gressiveness. In all the experiments, recovery was complete, and there were no mortalities.

Although preliminary observations would indicate that *B. splendens* responds differently to a diverse number of pharmacological agents, the tranquilizing agents do seem to induce a characteristic response. All such agents definitely suppressed the quality of pugnaciousness without necessarily impairing sensitivity and motor activity. It is felt that this preparation can be used in the partial evaluation of the tranquilizers, as well as related neurotropic agents.

E. J. WALASZEK
L. G. ABOOD
*Department of Psychiatry* and
*Department of Biochemistry,*
*University of Illinois*
*College of Medicine, Chicago*

### References and Notes

1. J. V. Brady, *Science* **123**, 1033 (1956).
2. This research was supported jointly by a contract between the U.S. Office of Naval Research and the University of Illinois and by a grant from the Illinois Department of Public Welfare.
3. H. A. Abramson and L. T. Evans, *Science* **120**, 990 (1954); L. T. Evans *et al.*, *Science* **123**, 26 (1956).
4. W. T. Innes, *Exotic Aquarium Fishes* (Innes, Philadelphia, Pa., 1955).
5. The observations were made independently by us and by two psychologists, Garth J. Thomas and Leon S. Otis, whose assistance is gratefully acknowledged.

27 July 1956

## Extracellular Deoxyribonucleic Acid of Bacteria and a Deoxyribonuclease Inhibitor

The slime layer of bacterial cells is an extracellular accumulation of viscous material, most commonly composed of some high-molecular-weight polysaccharide. Smithies and Gibbons (*1*) found that the slime layers of various halophilic bacteria contain deoxyribonucleic acid (DNA). The slime could be dispersed by the action of pancreatic deoxyribonuclease (DNase), with the liberation of soluble DNA-split products, without affecting cellular viability. More recently, the viscosity of the ropy sediments produced by a number of nonhalophilic bacteria has been shown to depend likewise on the presence of polymerized extracellular DNA (*2*). Investigations of *Brucella* (*3*) indicate the presence in certain cultures of extracellular DNA that is sufficiently polymerized to produce a fibrous precipitate in ethanol (*4*). Because of the interest attaching to the extracellular accumulation of a supposedly intracellular gene-associated type of material, DNA was isolated from cultures of various bacteria by methods designed to leave the cells intact. In further investigations (*5*), a mechanism leading to the accumulation of DNA slime was found to be the production of extracellular ribonucleic acid (RNA), which is capable of inhibiting DNase action.

Slime-layer DNA preparations were obtained from *Micrococcus citreus, M. pyogenes* var. *aureus* (three strains), *Alcaligenes faecalis* (two strains), *A. viscosus,* and three strains originating as laboratory contaminants, which were notable for their massive accumulation of slime. Of these, strain B is a species of *Pseudomonas* and strains C and E appear to be members of *Flavobacterium. Micrococcus pyogenes* var. *aureus,* which is pathogenic for human beings, grows readily in mediums containing 7.5-percent NaCl (1.3*M*) but usually is not considered to be a halophile. The other bacteria were considerably less salt tolerant, their growth being inhibited by 1.25*M* NaCl, and maximum growth was obtained only in broth containing less than 0.25 to 0.5*M* NaCl.

*Micrococcus pyogenes* var. *aureus,* which produces large quantities of an extracellular DNase under certain conditions (*6*), formed visible slime in young nonaerated brain-heart infusion (Difco) cultures; more massive accumulations were obtained when 1*M* NaCl was present or when the broth was buffered at *p*H 6.0 (succinate, 0.05*M*)—procedures designed to minimize DNase action. The other bacteria formed DNA slime under various conditions; commonly, a peptone-yeast extract broth was used. Cultures were harvested, usually after 2 to 4 days, by aspirating off the nonviscous broth from the mass of slime-covered cells. The slime was stirred with 0.41-percent sodium dodecyl sulfate for 3 hours, and NaCl was added (final concentration 1*M*) with further stirring. The viscous mixture was centrifuged and cells, which were revealed to be intact by microscopic examinations, were discarded. Nucleic acid was precipitated from the supernatant by addition of ethanol. Yields at this stage from strains B and C were commonly 200 mg of crude, dry product (containing 40 to 45-percent DNA) per liter of broth culture. One or two additional steps of purification with detergent were carried out (*7*).

Curves of the ultraviolet absorption spectra for solutions of each slime-layer preparation were quite similar to the curve obtained using thymus DNA; maxima for all occurred between 256 and 259 mμ. Comparisons of deoxyribose (*8*) and phosphorus values indicated that, with preparations from *Micrococcus citreus, Alcaligenes faecalis,* and strain B, more than 95 percent of the nucleic acid was DNA. The other preparations, however, showed considerably higher proportions of RNA (*9*). Furthermore, ratios of phosphorus to dry weight indicated that about half of each sample consisted of phosphorus-free ultraviolet-nonabsorbing material, believed to have been mainly polysaccharide (*9*).

