FREE RADICAL FORMATION AND SURVIVAL OF LYOPHILIZED MICROORGANISMS[1]

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ABSTRACT

HECKLY, ROBERT J. (University of California, Berkeley), R. L. DIMMICK, AND J. J. WINDLE. Free radical formation and survival of lyophilized microorganisms. J. Bacteriol. **85:**961–966. 1963.—A correlation between death and spontaneous free radical production, measured by an increase in the relative electron paramagnetic resonance (EPR) signal, was shown to exist for several species of microorganisms stored in the freeze-dried state, but the relationship between the free radical concentration and number of dead cells was not a simple proportion. Lactose added to *Sarcina lutea* reduced radical production and increased stability when dry preparations were stored in air. Death and free radical formation were more extensive when lyophilized *Streptococcus lactis* cultures were stored in air than in vacuum. Free radicals were also produced by dry yeast. Few, if any, free radicals were produced by bacteria or yeast stored in vacuum. It was shown that the observed free radical production was not caused by exposure to light. The EPR signal produced by dry *Serratia marcescens* decreased rapidly when cells were exposed to a humid atmosphere but the EPR signal slowly increased after cells were redried and exposed to oxygen.

Microorganisms preserved in the freeze-dried state are generally considered to be dormant, but there is evidence that some changes may occur during the storage period. Lion, Kirby-Smith, and Randolph (1961) showed, by means of electron paramagnetic resonance (EPR) that free radicals accumulated in dry *Escherichia coli* stored in the presence of oxygen. Dimmick Heckly, and Hollis (1961) found that the same phenomenon occurred in dry *Serratia marcescens* as a function of viability. It is well known that dry spores can be stored with little loss of viability. Windle and Sacks (1963), who investigated the EPR spectra of several species of bacterial spores, found no signal attributable to free radicals in clean, dry preparations, even after prolonged storage in air.

In an effort to extend these observations, and to investigate the effect of moisture, selected protective additives, and other environmental factors on the phenomenon of spontaneous free radical production in dry bacteria, we followed the changes in the intensity of EPR signals of several representative types of microorganisms, and made preliminary investigations of other factors influencing the characteristics of this effect.

MATERIALS AND METHODS

S. marcescens (8 UK) was prepared and examined as described by Dimmick et al. (1961). In one experiment, a suspension was divided; one portion was held in as near total darkness as possible during the drying, packing, and storage steps by wrapping the vials and tubes in aluminum foil and working in subdued light; the other portion was exposed to ambient light conditions. In another experiment, 0.1% cysteine hydrochloride was added to the cell suspension before the drying step. In a third, cells were subjected to incubation at 52 C for 10 min before being frozen. *S. marcescens* was grown at 35 to 37 C and washed in 0.05 M phosphate buffer (pH 7.0) to obtain the data in Fig. 5. One portion was resuspended in the buffer fortified with 0.5% lactose.

Media and growth conditions for the other organisms are listed in Table 1. Cells in the maximal stationary phase of growth were harvested by centrifugation and washed once with dilute buffer

[1] Some of the results were described in a discussion at the Specialists Conference on Culture Collections, sponsored by the Canadian Committee on Culture Collections of Microorganisms, 27 and 28 August 1962, at Ottawa, Canada.

TABLE 1. *Conditions for cultivation of microorganisms*

Organism	Medium	Incubation
Sarcina lutea strain 1957*	Heart Infusion Broth	32 C, 24 hr, aerated
Streptococcus lactis strain C2†	Glucose-beef extract broth	35 C, 48 hr, static
Saccharomyces cerevisiae (Fleischmann's yeast)	Glucose-yeast extract broth	35 C, 24 hr, aerated and static

* Kindly supplied by B. Lamb, Suffield Experimental Station, Alberta, Canada.

