SECRETION OF PYRUVATE

An Antioxidant Defense of Mammalian Cells

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Mammalian cells in vivo must cope with the effects of reactive oxygen intermediates generated by aerobic metabolism (1), by the catabolism of drugs and other xenobiotics (2), by x-rays (3), and by the respiratory burst of phagocytic cells (4). Similarly, cells in vitro encounter reactive products generated in the culture medium from the autooxidation of ascorbate (5) and sulfhydryls, notably cysteine (6–9), and photooxidation of medium components such as riboflavin, tyrosine, and tryptophan (10–12). Cells are usually cultured in 95% air, which provides an oxygen tension far higher than that in most body fluids, exacerbating oxidative stresses. Toxic reaction products generated include singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide (H_2O_2). Of these, only H_2O_2 has a long half-life and accumulates in the medium. There are major gaps in our understanding of the biochemistry of oxidative injury and antioxidant defense. For example, we cannot yet fully explain the striking variation in sensitivity of different cell types to lysis by H_2O_2 (13, 14). Differences in the function of catalase and of the glutathione $(GSH)^1$ redox cycle account for only a portion of this variation (13–14).

We are currently testing the hypothesis that α-ketoacids may be important targets for injury of cells by H_2O_2. It has been known since 1904 that H_2O_2 causes a rapid, nonenzymatic, and stoichiometric decarboxylation of pyruvate (15, 15a). Decomposition of pyruvate, oxaloacetate, and α-ketoglutarate could cripple cellular synthesis of ATP from both glucose and glutamine (16). A precipitous decline in ATP appears to be a critical early step in cytolysis induced by H_2O_2 (17). While testing these ideas, we questioned why many cell culture media formulations include pyruvate in addition to glucose. The practice stems

^{1} *Abbreviations used in this paper:* αMEM, alpha-modified minimum Eagle's medium; BSTFA, N,O-bis-(trimethylsilyl)trifluoroacetamide; DPBS, Dulbecco's PBS; GC-MS, gas chromatography–mass spectral analysis; GSH, glutathione; HS, horse serum; LDH, lactic dehydrogenase; M-5HS, αMEM and HS; MOX, hydroxylamine in pyridine; MSD, mass-selective detector.

from empiric observations a quarter century ago that pyruvate promotes the survival of cells at low plating densities (18–21). Contemporary studies demonstrate that pyruvate can substitute for conditioned medium (22) or serum (23–25) in supporting the growth of certain cells. The growth-promoting effect of conditioned medium has been attributed to release of endogenous pyruvate from one cell type, astroglia (26). Higuchi (27) observed that pyruvate countered the toxic effect of exogenous cysteine and that catalase could substitute for pyruvate. However, despite these observations, no explanations have emerged either for the similarity of the cytoprotective effect of most conditioned media and pyruvate, or for the mechanism of cell protection by pyruvate.

In the work reported below we found that H_2O_2 decarboxylated α-ketoacids in a physiologic cell culture medium and that the reaction was rapid enough for exogenous pyruvate to protect cells from lysis by H_2O_2. All five types of mouse and human cells tested released pyruvate until the extracellular concentration approximated that observed in plasma and serum. Among the eight Embden-Meyerhof and Krebs cycle intermediates measured in P815 cells, export was limited to pyruvate and lactate. Exogenous catalase enhanced the rate of accumulation of extracellular pyruvate. These results suggest that pyruvate is specifically secreted, and functions extracellularly as an antioxidant.

Materials and Methods

Materials. α-modified minimum Eagle's medium (αMEM), RPMI, sodium pyruvate (for tissue culture), and glutamine were from KC Biological, Inc., Lenexa, KS. Both media were supplemented with 0.1 mM nonessential amino acids (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin; RPMI was further supplemented with 1 mM glutamine. The following were obtained from the indicated sources: horse serum (HS) (HyClone Laboratories, Logan, UT); Dulbecco's PBS without calcium and magnesium (DPBS; Gibco Laboratories); $Na_2{}^{51}CrO_4$ (620 mCi/mg Cr; New England Nuclear, Boston, MA); H_2O_2 (30% superoxol; Fisher Chemical Co., Fairlawn, NJ). The following were from Sigma Chemical Co., St. Louis, MO: catalase (C 40) from bovine liver, 11,000 Sigma U/mg protein; lactic dehydrogenase (LDH) type XI (L 1245) from rabbit muscle, 75 U/mg protein; NADH grade III (N 8129) disodium salt from yeast; pyruvate (for biochemical studies), lactate, fumarate, citrate, succinate, α-ketoglutarate, malate, and oxaloacetate. For GC-MS experiments, oxaloacetic acid was 90–95% pure; all other acids were >98% pure. Octadecane, hydroxylamine hydrochloride, and anhydrous pyridine (stored over calcium hydride) were from Aldrich Chemical Co., Milwaukee, WI. 2% hydroxylamine in pyridine (MOX) was freshly prepared before use. N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was from Pierce Chemical Co., Rockford, IL.

Cell Cultures. Mouse cell lines used were: P815 mastocytoma (nonadherent), P388 lymphoma (nonadherent), and L929, a connective tissue line (adherent). Human cell lines used were: CCD-21SK, diploid skin fibroblasts (adherent), and SK-BR-2-III (nonadherent) and CAMA-1 (adherent) breast adenocarcinomas. Cells were cultured in a humidified atmosphere of 95% air, 5% CO_2. Adherent cells were grown to confluence in 60-mm polystyrene tissue culture plates. Cell counts were obtained by individually trypsinizing two plates and counting the cells in a hemocytometer. The mean of these counts was taken to be representative for the remaining plates. At the start of the experiment the medium was aspirated from the cells and they were washed three times with 3 ml RPMI at 37°C and then brought to the desired cell density (usually 2.5 × 10⁶/ml) by adding the appropriate volume of this medium. The time of this final addition was taken as time 0 for the experiment. Nonadherent cells were washed three times in 20 ml RPMI at 37°C by centrifugation at 550 g for 7 min and then they were suspended at the desired

density (usually 2.5 × 10⁶/ml) in RPMI. The time of this final resuspension was taken as time 0 for the experiment. Nonadherent cells were cultured in 13-ml polystyrene culture tubes (No. 2057; Falcon Labware, Oxnard, CA) secured at about 30° from the vertical on a platform rotating at 100 rpm.

