riboflavin, the reactants were the same except 0.5 ml of ACES plus salts (solution 1, ABCD broth) was substituted for EDTA. That the reduction of NBT was the result of reduction by O_2^- was ascertained by a comparison with duplicate reaction tubes to which 50 U of SOD per ml had been added to remove O_2^-.

All enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. When catalase, peroxidase, or SOD was added to media, it was prepared in a concentrated solution in water, filtered for sterilization, and added to a final concentration of 50 U/ml.

RESULTS

Comparative production of catalase, peroxidase, and SOD by *Legionella* species. We made a direct comparison of the effect of keto acids on enzyme production by three species of *Legionella* grown in the ABCD broth. The three species differed in their responses to the synthetic media with and without keto acids (Fig. 1 and 2). *L. bozemanii* showed little change in its catalase and SOD contents during the early stages of growth, but catalase or catalase and SOD production was strongly deregulated as the bacterium entered the stationary phase of growth. Both *L. pneumophila* and *L. dumoffii* showed very limited peroxidase formation in the basal ABCD medium. Although addition of keto acids stimulated the production of peroxidase by *L. dumoffii*, they inhibited peroxidase production by *L. pneumophila*. Catalase was not formed by *L. dumoffii* or *L. pneumophila* in

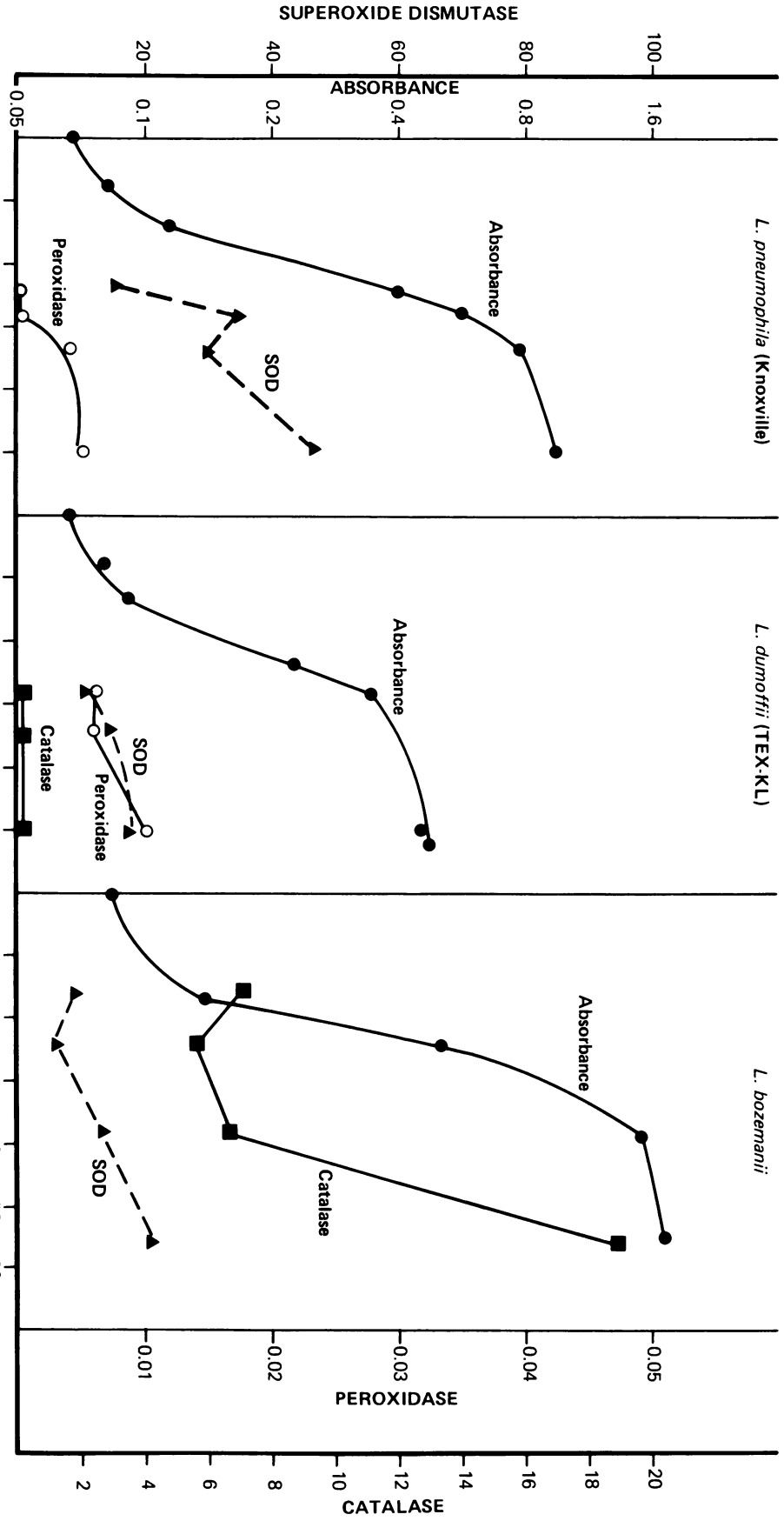

FIG. 1. Formation of reduced-oxygen-scavenging enzymes during growth in basal ABCD broth. All enzyme activities are expressed in units per milligram of protein.

