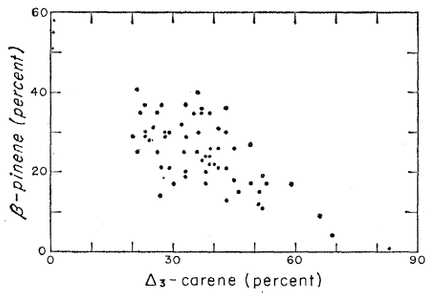

Fig. 1. Relationship of β-pinene and Δ_3-carene in the oleoresin of 64 ponderosa pines.

Table 2. Range in amount (percentages) of monoterpenes in the oleoresin of 64 ponderosa pines.

Terpene	Average	Minimum	Maximum
Δ_3-Carene	36.2	*	82.5
β-Pinene	26.4	*	57.5
Limonene	14.5	*	30.7
Myrcene	13.3	4.6	27.5
α-Pinene	6.3	1.5	13.3
β-Phellandrene	1.8	0.3	3.7
Unknown	1.5	0.0	3.1
Camphene and heptane	*	0.0	*

* Trace.

specific differences, most of which is expressed by optical rotation.

During studies on the relation of resin to the resistance of pines to bark beetles (*5*), large differences were found among ponderosa pine trees (*Pinus ponderosa* Laws.) in the monoterpene composition of their wood oleoresin. This tree is economically the most important and ecologically one of the most widespread pines in western North America. The 64 trees examined were located in several plots in the central Sierra Nevada in California from Lake Tahoe to Placerville and in one plot in the Warner Mountains north of Alturas. All resin was collected with a closed-face microtap (*6*). The freshly collected resin was processed for gas chromatographic analysis either by (i) petroleum ether extraction or (ii) molecular distillation with a Hickman still at 40°C for 24 hours at atmospheric pressure. There was essentially no difference in the terpene composition of the extract and the distillate.

The analyses were made with a thermal conductivity detector and a stainless-steel column (2.5 m by 0.6 cm) packed with 10-percent or 20-percent oxydipropionitrile on 60- to 80-mesh acid-washed chromosorb W (*7*). The conditions of operation were temperatures of 120° to 130°C on the injector, 55° to 60°C on the column, and 145° to 151°C on the detector; filament current, 200 ma; helium flow, 90 or 60 ml per minute at the outlet port; and sample size, 0.2 to 4.0 μl.

Qualitative determinations were made by comparing relative retention times for the oxydipropionitrile columns and for a LAC-446 column (*8*) with data from the literature, with known compounds, and by internal standardization with known compounds. Quantitative determinations were made by internal normalization of disk integrator values. The two columns agreed in both qualitative and quantitative analyses.

No qualitative and practically no quantitative differences in monoterpene composition could be associated with the way in which the sample was prepared, the variations of column and the instruments used within the stated limits, the time of the flow of resin after wounding the tree, circumferential and vertical position of the source of the resin within a tree, the time of sampling within and between seasons of the year, and the year of sampling. No differences were found in 60 duplicate sets of samples representing changes in time or place within trees.

There was, on the other hand, great variation in the quantitative terpene composition of the wood oleoresin between individual trees (Table 1) and a wide range of difference within the 64-tree sample (Table 2). Several of the values for the extremes were greater than those obtained by Mirov (*4*) in his analysis of bulk samples from 12 widely separated areas in western United States. There was a tendency for inverse relationship between the amounts of Δ_3-carene and β-pinene (Fig. 1).