Fluorimetric Assay for Pyruvate. To measure pyruvate in the medium, an aliquot (usually 200 μl) was withdrawn from the culture and centrifuged at 10,000 g for 10 s in a microcentrifuge (5414; Eppendorf; Brinkmann Instruments Inc., Westbury, NY). For adherent cells, a separate 60-mm plate was used for each measurement. From the centrifuged supernatant, 100 μl was transferred to a 5-ml glass tube and assayed immediately, or stoppered and frozen at −80°C for assay within 3 d. For the fluorometric assay, 100 μl of 0.5 M ice-cold perchloric acid was added to this 100-μl sample. After 5 min on ice the perchloric acid was neutralized with 40 μl 2.5 M KHCO₃. This solution was degassed under vacuum for 15 min on ice.

To measure intracellular pyruvate all medium was aspirated from adherent or pelleted nonadherent cells. The cells were then incubated in 500 μl of 0.25 M ice-cold perchloric acid for 5 min, transferred (adherent cells were scraped with a rubber policeman) to a 1.5-ml conical tube and centrifuged at 10,000 g for 10 s. An aliquot of the supernatant was neutralized with KHCO₃ and degassed as described above.

The fluorimetric assay was a modification of the method of Lowry and Passonneau (28). Pyruvate was converted to lactate by LDH in the presence of NADH. The loss of fluorescence (excitation 340 nm, emission 460 nm) as NADH was converted to NAD was followed using a SPEX F112A photon-counting spectrofluorometer fitted with a cooled photomultiplier (Spex Industries, Inc., Edison, NJ). The assay system consisted of 0.4 ml 0.125 M sodium phosphate, pH 7.4, containing 2.5 μmol NADH. To this we added 100 μl of the sample to be assayed and we established the baseline fluorescence. The reaction was started by adding 10 μl (400 μg/ml) LDH and fluorescence followed until no further change was observed. Pyruvate standards were carried through the same extraction procedure (perchloric acid, KHCO₃, degassing) and measured in the same manner to provide a standard curve. It was necessary to screen reagent stocks and to use acid-washed glassware to minimize fluorescent contamination.

The modifications implemented in the fluorescent pyruvate assay (28) resulted in linear and reproducible standard curves (3% SE among 19 standard curves) and dose-responsive measurements of released and cell-associated pyruvate. Addition of exogenous pyruvate to perchloric acid–treated cells resulted in a 97 ± 5% recovery (mean ± SE, n = 13). The limit of sensitivity of the modified assay was 0.084 nmol/100 μl sample, reflecting an original pyruvate concentration in a nonextracted sample of 2.0 μM. This limit is based on the mean value for mock samples (pyruvate-free samples brought through the extraction procedure) + 1 SD (n = 19). The modified fluorescent assay agrees with GC-MS measurements of the same samples (r = 0.98). Pyruvate in RPMI 1640 was stable at 37°C or −70°C for the periods of accumulation and storage used in this study.

Gas Chromatography—Mass Spectral Analysis (GC-MS). A stock solution of acids was prepared in water and neutralized with NaOH. Aliquots were diluted into vials and frozen in dry ice–acetone. Aliquots (0.5 ml) of culture medium were prepared in a similar manner. MOX (150 μl) was added to the vials after lyophilization for 1 h. After incubation at 4°C overnight, 250 μl BSTFA was added along with 2 μl of the internal standard solution in pyridine. The derivative mixture was allowed to stand another 30 min before a 2-μl sample was injected into the GC-MS system. Derivatives were prepared from lyophilized medium or cell residue in a similar manner, except that the vials of the latter were centrifuged briefly before removal of a sample for injection.

GC-MS analyses of derivatives were performed using a HP5890A gas chromatograph and HP5970B mass-selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA). The HP59970A workstation is controlled by a series 200 computer with software for acquisition and analysis of GC-MS data. MSD source temperature was 200°C and electron ionization energy was 70 eV. The 0.2-mm inner diam glass capillary column, either 12.5 or 15 m in length, was coated with a 0.33 μm crosslinked dimethyl silicone film. The column oven was maintained at 70°C for 3.5 min after injection, increased at 5°C/min
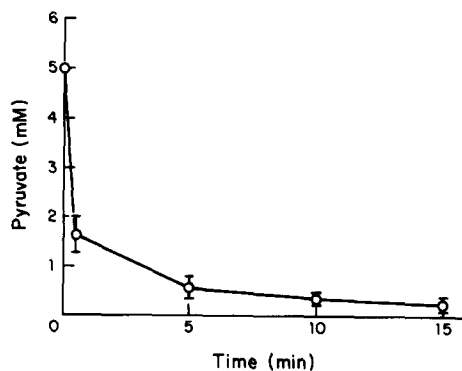
FIGURE 1.   The reaction of H_2O_2 and pyruvate in RPMI 1640 medium. Pyruvate (5 μmol) and H_2O_2 (5 μmol) in 1 ml RPMI 1640 were incubated at 37°C and the reaction was stopped at indicated times by addition of 1,000 Sigma units of catalase. Residual pyruvate was determined fluorometrically. Means ± SE, $n = 6$.

to 190°C and then rapidly increased to 240–260°C to remove later-eluting components. The helium flow rate through the column was 1 cc/min and total carrier gas flow rate was 20 cc/min. Samples were injected using the splitless mode with a 2-min purge activation time and an injection port temperature of 250°C.

The acid derivatives and hydrocarbon internal standards were identified by comparison of their retention times and mass spectra with those of authentic compounds (29, 30), and those of the hydrocarbons showed characteristic fragmentation patterns. All spectra also contained the predicted parent ion, and matched reference spectra in the National Bureau of Standards mass spectral data base.

Cytolysis Assay.   Susceptibility of cells to lysis by H_2O_2 was determined by a ^{51}Cr release assay, as previously described (31).

Cell Water Space.   Cell water space was estimated by the method of Rottenberg (32) using ^3H_2O and [^{14}C]sorbitol and related to cell protein in each assay.

Protein.   We measured protein by the method of Lowry et al. (33) with BSA as the standard.

H_2O_2 Measurement.   Concentrations of H_2O_2 were measured using an oxygen electrode (model 53 Oxygen Monitor; Yellow Springs Instrument Co., Yellow Springs, OH) as twice the O_2 generated by catalase (14) or by the scopoletin assay (34).

Results

Reaction of H_2O_2 with Pyruvate and Other α-Ketoacids.   Using a complex cell culture medium, we confirmed the original reports (15, 15a) that pyruvate in the millimolar range reacts nonenzymatically with H_2O_2 in a 1:1 stoichiometry (data not shown). The $t_{1/2}$ of the reaction was less than 30 s when both reactants were initially present at 5 mM (Fig. 1).