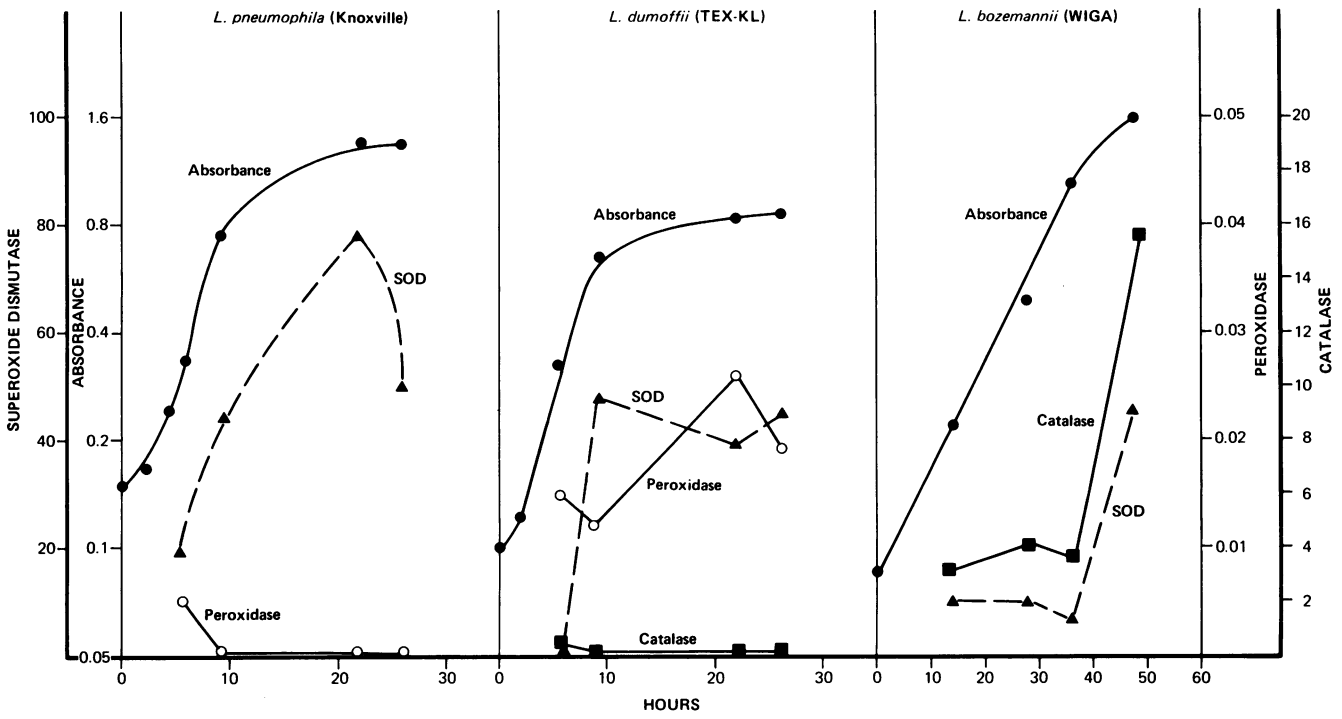

FIG. 2. Formation of reduced-oxygen-scavenging enzymes during growth on ABCD broth (containing 0.1% sodium pyruvate and 0.1% alpha-ketoglutaric acid). All enzyme activities are expressed in units per milligram of protein.

either the basal or the complete ABCD medium. In contrast to the results obtained with *L. bozemanii*, the addition of keto acids stimulated a three- to eightfold increase of SOD by both *L. pneumophila* and *L. dumoffii* during the late lag and logarithmic phases of growth. Although not readily apparent from Fig. 1 and 2 because of differences in their time scales, the presence of keto acids stimulated the growth of all three species; this is more clearly shown below (Fig. 3).

Effect of reduced-oxygen-scavenging enzymes added to media. In many experiments, the addition of charcoal to synthetic or semisynthetic media permitted the growth of higher dilutions of inoculum in broth or gave 2- to 4-log greater colonies on agar media. Because we recognized the catalase and SOD activities of charcoal (28), we determined the effects of the separate or combined additions of catalase, peroxidase, and SOD on the growth of *L. pneumophila*. There were no consistent, definitive patterns of growth stimulation by the addition of these enzymes when the cultures were incubated in the dark or in the light.