RICHARD H. SMITH

Pacific Southwest Forest and Range Experiment Station, U.S. Department of Agriculture, Berkeley, California

References and Notes

1. A. J. Hagen-Smit, C. T. Redemann, T. H. Wang, N. T. Mirov, *J. Pharm. Sci.* **39**, 260 (1950).
2. M. H. Bannister, H. V. Brewerton, I. R. C. MacDonald, *Svensk. Papperstidning* **62**, 567 (1959).
3. W. Sandermann, *Holzforschung* **16**, 65 (1962).
4. N. T. Mirov, *U.S. Dept. Agr. Tech. Bull. 1239* (1960).
5. R. H. Smith, *J. Econ. Entomol.* **54**, 365 (1961).
6. ———, *ibid.*, p. 359.
7. M. H. Klouwen and R. ter Heide, *J. Chromatog.* **7**, 297 (1962).
8. R. A. Bernhard and B. Scrubis, *ibid.* **5**, 137 (1961).

28 October 1963

Dissociation of Olfactory Neural Response and Mucosal Potential

Abstract. *The olfactory mucosal slow potential decreased remarkably or disappeared when part of the mucus on the olfactory epithelium was removed with absorbent paper. In contrast, the neural response was not changed much, while unitary spikes in response to odor also appeared in the epithelium. The paper reduced the thickness of the mucus by about half, but olfactory cilia were still present. This evidence suggests that the slow potential is not the generator potential.*

Slow potentials in the olfactory mucosa in response to odors have been recorded by several investigators (*1–6*). Ottoson (*1*) proposed the name electro-olfactogram for the record of change in electrical potential led from the surface of the olfactory mucosa. This phenomenon is naturally expected to bear some causal relation to the initiation of olfactory neural activity, perhaps quite directly related to the generalized concept of a generator potential responsible for initiation of propagated action potentials which are conducted to the central nervous system by sensory nerve fibers (*7*). Indeed, Ottoson (*8*) recently interpreted the electro-olfactogram as a mass response due to contributions from many individual receptors, and he stated that it is "the generator potential of the olfactory organ." However, a few workers occasionally observed that the slow potential did not coincide with the neural discharge or the response of the olfactory bulb (*2, 4*).

Experiments to ascertain the influence of mucus thickness over the olfactory receptors on their response parameters yielded the unexpected result that the electro-olfactogram can be practically abolished without significantly affecting the neural activity. The tortoise (*Gopherus polyphemus*) preparation described by Tucker (*9, 10*) was used. The small area of the sensory epithelium to which the nerve twig projected was detected by means of mechanical stimulation (probing with a human hair) of the epithelium or by antidromic electrical stimulation of the nerve twig (*10*). To record slow potentials a macroelectrode (glass pipette with tip diameter of about 200 μ filled with Ringer's solution) and a microelectrode (less than 1 μ in tip

diameter filled with 3*M* KCl or 15-percent potassium ferricyanide) were used. Negative slow potentials of 2 to 5 mv appeared in the olfactory mucosa in response to stimulation with puffs of odor, and neural activity appeared in the nerve twig (Fig. 1*A*). After the control records were taken, a small piece of absorbent paper (a 3- by 5-mm rectangle of thin filter paper, Kimwipes, or Kleenex) was put on the mucosa for about 1 to 4 minutes. Then the paper was carefully removed and electrodes were replaced at the same points. The slow potentials recorded with both mucosal electrodes decreased remarkably or disappeared (Fig. 1*B*).