To examine the specificity of this reaction for pyruvate, a mixture of 10 mM each of pyruvate, oxaloacetate, fumarate, malate, lactate, and citrate was exposed to 45 mM H_2O_2. Fig. 2 shows the GC-MS chromatogram of the organic acids before and 10 min after exposure to H_2O_2. The three α-ketoacids were totally degraded. In contrast, the acids lactate and malate were quantitatively recovered (100% and 101%, respectively), and the recovery of citrate was 92%. Some loss of fumarate (42%) may be attributable to the double bond on the α-carbon. The postreaction mixture showed the appearance of two new acids, malonate and succinate, the decarboxylation products of oxaloacetate and α-ketoglutarate,
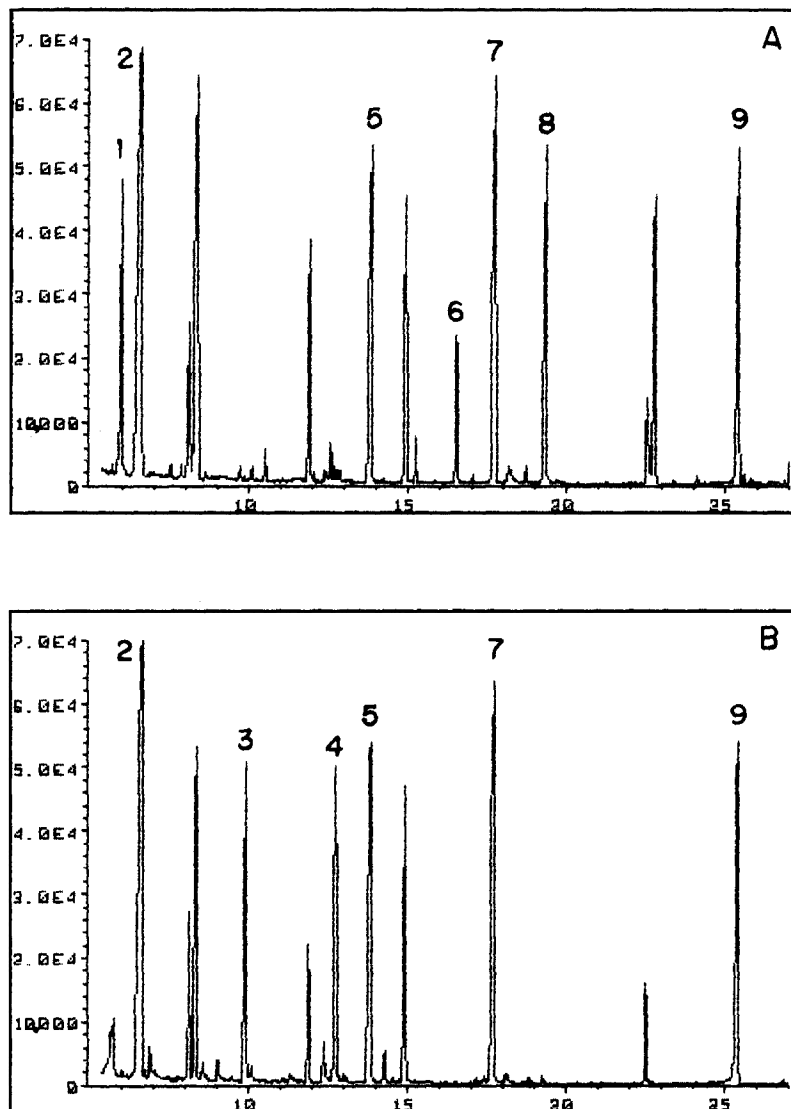
FIGURE 2. Specificity of reaction with H_2O_2 for α-ketoacids. A mixture of organic acids (pyruvate [1], lactate [2], fumarate [5], oxaloacetate [6], malate [7], α-ketoglutarate [8], and citrate [9]), each at 10 mM, was exposed to 45 mM of H_2O_2 for 10 min. Samples of the mixture before (A) and after (B) exposure to H_2O_2 were derivitized and analyzed by GC-MS. Succinate (4) and malonate (3) are the decarboxylation products of oxaloacetate and α-ketoglutarate, respectively. Acetate, the decarboxylation product of pyruvate, is too volatile to be carried through the derivitization. Ordinate, abundance; abscissa, retention time (min).

respectively. Acetate, the decarboxylation product of pyruvate, is too volatile to carry through the derivitization procedures, and was not detected.

Exogenous α-Ketoacids Protect Cells from Cytolysis by Exogenous H_2O_2. When ^{51}Cr-labeled, P388 mouse lymphoma cells were exposed to H_2O_2, the addition of pyruvate to the culture medium protected the cells against cytolysis in a dose-dependent manner. Fig. 3a shows the H_2O_2-induced cytolysis curves for P388
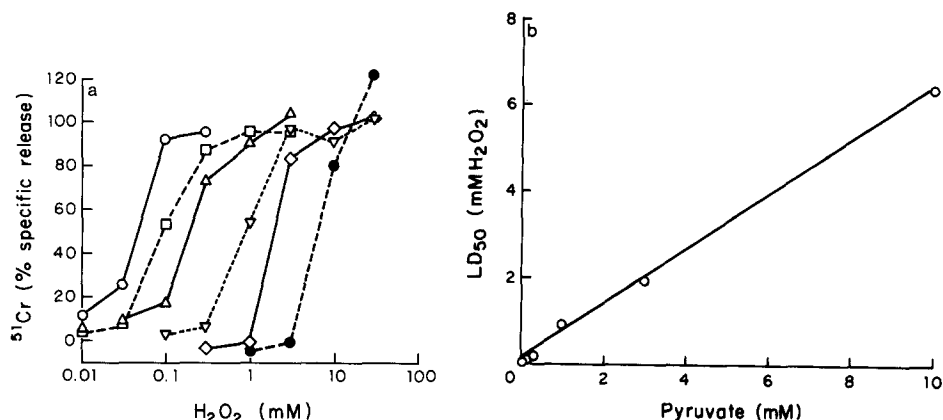
FIGURE 3. Effect of pyruvate on sensitivity of P388 cells to H_2O_2. 2×10^5 ^{51}Cr-labeled cells/ml in M-5HS were incubated in the presence or absence of H_2O_2 (10^{-5} to 3×10^{-2} M) and various concentrations of pyruvate for 3 h in 5% CO_2/95% air. (*a*) Specific ^{51}Cr release was plotted vs. H_2O_2 concentration. Added pyruvate (mM): 0 (O), 0.1 (□), 0.3 (△), 1.0 (▽), 3.0 (◇), 10 (●). (*b*) LD_{50} for H_2O_2 concentration was plotted vs. the pyruvate concentration added. Means of triplicates.

