however, apparently did not affect the enzymatically active sites of the particles, since there were only slight changes in oxidative and phosphorylative capacities. From the little change in the ascorbic acid oxidase activity of the preparations with and without pectinase treatment, the ascorbic acid oxidase activity seemed intrinsic to the particles of tobacco-tissue cultures, as was reported for mitochondrial preparations from tomato-tissue cultures (*5*).

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Isolation of Listeria monocytogenes from Oat Silage

Abstract. A culture of *Listeria monocytogenes* isolated from the brain of an infected sheep from a flock fed contaminated silage and cultures isolated from this silage were all serological type 4b, establishing a definite epidemiological relationship.

In spite of considerable effort by a number of investigators, the epidemiology and pathogenesis of all forms of infection due to *Listeria monocytogenes* remain obscure (*1*, *2*). Of the more than 700 confirmed cases of listeric infection in man (*1*) and the several thousand in animals, only in a few instances has it been possible to trace the source of infection. An apparent relationship between silage feeding and listeric infection in ruminants has been mentioned often during the two decades since Olafson (*3*) first suggested it. In Iceland this relationship is so striking that listeriosis is known as *votheysveiki*, or silage sickness (*4*). Recently Gray (*5*) reported the isolation of *L. monocytogenes* from the viscera of two out of 20 mice fed corn silage thought to have been responsible for an outbreak of listeric abortion in range cattle. Serological typing of the cultures isolated from the mice and from the aborted bovine fetuses revealed that all were type 1 (*1*), strongly suggesting an epidemiological relationship.

Early in March 1960 oat silage was given as supplementary feeding to a flock of 900 sheep in northwestern Montana. About 1 week later 16 sheep showed symptoms suggestive of listeric encephalitis and some died. The brain of one sheep killed in the terminal stage of the disease was cultured, and *L. monocytogenes* was isolated from it. Unfortunately, only one other sheep brain was submitted for culture. This sheep died considerably later, and all attempts to isolate the bacterium from it failed.

Silage from the same area of the pit as the silage which had been fed to the sheep was divided into several portions. One of these was fed to 15 mice. Although all died within 14 days, there were no gross lesions suggestive of listeric infection at necropsy, and the bacterium was not isolated from any of the mice, even though suspensions of the liver and spleen were refrigerated for longer than 4 months (*6*).

Extracts of four other portions of silage taken at random from the sample submitted were prepared by suspending approximately 25 g of silage in 100 ml of sterile distilled water. These extracts were held at 4°C for at least 48 hours. Five mice were then inoculated intraperitoneally with 0.5 ml of each extract. This was repeated with three extracts after a 1-month interval. Generally, within 24 hours after exposure some mice in each group were lethargic and a few appeared acutely ill. Of 35 mice exposed, 32 died. First deaths usually occurred on the 2nd postexposure day, and only a few mice survived more than 4 days. Necropsy usually revealed moderate to severe focal hepatic necrosis characteristic of listeric septicemia. Even though a few dead mice showed no gross lesions, *Listeria monocytogenes* was isolated in essentially pure culture from a liver and spleen pool of every mouse that died. Three mice in one group remained asymptomatic and were killed on the 8th postexposure day. In spite of their healthy appearance, two of these had a few necrotic foci on the liver, and the bacterium was isolated from all three.

The four silage extracts also were plated on tryptose agar (Difco). The plates were incubated for 24 hours at 37°C and examined for the presence of characteristic blue-green colonies of *L. monocytogenes* with a binocular scanning microscope and obliquely transmitted light (*6*). In general, it was not possible to detect colonies of *L. monocytogenes* in extracts that had been refrigerated less than 10 days, as they were often rather heavily contaminated by a variety of bacteria and molds. Cultures prepared after 10 days of refrigeration usually revealed from five to 15 or more colonies of *L. monocytogenes*. The bacterium was isolated in this manner from all the extracts prepared.

The fact that all cultures isolated during this study were type 4b (*1*) strongly suggests that the sheep actually were infected by ingestion of the contaminated silage and establishes an important link in the epidemiology of listeric infection in ruminants.