† Kindly supplied by E. B. Collins, University of California, Davis.

solution; *Sarcina lutea* was washed and resuspended in 0.1 M phosphate buffer (pH 7.0) with or without 1% lactose. *Streptococcus lactis* and *Saccharomyces cerevisiae* were washed and resuspended in 0.01 M phosphate buffer (pH 7.0) containing 0.05% lactose.

For studies of viability, slurries of resuspended organisms in 6-ml spherical vials were frozen by slow cooling to about −15 C and dried by evacuating the vials, attached to a manifold, to pressures of 5 to 20 μ of Hg for 18 to 24 hr at room temperature. Reconstitution was accomplished by adding an appropriate volume of sterile distilled water to the dried material. Suspensions were held for 10 to 20 min before a sample was taken for routine assay of viability.

For the EPR studies, two methods were used to prepare and pack samples into the small 4-mm tubes. In the first method, organisms were dried in 60-ml vaccine bottles, then transferred to quartz EPR tubes in a dry box. In the other procedure, a device (Fig. 1) was used to dry and pack the organisms without exposing them to air. About 2 ml of a cell suspension were frozen and dried in the bulb portion, with the device in a horizontal position. After drying was complete, the device was rotated to a vertical position and tapped to dislodge the pellet. The cells were then compressed into the bottom of an EPR tube with a 2.5-mm stainless steel rod. Thus, cells could be packed under any desired gaseous system without atmospheric contamination.

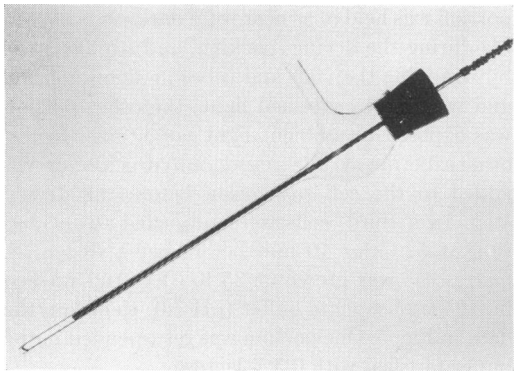

FIG. 1. *Device used for drying and packing organisms for free radical studies. Organisms were packed into the distal end, after which we flame-sealed and removed the tube.*

An X-band EPR spectrometer of our own construction (employing crystal detection, a Varian 6-in. electromagnet, and a 100 kc/sec multipurpose cavity) was used to obtain the EPR spectra of the samples. The first derivative of the EPR absorption curves was recorded, and the peak-to-peak amplitude, in arbitrary units, was used as a measure of the relative free radical concentration. Although instrument settings remained constant during the collection of data pertinent to each figure shown, conditions were not necessarily the same for the different experiments, and hence the relative signal intensity between figures cannot be directly compared.

RESULTS AND DISCUSSION

Lactose has been shown to preserve viability of dry bacteria during storage (Heckly, Anderson, and Rockenmacher, 1958; Heckly, 1961). In Fig. 2, we have compared the viability of cells with the formation of an EPR signal when *S. lutea* was dried with and without lactose and stored in air at room temperature. In the absence of lactose, both loss of viability and free radical production were greater than when lactose was present. Similar results were obtained when *S. marcescens* preparations were supplemented with lactose or cysteine; this effect is illustrated in Fig. 3. The presence of these additives markedly reduced the free radical production. The EPR signal strength was inversely related to the percentage of bacteria that survived after 6 days of storage (0.25% without additive, 7% with cysteine, and 15% with lactose), but the relationship was not one of simple proportionality.

