segment of the alpha cycle where Lansing found stimuli associated with slowest responses.

These data indicate a more than chance relationship between visual reaction time and alpha phase at stimulation. Finer details of the relationship between alpha phase and reaction time must wait further investigation.

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Use of Pectinase in Preparation of Mitochondria from Tobacco-Tissue Cultures

Abstract. Mitochondrial particles were isolated from tobacco cells in tissue cultures which were disintegrated with pectinase. The pectinase treatment of the tissue cultures reduced the dry weight of the particulate preparations and eliminated glutinous material, but caused little change in oxidative and phosphorylative activities. The pectinase apparently did not affect the enzymatically active sites of the particles.

Pectinase has been used to disintegrate tobacco cells in tissue cultures for the preparation of mitochondria. The hope was that pectinase would eliminate the glutinous nature of the pellet secured from centrifuging the homogenate of the tissue cultures. Thus, uniform suspensions of mitochondria could be obtained readily in the reaction medium. This approach to enzymic disintegration of tissue seemed promising because of the successful isolation of mitochondria from pigeon heart muscle and rat liver by the use of proteinase (*1*). Prior incubation of such tissues with proteinase facilitated the grinding procedure, minimized mechanical damage to the particles, and produced particles with a higher P/O ratio, greater stability, and a greater general reactivity.

The tobacco tissue used in this experiment (*2*) was a single cell clone (H196) from a stem of *Nicotiana tabacum* × *N. glutinosa,* and was grown on a modified White's agar medium (*3*). Mitochondria were prepared with pectinase as follows: 150 g of tissue, 2 weeks old, was washed by suspending it in 400 ml of 0.5*M* mannitol, and was separated from the mannitol solution by filtration on two layers of cheesecloth. The washed cells then were suspended in 400 ml of 0.5*M* mannitol to which 600 mg of pectinase (Nutritional Biochemicals Corp.) was added; the suspension was kept at 2°C for 45 min with gentle stirring with a magnetic stirrer. The suspension became more acidic with time and was adjusted to *p*H 7.2 with 0.5*M* potassium hydroxide as needed. After incubation, the suspension was homogenized in a glass homogenizer with a Teflon pestle. During grinding, the *p*H was again adjusted with potassium hydroxide. The pectinase-treated tissue was soft and was readily ground by three in-and-out movements of the pestle in the mortar as compared to five in-and-out movements with nontreated tissue suspension. The particles were separated and washed by centrifugation (*4, 5*). Oxygen consumption was measured with an oxygen electrode apparatus (*6*), and phosphate was determined by King's method (*7*).

Table 1 shows typical results of analyses of mitochondria prepared from pectinase-treated tissue as compared to mitochondria prepared from nontreated tissue. The yield of mitochondria on the basis of wet and dry weights was decreased to less than half by the pectinase treatment. At the same time, the glutinous material in the original preparation was removed, and the particles could be suspended easily in the reaction medium. The protein content in particulate preparations was little affected by the pectinase treatment; the ratio of nitrogen to dry weight was almost doubled by the treatment.

The particulate preparations from both pectinase-treated and nontreated tissues oxidized succinate, citrate, glutamate, α-ketoglutarate, reduced diphosphopyridine nucleotide (DPNH) and ascorbate. For comparison, oxidation rates for succinate, DPNH, and ascorbate are shown in Table 1. The pectinase treatment apparently caused no significant change in oxidative activities and in the relative activities of the enzymes. The P/O ratio also was unchanged by the treatment. Essentially the same results were obtained with mitochondria originally isolated from nontreated tissue but incubated with pectinase (1 mg of pectinase per milliliter of mitochondrial suspension) for 15 minutes at 2°C after isolation (see column 9, Table 1). After incubation with pectinase, the wet weight of the mitochondrial preparation decreased to less than half, and the cohesive nature of the original preparation disappeared. The oxidative and phosphorylative activities of mitochondria treated with pectinase were comparable to those of the particles prepared from either pectinase-treated or nontreated tissue cultures. Mitochondria isolated from pectinase-treated tissue cultures or mitochondria treated with pectinase after isolation responded more to adenosine diphosphate (ADP) than mitochondria isolated from nontreated tissue.

The pectinase treatment seemed to eliminate polysaccharide substances which otherwise might contaminate the pellet of mitochondria and cause them to aggregate tenaciously. This treatment,

Table 1. Comparison of mitochondrial preparations from pectinase-treated and nontreated tissue cultures of tobacco and a mitochondrial preparation treated with pectinase after isolation.

Item	Mitochondria from				Mitochondria* treated with pectinase after isolation	
	Nontreated tissue		Pectinase-treated tissue			
Wet weight	2.36 g		0.91 g		0.79†	
Dry weight	209 mg		91.5 mg			
Protein–N	2.52 mg		2.24 mg			
Protein–N/dry weight	0.0125		0.0243			
(Oxidative activities)	(mμatoms/min per mg N)	(Relative activity)	(mμatoms/min per mg N)	(Relative activity)	(mμatoms/min per mg N)	(Relative activity)
Succinate oxidation	18.7	1.0	14.1	1.0	16.6	1.0
Succinate oxidation in presence of ADP	105	5.6	102	7.3	133	8.0
DPNH oxidation	342	18.3	293	20.8	293	17.7
Ascorbate oxidation	1790	95.7	1500	106.1	1540	92.8
P/O ratio with succinate	0.85–1.0		0.84–1.0		0.87–1.0	

* Mitochondria originally isolated from nontreated tissue cultures. † Wet weight before pectinase treatment was 1.79 g.

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.*