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Terminal Oxidase of Orchard Grass

Abstract. The response of infiltrated surviving green leaves to HCN, 1-phenyl-2-thiourea, and sodium diethyldithiocarbamate is consistent with a functional role for cytochrome oxidase in respiration. Polyphenol oxidase does not function as a terminal oxidase in orchard grass.

The identity of the functioning terminal oxidase in green leaves of grasses has not been clearly established. While studying losses of dry matter in the curing of hay, I therefore examined the terminal oxidase responsible for respiration in the leaves of green grass. Cytochrome oxidase has been reported in various plant tissues, but the pathway of electron transfer varies among different plants (*1*). Polyphenol oxidase has been regarded by many as a possible terminal oxidase, although there is little direct evidence of its primary function in undamaged cells (*2*). However, reports that it is at least partially functional continue to appear (*3*), so that it appears desirable to offer contrary evidence.

Daly *et al.* (*4*) reported that respiration of young leaves of barley was mediated by cytochrome oxidase. However, in older leaves respiration was not inhibited by carbon monoxide; thus the functional oxidase in mature leaves was left unknown. Deijs and his co-workers (*5*) showed that, as rye grass dried, its decline in respiration was paralleled by a similar decline in polyphenol oxidase activity. These workers attributed HCN inhibition of grass respiration to the effect of cyanide on polyphenol oxidase.

Orchard grass (var. Potomac) contains cytochrome oxidase and abundant amounts of polyphenol oxidase. Neither the intact leaf nor homogenates of it oxidizes ascorbic acid. Cytochrome oxidase appears to be the principal functioning terminal oxidase.

Grass of height 10 to 20 cm was homogenized for 40 seconds in a Waring blender with cold 0.1*M* phosphate (5 ml of buffer per gram of grass). For the assay of polyphenol oxidase the phosphate buffer was *p*H 6.5, for cytochrome oxidase, *p*H 6.8. Since addition of 0.2*M* sucrose and 0.001*M* ethylenediaminetetraacetate did not increase cytochrome oxidase activity, these compounds were usually omitted (*6*). For ascorbate oxidation, the homogenizing medium was 0.1*M* citrate-phosphate, *p*H 5.7 (*7*). Glass-distilled water was used throughout.

Cytochrome oxidase was manometrically measured in darkness at 30°C with 0.014*M* *p*-phenylenediamine as substrate. This enzyme is stimulated two- to threefold by the addition of $10^{-5}M$ exogenous cytochrome *c*. The most active fraction of cytochrome oxidase is sedimented in 20 minutes at 6230 *g* (average). For the assay of polyphenol oxidase, oxidation of catechol was followed manometrically (*8*). Polyphenol oxidase activity is not sedimented by 16,700 *g* in 20 minutes.

Several enzyme inhibitors were studied both in vitro and in surviving leaves. Grass was cut into pieces 1 to 2 cm long and vacuum infiltrated with inhibitors in 0.1*M* phosphate, plus 0.2*M* sucrose at *p*H 6.8, prior to respiration measurements. Table 1 shows that the effects of inhibitors in vivo are similar to those obtained with the cytochrome oxidase preparation but that they differ from those obtained with polyphenol oxidase. The stimulation of cytochrome oxidase and leaf respiration by diethyldithiocarbamate could be caused by its acting as a substrate for this oxidase (*9*). The response of intact green leaves to inhibitors is consistent with a functional role for cytochrome oxidase in respiration. Polyphenol oxidase cannot be the terminal oxidase in this issue.

Table 1. Effect of inhibitors on oxidases and on respiration of orchard grass.

Inhibitor	Percentage change in activity from control*		
	Polyphenol oxidase (in vitro)	Cytochrome oxidase (in vitro)	Infiltrated surviving leaves
$10^{-3}M$ HCN	− 90	− 100	− 40
1-phenyl-2-thiourea, saturated (*8*)	− 100	0	0
$10^{-3}M$ Na diethyldithiocarbamate	− 100	+ 16	+ 60

* The endogenous respiration of grass infiltrated with 0.1*M* phosphate, *p*H 6.8, averaged 270 μl of O_2 per hour per gram of fresh grass. Oxygen uptake per Warburg flask averaged 111 μl/hr for cytochrome oxidase, and 330 μl/hr for polyphenol oxidase.