Free radicals are produced by irradiation of many biological substances (Blois et al., 1961;

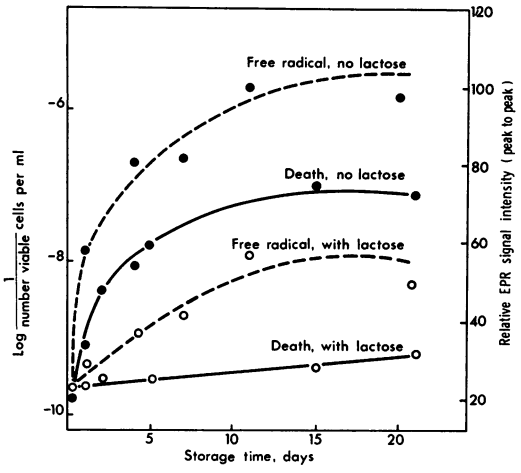

FIG. 2. *Effect of lactose on free radical formation and death of lyophilized Sarcina lutea stored in air.*

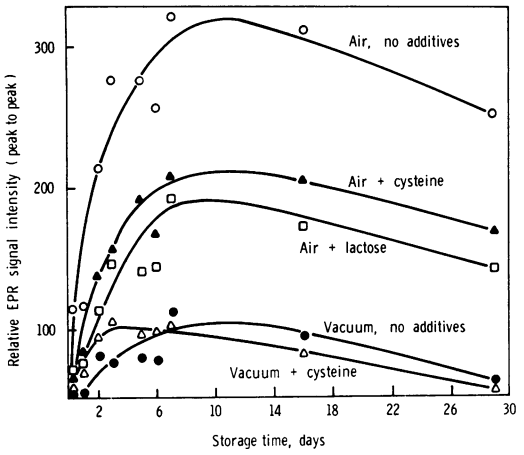

FIG. 3. *Effects of air, cysteine, and lactose on free radical production by dry Serratia marcescens.*

Powers, 1962). With the possibility in mind that ambient room light may have induced free radicals, we compared the signal intensity of foil-covered samples of *S. lutea* and *S. marcescens* with uncovered samples, and found that a significant signal appeared in all instances where cells had been stored in air. No significant signal appeared during the 40-day observation period in which cells had been stored in vacuum, regardless of irradiation. We conclude, therefore, that ambient room light was not responsible for free radical production in the dried organisms.

Dimmick et al. (1961) suggested that the oxygen transport mechanism was involved in spontaneous free radical production by dry bacteria. If this hypothesis were true, then no signal should arise in dried preparations of an anaerobe. The results of an experiment to test this hypothesis by following the change in signal intensity of *S. lactis* preparations are shown in Fig. 4. In the presence of air, most of the cells died, and the signal increased markedly, whereas in vacuum there was little loss of viability, and only a slight change in EPR signal intensity. Therefore, it is unlikely that the oxygen transport mechanism alone is responsible for the increase in EPR signal.

As with the bacteria, free radical production by dry yeast was correlated with death. The number of viable yeast cells decreased from 10^9 to 10^7 per ml of reconstituted sample during 80 days of storage in air, and the EPR signal increased markedly. There was no significant loss of viability or change in the signal intensity of samples stored in vacuum.

The effect of air, moisture, and lactose on the free radical concentration of *S. marcescens* preparations is illustrated in Fig. 5. There was no significant change in the minimal EPR signal of samples while under vacuum (E and F), but the signal of samples exposed to air (A, B, C, and D) increased rapidly, and reached a maximum after 8 to 12 days. The EPR signal of samples packed and sealed in air (C and D) increased at essen-

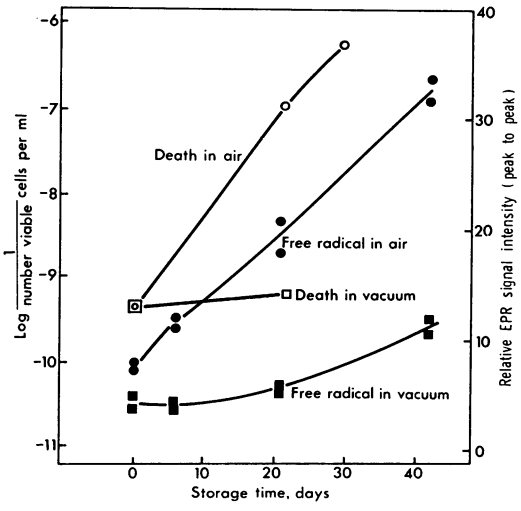

FIG. 4. *Effect of air on viability and free radical formation by Streptococcus lactis. Organisms were washed and suspended in 0.01 M phosphate buffer (pH 7.0) containing 0.5% lactose.*