Other experiments on the growth of *L. pneumophila*, *L. dumoffii*, and *L. bozemanii* in filtered yeast extract broth (47) and in the basal ABCD broth showed that growth of these species was totally inhibited by incubation under fluorescent light. Removal of riboflavin from the ABCD broth permitted equal growth of strain Knoxville 1 in the presence or absence of light. In the presence of riboflavin, the additions of SOD, catalase, or peroxidase, individually or in combination, did not permit growth in the basal ABCD broth under light, although good growth in the presence of these enzymes was obtained in the absence of light. Similarly, addition of 0.2% mannitol to the basal ABCD broth as a scavenger of hydroxyl radical (11) did not permit growth under light.

Effect of keto acids on growth of *Legionella* species. Although the addition of reduced-oxygen-scavenging enzymes did not permit growth of the *Legionella* species under fluorescent light, excellent growth of all species was obtained under light when pyruvate or alpha-ketoglutarate was present in the ABCD broth (Fig. 3). Pyruvate was the most effective protector against the effect of light, permitting almost twice the rate of growth of *L. pneumophila* as that obtained with alpha-ketoglutarate. But we retained alpha-ketoglutarate in the medium since it behaved differently from pyruvate. Thus pyruvate was rapidly oxidized by cell suspensions, whereas alpha-ketoglutarate was not (29), and, although pyruvate or alpha-keto-glutarate by itself increased the production of acid SOD of strains Knoxville 1 grown in the ABCD broth, stimulation of SOD by alpha-ketoglutarate was greater than that obtained with pyruvate (data not shown). That the protective effect of the keto acids was not due to the stimulation of either catalase or peroxidase was shown above; that their protective effect was not due to stimulation of SOD was supported by the fact that these acids did not stimulate SOD in *L. bozemanii* during its lag and logarithmic phases of growth (Fig. 2).

Experiments were done to determine if the protective effects of the keto acids could be due to (i) a metabolic change within the bacterium, (ii) the protection of a required component of the medium such as cysteine, or (iii) the removal of a toxic product such as hydrogen peroxide (45). The effects of the keto acids on growth with and without light were determined for *L. pneumophila*, *L. dumoffii*, and *L. bozemanii*. In the presence of keto acids in light and in the absence of light, the lag periods for each of the three species were shortened or eliminated, and the rates of growth were

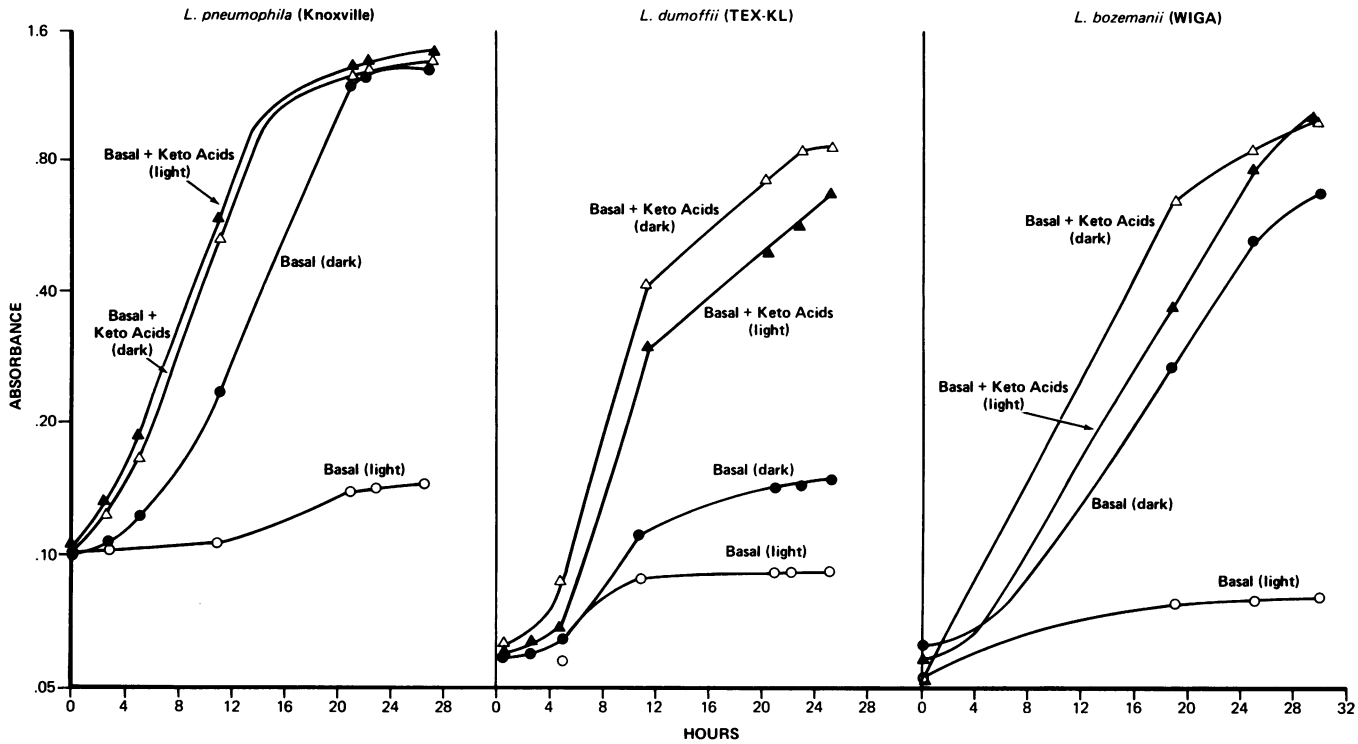

FIG. 3. Effect of light on growth of *Legionella* species in basal ABCD broth and ABCD broth with keto acids. Cultures were grown in 25 ml of broth in 125-ml side-arm flasks. Flasks incubated in light were 12 in. below two 14 in., 15-W fluorescent lights. Control flasks were wrapped in aluminum foil.

greater than that observed in the absence of keto acids (Fig. 3).