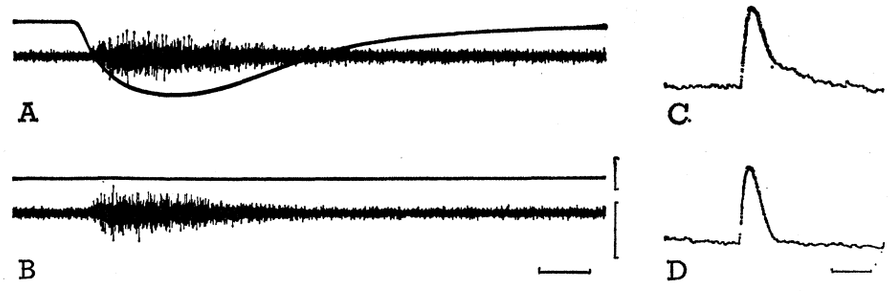

Fig. 1. *A*, Negative slow potential and nerve twig response in the tortoise produced by small puff of amyl acetate near vapor saturation. *B*, Slow potential and twig response recorded after absorbent paper was put on the epithelium for 4 minutes and then taken off. The slow potential nearly disappeared, but the nerve twig response did not change much. Calibrated voltage: above, 1 mv; below, 40 μv. Time trace, 0.5 second. *C*, Integrated curve of nerve twig response in (*A*). *D*, Integrated curve of nerve twig response in (*B*). Time trace, 2 seconds.

In one instance, when a small piece of paper was applied for 1 minute, the slow potential decreased to about one-fourth of that of the control; then, when paper was put on the same area for 3 minutes the slow potential disappeared completely, but another area of the mucosa that had not been covered with paper produced normal slow potentials. Nerve twig responses were still recorded as long as the epithelium was not injured, in spite of the profound decrement of the slow mucosal potential led from the area of innervation. Integration, or averaging, of the neural response showed that its maximum value was unchanged, but that its time course was faster (Fig. 1, *C* and *D*). Thinning of the mucous layer should accelerate transport of stimulus molecules to the receptor sites (*9*).

Pieces of paper that had been washed with distilled water and dried were still effective. Tests with Teflon and vinyl plastic sheets revealed no consistent decrease of the slow potentials and sometimes they were augmented. These observations suggest that the slow potential disappeared because of the removal of mucus which stuck to the absorbent paper, since practically none of the tenacious mucus adhered to the plastics. A species peculiarity was ruled out by investigating the mucosa, but not the nerve, of the bullfrog (*Rana catesbeiana*). The effect was somewhat easier to obtain in the bullfrog than in the tortoise.

The area of the olfactory mucosa from which part of the mucus was removed with absorbent paper, and from which the slow potential had disappeared, was isolated carefully and sections made with small scissors were observed under a microscope. The thickness of the mucus covering the epithelium had been reduced by about half, but olfactory cilia were still present. The motility of olfactory cilia of the short type in the tortoise and cilia in the bullfrog had not changed. (There are two types of cilia on the olfactory epithelium of the tortoise. One is 40 to 50 μ long and another is 20 to 25 μ long; only the short type is motile. The olfactory cilia of the bullfrog are about 30 μ long.) Accordingly, it appears likely that the olfactory cilia were not damaged by the treatment with small pieces of paper, though damage to the tips might not be evident with ordinary microscopy (*11*).

On- and off-slow potentials recorded from the olfactory mucosa of the frog have been described (*3, 4*). In the tortoise and the bullfrog, off-slow potentials appeared with ether stimulation. The off-potential disappeared together with the on-potential by removing some of the mucus with paper. It is presumed that both components originated at the same layer.

Unitary spike activity in response to odors has been recorded from the olfactory epithelium of the tortoise, frog, and toad by means of microelectrodes (*5, 6, 12*). Moreover, a small, positive slow potential often appeared from about the same layer as spikes during odor stimulation (*13*). When the microelectrode penetrated into the epithelium from which part of the mucus was removed, spikes and a small positive potential appeared. The positive slow potential described here may be related to the positive component of mucosal slow potential described by Gesteland (*14*).

In earlier studies, when a micropipette electrode was slowly lowered from the surface of the mucus to the basal membrane, the height of the slow potential decreased gradually (*1, 5*). It was presumed that the origin of the slow potential was near the surface of the olfactory epithelium. The results reported here suggest that the slow potential may originate from the mucous layer because of the disappearance after removal of the superficial mucus and because the olfactory cilia are still motile. On the other hand, even though the slow potential disappeared, nerve twig response and unitary spikes in response to odor stimulation were recorded. Therefore, it is presumed that the negative slow potential recorded from the olfactory mucosa is not the generator potential.

