plying the necessary tanning agent is the product of the underlying secretory cells. A positive argentaffin reaction plus the identification of a protein substrate and a phenol oxidase provide support for the existence of a complete quinone tanning system (*11*). On the basis of the present research it can be postulated that the hardening of the periostracum of *M. mercenaria* may be brought about by the action of an orthoquinone produced by the oxidation of the side chains of the phenolic compound secreted by the underlying cells of the first fold. The combination of this oxidation product with either the main chains of the polypeptides, or the side chains, results in the tanning reaction. Brown (*2*) has suggested a self-tanning, that is, one in which the phenolic protein acts as both a substrate and a tanning agent, for the byssus of *Mytilus edulis*. Whether the product of the secretory cells undergoes self-tanning or, in an oxidized state, aids in tanning another protein, perhaps the product of the epithelial cells themselves, is not clear.

It is interesting that, except for the sulfide link found in keratinized proteins, the quinone bonding mentioned above is the only other covalent link found so far in skeletal proteins (*12*), and might possibly be responsible for the sclerotization of such proteins throughout the animal kingdom (*11; 13*).

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19 July 1961

Luminescence Potency of the Cypridina System

Abstract. Visible light is emitted on oxidation of 0.00001 μg/ml ($2 \times 10^{-11}M$) of *Cypridina* luciferin with 0.01 mg/ml of luciferase protein, or with 1 μg/ml of luciferin with 0.0000001 μg/ml ($2 \times 10^{-15}M$) of luciferase. Data on yields indicate the average content of luciferin and luciferase to be, at best, about 1 μg of each in a single living organism.

In early studies on the biochemistry of the light-emitting enzyme-substrate ("luciferase-luciferin") system of the small ostracod crustacean *Cypridina*, Harvey (*1*) estimated that the addition of crude luciferase to a solution containing 1 part of luciferin in from 4 to 40 billion parts of water resulted in the production of light visible to the dark-adapted eye. This estimate was based on the assumption that the raw material, consisting of whole dried organisms, contained between 1 and 10 percent luciferin by weight. Since pure, crystalline luciferin has recently been isolated from dried specimens (*2*) as well as from initially living organisms preserved in Dry Ice (*3*), and a tentative description of its structure has been made (*4*), more definitive estimates can now be made regarding the actual luciferin content of the organism as well as the minimal concentration, in terms of number of molecules (molecular weight 469) required for visible luminescence. The same is true with respect to luciferase; it has very recently been obtained in an essentially pure state, and its major properties have been determined (*5*). Moreover, when both the enzyme and substrate are pure, quantitative errors due to the influence of unknown substances, which might exert inhibitory effects on the activity of luciferase or quenching effects on the total light emission, can be eliminated.

For the experiments reported here (*6*), a methanolic solution of crystalline luciferin was calibrated by optical density at 435 mμ in a Beckman spectrophotometer. Pure luciferin is relatively stable in methanol, although it rapidly autoxidizes in aqueous solution exposed to air. Therefore, dilutions were made in methanol; and in accordance with Harvey's observations (*1*), extreme precautions (using all new glassware, and so forth) were exercised to avoid errors due to adsorption of luciferin on, and its release from, glass surfaces of pipettes and containers. Tests for light emission were made by adding, in total darkness, 4.9 ml of a solution of 0.5 mg protein of practically pure luciferase in 0.1M sodium chloride plus 0.05M sodium phosphate buffer, pH 7.0, to 0.1 ml of a given dilution of luciferin, for simultaneous observation by three or four persons with dark-adapted eyes. A faint, momentary luminescence was clearly seen at a dilution of 1 in 10^{11} ($2 \times 10^{-11}M$ luciferin), but none at 1 in 10^{12}.

The potency of luciferase was similarly tested by adding dilutions of the enzyme in the aforementioned salt solution (glassware and blank solutions were first autoclaved) to 1 μg of luciferin in 0.1 ml of methanol. Light was observed at a final concentration of 1 part of luciferase in 10^{13} parts of solution, though not with certainty at 1 part in 10^{14}. On the basis of 50,000 as the molecular weight (*5*), a luciferase concentration of somewhat less than $10^{-15}M$ ($<10^{-7}$ μg/ml) is thus sufficient to yield visible light.