But the cell yields obtained in the absence of keto acids might also reflect the vigor of the inoculum so that strains which exhibited an initial slow growth in the absence of keto acids showed, on occasion, a strong depression of maximum cell yields (TEX-KL, Fig. 3). On the basis of several experiments, this failure to reach maximum growth was attributed to the rapid oxidation of cysteine to cystine before bacterial growth was initiated, i.e., during the lag period of growth (Table 1). However, if a prior incubation of the basal ABCD broth was made for 24 h with shaking in the dark, little or no difference of growth was observed for TEX-KL than was observed on the basal medium inoculated immediately after preparation (Fig. 4, I and II). But basal ABCD broth exposed for 24 h to light under growth conditions prior to bacterial inoculation gave a much reduced rate of growth of TEX-KL and approximately half the cell yields of those obtained in the medium incubated without light (Fig. 4, III). Under both conditions, cysteine was depleted within 6 h to levels which were considered to be limiting (41), although in light the rate of the cysteine disappearance was greater (Table 1). These results suggested that cysteine of the medium incubated in the light underwent a different chemical change from that observed without light. All media, when incubated without light for 30 days at 5°C, deteriorated and supported only 25 to 50% of maximum growth in the absence or presence of keto acids, respectively.

TABLE 1. Effect of light and keto acids on concentration of cysteine in ABCD broth[a]

Medium	Concn (μmol/ml)						
	0.0 h	5.5 h		16.0 h		22.0 h	
		Dark	Light	Dark	Light	Dark	Light
ABCD	3.60	0.95	0.10	0.15	0.10	0.12	0.10
ABCD + kg	3.55	1.0	0.25	0.70	0.15	0.60	0.15
ABCD + pyr	3.45	1.40	0.80	0.85	0.40	0.65	0.35
ABCD + kg + pyr	3.25	1.55	1.70	1.45	0.85	1.25	0.70

[a] The media were prepared and zero-time samples were immediately frozen at −10°C. The remained media were dispensed in shake flasks and incubated, uninoculated, for the times indicated as described for Fig. 3. All flasks were then analyzed for cysteine (13). kg, alpha-ketoglutarate; pyr, pyruvate.

Quantitative changes of pyruvate and alpha-ketoglutarate during growth. Hydrogen peroxide has been described as a product of iron-catalyzed oxidation of cysteine in diverse complex organic media (7, 8, 36, 37). Recognizing that pyruvate and alpha-ketoglutarate are strong scavengers of hydrogen peroxide (45), we analyzed several combinations of media for hydrogen peroxide after incubation with and without light. Although we were unable to demonstrate hydrogen peroxide in the basal ABCD broth, traces of acetic acid were formed when pyruvate-containing media were incubated in the light. Similarly, traces of succinic acid were formed when alpha-ketoglutarate-containing ABCD broth was exposed to light. These products were not formed in the absence of light and it is suggested that trace amounts of hydrogen peroxide formed when the basal ABCD broth was incubated in the light reacted with pyruvate and alpha-ketoglutarate to form acetate and succinate, respectively.

Analyses of uninoculated media which had been incubated in the absence of light for 24 h under growth conditions showed no formation of acetate or of succinate in the basal or the complete ABCD medium. However, when incubated