These findings indicate not only that this silage contained rather large numbers of *Listeria monocytogenes* but also that it was of sufficient pathogenicity to incite active infection both in the naturally exposed sheep and in artificially exposed mice. Further proof of its pathogenicity was the rapid production of a marked purulent conjunctivitis and keratitis after instillation of the culture into the conjunctival sac of rabbits.

These findings, coupled with the earlier observations of Gray (*5*) and of Gislason and Vigfusson (*7*), not only support but also offer a possible explanation for the apparent higher incidence of listeric infection among ruminants fed silage than among those fed other rations. Superficially, these findings tend to support the concept that in listeric encephalitis the bacterium enters through the digestive tract; however, this is contradicted by observations during naturally induced outbreaks of the disease, by the consistent failure to produce the disease artificially through oral exposure (*1*, *2*), and by the failure to infect mice fed this silage. The findings reported here are not necessarily at variance with the view of those who hold that listeric encephalitis results either from exposure through the upper respiratory tract or by migration of the bacterium along branches of the trigeminal nerve (*2*), since either system would be vulnerable during ingestion of a forage such as silage.

Although these results establish that silage may contain *Listeria monocytogenes* in sufficient numbers to cause infection under field conditions, they give no clue to the manner in which the silage becomes contaminated. It is possible that *L. monocytogenes* is a soil bacterium or even a common inhabitant of some plant life (*1*). Also, silage could be contaminated by ground game or birds, which are known to be potential carriers of the bacterium (*1*, *2*).

This investigation reemphasizes the importance of refrigeration of material to be cultured for the presence of *L.*

monocytogenes and stresses the inadequacy of present isolation techniques in which a nonliving culture medium is used.

Studies identical with those described above have been carried out on silage from other areas where listeric infection developed shortly after the initiation of silage feeding, but the bacterium has not been isolated from any of these, to date (*8*).

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8. The technical assistance of Mrs. Jean Martin is gratefully acknowledged. This work was supported in part by research grant No. E-2571 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. This report is a contribution (paper No. 515, journal series) from the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating), Montana State College, Agricultural Experiment Station.

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Thermal Annealment and Nitric Oxide Effects on Free Radicals in X-irradiated Cells

Abstract. Four kinds of radicals are identified in dry spores *(Bacillus megaterium)* after x-irradiation: those associated with singlet, doublet, triplet, and oxygen-complex types of spectra. The singlet, present only at low temperatures, gives rise irreversibly to the doublet at 25°C. All hyperfine structure is depressed after annealment at 100°C and is lost when oxygen or nitric oxide is added. The physical results support the hypothesis that reactions of long-lived free radicals can account for the radiobiological phenomena of thermorestoration, nitric oxide protection, and latent oxygen effect.

Studies by Powers *et al.* (*1*) on the influence of physical and chemical factors on the response of nonmetabolizing dry spores to x-irradiation, as measured by colony-forming capacity, have revealed a systematic sensitivity to such factors as gas and temperature not only during but also after irradiation. These results suggest several classes of damage, one of which is thought to be due to long-lived free radicals. Upon reaction with oxygen these radicals may form lethal complexes; alternatively, they may be rendered harmless if scavenged by chemical recombination or annealed by thermal energy. This report presents physical evidence for the existence of such long-lived radicals with the postulated properties in irradiated dry spores.

The electron spin resonance spectrometer was a conventional microwave system (9.35 kMcy/sec) with a reflection cavity; a double modulation technique was used. The resonance patterns are approximately the second derivative of the absorption signal (*2*). The basic theory and practice of electron spin resonance spectroscopy have been treated by Ingram (*3*). Lyophilized bacterial spores were irradiated in 25-mg lots in a beryllium-window exposure chamber in an atmosphere of helium gas, at a temperature of −195°C. The x-ray source was equipped with a wolfram target and a beryllium window, operated at 50 kv and 30 ma (constant potential), at a dose rate of 50 kr/min. After irradiation the spores were quickly dumped into another section of the chamber, composed of a quartz tip (protected from radiation), shaped like the spectrometer cavity. The tip was then transferred into the cavity (also at —195°C). The spectra shown in this study were obtained after irradiation at doses of 4000 kr; essentially similar but quantitatively weaker signals were obtained at doses as low as 250 kr. No signals were found with unirradiated spores.