TABLE I

Effect of α- and β-Ketoacids on Sensitivity of P815 to H_2O_2

Acid	LD_{50}	Fold shift to right of dose-response curve
	mM	
None	0.08	1
Pyruvate	6.64	83
Oxaloacetate	5.19	65
α-Ketobutyrate	6.32	79
α-Ketocaproate	6.03	75
α-Ketoadipate	6.18	77
β-Ketoadipate	0.17	2

2×10^5 ^{51}Cr-labeled cells/ml in M-5HS were incubated with 10 mM of the indicated agents in the presence of various concentrations of reagent H_2O_2 for 3.5 h at 37°C in 5% CO_2, as shown in Fig. 3. Specific ^{51}Cr release was measured (31) and LD_{50} levels were calculated by interpolation. The fold shift to right in the H_2O_2 dose-response curve is the ratio of the LD_{50} in the presence of the indicated agent divided by that in the absence.

in the presence of 0–10 mM pyruvate. The H_2O_2 concentration resulting in 50% specific release of ^{51}Cr (LD_{50}) was a linear function of the concentration of pyruvate added ($r = 0.999$) (Fig. 3*b*). The inability of pyruvate to provide complete protection against the cytolytic effect of equimolar H_2O_2 presumably reflects the fact that H_2O_2 reacts simultaneously with both extracellular pyruvate and critical cellular components. We have previously shown (35) that exposure of P388 cells to H_2O_2 commits them within a few minutes to subsequent lysis. Exogenous pyruvate protected P815 mouse mastocytoma cells from H_2O_2 in a similar manner (Table I). All four other α-ketoacids tested substituted for pyruvate in this protective role, but a β-ketoacid was ineffective (Table I).
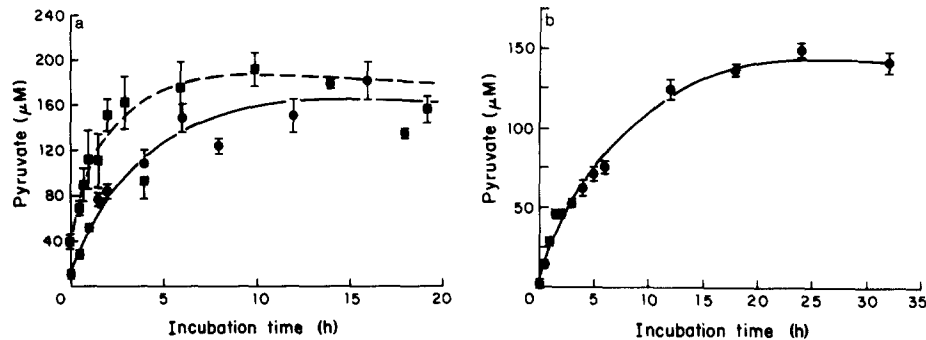
FIGURE 4. Release of pyruvate into the extracellular medium. Murine P815 cells (a) or normal diploid human fibroblasts (b) were incubated at 2.5 × 10⁶/ml in RPMI 1640 and then the medium and cells were prepared for fluorometric assay of pyruvate at the times indicated. (●) Extracellular pyruvate (μM), (■) cell-associated pyruvate (μM) as determined by measurements of nanomoles of pyruvate per microliter of water in a packed pellet. Means ± SE, n = 3–27.

Release of Pyruvate by Cells in Culture. Intracellular pyruvate dropped precipitously when cells were transferred to pyruvate-free medium. As the cells replenished their stores, equilibrium between intracellular and extracellular pyruvate was maintained (Fig. 4a). The kinetics of pyruvate release showed three phases (Fig. 4, a and b). A rapid, early release lasted ~2 h and averaged 9.4 μM/h/10^6 human diploid fibroblasts and 19.4 μM/h/10^6 P815 cells (cells cultured at 2.5 × 10^6/ml). This was followed by a slower phase lasting 4–10 h and averaging 3.1 μM/h/10^6 fibroblasts and 6.5 μM/h/10^6 P815 cells. Finally, a plateau was reached by 6–12 h, corresponding to reported levels of pyruvate in normal human plasma and serum (90–120 μM) (36, 37). Plateau values (micromolar pyruvate in medium, means ± SE for n experiments, cells cultured at 2.5 × 10^6/ml) were 138.0 ± 3.1 (n = 27) for fibroblasts; 60.4 ± 4.6 (n = 10) for CAMA-I human breast adenocarcinoma; 82.4 ± 5.2 (n = 3) for SK-BR-2-III human breast adenocarcinoma; 149.9 ± 8.0 (n = 15) for P815 cells; and 87.9 ± 4.0 (n = 2) for the L929 mouse connective tissue cell line.

The rate of accumulation of pyruvate in the medium was dependent on the initial cell density in the range of 10^4–10^6 cells/ml (Fig. 5a). At densities below 10^4 cells/ml, the release of pyruvate over 9 h was below the detection limit of our assay (Fig. 5b).

Other α-ketoacids and related metabolic intermediates, with the exception of lactate, were not released from P815 cells over the 4-h time course studied. GC-MS analysis of initially pyruvate-free medium, conditioned by P815 cells, showed that when extracellular pyruvate concentrations had risen close to plateau levels, there was almost no detectable extracellular succinate, fumarate, citrate, oxaloacetate, α-ketoglutarate, or malate (Table II).