Although the microbial preparations were not pure DNA, the viscosity of the solutions was practically the same as that of solutions of thymus DNA (*7*) having equivalent deoxyribose (Dische reaction, *8*) content. By means of small Ostwald-type viscometers (*10, 11*), determinations were made of the rates of viscosity reduction by crystalline pancreatic DNase (Worthington) of each of the slime-layer preparations and of thymus DNA. The reaction mixtures at 37°C contained $10^{-6}$ mg/ml of pancreatic DNase, 0.8 to 1.2 mg/ml of DNA, and imidazole buffer at *p*H 7.3, with 0.025*M* $MgCl_2$. The rates of depolymerization were essentially the same for all the samples (and their viscosities were reduced to that of water in each case, except *Alcaligenes viscosus*), thus verifying the presence of highly polymerized DNA.

Such DNA could not accumulate in the presence of active depolymerizing enzyme. Investigations of strain B showed that, under certain conditions, broth cultures may evidence depolymerizing activity when tested against thymus DNA but little or no activity against strain B DNA. This suggested that strain B and possibly the other bacteria also, may produce a deoxyribonuclease inhibitor (*11, 12*).

Table 1 shows the effects of treating strain B DNA with crystalline ribonuclease (RNase, Worthington), which was found to be free of DNase activity. The DNase of strain B, which resembles pancreatic DNase in being activated by Mg at *p*H 7.3, was obtained from cells (shaken with ballotini in a Mickle disintegrator) of a young culture. Using a fresh dilution (1/20 in 0.1-percent gelatin) of this disintegrated-cell preparation each time, parallel viscometric tests were carried out with DNA from two sources.

Table 1. Effect of ribonuclease on susceptibility of a preparation of slime-layer DNA to the subsequent action of DNase.

| RNase treatment of strain B DNA (before adding DNase) | | Activity (units) of strain B DNase against | |
|---|---|---|---|
| RNase (mg/ml) | Time incubated (min) | Strain B DNA | Thymus DNA (control) |
| No RNase treatment | | 1.3 | 12 |
| $5 \times 10^{-4}$ | 1 | 10 | 13 |
| | 120 | 12 | 12 |
| $1 \times 10^{-5}$ | 2 | 7 | 13 |
| $1 \times 10^{-6}$ | 7 | 6 | 13 |
| $1 \times 10^{-7}$ | 2 | 1 | 12 |
| | 65 | 2 | 12 |
| | 2880 | 6 | 13 |

Exposure of strain B DNA to $5 \times 10^{-4}$ mg/ml of RNase for 1 minute before testing allowed the DNase to exhibit 10 units (instead of 1.3 units obtained in tests without preliminary RNase treatment), or 77 percent of the activity which it showed against thymus DNA. (If, in addition, the DNase preparation itself was subjected to RNase action, a higher DNase activity was demonstrated.) Lower concentrations of RNase also evidenced some inhibitor-destroying capacity, justifying the conclusion that this DNase inhibitor is a ribonucleic acid.

B. WESLEY CATLIN

*Department of Microbiology, Marquette University School of Medicine, Milwaukee, Wisconsin*

**References and Notes**

1. W. R. Smithies and N. E. Gibbons, *Can. J. Microbiol.* 1, 614 (1955).
2. B. W. Catlin, *Bacteriol. Proc.* 123 (1956).
3. W. Mauzy, W. Braun, J. Whallon, *ibid.* 46 (1955).
4. W. Braun, personal communication.
5. Supported by research grant C 2405 from the National Institutes of Health, U.S. Public Health Service.
6. L. Cunningham, B. W. Catlin, M. Privat de Garilhe, *J. Am. Chem. Soc.*, in press.
7. R. M. Kay, N. S. Simmons, A. L. Dounce, *ibid.* 74, 1724 (1952).
8. Z. Dische, *The Nucleic Acids*, E. Chargaff and J. N. Davidson, Eds. (Academic, New York, 1955) vol. 1, p. 285.
9. Details of these analyses are in preparation.
10. M. Laskowski and M. K. Seidel, *Arch. Biochem.* 7, 465 (1945).
11. S. Zamenhof and E. Chargaff, *J. Biol. Chem.* 180, 727 (1949).
12. A. W. Bernheimer and N. K. Ruffier, *J. Exptl. Med.* 93, 399 (1951); A. W. Bernheimer, *Biochem. J. (London)* 53, 53 (1953); L. M. Kozloff, *Cold Spring Harbor Symposia Quant. Biol.* 18, 209 (1953); M. N. Swartz, N. O. Kaplan, M. E. Frech, *Science* 123, 50 (1956).