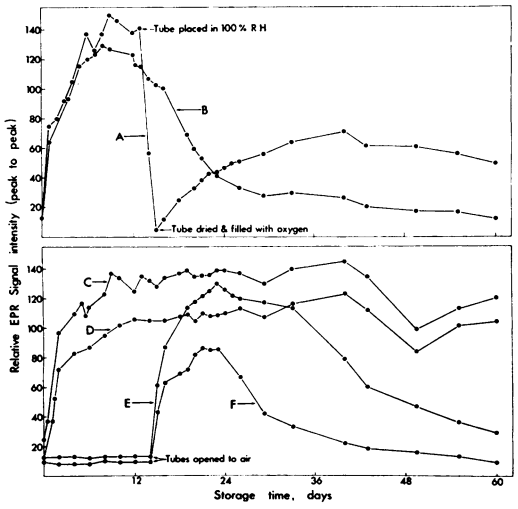

FIG. 5. *Effects of lactose, air, and moisture on free radical formation by lyophilized Serratia marcescens* A*: cells suspended in buffer, dried, and packed in vacuum, then opened to room air for 13 days.* B*: cells suspended in buffer containing 0.5% lactose, packed in vacuum, then opened.* C*: same as sample* A *but cells packed and sealed in air.* D*: same as sample* B *but packed and sealed in air.* E*: same as sample* A *but not opened until after 14 days.* F*: same as sample* B *but not opened until after 14 days.*

tially the same rate as those that were left open (A and B). The free radical concentration in samples sealed in air remained high for the duration of the experiment, whereas the EPR signal of samples in open tubes slowly decreased to the original level after about 60 days. In each instance, fewer free radicals were produced by preparations containing lactose than by those without lactose.

The decay of the signal in the specimens left open to the air is attributed to absorption of moisture by the sample. The effect of moisture on free radical concentration is clearly shown by sample A (Fig. 5). After the EPR signal had developed, this specimen was placed in an evacuated chamber saturated with water vapor; within 48 hr, the signal dropped from approximately 140 to 5 units. We suggest that, as the moisture content increased, the free radicals disappeared by reacting with the absorbed moisture, or with other components in the preparation. When this sample was again evacuated and sealed in an atmosphere of oxygen, the EPR signal increased a second time, though not as rapidly as initially. The slower rate of decay of the free radicals in specimens exposed to ambient relative humidity (RH) is attributed in part to continued signal production during the period of decay, and in part to the fact that air reduced the diffusion rate of moisture into the tightly packed cells.

No change in the EPR signal was observed in samples E and F (Fig. 5) during 14 days of storage in vacuum, although viability decreased to approximately 0.1 and 1%, respectively. At this time, the EPR tubes were opened to the air, and the EPR signal then increased at a rate comparable with that in the corresponding samples (A and B) that had been opened within 1 hr after being packed into the sample tube.

Previously, no free radicals had been observed in cells of *S. marcescens* killed by mercuric chloride or autoclave temperatures (Dimmick et al., 1961). The present experiment, wherein we found an increased signal from apparently dead *S. marcescens* cells, is presumptive evidence that free radicals are not directly associated with death of this organism. At present, we cannot differentiate between the possibility that living cells react with oxygen in the presence of some limiting moisture concentration to produce free radicals, and thus die, or the possibility that the free radicals are only the result of a reaction between oxygen and certain labile components of dead cells. However, it is also possible that the cells were still alive at the end of the initial storage interval but were unable to form colonies when resuspended and plated by our methods.