Interrupting the conditioning of the medium with pyruvate by transferring the cells to fresh pyruvate-free medium resulted in a restart of the process. Cells interrupted in the steady-state condition (8 h after transfer) immediately resumed the rapid release characteristic of their first introduction into pyruvate-free medium (Fig. 6). Similar results were obtained when cells were transferred during the early build-up of pyruvate, 2–4 h after transfer to fresh medium (data
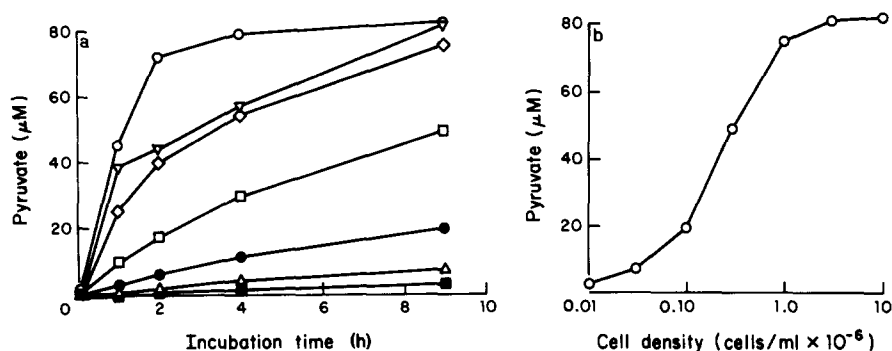
FIGURE 5.   Pyruvate release in relation to cell density. P815 mastocytoma cells were cultured at various densities in RPMI 1640 and pyruvate in the medium measured at various times. (a) Pyruvate (μM) in medium vs. time after plating, initial cell density per ml: 10^4 (■), 3×10^4 (△), 10^5 (●), 3×10^5 (□), 10^6 (◇), 3×10^6 (▽), 10^7 (○). (b) Pyruvate (μM) in medium at 9 h vs. initial plating density. Data are means of duplicates from one representative experiment.

TABLE II

GC-MS Analysis of Compounds Released into the Culture Medium by P815 Cells

Compound	Release after incubation (h)*				Reported intracellular concentration[‡]
	1	2	3	4	
	μmol/liter				μmol/liter
Pyruvate (fluorimetric)	41.9[§]	65.3	87.3	106	
Pyruvate	35.8	70.6	96.8	109	90
Succinate	0.3	1.1	1.8	2.7	
Fumarate	0.1	0.5	0.3	0.7	
Citrate	0.2	0.7	1.4	2.7	280
Oxaloacetate	ND[‖]	ND	ND	ND	
α-Ketoglutarate	ND	ND	ND	ND	380
Malate	ND	ND	ND	ND	1,320
Lactate	117	852	1,388	1,454	

* P815 cells were placed in RPMI (pyruvate-free) at 2.5×10^6 cells/ml. At the indicated times samples were removed and prepared for GC-MS, or where indicated for fluorimetric analysis.

‡ Values expressed in micromoles/liter under comparable experimental conditions are not readily available. Examples are included for HeLa cells (16) and for guinea pig heart (36).

§ Means of duplicates less medium blank.

‖ Below the limit of detection (0.1–0.5 μmol/liter of medium, depending on the compound).

not shown). Cells placed in medium containing 150 μM pyruvate at time 0 maintained the pyruvate concentration at this initial level (Fig. 6).

We hypothesized that the slowed rate of accumulation of pyruvate after 2 h (second phase, Fig. 4) represented a loss of pyruvate to a side-reaction, rather than a change in the rate of production or release by the cells. This could be accounted for either by delayed onset of H_2O_2 production in the cultures or by exhaustion of another antioxidant in the medium that protects pyruvate from reaction with H_2O_2 during the first 2 hours. To test this hypothesis we followed the accumulation of pyruvate in P815 cultures after transfer into pyruvate-free
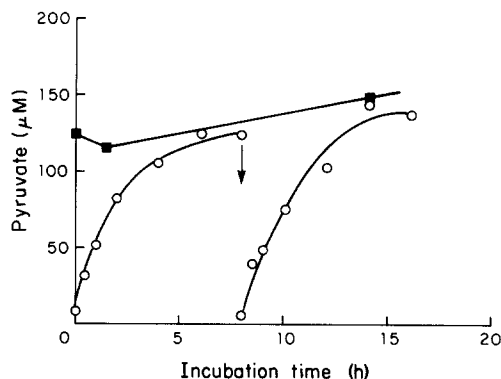
FIGURE 6. Release of pyruvate after transfer into fresh medium. P815 cells cultured as described for Fig. 4 were transferred into fresh pyruvate-free medium at the start of the experiment and again 8 h later (*arrow*). Medium was collected for fluorimetric pyruvate assay at the times indicated (O). A partner set of cells was placed in RPMI 1640 supplemented with 150 μM pyruvate at time 0 (■). Data are means of 2–4 samples from one representative experiment.
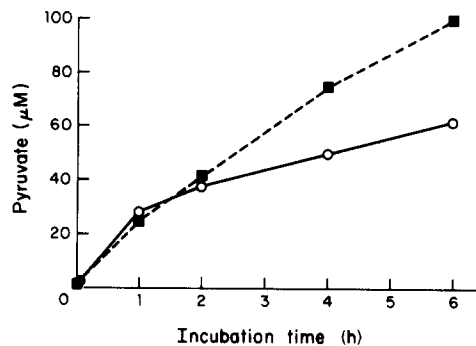

FIGURE 7. Enhanced pyruvate accumulation in the presence of catalase. Fibroblasts were cultured as for Fig. 4 in the presence (■) or absence (O) of 1,000 Sigma U/ml catalase and medium was collected for fluorometric assay at the times indicated. Means of duplicates from one representative experiment.

medium with and without 1,000 Sigma U/ml of added catalase. The presence of catalase had little effect on pyruvate accumulation over the first 2 h (Fig. 7), but thereafter the rate of accumulation of pyruvate in the presence of catalase was increased 76 ± 28% (n = 6) compared with the noncatalase controls.

Discussion

It is surprising that mammalian cells in culture divert a substantial portion of their potential energy supply to the export of pyruvate, and that this has apparently been documented for only one cell type (26). Because the export of pyruvate appears to be specific and widespread among cells of at least two species, we presume it subserves a physiological function commensurate with its cost to the cell.

In 1962, Eagle and Piez (38) summarized several reported demonstrations of a pyruvate requirement for the replication of cells at low population densities.