TATSUAKI SHIBUYA

Department of Biological Sciences, Florida State University, Tallahassee

References and Notes

1. D. Ottoson, *Acta Physiol. Scand.* Suppl. 122, **35**, 1 (1956).
2. S. F. Takagi, T. Shibuya, S. Higashino, T. Arai, *Jap. J. Physiol.* **10**, 571 (1960).
3. S. F. Takagi and T. Shibuya, *ibid.*, pp. 99 and 385.
4. M. M. Mozell, *Am. J. Physiol.* **203**, 353 (1962).
5. T. Shibuya and S. Shibuya, *Science* **140**, 495 (1963).
6. R. C. Gesteland, J. Y. Lettvin, W. H. Pitts, A. Rojas, in *Olfaction and Taste*, Y. Zotterman, Ed. (Macmillan, New York, 1963), p. 19.
7. H. Davis, *Physiol. Rev.* **41**, 391 (1961).
8. D. Ottoson, in *Olfaction and Taste*, Y. Zotterman, Ed. (Macmillan, New York, 1963), p. 37.
9. D. Tucker, thesis, Florida State Univ. (1961), p. 188.
10. ———, *J. Gen. Physiol.* **46**, 453 (1963).
11. Since this paper was submitted, I have seen long nonmotile cilia on the olfactory epithelium of the frog, as described by A. E. Hopkins [*J. Comp. Neurol.* **41**, 253 (1962)]. Long cilia were much more frequent on the dorsal olfactory mucosa.
12. S. F. Takagi and K. Omura, *Proc. Japan Acad.* **39**, 253 (1963).
13. T. Shibuya, unpublished results.

14. R. C. Gesteland, *Ann. N.Y. Acad. Sci.*, in press.
15. I thank L. M. Beidler for his support and advice and D. Tucker for his technical advice on the twig preparation. Supported by U.S. Public Health Service grant NB 5258-05.

30 December 1963

Persistence of Bacteria in "Protoplast" Form after Apparent Cure of Pyelonephritis in Rats

Abstract. *Appropriate antibiotic treatment of experimental enterococcal pyelonephritis in the rat appears to effect the removal of the infecting organisms from the kidney, as judged by the culturing of kidney homogenates on standard bacteriological media. When the homogenates were cultured on media containing 0.3*M *sucrose, osmotically stabilized, it was demonstrated that bacteria were present in the "protoplast" form, and that this form persisted in the kidney for at least 13 weeks after therapy.*

The course of pyelonephritis may be characterized by frequent recurrences despite seemingly adequate therapy. Furthermore, in many cases, the disease appears to progress histologically despite the absence of bacterial infection as determined by either urine cultures or bacteriological examination of biopsy specimens from the kidney. A possible explanation of these findings is that bacteria persist in the kidney, even after appropriate antibiotic therapy, in a form that cannot be cultured on standard bacteriologic media. It has recently been reported from this laboratory that enterococcal pyelonephritis in the rat can apparently be cured by penicillin therapy (*1*). Experiments were designed to determine if renal persistence of bacteria in the form of "protoplasts" (*2*) occurred.

Ninety-four white, male Wistar rats weighing 100 to 115 g were injected intravenously with 1.0 ml (4.0×10^8 bacteria) of an 18-hour broth culture of *Streptococcus faecalis* (*3*). One day after inoculation, 49 of these rats were started on therapy which consisted of 100,000 units of procaine penicillin injected intramuscularly twice daily for 3 weeks. Groups of treated and untreated rats were killed at intervals during and up to 13 weeks after cessation of treatment, and the kidneys were removed for bacteriological study. Each kidney was divided into three equal pieces; each piece was homogenized and the homogenate was serially diluted in distilled water, 0.85 percent sodium chloride solution, or 0.3*M* sucrose solution in heart infusion broth. For cell counts of viable bacteria portions of the diluted homogenates made with 0.85 percent sodium chloride solution or distilled water were incorporated into pour plates containing blood agar base (Difco). Portions of the suspensions diluted in 0.3*M* sucrose were incorporated into pour plates made with blood agar base that also contained 0.3*M* sucrose. This concentration of sucrose, 0.3*M*, acts as a stabilizing agent for the osmotically fragile "protoplasts." All plates were incubated at 37°C for 48 hours. Several colonies were picked from each plate and the organisms were identified as *S. faecalis* by the following criteria: typical appearance on gram stain, growth in 6.5 percent NaCl in heart infusion broth, growth in and reduction of the dye in *S. faecalis* medium (BBL), negative catalase test and fermentation of lactose, mannitol, glucose and sorbitol.