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3 December 1958

Transmission of Rabies to Laboratory Animals by Bite of a Naturally Infected Bat

Abstract. An insectivorous bat that attacked a man in western Montana was induced to bite suckling mice. Subsequently the bat died, and brain and salivary gland suspensions were inoculated into other mice. Rabies virus was isolated from all three groups of mice.

In a review of the relationship between bats and rabies, Enright (*1*) noted that transmission of rabies by bite of insectivorous bats had not been demonstrated. Burns (*2*) failed to obtain transmission to monkeys, guinea pigs, and white mice by bites of experimentally infected *Tadarida mexicana* and *Antrozous pallidus*, although he found the saliva infectious by intracerebral inoculation. Stamm *et al.* (*3*) also found the saliva of one experimentally infected bat (*Myotis lucifugus*) infectious on intracerebral inoculation. However, what evidence exists for transmission of infection by the bites of insectivorous bats rests upon the occurrence of infection in human beings. At the present time, one well substantiated (*4*) and two possible (*5*) infections have been reported in North America. The present report records infection in white mice resulting from the bite of a naturally infected little brown bat.

The bat (*Myotis californicus californicus*) (*6*) was captured by elk hunters on 21 September 1958 in the Bitterroot Mountains of western Montana, where it attacked one of them twice. The first attack occurred at midday of a sunny day while the hunter was standing in camp. The bat suddenly appeared, lit on the front of his shirt, and bit the fabric. The hunter, a technician in the Rocky Mountain Laboratory, was aware

of the infectious potential of the animal and therefore did not touch it. In a moment the bat flew away and the men entered their cabin. When they emerged about 3 or 4 minutes later, the bat again lit on the shirt and again bit it. It was captured in a jar and brought to the laboratory the next day.

The bat was induced to bite one thigh of each of three 2-day-old mice. Its attack upon the mice was not exceptionally vicious. Three other suckling mice of the same litter were not submitted to bites but were kept in the litter as controls. This measure was found necessary because of occasional nonspecific mortality in litters. Neither the injected nor the normal mice were marked to distinguish them, since this might induce cannibalism in, or abandonment by, the mother.

The bat was kept in a glass jar with water and canned dog food available. Under these conditions, normal bats usually survive for a month or more but this one died 7 days after capture. The carcass was stored in Dry Ice until it could be examined.

On the 13th day after being bitten, two mice, and on the 14th day a third mouse, exhibited partial paralysis of the hindquarters. Their brains were removed for transmission. The remaining mice were observed for 28 days and remained normal throughout that period.

When it appeared that an infectious agent was present in the bitten mice, brain tissue and salivary glands of the bat were triturated separately in albumen-saline diluent and injected intracerebrally into 21-day-old white Swiss mice. Salivary-gland suspension was also injected intramuscularly. All injections were 0.02-ml volumes of 5- to 10-percent suspensions. On the 10th day afterward, four of six mice injected intracerebrally with brain suspension were obviously ill and were killed. Another mouse was sick on the 11th day, the sixth mouse on the 12th day; both were dead on the 14th day. None of the mice injected with salivary-gland suspension showed signs of illness during a 28-day period of observation.

An aliquot of the salivary-gland suspension not used in the first injections and which had been stored in a Dry Ice chest was then injected intracerebrally into six 21-day-old mice and into a litter of five 2-day-old mice. One of the litter became ill on the 14th day and was killed. Another, sick on the 21st day and held for observation, was dead the next day and was discarded. Three of the litter remained normal. None of the 21-day-old mice showed evidence of infection.