To explain the paradox that cells required something they themselves produced, the authors theorized that at low but not high cell population densities, pyruvate was lost to the medium faster than cells could make it. This requirement "disappeared at cell densities sufficiently large to bring the concentration in the medium and in the cellular pool to metabolically effective levels before the cell died of the specific deficiency" (38). However, the postulated release of pyruvate was never demonstrated. This may have been due in part to the technical difficulty of measuring pyruvate by NADH-linked enzymatic assays in the presence of interfering substances, including LDH, in cell-conditioned culture media. We have carefully optimized an enzymatic assay for pyruvate in cell culture medium, and validated it by reference to GC-MS analysis. In agreement with Eagle's and Piez's hypothesis, we have found that (a) cells rapidly release pyruvate into the extracellular medium, (b) the rate of accumulation is dependent on cell density, and (c) the concentration of intracellular pyruvate corresponds closely to that of extracellular pyruvate at all times. At low plating density, the increased time required by cells to condition the extracellular medium to homeostatic levels of pyruvate prolonged the depletion of intracellular pyruvate, thus exposing cells to the deleterious effects of oxidants, as well as to other metabolic constraints, for many hours. Further, we have used the GC-MS technique to analyze cell-conditioned medium simultaneously for eight glycolytic and Krebs cycle intermediates. Only pyruvate and lactate were released from P815 cells, even though the intracellular concentrations of some of the other intermediates were expected to exceed that of pyruvate. Thus, neither cell lysis nor nonspecific permeability of the plasma membrane could account for the accumulation of pyruvate in cell-conditioned medium. Pyruvate release appears to be a specific secretory phenomenon.

All the mouse and human cells tested, whether normal or malignant, were able to bring both intracellular and extracellular pyruvate to a homeostatic level (60–150 μM), corresponding approximately to that in human plasma and serum (36, 37) as long as they were plated at $\geq 10^4$ cells/ml. When we replaced conditioned medium with pyruvate-free medium, extracellular accumulation of pyruvate commenced by the first time point studied and paralleled intracellular levels until both had reached homeostatic levels. When conditioned medium was replaced with fresh medium supplemented with 150 μM pyruvate, no accumulation of pyruvate was induced. When we varied the ratio of cell number to volume of the medium, the amount of pyruvate secreted per cell was not fixed, but varied in such a way as to bring the extracellular concentration of pyruvate to the plateau level found intracellularly for each cell type. We have no understanding of what determines the homeostatic set point for intracellular/extracellular pyruvate. The plateau was not due to exhaustion of glucose in the medium (data not shown). Extracellular pyruvate could not arise from secreted lactate via the action of LDH and NAD released from dying cells, because the equilibrium constant of the reaction is 2×10^{-12} M in favor of lactate (39).

Other carboxylated metabolic intermediates (succinate, fumarate, citrate, oxaloacetate, α-ketoglutarate, malate) were not exported from P815 cells in significant amounts, except lactate. The flux of lactate was even greater than that of

pyruvate. The operation of a specific transmembrane carrier system for these two compounds would be consistent with these findings. Because of the close similarity of pyruvate and lactate, a carrier system might mediate the movement of both. A pyruvate carrier has been demonstrated in mitochondria (40–45), erythrocytes (40), and intestinal epithelial cells (46). The lactate carrier in hepatocyte membranes binds pyruvate, and this inhibits lactate transport (47–49). The probable role of the lactate carrier as a pyruvate transporter has apparently not been considered.

Antioxidant functions have been ascribed to many molecules, but few of them are known to appear extracellularly in the absence of cell lysis. Plasma urate (0.3 mM) is prominent among the components of extracellular fluid that react nonspecifically with hydroxyl radical (50). Ceruloplasmin serves as a superoxide dismutase (51) in plasma. Some cells may secrete a novel superoxide dismutase (52). Plasma ascorbate (27–50 μM) (53) and tocopherol (24 μM) (54) may scavenge H_2O_2. Plasma glutathione is reported to be 21 μM in the reduced form (55) that is reactive with H_2O_2. Thus, pyruvate plasma or serum levels (90–120 μM [36, 37]) may be the major scavenger of H_2O_2 in extracellular fluid. The observation that exogenous catalase enhanced the rate of accumulation of secreted pyruvate strongly suggests that pyruvate actually functions as an extracellular scavenger of H_2O_2 arising in cell culture.

The ability of exogenous pyruvate to protect bacteria from endogenous or exogenous oxidants has been recognized since 1934 (56–64). However, the ability of exogenous pyruvate to protect mammalian cells from H_2O_2 was apparently first reported only in 1985 (65), while this study was in progress. Indeed, exogenous pyruvate has probably acted as an unrecognized antioxidant in many studies of the toxicity of H_2O_2 to mammalian cells. Moreover, the use of pyruvate-free medium in many other studies has no doubt imposed metabolic stress. These points should now be considered in the design, interpretation, and comparison of studies of oxidant injury. More generally, cell culture techniques should now take the role of pyruvate into account on a rational basis, rather than on the empiric basis formerly used. This may be especially important in cloning and in establishing cultures ex vivo. Finally, because pyruvate is presumably nontoxic, and because it is freely diffusible from the intravascular to the extravascular, intercellular, and intracellular spaces, consideration should be given to testing whether administration of pyruvate might afford a beneficial antioxidant effect in certain pathophysiologic states.

There is an obvious advantage to cells in scavenging exogenous H_2O_2 before it reaches the cell. It is likely that intracellular α-ketoacids also scavenge intracellular H_2O_2, but this remains to be demonstrated. Future studies will analyze whether differences in the capacity to synthesize and secrete pyruvate during oxidant stress may underlie some of the hitherto unexplained variation among cell types in susceptibility to oxidant injury.

Summary

Cells in culture are exposed to marked oxidative stress, H_2O_2 being one of the predominant agents. Pyruvate and other α-ketoacids reacted rapidly, stoichiometrically, and nonenzymatically with H_2O_2, and they protected cells from its

cytolytic effects. All five human and murine cell types studied, both malignant and nonmalignant, released pyruvate at an initial rate of 35–60 μM/h/2.5 × 10^6 cells when placed in 1 ml pyruvate-free medium. After 6–12 h a plateau of 60–150 μM pyruvate was attained, corresponding to concentrations reported for normal human serum and plasma. The rate of pyruvate accumulation was almost doubled in the presence of exogenous catalase, suggesting that released pyruvate functions as an antioxidant. The rate of pyruvate accumulation was dependent on cell number. Succinate, fumarate, citrate, oxaloacetate, α-ketoglutarate, and malate were not secreted in significant amounts from P815 cells; export was specific for pyruvate and lactate among the metabolites tested. Extracellular pyruvate was in equilibrium with intracellular stores. Thus, cells conditioned the extracellular medium with pyruvate at the expense of intracellular pyruvate, until homeostatic levels were attained in both compartments. We propose that cells plated at low density in the absence of exogenous pyruvate fail to thrive for two reasons: prolonged depletion of intracellular pyruvate and prolonged vulnerability to oxidant stress.