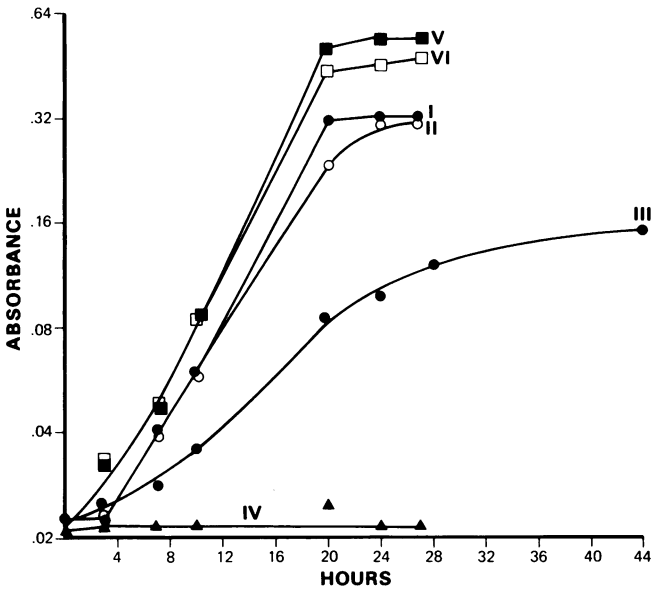

FIG. 4. Effect of light exposure of basal ABCD broth on subsequent growth of *L. dumoffii* (TEX-KL). I. Basal ABCD broth was inoculated and incubated in the absence of light. II. Basal ABCD broth, incubated in the absence of light under cultural conditions for 24 h, was inoculated and incubated in the absence of light. III. Basal ABCD broth, incubated under fluorescent light for 24 h, was inoculated and incubated in the absence of light. IV. Basal ABCD broth was inoculated and incubated under fluorescent light. V. Culture in ABCD broth was 0.1% sodium pyruvate and 0.1% alpha-ketoglutaric acid was incubated in the absence of light. VI. As V, incubated under light.

under light, all uninoculated media having pyruvate formed approximately 0.6 μmol of acetic acid per ml and all media having alpha-ketoglutarate formed 0.6 μmol of succinic acid per ml.

However, when cultures of *L. pneumophila* and *L. dumoffii* were incubated in the absence of light in the basal ABCD broth, 3 to 4 μmol of pyruvic acid per ml was formed, and this production of pyruvate remained unchanged in the media to which pyruvate or alpha-ketoglutarate had been added. Without light, traces of succinic acid were formed in these cultures to which alpha-ketoglutarate had been added. But cultures incubated in the light showed a decreased production of pyruvate in all cultures, and there was an increase in succinate formed in the alpha-ketoglutarate-containing media (to approximately 2 μmol/ml); no acetic acid was found in any of the cultures. Since the initial concentrations of pyruvate and alpha-ketoglutarate were 9.1 and 6.8 μmol/ml, the overall utilization of keto acids or formation of products from them was minor if not negligible. The results suggest that, under the influence of light, greater amounts of succinate were formed as a result of cell metabolism than those formed in the absence of light.

Effect of ABCD components on production of superoxide radical. The ABCD broth was prepared as a basic experimental medium to evaluate the growth requirements of *Legionella* species. Although it supports excellent growth of many strains of several different species, it fails to grow certain of the more recently described species. As an experimental medium, a vitamin supplement was included, although *L. pneumophila* has no reported vitamin requirements (48, 53). This vitamin supplement, which includes riboflavin, was found to stimulate growth in certain synthetic media; in many experiments conducted under normal laboratory conditions, this supplement did not inhibit growth. However, when cultures were incubated in light with the vitamin mix containing riboflavin, growth was inhibited. In the presence of light and riboflavin, certain nitrogenous compounds such as EDTA, methionine, dimethylglycine, and sarcosine are known to form superoxide radical and formaldehyde (2, 12, 16) and in the presence of iron or other transition metals, hydroxyl radical (OH ·), hydroxyl ion [$(OH)^-$], and hydrogen peroxide (H_2O_2) are formed as products of O_2^- decomposition (18, 19, 35). Using the NBT reduction as an indicator of O_2^- formation, we assessed the role of pyruvate and alpha-ketoglutarate in the reversal of the riboflavin-light inhibition of the growth of *Legionella* species.

In a typical standard assay for O_2^- formation, using EDTA as the substrate for the reduction of activated riboflavin, the final absorbances read at 2 h were as follows: no additions, 1.38; basal ABCD, 0.20; basal ABCD plus alpha-ketoglutaric acid, 0.19; basal ABCD plus pyruvate, 0.20; and complete ABCD, 0.14. Thus, approximately 85% of the inhibition of O_2^- formation was attributed to medium components which were not keto acids.

When the medium components were tested with and without SOD for their effect upon the production of O_2^-, certain of them strongly supported the production of O_2^-; others prevented its production, although NBT might still be reduced. Initially, EDTA was used as substrate, but it was found that ACES buffer itself could replace EDTA, although the rate of reaction was slower. With ACES as the substrate, the individual addition of the vitamin solution or cysteine strongly increased the rate of production of O_2^-, whereas the addition of the amino acid solution or $FeSO_4$ strongly inhibited O_2^- production (Table 2). Without EDTA in the assay, 82 to 96% of the reduction of NBT in ABCD broth without added Fe was due to the production of O_2^- as determined by the addition of SOD (Fig. 5). With added Fe, formazan production (i.e., reduced NBT) was lowered 50%,

TABLE 2. Effect of addition of ABCD components on reduction of NBT in the absence of EDTA

