increasingly approximated pecking. In the following three sessions, reinforcement was contingent upon the first response that occurred after a variable interval of time; this interval had a mean, initially, of 30 seconds and was progressively lengthened to 180 seconds. After a total of 15 conditioning sessions, a high constant rate of responding was maintained, and this rate was independent of the intensity of the key illumination.

In the following session a test for stimulus generalization was conducted: three reinforcements were presented for responses in the presence of the training stimulus (589 mμ) and then, during the remainder of the session, no more reinforcements were presented and each of eight different wavelengths, including 589 mμ, was projected in irregular order onto the key. These test wavelengths were produced by interposing gelatin filters between a tungsten filament lamp and the response key. Stimulus wavelength and intensity changed concurrently every 30 seconds during the 3-second blackout.

The number of responses emitted at each wavelength during the test session for each subject is shown in Fig. 1. Figure 2 shows the mean stimulus generalization gradient for each of the two groups of animals; individual gradients were normalized and averaged. Wavelength exerted no systematic control over response emission for birds 1 to 4, which were raised in monochromatic light. For those birds raised in white light (5 and 6) the number of responses emitted at each wavelength was a function of the difference in millimicrons between the conditioning and test stimuli.

In a second experiment, it was demonstrated that ducklings raised in a monochromatic environment could be conditioned to respond differentially to stimulus wavelength. Initially the subjects were trained to respond in the presence of a key illuminated with the variable-intensity, 589 mμ stimulus of the first experiment. An identical conditioning procedure was employed. Then followed 15 sessions of conditioning with reinforcement contingent upon the completion of a fixed number of responses. Finally, a multiple schedule (*1*) was introduced in which a second stimulus (610 mμ) was alternated randomly with the training stimulus (589 mμ). In the presence of the new stimulus (610 mμ), reinforcement was contingent upon a pause (that is, cessation of pecking) of 45 seconds; in the presence of the training stimulus (589 mμ), reinforcement was contingent upon completion of 20 responses. For both subjects, there was a marked decrease in the rate of responding in the presence of the stimulus correlated with reinforcement for not responding within the first session in which reinforcement was differentially correlated with wavelength (*2*).

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Parotoid Secretions of Indonesian Toads

Abstract. A preliminary study of the parotoid secretions of *Bufo melanostictus* and *B. asper* shows that a methanol extract is rich in digitalis-like bodies (bufodienolides) and that an acid extract contains indolethylamine derivatives.

During the last three decades we have been studying the pharmacological properties of the constituents of 14 species of toads (*Bufo*) (*1, 2*). Church (*3*) recently reported that the erythrocytes of *Bufo melanostictus* in Java increased in size continuously throughout the life of the animal. He attributed this observation to the unchanging climatic conditions of the tropical Indonesian Archipelago. Through the cooperation of Church, who was then at the Institute of Technology, Bandung, we obtained small supplies of the dried parotoid secretions of two species of Javanese toads—*B. melanostictus* and *B. asper*. A preliminary study of each specimen was made by the same procedures employed previously (*2*). *Bufo asper* is the larger of the two; the longitudinal axis of its parotoid glands is perpendicular to the body axis (*4*). *Bufo melanostictus* is not only common in Java, but also is indigenous to Canton, China (*5*), Assam, India (*6*), and Bangkok, Thailand (*7*). Van Gils (*8*) isolated melanobufagin from the parotoid secretion of this species.

The sample from ten specimens of *B. melanostictus* weighed 91 mg and that from three specimens of *B. asper*, 27 mg. Both samples were easily pulverized, and grayish in color. A methanol extract of the venom from *B. melanostictus* was diluted to 1:10,000, and infused at the rate of 1 ml per minute into the femoral vein of two etherized cats. Electrocardiograms were recorded from Lead II. Sample tracings are shown at the top of Fig. 1. Like any active digitalis-like substance, the extract produced slowing of heart rate, P-R prolongation, inversion of T wave, ventricular rhythm, bundle branch block, and ventricular fibrillation. The electrocardiographic changes of the second cat were similar. The high potency of the as yet unidentified bufodienolides was indicated by the small lethal dose (LD): one was equivalent to 0.855 mg of the dried parotoid secretion per kilogram of body weight, and the other, to 0.709 mg/kg. The lower half of Fig. 1 is the result of an experiment with the methanol extract of the venom of *B. asper*, demonstrating its digitalis-like action electrocardiographically. The dilution was 1:20,000. Two cats succumbed to doses equivalent to 0.721 and 0.618 mg/kg.

Another extract was made with 0.1*N* HCl (*2*) and an aliquot was tested for color reactions. The venom extract of *B. melanostictus* reacted with *p*-dimethylaminobenzaldehyde (*9*) to give a dark green color, slightly greener than that produced by serotonin. The addition of Steensma reagent (*10*) resulted

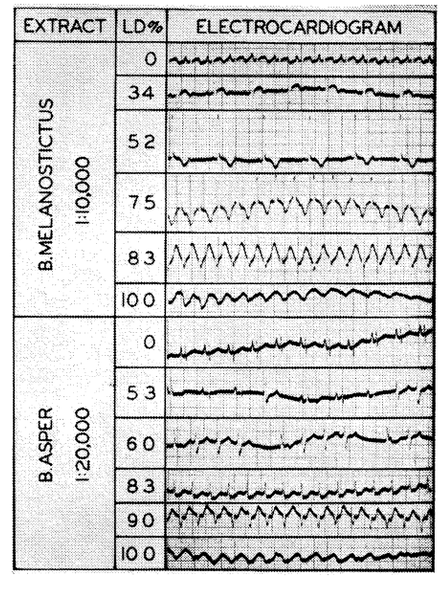

Fig. 1. Electrocardiographic changes produced by extracts of toad venom.

in a purple color similar to that caused by serotonin. The acid extract of the secretion of *B. asper* gave a light violet color with *p*-dimethylaminobenzaldehyde, and a faint purple color with the Steensma reagent, much less intense than that of *B. melanostictus*.