References

1. Chance, B., H. Sies, and A. Boveris. 1979. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* 59:527.
2. Hassan, H. M., and I. Fridovich. 1979. Intracellular production of superoxide radical and hydrogen peroxide by redox active compounds. *Arch. Biochem. Biophys.* 196:385.
3. Pryor, W. A. 1976. The role of free radical reactions in biological systems. *In* Free Radicals in Biology. Vol. 1. W. A. Pryor, editor. Academic Press, New York. 1–49.
4. Badwey, J. A., and M. L. Karnovsky. 1980. Active oxygen species and the functions of phagocytic leukocytes. *Annu. Rev. Biochem.* 49:695.
5. Bielski, B. M. J. 1982. Chemistry of ascorbic acid radicals. *In* Ascorbic Acid: Chemistry, Metabolism, and Uses. P. A. Seib and B. M. Tolbert, editors. American Chemical Society, Washington, DC. 81–100.
6. Cavallini, D., C. DeMarco, and S. Dupre. 1968. Luminol chemiluminescence studies on the oxidation of cysteine and other thiols to disulfides. *Arch. Biochem. Biophys.* 124:18.
7. Carlsson, J., G. P. D. Granberg, G. K. Nyberg, and M. K. Edlund. 1979. Bactericidal effect of cysteine exposed to atmospheric oxygen. *Appl. Environ. Microbiol.* 37:383.
8. Misra, H. P. 1974. Generation of superoxide free radical during the autooxidation of thiols. *J. Biol. Chem.* 249:2151.
9. Arrick, B. A., W. Griffo, Z. Cohn, and C. Nathan. 1985. Hydrogen peroxide from cellular metabolism of cystine. A requirement for lysis of murine tumor cells by vernolepin, a glutathione-depleting antineoplastic. *J. Clin. Invest.* 76:567.
10. McCormick, J. P., J. R. Fischer, J. P. Patchlatko, and A. Eisenstark. 1976. Characterization of a cell-lethal product from the photooxidation of tryptophan: hydrogen peroxide. *Science (Wash. DC).* 191:468.
11. Wang, R. J., and B. T. Nixon. 1978. Identification of hydrogen peroxide as a photoproduct toxic to human cells in tissue-culture medium irradiated with "daylight" fluorescent light. *In Vitro (Rockville).* 14:715.

12. Jernigan, H. M., Jr. 1985. Role of hydrogen peroxide in riboflavin sensitized photodynamic damage to cultured rat lenses. *Exp. Eye Res.* 41:121.

13. Nathan, C. F., B. A. Arrick, H. W. Murray, N. M. DeSantis, and Z. A. Cohn. 1981. Tumor cell anti-oxidant defenses. Inhibition of the glutathione redox cycle enhances macrophage-mediated cytolysis. *J. Exp. Med.* 153:766.

14. O'Donnell-Tormey, J., C. J. DeBoer, and C. F. Nathan. 1985. Resistance of human tumor cells *in vitro* to oxidative cytolysis. *J. Clin. Invest.* 76:80.

15. Holleman, A. F. 1904. Notice sur l'action de l'eau oxygénée sur les acides α-cétoniques et sur les dicétones 1.2. *Recl. Trav. Chim. Pays-bas Belg.* 23:169.

15a. Hatcher, W. B., and A. C. Hill. 1928. Hydrogen peroxide as an oxidizing agent in acid solution. IX. The oxidation of keto acids. *Trans. R. Soc. Can.* 22:211.

16. Reitzer, L. J., B. M. Weiss, and D. Kennell. 1979. Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J. Biol. Chem.* 254:2669.

17. Spragg, R. G., D. B. Hinshaw, P. A. Hyslop, I. U. Schraufstatter, and C. G. Cochrane. 1985. Alterations in adenosine triphosphate and energy charge in cultured endothelial and P388D$_1$ cells after oxidant injury. *J. Clin. Invest.* 76:1471.

18. Neuman, R. E., and T. A. McCoy. 1958. Growth-promoting properties of pyruvate, oxalacetate and α-ketoglutarate for isolated Walker carcinosarcoma 256 cells. *Proc. Soc. Exp. Biol. Med.* 98:303.

19. Herzenberg, L. A., and R. A. Roosa. 1960. Nutritional requirements for growth of a mouse lymphoma in cell culture. *Exp. Cell Res.* 21:430.

20. Moser, H. 1960. Modern approaches to the study of mammalian cells in culture. *Experientia (Basel).* 16:385.

21. Neuman, R. E., and A. A. Tytell. 1961. Partial replacement of serum and embryo extract requirements for growth of avian cell cultures. *Proc. Soc. Exp. Biol. Med.* 106:857.

22. Ellem, K. A. O., and G. F. Kay. 1983. The nature of conditioning nutrients for human malignant melanoma cultures. *J. Cell Sci.* 62:249.

23. Wasilenko, W. J., and C. Marchock. 1984. Pyruvate regulation of growth and differentiation in primary cultures of rat tracheal epithelial cells. *Exp. Cell Res.* 155:507.

24. McKeehan, W. L., and K. A. McKeehan. 1979. Oxycarboxylic acids, pyridine-nucleotide-linked oxidoreductases and serum factors in regulation of cell proliferation. *J. Cell. Physiol.* 101:9.

25. McKeehan, W. L., and K. A. McKeehan. 1980. Serum factors modify the cellular requirement for Ca^{2+}, K^+, Mg^{2+}, phosphate ions, and 2-oxocarboxylic acids for multiplication of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 77:3417.

26. Selak, I., S. D. Skaper, and S. Varon. 1985. Pyruvate participation in the low molecular weight trophic activity for central nervous system neurons in glia-conditioned media. *J. Neurosci.* 5:23.

27. Higuchi, K. 1963. Studies on the nutrition and metabolism of animal cells in serum-free media. I. Serum-free monolayer cultures. *J. Infect. Dis.* 112:213.

28. Lowry, O. H., and J. V. Passonneau. 1972. A Flexible System of Enzymatic Analysis. Academic Press. New York. 1–291.

29. Horning, M. G., E. A. Boucher, A. M. Moss, and E. C. Horning. 1968. Gas chromatographic study of derivatives of acids of the Krebs cycle and related compounds. *Anal. Lett.* 1:713.

30. Lawson, A. M., R. A. Chalmers, and R. W. E. Watts. 1974. Studies of O-substituted oxime-trimethylsilyl derivatives of some metabolically-important oxocarboxylic acids. *Biomed. Mass. Spectrom.* 1:199.