The initial spectrum (no warm-up) is shown at upper left in Fig. 1*A*. The spores were then warmed up to 25°C for specified intervals and returned to the cavity for measurement at −195°C. On warming over a period of 22 minutes, a singlet component of the initial spectrum appears to be converted into a doublet with 20-gauss spacing. A triplet present in the initial spectrum remains unchanged. Growth of the doublet at the expense of the singlet at 25°C is shown in Fig. 1*B*. the five-line spectrum at 22 minutes (Fig. 1*A*), consisting of a 1:2:1 triplet with 30-gauss spacings and the 1:1 doublet superimposed, is essentially the same as that seen when spores are irradiated at room temperature (25°C, Fig. 2*a*).

In viability studies, exposure of the spores to higher temperatures after anoxic irradiation results in thermorestoration of a considerable fraction (*4*). The physical results are similar in that a reduction of the number of radicals (electron spin resonance) by as much as 50 percent is effected by annealment at 100°C for 10 minutes (Fig. 2*c*). Furthermore, in the viability studies, if the anaerobically irradiated spores are exposed to oxygen before annealment, no part of the potential damage is reversible. In these studies, if the anaerobically irradiated sample is first exposed to oxygen at 25°C, the spectra shown in Fig. 2*b* result (dotted line, 1.5 minutes; solid line, after 3 minutes). This represents a new oxygen-complex radical resulting from the reaction of radicals *R2* and *R3* (Fig. 1*A*) with oxygen. The residual radicals in the annealed system (Fig. 2*c*) also react with oxygen to form a typical oxyradical spectrum (Fig. 2*d*) but of reduced strength. This is in general agreement with the biological results.

Again, the discovery from the viability studies that nitric oxide enhanced the survival of anaerobically irradiated spores (*5*) led to the hypothesis that the protective action of nitric oxide could be explained by its scavenging action upon long-lived oxygen-reacting radicals. The physical results are in accord with this hypothesis in showing nearly total obliteration of the hyperfine spectra by nitric oxide (50 percent

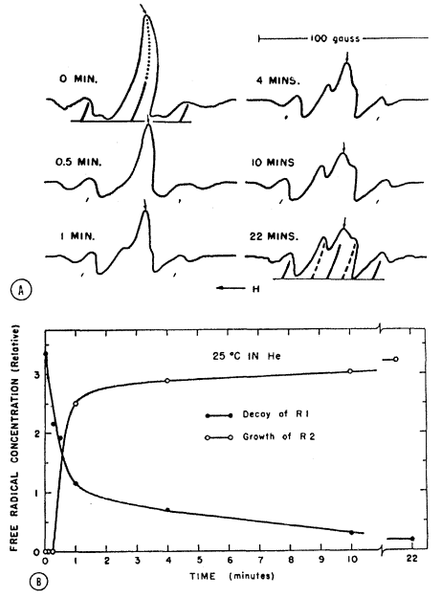

Fig. 1. (*A*) Paramagnetic resonance measured at −195°C in dry bacterial spores previously irradiated anaerobically (4×10^6 r in He at −195°C; warmed in He at 25°C). A hyperfine doublet (broken lines) appears between 1 and 22 minutes, lateral to the center line of an initial triplet (solid lines), while the superimposed initial singlet (dotted line) disappears. Arrow represents position of g (= 2.003). (*B*) Kinetics of destruction of the singlet component (*R1*) of the center line and growth of the doublet (*R2*) at 25°C anaerobically. [The amplitude of meter deflection is plotted directly for *R2*; the asymptotic values of the triplet (*R3*) center line were subtracted in computing *R1*.]