The survival of *S. marcescens* cells that had been heated at 52 C for 10 min before drying is shown in Fig. 6. A recuperation period, similar to that found by Dimmick (1961) for *S. marcescens* during thermal stress, was evident in both the heat-treated and control cultures. Such phenomena, wherein some cells apparently regain the ability to form colonies after a period of "dormancy," provide a measure of support for the suggestion that cells may sometimes be "alive" yet not able to form colonies at the time of sampling. This concept is not new, and increasing attention to its importance is being shown (Ersman, 1961).

Heating at 52 C also affected free radical production. In contrast to all our previous findings, treated cells stored in vacuum produced a greater EPR signal after 1 day of storage than either the heat-treated or control cultures stored in air, but after 3 days the signals were of about equal in-

tensity. This result was not the same as the slight increase in free radical concentration observed in samples stored in vacuum in experiments summarized in Fig. 3 and 4. The rate of free radical formation in the latter was much slower, and can be attributed to oxygen absorbed by cells during the time powders were exposed to air in the dry box during packing.

Heat treatment also caused some cells to form diminutive colonies when plated on agar, and this tendency was transmitted through the lyophilization step; yet treated cells apparently survived storage almost as well as unheated cells. Evidently, the cells were injured enough to cause a change in their colony-forming characteristics, but not enough to destroy their ability to survive. The production of free radicals by cells in vacuum may be another expression of this injury.

All the EPR spectra obtained appeared to be simple derivative curves, except those associated with the experiment summarized in Fig. 5, wherein a slight but definite indication of an underlying hyperfine structure was evident in the spectra. The two EPR spectra shown in Fig. 7 are representative of those from which the data plotted in Fig. 5 were obtained. The only difference between these preparations and others that yielded only simple derivative curves was that the cells were grown at a slightly higher temperature and were packed more densely than before. If the hyperfine structure can be resolved by manipulating the conditions of either sample preparation or measurement, it may be possible to gain information about the identity of the radical as well as the specific site of formation or entrapment.

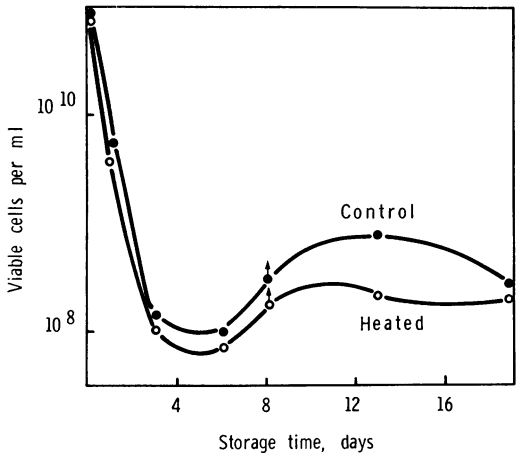

FIG. 6. *Viability of lyophilized Serratia marcescens held at room temperature in air. The 8-day samples contained too many cells to count in the highest dilution tested. Heating was at 52 C for 10 min before drying.*

FIG. 7. *Derivative of electron paramagnetic resonance spectra of Serratia marcescens specimens from the experiment summarized in Fig. 5.* (a) *Specimen corresponding to curve* C; (b) *specimen corresponding to curve* B. *The spectra were obtained after 6 months of storage at room temperature. The vertical arrows indicate a gauss value of 2.00. The shoulder which is evident where the derivative crosses the axis is indicative of the presence of hyperfine structure.*

These experiments show possible applications of EPR to the detection of reactions that occur in biological systems during storage in the dry state, and consequently to the evaluation of additives, to lyophilization procedures as related to viability, and to studies of species characteristics. They show that a correlation exists between the death of dry cells and the rate or extent of free radical formation, although the exact relationship is still obscure. The phenomenon is one that is worthy of continued intensive investigation.

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