A transmissible agent was demonstrated in tissues of mice injected with tissues from, or bitten by, the bat. These tissues included brain of mice infected by biting or by injection of salivary glands and brain, and salivary glands of mice infected by biting. The isolates were neutralized by immune serum prepared in rabbits by hyperimmunization with PV-1 strain of rabies virus. Mice immunized with H.E.P. Flury strain virus (*7*) were immune to intracerebral injection of the bat isolates.

A hemisphere of brain from a mouse bitten by the bat was fixed and stained with William's stain. It contained numerous Negri bodies (*8*).

The occurrence of human rabies following bat bite and this demonstration of infection in bitten laboratory animals establish the infectiousness of bites of insectivorous species. While little doubt existed that such would be found eventually, the potential is now clearly demonstrated. The fact that three mice were bitten and three, presumably the same ones, succumbed at about the same time probably indicates that all were infected. Greater susceptibility of young mice (*9*) infected by any route may have, in part, contributed to the efficacy of transmission in this case. This certainly seemed to be true when a salivary gland suspension was injected into suckling and into weanling mice. Perhaps the thin skin of 2-day-old mice may also have facilitated transmission.

Because the bat died before the bitten mice became ill, a second test of the bite to determine persistence of infectiousness was not made. Unusual behavior in captivity was not noted, but opportunities for activity were limited in the close confines of the jar. It has yet to be demonstrated that insectivorous bats can become true carriers of infection—that is, infective but symptom-free. If such were the case, and if they lacked the aggressive urge of furious rabies, they, unlike the vampire bats, would not constitute a direct and serious menace to animals other than bats. However, the potential for spread within a colony as a result of intraspecific strife might be increased.

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28 January 1959

Identification and Assay of 5-Hydroxytryptamine in Molluscan Tissues by Fluorescence Method

Abstract. The fluorescence assay method has been used in the identification and quantitative estimation of 5-hydroxytryptamine (5-HT) in mollusks. Ganglia of a representative pelecypod contain more 5-HT than those of a gastropod. Most non-nerve tissues have low levels of 5-HT, which, except in "kidneys," may derive from nerve endings.

By means of paper chromatography and bioassay, 5-hydroxytryptamine (serotonin, enteramine) was found in nerve tissue of the mollusks *Venus mercenaria* and *Busycon canaliculatum* (*1*). This earlier identification has now been confirmed, non-nerve tissues have been examined, and the quantities of 5-HT have been more precisely determined, by the spectrofluorometric method developed at the National Institutes of Health (*2*). We have followed the analytical procedure outlined by Bogdanski *et al.* (*3*), with minor modifications, such as a lower ratio of tissue weight to solvent

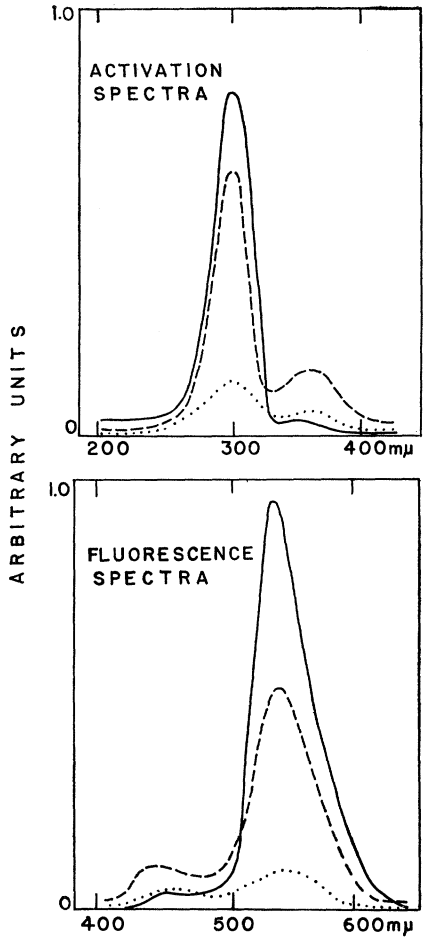

Fig. 1. Activation and fluorescence spectra of authentic 5-HT (solid line), extract of *Venus* ganglia (dashed line), and extract of *Busycon* ganglia (dotted line), all in 3*N* HCl.