tides (DPNH, TPNH), or reduced redox substances with potentials more negative than −81 mv can serve as electron sources for the reduction of flavin mononucleotide (FMN) (*4*).

Redox substances that react with the electron carriers involved in bioluminescence divert the electron flow from the light reaction and thus inhibit bioluminescence. For example, Spruit and Schuiling (*5*) found marked inhibition of bacterial luminescence by certain naphthoquinones and dyes whose redox potentials range from −33 to +65 mv, and McElroy and Strehler (*3*) point to the inhibition of luminescence by riboflavin or flavin adenine dinucleotide, substances which are thought to compete with FMN for the electrons from DPNH; a similar action is exerted by ferricyanide (*4*).

The study described in this report deals with the action on bacterial luminescence of redox indicators whose potentials range from −125 to +217 mv and shows that luminescence was inhibited by substances with redox potentials between +11 and +217 mv.

Suspensions of *A. fischeri,* prepared as described previously (*6*), were diluted 1:1.5 with 2.5 percent NaCl, buffered with 0.06*M* phosphate at *p*H 7.4. To these suspensions in test tubes were added the redox substances listed in Table 1. Bacterial luminescence and reduction of the indicator dyes were observed visually.

Reversible redox substances with potentials lower than +11 mv did not influence the luminescence of the bacteria, while above this potential level, luminescence was abolished and the dyes were reduced to their colorless leuco forms except for a narrow zone at the surface of the experimental mixtures.

The irreversible reduction indicator, triphenyltetrazolium chloride (TTC), was reduced to the red, water-insoluble triphenyl formazan by suspensions of *A. fischeri,* while bioluminescence was strongly but not completely inhibited. Triphenyltetrazolium chloride is known to be reduced by bacterial flavoproteins (*7*).

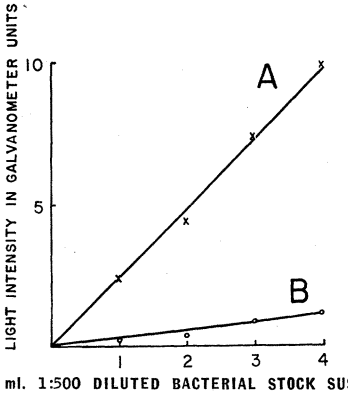

Fig. 1. Reversible inhibition of bioluminescence by methylene blue. (A) No inhibitor; (B) $(1.6 \times 10^{-5})M$ methylene blue added.

Table 1. Interaction of reversible redox substances with the bioluminescence of *Achromobacter fischeri.*

Dye and molar concn.	Redox potential (mv)	Inhibition of luminescence	Reduction of dye
Nile blue (9.1×10^{-5})	−125	−	−
Indigo tetrasulfonate (4.5×10^{-5})	− 46	−	slight
Methylene blue (1.0×10^{-4})	+ 11	+	+
Thionine (1.5×10^{-4})	+ 62	+	+
Toluylene blue (1.1×10^{-4})	+115	+	+
2,6-Dichlorphenol indophenol (1.2×10^{-4})	+217	+	+

Competition for the electrons of $FMNH_2$ between the light reaction and a redox substance of suitable potential could be expected to produce the phenomenon of reversible inhibition of bioluminescence. This hypothesis was tested for methylene blue as the alternate carrier, an experimental design outlined by Ackermann and Potter (*8*) being used. Bioluminescence was measured quantitatively in a special photometer, which has been described previously (*6*).

Curve A in Fig. 1 shows that the light production in appropriately diluted suspensions of *A. fischeri* was directly proportional to the concentration of bacteria in suspension. The addition to these suspensions of methylene blue to a molar concentration of 1.6×10^{-5} resulted in approximately 90 percent inhibition of luminescence, regardless of the bacterial concentration (curve B, Fig. 1). This type of response is indicative of reversible inhibition (*8*).

Reduction of reversible redox indicators could well be brought about by tapping the electron flow of the main respiratory pathway of the bacteria; however the parallelism between inhibition of luminescence and reduction of dyes in this study suggests that the two phenomena may be related. While it is thought that TTC and methylene blue react with a flavin enzyme of the diaphorase type, the inhibition of bioluminescence by substances with more positive redox potentials suggests an interaction with at least one additional component of the light-producing system whose redox potential could be as high as +217 mv. It has been suggested that palmitic aldehyde may be such a cofactor (*9*), although enzymatic oxidation of aldehyde groups usually proceeds at less positive potentials.

McElroy and Green have propounded a hypothesis according to which two reduced flavin molecules are required for light production—one which combines with the aldehyde and a second one which forms an organic peroxide; oxidation of the aldehyde by the peroxide is considered to be the reaction that yields the energy for light production (*4*). The two authors have stated that peroxidation of the aldehyde would yield approximately 75 kcal/mole, which would be in excess of the 60 kcal required for the bacterial emission of light with a peak around 480 mμ.