Product added[a]	Description of medium component[b]	Absorbance[c]
1. Riboflavin + assay buffer		0.00
2. 1 + ACES salts	Soln 1	1.01
3. 2 + amino acid mix	Soln 5	0.07
4. 3 + pyruvate	Soln 6	0.08
5. 4 + α-ketoglutaric acid	Soln 7	0.07
6. 5 + vitamin mix	Soln 8	0.41
7. 6 + thioctic acid	Soln 9	0.37
8. 7 + coenzyme A	Soln 10	0.41
9. 8 + cysteine		1.25[a]
10. 9 + glutathione		1.23
11. 10 + tyrosine		1.24
12. 11 + Ca^{2+}, Mg^{2+}, VO_3^-, Zn^{2+}	Soln 2	1.27
13. 12 + Co^{2+}, Cu^{2+}, Mn^{2+}, Mo^{7+}, Ni^{2+}	Soln 3	1.32
14. 13 + Fe^{2+}	Soln 4	0.45
15. 14 + hemin	Soln 11	0.58

[a] Assay was as described in Materials and Methods with 0.1% starch present. Tubes were shaken and read at 15-min intervals. Total incubation time was 150 min. Effects of SOD on the overall reactions are given in Fig. 7. Reaction 9 above (8 + cysteine) was 0.00 with added SOD (50 U/ml). All reactants were also tested in the dark; no reduction of NBT was observed.

[b] Solutions are described in Materials and Methods for the preparation of ABCD broth.

[c] Average value of duplicate tubes.

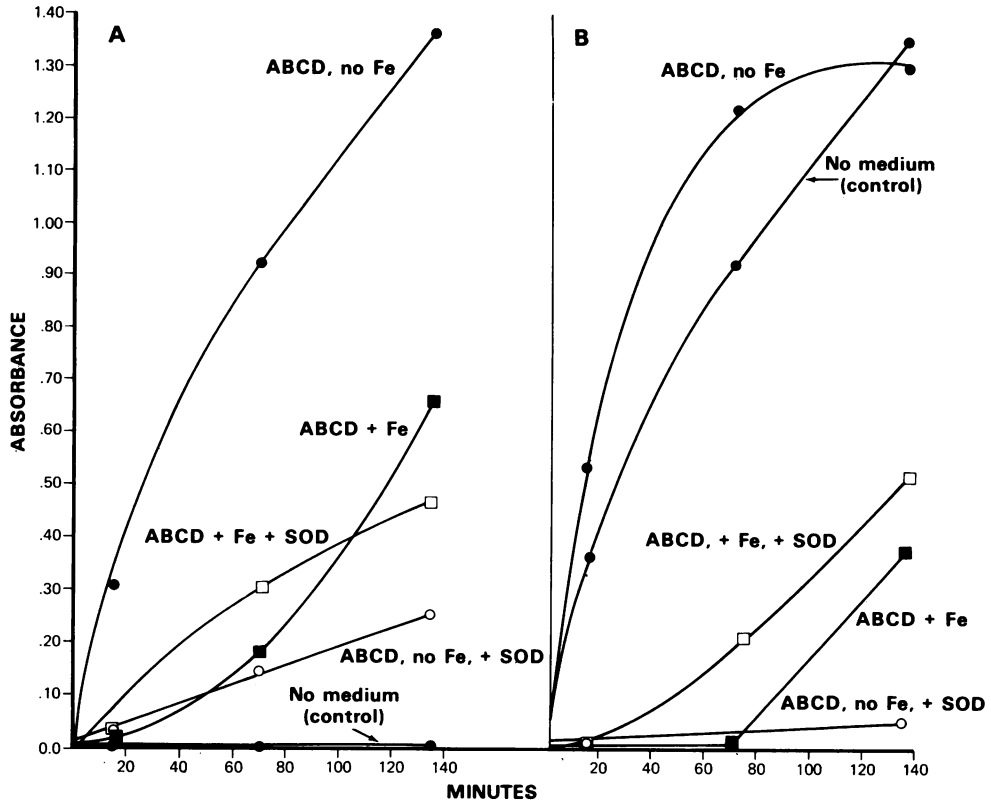

FIG. 5. Production of superoxide radical in the presence and absence of added iron ($FeSO_4$) in ABCD broth with 0.1% sodium pyruvate and 0.1% alpha-ketoglutaric acid. (A) Assay without EDTA as substrate for the generation of the superoxide radical. (B) Assay with EDTA (see Materials and Methods). Superoxide dismutase was added at 50 U/ml; Fe^{2+} was added at 100 μg/ml.

and, of the formazan formed, only 30% was attributed to the production of O_2^- (Fig. 5). Evaluation of the data in the presence and absence of EDTA clearly showed that two competing reactions occurred which reduced NBT under fluorescent light; one involved the formation of O_2^- from cysteine and ACES buffer, and the second, also light induced, occurred in the presence of SOD. Additional tests clearly showed that the second reaction required the combined presence of cysteine, pyruvate, and Fe, or cysteine, alpha-ketoglutarate, and Fe (Fig. 6). In the presence of these latter combined constituents, the production of O_2^- was essentially eliminated. Although the mechanisms of action are unknown, the results together with those presented above (Fig. 4) support the conclusion that, in the absence of keto acids, cysteine was oxidized under the influence of O_2^- to some product not used by the cells but that in their presence O_2^- formation was suppressed. It is known that cysteine may be oxidized directly or indirectly to sulfite derivatives through the mediation of Cu or Fe and the dismutation of O_2^- (1, 8, 34). Thus keto acids stimulated the production of SOD and functioned directly with cysteine and Fe to inhibit O_2^- formation under light (Fig. 6); they maintained concentrations of cysteine which supported maximum growth; and under light and in the absence of light without being metabolized to any major extent, they caused a decreased lag phase and increased the rate of growth (Fig. 3).