A second portion of the acid extract was injected intravenously into pithed cats. Rise of carotid blood pressure occurred with both *B. asper* and *B. melanostictus*. Equivalent doses showed that the extract of *B. melanostictus* was more active than that of *B. asper*. The pressor action was not blocked by ergotoxine or dibenamine. When the methanol extract was applied to the isolated rabbit ileum and uterus and the isolated guinea pig ileum and uterus, stimulation was observed with concentrations of 1:10,000 and 1:5000. These pharmacological results and color reactions are suggestive of the presence of indolethylamines, probably analogues of serotonin, in the parotoid secretions of the two species of Javanese toads. These bases also occur in the secretion of other toads (*11*). Of special interest is the absence of catecholamines, since no color was produced by Ewins' reaction (*12*) for epinephrine detection, even though concentrations of epinephrine of 1:500,000 gave a red color. These adrenergic substances have been found in certain species of toads (*1, 2, 13*).

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Detection of Aspergillus flavus in Soil by Immunofluorescent Staining

Abstract. Strains of *Aspergillus flavus* grown in soil in the presence of buried slides could be detected on the slides by fluorescent antibody techniques. Staining with *A. flavus* antiserum labeled with fluorescein, followed by examination with fluorescence microscopy revealed characteristic fluorescence at sites of distribution of the homologous fungus. The specificity of the reaction and the absence of nonspecific absorption of antibody to soil materials suggest that the method may be useful in studying the ecology of the soil microflora.

Knowledge of ecological relationships among microorganisms in the soil is fundamental to the disciplines of microbiology, soil science, and plant pathology. Little specific information has accumulated as the techniques available to soil ecology sharply restrict the relationships that can be studied. The complexity of the soil environment, the small size and nondescript morphology of most soil microflora, and a lack of distinctive physiological features for much of the indigenous microflora, present inherent difficulties.

Soil ecology problems may be approached by both direct and indirect means. Among the indirect methods, elective culture techniques have been exploited most extensively in recent years in connection with interest in the ecology of soil fungi (*1*). Information obtained by elective culture and other indirect methods is derived at the expense of separating a given isolate from its soil environment. Direct methods also have intrinsic limitations but often afford a realistic view of microorganisms *in situ*. The Cholodny buried (contact) slide technique provides one of the best means for viewing some interrelationships in the soil environment, but the organisms viewed cannot be cultured and only rarely can be identified.

The usefulness of the buried slide would be greatly extended if microorganisms could be detected and recognized directly on the slide. Application of the immunofluorescent staining technique was considered as a possible means of detecting a particular microorganism on the buried slide. The promising results obtained thus far with this approach, substantial interest following an oral report (*2*), and the possible application of the technique to numerous ecological problems prompt this preliminary report.

Interest was centered on the soil fungus *Aspergillus flavus*. As this heterotrophic organism is capable of nitrate formation in pure culture, its ecology in natural environments is worthy of attention (*3*). The antigen was mycelium of *A. flavus* strain CS, cultured in a glucose, dicalcium glutamate, inorganic salts medium in shaken culture at *p*H 7.3. Mycelium was filtered, washed with sterile saline, minced in a blender, and suspended in saline at a concentration of 0.1 mg/ml (based on dry weight of mycelium) for intravenous injection into rabbits. Antiserum with agglutination titers of at least 1280 was fractionated with ethanol, and the crude gamma globulin was conjugated with fluorescein isothiocyanate (*4*).

A simple soil system was adopted to permit the development of the technique with a single known culture present on the slide. Centrifuge tubes, 25 to 30 cm in diameter, were half filled with air-dry loam soil and then sterilized. Sterile microscope slides were then inserted part way into the soil. The soil was moistened slightly with 3 to 5 ml of sterile glucose (0.1 percent) solution and inoculated with a few drops of a spore suspension of the test fungus. After 2 to 4 days of incubation, one surface was washed clean, and the other carefully flooded with distilled water to remove larger soil particles only. After drying briefly, the slide was gently heat fixed, placed in a moist chamber, and the unwashed face was flooded with labeled antiserum. After 30 minutes of contact with the antiserum, the slide was washed carefully with distilled water and dried at room temperature.

Preliminary examination of slides with bright-field microscopy and ordinary light often was helpful in locating fields of particular interest. Mycelium and spores usually were plentiful on the slides together with the soil minerals and the amorphous organic matter that is normally present on buried slides.

Slides were examined for fluorescence by means of a dark-field condenser with an HBO-200 mercury vapor light source. A primary filter (Corning 5840 or Corning 5860) and a colorless eyepiece barrier filter (Wratten 2B) were effective in suppressing the autofluorescence of soil particles and microorganisms, and they allowed the detection of the yellow green color emitted by the fluorescein. Fields were