If an additional electron carrier with a potential considerably more positive than that of FMN were involved in light production, it would be difficult to visualize how one pair of electrons might pass in one step across the potential difference of 1200 mv to produce the approximately 60 kcal/mole of quanta (Einstein) which correspond to the emitted wavelength around 480 mμ.

FRED E. HAHN

Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C.

References

1. M. J. Cormier and B. L. Strehler, *J. Cellular Comp. Physiol.* 44, 277 (1954).
2. B. L. Strehler, *J. Am. Chem. Soc.* 75, 1264 (1953).
3. W. D. McElroy and B. L. Strehler, *Bacteriol. Rev.* 18, 177 (1954).
4. W. D. McElroy and A. A. Green, *Arch. Biochem. Biophys.* 56, 240 (1955).
5. C. J. P. Spruit and A. L. Schuiling, *Rec. trav. chim.* 64, 219 (1945).
6. F. E. Hahn, C. L. Wisseman, Jr., H. E. Hopps, *J. Bacteriol.* 69, 215 (1955).
7. A. F. Brodie and J. S. Gots, *Science* 116, 588 (1952).
8. W. W. Ackermann and V. R. Potter, *Proc. Soc. Exptl. Biol. Med.* 72, 1 (1949).
9. B. L. Strehler and M. J. Cormier, *J. Biol. Chem.* 211, 213 (1954).

4 October 1957

Isolation of Cytoplasmic Particles with Cytochrome Oxidase Activity from Apples

The apparent absence of cytochrome oxidase activity and the simultaneous presence of very high phenolase activity in particles isolated from plant tissues should not, without further careful scrutiny, be considered sufficient evidence for the nonparticipation of the cytochromes in the respiration of these tissues. In fact, we have found that cytochrome oxidase activity in young apples can be completely masked by the effects of low *p*H of the extracting medium.

When young Rome Beauty apples about 4 to 5 cm in diameter were homogenized in a Waring blender at 2° to 4°C

in a medium containing 0.25*M* sucrose, 0.01*M* ethylenediaminetetraacetic acid (EDTA), and 0.1*M* phosphate buffer at *p*H 7.0, no cytochrome oxidase activity was found (Fig. 1) (*1*). The homogenate rapidly turned brown during blending (30 seconds at top speed and 1 minute at a slow speed at approximately 30 v), and after centrifugation of the homogenate at 1000*g* the pellet isolated at 16,000*g* was also brown. The *p*H of the homogenate obtained by such a procedure was 6.0, and the *p*H of the particles suspended in 8 ml of 0.25*M* sucrose and 0.001*M* adenosine triphosphate (ATP) was 5.2 (*2*). The nitrogen content of 0.5 ml of the particulate suspension (derived from 200 g of tissue and 300 ml of homogenizing medium) was about 260 μ. All the phenolase activity from such a preparation was associated with the particulate fraction (Fig. 1), which showed considerable activity.

Quite a different picture emerged when the apples were homogenized in a solution containing 0.15*M* sucrose, 0.01*M*

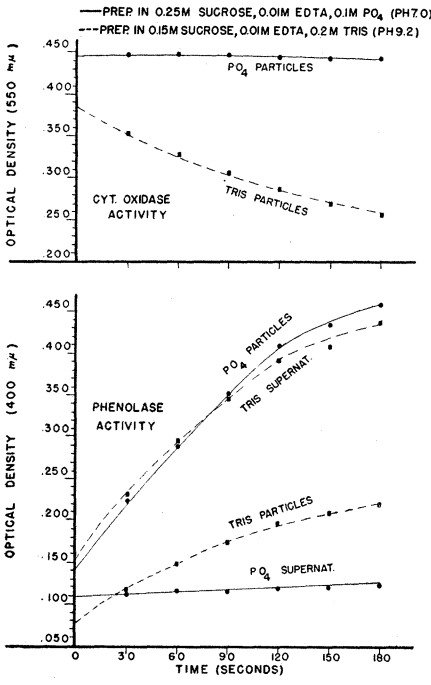

Fig. 1. (Top) Cytochrome oxidase activity of cytoplasmic particles from apples. Activity was determined by the method of Cooperstein and Lazarow (*1*). The crude enzyme used in the assay consisted of 0.03 ml of the particulate suspension. (Bottom) Phenolase activity of cytoplasmic particles and supernatant from apples. Activity was determined by the method of Ponting and Joslyn (*3*) with modifications as follows: 2.99 or 2.90 ml of 0.05*M* catechol in acetate buffer at *p*H 5.0 was the substrate, and 0.01 ml of the particulate suspension or 0.1 ml of the supernatant was added at zero time to a 3-ml cuvette. All assays were made with a Beckman DU spectrophotometer. The values shown are averages of three experiments.