DISCUSSION

The results which we present here are based upon the use of an experimental medium, the ABCD broth. In direct comparisons, this broth was found to be equal or superior to previously described chemically defined media (41, 46, 48). However, the medium fails to support maximum growth of two strains of *L. dumoffii* (NY-23 and TEX-KL) due to a requirement for increased levels (to 0.02%) of phenylalanine and proline. Other species, more recently described, fail to grow in sequential transfer due to a requirement for yeast extract dialysate; this latter requirement is satisfied by an equivalent substitution of guanine (L. Pine, M. J. Franzus, and G. B. Malcolm, unpublished data). These and other adjustments of the ABCD broth are to be presented in the future, but the results presented here are believed to represent general phenomena which occur in *Legionella* sp. media such as the yeast extract broth or BCYE agar. The background for the addition of pyruvate and alpha-ketoglutaric acid is based on our earlier results (41) and has been discussed by Weiss and Westfall (56).

Several authors have demonstrated the rapid stimulation of SOD within cells by hyperbaric oxygen (15, 17, 44, 49) but the use of common metabolites have also been shown to increase the production of SOD. Thus, increased SOD production has been reported in association with ethanol as a substrate (20) and with increased metal supply (17); Hoffman et al. (27) reported increased SOD production by *Campylobacter* spp. grown in *Brucella* sp. media containing sulfite, $FeSO_4$, and pyruvate. Also, specific increases in the production of SOD have been related to the presence of redox active dyes which disrupt the usual course of catabolism to produce O_2^- internally (21–24). Conceivably, the function of the keto acids in (i) stimulating growth, (ii) stimulating the production of SOD in the absence of light,

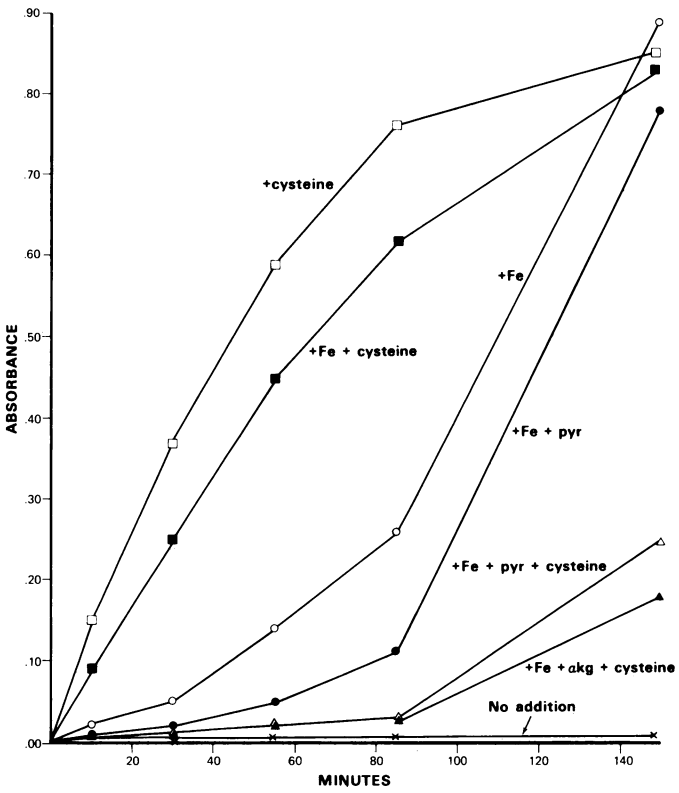

FIG. 6. Reaction of Fe, cysteine, and keto acids of ABCD broth on reduction of NBT with riboflavin and fluorescent light. The "no addition" basal reaction mixture contained only riboflavin, NBT, and starch (see Materials and Methods). Single or combined additions were then made to the final concentrations of the ABCD broth; all curves depict reaction mixtures to which ACES buffer plus salts (solution 1) was added. The reaction curves for ACES buffer plus salts alone; plus pyruvate; plus alpha-ketoglutarate (α-kg); or plus Fe plus α-kg lie between the boundaries depicted by +Fe and +Fe + pyruvate (pyr). No other medium components such as vitamins, trace metals, or amino acids were tested. All reaction curves depicted are with uninoculated media.

and (iii) stimulating resistance to the toxic effects of external light-induced O_2^- in *L. pneumophila* and *L. dumoffii* is related to the potential chelating activities of these two acids. Although we have used 0.1% keto acids, the acids themselves stimulate growth at 0.02 to 0.04% in the ABCD broth, and it would also appear possible that the primary effect of pyruvate or alpha-ketoglutaric acid or both related more directly to one or more internal metabolic steps. Pyruvate is rapidly oxidized by cell suspensions, whereas alpha-ketoglutarate is used poorly or not at all (29, 51, 56). By virtue of their transamination reactions, these keto acids may function in the mechanism by which the amino acid substrates enter the cell (25).