15 June 1956

## Second Pain: Fact or Artifact?

The phenomenon of "double pain," two temporally discrete and sometimes qualitatively different responses to a single noxious stimulus, has been reported intermittently for the past 75 years. Although there is a good deal of evidence that the phenomenon may be an artifact, it is accepted as genuine by many physiologists. Sinclair, who has been associated with much recent work on second pain, has concluded that it needs reinvestigation (*1*).

The problem is not simple. There are two quite separate and distinct aspects —sensation, a psychological term implying awareness, and afferent impulses, a physiological phenomenon revealed by electric recording techniques. These are often treated as though they were one and the same thing—indeed, it has recently become the fashion to speak of "C-fiber pain." The first question to be asked is whether there is a genuine sensory phenomenon that can be called "second pain." The present experimental data bear on this question only.

The experimental work consisted of three successive attempts to elicit double pain by stimulation of cutaneous receptors of the arm. In the first series, contact heat thresholds (time for a known temperature) were determined for eight subjects for hand, forearm, and elbow. In about one-fourth of the trials, two pains could be inferred—either one brief flash (which disappeared before the subject could respond) followed by a second pain, or fluctuation in the intensity of the pain. It seems obvious that any stimulus of this nature is essentially uncontrolled at the receptor level, and that heat continues to penetrate deeper into the tissues and to stimulate more remote receptors as well as deeper ones, even after the stimulator is removed. For this reason, no further work was done with this type of stimulus. The literature shows this to be one of the most effective stimuli in eliciting double pain.

In the second series, mechanical pain thresholds for three spots on the dorsal forearm were first determined for eight subjects. A rigidly-mounted, needle algesimeter, calibrated in ¼-g steps, was used. The needle was sharpened under a microscope to minimize the stimulation of pressure, a probable reason for some of the reports of double pain, as von Frey contended (*2*). No double pains were reported. Adaptation trials at threshold and at 1 g above threshold (thresholds range from 2.5 to 5.0 g) were made. The course of adaptation showed fluctuations. In about one-fifth of the trials, there were only two peaks, which naive observers might possibly have interpreted as double pains, although ours did not. This work was repeated with four subjects who were highly practiced in psychophysical observation, using three spots on the dorsal forearm and three spots on the dorsal aspect of the middle finger. Additional adaptation trials were made at 2 g above threshold. Again no double pains were observed.

It is common knowledge in psychophysical research on pain that the stimulus must be unvarying. This would rule out handheld needles and similar apparatus, the sort of equipment which appears frequently in positive reports of double pain. If one permits the needle to remain in place, one finds fluctuations in sensation, as illustrated in the preceding paragraph. If one pulls it out, one has restimulated the receptor, as shown electrophysiologically by Zotterman (*3*). These phenomena will be intensified with suprathreshold stimuli. At or near threshold, double pains are not reported for mechanical stimulation. Where thresholds have not been experimentally determined (in the majority of studies), reports of double pain are suspect.

A further difficulty besets mechanical stimulation with suprathreshold stimuli —namely, the possibility of stimulating two discrete receptors sequentially. The fact that it takes appreciable time for a needle to penetrate to its maximum depth (*4*), makes this possibility very real. When our four experienced subjects were requested to observe carefully for a possible double pain and then were stimulated with suprathreshold stimuli, there were four reports of double sensations out of 20 trials. But two of these involved other cutaneous senses; one was cold and pain, and one was pressure and pain. Only two were double pains. This lends support to the hypothesis that two discrete receptors may be stimulated at different times under these conditions.

The third series consisted of 120 electric pain thresholds on the dorsal forearm for the group of four experienced subjects. A square-wave pulse from a Grass stimulator was used, the duration being physiologically infinite. There were no double pains, and no single, delayed pains.

In many ways, electric stimulation is less subject to artifacts than other kinds —at least, the time of action of the stimulus is constant, so that if two receptors are stimulated, they are stimulated simultaneously. In addition, it is possible to stimulate pain without the concomitant arousal of other cutaneous senses. It is significant that no experimenter has reported double pain with a single electric stimulus. Since all other senses are stimulable electrically, it seems difficult to believe that if there is a second, slower pain system leading to sensation, it would not appear in 480 separate determinations. The argument that a second pain system is suppressed by the faster system lacks evidence.

Double pain was not found with normal subjects under controlled conditions. Landau and Bishop also obtained somewhat similar results with their normal subjects (*5*).

Other lines of evidence have been thoroughly reviewed recently (*6*). One of the prime lines of evidence for double pain has been the order of loss of sensations during nerve blocks. Careful experimentation on several different areas, coupled with statistical analysis of the significance of the order of loss, shows this evidence to be unreliable (*6, 7, 8*).

A second line of defense has been the delay in pain perception under ischemic conditions, often interpreted as a dropping out of one pain system while a