The results, presented in Table 1, (*4*) indicated that the bacterial population in the kidneys of infected, untreated rats persisted in relatively constant numbers throughout the period of observation. These data do not indicate that significant "protoplast" formation occurred in the untreated animals. In those infected animals which received penicillin treatment, bacteria were not found in the kidneys at the end of therapy when homogenates of these organs were cultured on standard, nonstabilized medium. However, when 0.3*M* sucrose medium was used, "protoplasts" were found in the kidneys of the majority of animals up to 13 weeks after cessation of therapy. It is clear that the bacteria persisted in the tissues of the penicillin-treated animals in the form of "protoplasts," as indicated by the lack of growth in the nonstabilized medium. However, when grown on 0.3*M* sucrose medium, the "protoplasts" reverted to standard bacterial forms as manifested by growth in subculture in the various nonstabilized media used for identification.

Two additional control groups of animals were studied. Twelve normal (uninfected and untreated) rats and eight treated (uninfected but given penicillin for 3 weeks) rats were killed at times corresponding to 1 and 2 weeks after treatment and homogenates of the kidney were cultured. In no instance were bacterial or "protoplast" forms isolated. Thus, "protoplasts" developed only in the kidneys of animals infected with *S. faecalis* and subsequently treated with penicillin. Although penicillin therapy accounted for the conversion of bacterial forms to "protoplasts," it should be recognized that the osmolar concentration of renal tissue and urine is sufficiently high to protect these "protoplasts" from osmotic shock (*5*). This suggests that "protoplasts" might persist in renal tissue for long periods of time. The reason that "protoplasts" persist without reversion to the bacterial form is unknown but there may be a relation to the part played by serum antibody. Muschel *et al.* (*6*) have shown that exposure of bacteria to antibody, complement, and lysozyme can result in "protoplast" formation. Antibacterial antibody has been found in rats with *S. faecalis* pyelonephritis (*7*)

Table 1. Average of the logarithm of the number of bacterial and "protoplast" forms, per gram of kidney, isolated from treated (with penicillin) and untreated pyelonephritic rats. The numbers in parentheses indicate the proportion of kidneys containing organisms. Since all kidneys of untreated animals examined contained bacteria, the fraction is not presented in this group.

Time*	Treated			Untreated		
	No. of rats	Culture medium		No. of rats	Culture medium	
		Standard	0.3*M* sucrose		Standard	0.3*M* sucrose
2 days†	5	2.35 (4/5)	4.39 (5/5)	5	3.86	5.70
8 days†	6	1.02 (3/6)	2.49 (6/6)	6	6.39	6.60
3 wk	6	0.14 (1/12)	3.64 (12/12)	6	6.04	5.91
4 wk	6	0	3.82 (12/12)	6	6.46	6.53
5 wk	6	0	1.95 (6/12)	6	6.18	6.50
6 wk	6	0	2.64 (11/12)	6	5.42	5.83
11 wk	6	0	1.06 (4/12)	6	5.19	5.44
16 wk	8	0	2.01 (12/16)	4	4.92	5.61

* Interval between intravenous infection and sacrifice. † In the 2- and 8-day experiments only alternate kidneys were studied. At subsequent times, both kidneys were examined for bacterial and "protoplast" forms.