That the basal ABCD medium itself might be a source of hydrogen peroxide which limited growth of *Legionella* spp. was suggested by numerous reports in which hydrogen peroxide was directly or indirectly demonstrated in bacteriologic media (5, 7, 10, 28, 36, 37). In certain of these reports, the addition of catalase to the medium relieved the inhibition of growth (5, 7, 10, 14, 28, 37). However, the addition of catalase or peroxidase to synthetic media has not stimulated growth in the experiments reported here or previously (41). However, others (30, 31) have shown that addition of catalase, SOD, mannitol, and ethanol inhibits the killing of *Legionella* cells by an in vitro myeloperoxidase system (30, 31). Cysteine rapidly scavenges hydrogen peroxide, and, conversely, cysteine, in association with tryptophane or methionine, or in association with Fe and Cu, is known to form hydrogen peroxide (7, 8, 36, 37). We did not demonstrate directly the production of hydrogen peroxide, but more sensitive methods may have been required (37). However, an indirect suggestion of hydrogen peroxide formation was made with the observation of acetate and succinate formed in the presence of pyruvate and alpha-ketoglutarate, respectively. These products were seen only in light-exposed, uninoculated media. Hydrogen peroxide might arise from the oxidation of thiols in the presence of light (8) or from an Fe complex-catalyzed dismutation of O_2^- (18) formed by the reaction of light-activated riboflavin with methionine, glycine, or cysteine of the medium (12, 16). That Fe^{2+} is rapidly oxidized to Fe^{3+} during the preparation of the ABCD broth is readily apparent from the deep blue-purple color (Fe^{3+}-cysteine complex) (50) formed as the medium is brought from a strong acid pH to pH 6.5.

The very strong inhibition by added Fe^{2+} of the O_2^--generating systems with ACES has a parallel in the inhibition of O_2^- production by Fe-EDTA complexes rather than free Fe^{2+} or EDTA alone, and the differential abilities of these complexes to compete with other electron-accepting systems has been well described (2, 4, 18, 19, 35). The effect of Fe^{2+} in the presence of EDTA on the NBT assay for O_2^- production (1) was similar to that reported by Halliwell (18). But the reduction of NBT may occur directly without the mediation of O_2^- (39). Thus the formation of formazan by the addition of cysteine to the ACES salts plus keto acids can be attributed to the formation of O_2^- since the addition of SOD essentially eliminated this reduction of NBT (Table 2). The addition of Fe^{2+} to this system of cysteine plus keto acids in the ABCD broth did not completely inhibit the reduction of NBT. The reduction of NBT which did occur in the presence of Fe^{2+} was not inhibited by SOD and consequently this reduction does not appear to be O_2^- mediated. However, it too was light mediated. Although the potential of the ABCD broth to produce the highly toxic OH · radical was recognized (1, 2, 19, 35), we observed no effect on growth by the addition of mannitol or combined additions of reduced-oxygen-scavaging enzymes.

In summary, the results presented permit several conclusions pertaining to the growth of *Legionella* spp. (i) The growth responses of *Legionella* species to the basal or complete ABCD broths cannot be correlated with their individual synthesis of catalase, peroxidase, and SOD since each of the species reacted differently. (ii) That the additions of catalase, peroxidase, and SOD, individually or in combination, did not stimulate growth under light or without light suggests that hydrogen peroxide and O_2^- do not play a metabolic role in the cells' environment as we have tested it and most likely reduced oxygen radicals external to the cell are not responsible for the observed enzymatic responses observed. (iii) By some unknown mechanism, pyruvate and alpha-ketoglutarate shorten the lag period and increase the rate of growth in the absence of light. (iv) These two keto acids reduce the rate of cysteine oxidation in the presence or absence of light, and they scavenge hydrogen peroxide produced in the ABCD broth incubated under light. (v) Growth of *Legionella* species is strongly inhibited in the ABCD broth by the reaction of light; this inhibition is correlated with the reaction of light, riboflavin, and ACES to form O_2^-. This latter reaction is stimulated by cysteine but is

somewhat reduced by added Fe^{2+}. (vi) The addition of pyruvate or alpha-ketoglutarate to the ABCD broth has little effect on the formation of O_2^- in the presence of cysteine with no added Fe^{2+}, but there is a very strong repression of O_2^- formation when these acids are added to cysteine with added Fe^{2+}, and there is also a large decrease in the formazan produced by a second reaction involving light, cysteine, and added Fe^{2+}. These results suggest that keto acids, added Fe^{2+}, and cysteine function together to stimulate growth of *Legionella* spp. in the absence of light.

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