EDTA, and 0.2*M* tris-hydroxymethyl aminomethane (Tris) at *p*H 9.2. The homogenate in the Tris medium had a *p*H of 9.0 and remained green during blending. The isolated particles were green, and the particulate suspension had a *p*H of 6.2. The nitrogen content of 0.5 ml of this particulate suspension (obtained in exactly the same manner as particles isolated in sucrose, EDTA, and phosphate) was about 140 μg. Cytoplasmic particles from apples isolated in this way exhibited good cytochrome oxidase activity (Fig. 1) and showed a different distribution pattern for phenolase activity (*3*). Much of the phenolase activity in this preparation was associated with the supernatant from the high-speed centrifugation, but the cytoplasmic particles also showed significant phenolase activity (Fig. 1).

Three possible explanations for the altered pattern of phenolase activity and for the considerable difference in the nitrogen content of the particulates isolated at the two *p*H levels may be proposed. If it is assumed that phenolase is localized on the cytoplasmic particles *in vivo*, then the enzyme may be removed from the particles by solubilization at *p*H 9.2 but not at *p*H 7.0. A second possibility is that phenolase, present in both soluble and particulate fractions, is agglutinated when apple cells are ruptured in a homogenizing medium at a *p*H of 7.0, but not at the higher *p*H. The third possibility is that phenolase is preferentially adsorbed on the particles in an extracting medium at *p*H 7.0, but that there is no adsorption at *p*H 9.2. Ponting and Joslyn (*3*) found that apple phenolase tightly adsorbed on particles could be solubilized by using a 2 percent sodium carbonate solution of *p*H 11.3. Hagen and Jones (*4*) observed a similar *p*H effect on catalase activity in chloroplast particles and in supernatant fractions from wheat leaves.

Hackney (*5*) reported no cytochrome *c* in apples and concluded that cytochrome oxidase is absent from them. Webster (*6*) could not demonstrate the occurrence of cytochrome oxidase in apple tissue and failed to show a reversal of carbon monoxide inhibition. This report demonstrates cytochrome oxidase in apple particulates by spectrophotometric methods. There are two additional reports (*7*) in which cytochrome oxidase activity was demonstrated by manometric techniques in tissue slices and in cell-free extracts from apples. In our work with apples it appeared that use of homogenizing medium of high *p*H (9.2) is necessary for isolating particulates in which cytochrome oxidase activity can be demonstrated. The cells of the apple fruit have a sap *p*H of 3.5 (*8*) and contain a very active phenolase system (*9*). It seems necessary to counteract the acidity and remove most of the phenolase from the particulates before good cytochrome oxidase activity appears. Since the *p*H of the cell sap of many fruit tissues is very low, and since these tissues usually show high phenolase activity, it may be necessary to use homogenizing media of high *p*H values to isolate cytoplasmic particles that show cytochrome oxidase activity (*10*).

MORRIS LIEBERMAN

Biological Sciences Branch,
Agricultural Marketing Service,
Beltsville, Maryland

References and Notes

1. S. J. Cooperstein and A. Lazarow, *J. Biol. Chem.* 189, 665 (1951).
2. In the preparation procedure, the pellet obtained at 16,000*g* was washed once with 0.25*M* sucrose and resedimented at 16,000*g* before it was finally suspended in sucrose-ATP solution. The final suspension was homogenized with a motor-driven Teflon pestle in a snug-fitting homogenizing tube.
3. J. D. Ponting and M. A. Joslyn, *Arch. Biochem.* 19, 47 (1948).
4. C. E. Hagen and V. V. Jones, *Botan. Gaz.* 114, 130 (1952).
5. F. M. V. Hackney, *Proc. Linnean Soc. N. S. Wales* 73, 439 (1949).
6. G. C. Webster, *Am. J. Botany* 39, 739 (1952); *Plant Physiol.* 29, 399 (1954).
7. B. A. Rubin and M. E. Ladygina, *Biochemistry* (U.S.S.R.) (English translation) 21, 549 (1956); T. Nakabayashi, *J. Agr. Chem. Soc. Japan* 28, 212 (1954).
8. In *Handbook of Biological Data,* W. S. Specter, Ed. (Saunders, Philadelphia, Pa., 1956), p. 83.
9. H. W. Siegelman, *Arch. Biochem. Biophys.* 56, 97 (1955).

10. The assistance of Edwin H. Cumings is gratefully acknowledged.

4 October 1957

Alteration of Clay Minerals by Digestive Processes of Marine Organisms

The possible importance of the highly acid chemical environment in animal stomachs upon clay minerals suspended in marine water was brought to our attention by two papers (*1*) which were concerned primarily with the effects of inorganic particles upon organisms. These papers demonstrated that many plankton-feeding and ooze-feeding fishes, clams, plankton animals, and other organisms are apparently unable to separate organic food particles from inorganic clay particles of the same size and cannot avoid passing suspended clay material through their digestive organs. An analysis of the stomachs of mullet (*Mugil cephalus*) from the surf at Port Aransas, Texas, showed that each fish contained an average of 1.6 g of clay within its stomach and intestine. Similar clay-filled guts were observed in menhaden, shrimp, and anchovies. That changes in the clay mineral composition of sediments being brought to the bays