31. Nathan, C. F., L. H. Brukner, S. C. Silverstein, and Z. A. Cohn. 1979. Extracellular

cytolysis by activated macrophages and granulocytes. I. Pharmacologic triggering of effector cells and the release of hydrogen peroxide. *J. Exp. Med.* 149:84.

32. Rottenberg, H. 1979. The measurement of membrane potential and ΔpH in cells, organelles, and vesicles. *Methods Enzymol.* 55:547.

33. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.

34. Nathan, C. F., and R. K. Root. 1977. Hydrogen peroxide release from mouse peritoneal macrophages. Dependence on sequential activation and triggering. *J. Exp. Med.* 146:1648.

35. Nathan, C. F., L. H. Brukner, S. C. Silverstein, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. *J. Exp. Med.* 149:100.

36. Dijkstra, U., F. Gabreels, E. Joosten, R. Wevers, K. Lamers, W. Doesberg, and W. Renier. 1984. Friedreich's ataxia: intravenous pyruvate load to demonstrate a defect in pyruvate metabolism. *Neurology.* 34:1493.

37. Bunger, R. 1985. Compartmented pyruvate in perfused working heart. *Am. J. Physiol.* 249:H439.

38. Eagle, H., and K. Piez. 1962. The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize. *J. Exp. Med.* 116:29.

39. Passonneau, J. V., and O. H. Lowry. 1974. Pyruvate. Fluorimetric assay. Methods of Enzymatic Analysis. Vol. 3. H. U. Bergmeyer, editor. Academic Press, New York. 1452–1456.

40. Halestrap, A. P., and R. M. Denton. 1974. Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by α-cyano-4-hydroxycinnamate. *Biochem. J.* 138:313.

41. Papa, S., and G. Paradies. 1974. On the mechanism of translocation of pyruvate and other monocarboxylic acids in rat-liver mitochondria. *Eur. J. Biochem.* 49:265.

42. Halestrap, A. P. 1975. The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. *Biochem. J.* 148:85.

43. Halestrap, A. P. 1976. The mechanism of the inhibition of the mitochondrial pyruvate transporter by α-cyanocinnamate derivatives. *Biochem. J.* 156:181.

44. Paradies, G., and S. Papa. 1977. On the kinetics and substrate specificity of the pyruvate translocator in rat liver mitochondria. *Biochim. Biophys. Acta.* 462:333.

45. Halestrap, A. P., R. D. Scott, and A. P. Thomas. 1980. Mitochondrial pyruvate transport and its hormonal regulation. *Int. J. Biochem.* 11:97.

46. Lamers, J. M. J. 1975. Some characteristics of monocarboxylic acid transfer across the cell membrane of epithelial cells from rat small intestine. *Biochim. Biophys. Acta.* 413:265.

47. Monson, J. P., J. A. Smith, and R. A. Iles. 1982. Evidence for a lactate transporter in the plasma membrane of the rat hepatocyte. *Clin. Sci. (Lond.).* 62:411.

48. Welch, S. G., H. K. Metcalfe, J. P. Monson, R. D. Cohen, R. M. Henderson, and R. A. Iles. 1984. L(+)-Lactate binding to preparations of rat hepatocyte plasma membranes. *J. Biol. Chem.* 259:15264.

49. Fafournoux, P., C. Demigne, and C. Remesy. 1985. Carrier-mediated uptake of lactate in rat hepatocytes. Effects of pH and possible mechanisms of L-Lactate transport. *J. Biol. Chem.* 260:292.

50. Ames, B. N., R. Cathcart, E. Schwiers, and P. Hochstein. 1981. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc. Natl. Acad. Sci. USA.* 78:6858.

51. Bieri, J. G., L. Teets, B. Belavady, and E. L. Andrews. 1964. Serum vitamin E levels

in a normal adult population in the Washington, DC area. *Proc. Soc. Exp. Biol. Med.* 117:131.

52. Marklund, S. L. 1984. Extracellular superoxide dismutase in human tissues and human cell lines. *J. Clin. Invest.* 74:1398.

53. Spathis, G. S., and J. F. Hallpike. 1961. Observations upon the distribution of ascorbic acid in human blood. *Guy's Hosp. Rep.* 110:148.

54. Goldstein, I. M., H. B. Kaplan, H. S. Edelson, and G. Weissmann. 1979. Ceruloplasmin. A scavenger of superoxide anion radicals. *J. Biol. Chem.* 254:4040.

55. Anderson, M. E., and A. Meister. 1980. Dynamic state of glutathione in blood plasma. *J. Biol. Chem.* 255:9530.

56. Sevag, M. G., and Maiweg, L. 1934. The respiration mechanism of pneumococcus. III. *J. Exp. Med.* 60:95.

57. Baird-Parker, A., and E. Davenport. 1965. The effect of recovery medium on the isolation of *Staphylococcus aureus* after heat treatment and after storage of frozen or dried cells. *J. Appl. Bacteriol.* 28:390.

58. Martin, S. E., R. S. Flowers, and Z. J. Ordal. 1976. Catalase: its effect on microbial enumeration. *Appl. Environ. Microbiol.* 32:731.

59. Rayman, M. K., B. Aris, and H. B. El Derea. 1978. The effect of compounds which degrade hydrogen peroxide on the enumeration of heat stressed cells of *Salmonella senftenberg. Can. J. Microbiol.* 24:883.

60. Hoffman, P. S., H. A. George, N. R. Krieg, and R. M. Smibert. 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni.* II. Role of exogenous superoxide anions and hydrogen peroxide. *Can. J. Microbiol.* 25:8.

61. Pine, L., J. R. George, M. W. Reeves, and W. K. Harrell. 1979. Development of a chemically defined liquid medium for growth of *Legionella pneumophila. J. Clin. Microbiol.* 9:615.

62. McDonald, L. C., C. R. Hackney, and B. Ray. 1983. Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. *Appl. Environ. Microbiol.* 45:360.

63. Bolton, F. J., D. Coates, and D. N. Hutchinson. 1984. The ability of campylobacter media supplements to neutralize photochemically induced toxicity and hydrogen peroxide. *J. Appl. Bacteriol.* 56:151.

64. Pine, L., P. S. Hoffman, G. B. Malcom, R. F. Benson, and M. J. Franzus. 1986. Role of ketoacids and reduced-oxygen scavenging enzymes in the growth of *Legionella* species. *J. Clin. Microbiol.* 23:33.

65. Andrae, U., J. Singh, and K. Ziegler-Skylakakis. 1985. Pyruvate and related α-ketoacids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity. *Toxicol. Lett. (Amst.).* 28:93.