Analogous experiments with respect to the actual visibility of luminescence at great dilutions of firefly luciferin and luciferase have not been reported, but the quantum efficiency of the firefly system has been measured with the lu-

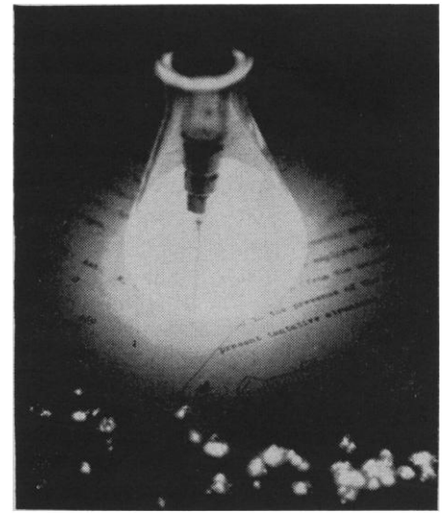

Fig. 1. Photograph taken by the light of the *Cypridina* reaction. The foreground shows individual specimens and small clumps of the organisms luminescing after removal from Dry Ice in which they were stored for about 2 months. The reaction mixture in the flask consisted of 28 ml of buffered salt solution containing 0.002 mg of pure luciferin to which was added, by the hypodermic syringe, 2 ml of salt solution containing 2 mg of practically pure luciferase. Exposure time, 1 minute at $f/2$; Tri-X Pan film.

ciferin in a concentration of $2 \times 10^{-11} M$ and found to be one quantum of light per molecule of luciferin oxidized, with an accuracy of $\pm$ 28 percent (*7*). The quantum efficiency of the *Cypridina* system remains to be determined. It is noteworthy, however, that a luciferin concentration of the order $10^{-11} M$, in both systems referred to above, is unusually low for a chemical test. The most sensitive qualitative tests, involving color reactions, precipitation, or fluorescence, are generally not useful at dilutions greater than 1 part in 10^{10}. Luminescence as an indicator for substances other than the specific enzyme or substrate is apparently not as sensitive; for example, the firefly system has been used to detect the cofactor adenosine triphosphate in concentrations down to about $10^{-9} M$ (*8*), and the "luminol reaction" to detect hydrogen peroxide in concentrations down to $10^{-7} M$ (*9*).

Quantitative data on the yield of pure luciferin (*3*, *10*) and luciferase (*5*) from *Cypridina* indicate that the living organisms, averaging about 4 mg (wet weight) each, contain at best 1 μg of luciferin and 1 μg of luciferase, in a molecular ratio of 100 to 1. The impressively bright luminescence of these small crustaceans, amply sufficient for a self-photograph (Fig. 1), is understandable in terms of the light-emitting potency of the system, despite the seemingly minute quantities of the essential components involved.

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10. We have recently obtained 60 mg of crystalline luciferin, in an approximately 50-percent yield, per kilogram (wet weight) of winter *Cypridina* preserved in Dry Ice.

14 August 1961

Glucose-6-Phosphate Dehydrogenase and Detoxification of Hydrogen Peroxide in Human Erythrocytes

Abstract. Human erythrocytes with deficient glucose-6-phosphate dehydrogenase levels are unable to maintain their levels of reduced glutathione in the presence of low-level, steady-state concentrations of hydrogen peroxide. This finding has bearing on the biochemical mechanisms of drug-induced hemolytic anemia.

Individuals with hereditary deficiencies in glucose-6-phosphate dehydrogenase (G-6-PD) are known to be sensitive to the hemolytic effects of fava bean and a wide variety of drugs, including primaquine, phenylhydrazine, and menadione (*1*). However, the biochemical mechanisms underlying the cellular damage induced by such compounds have not yet been clarified.

Suggestions that hydrogen peroxide might play a critical role in drug-induced hemolysis have in general been dismissed on the grounds that the high catalase activity of erythrocytes would result in rapid destruction of peroxide. Indeed, it may be readily observed that the addition of concentrated hydrogen peroxide directly to erythrocytes causes very little injury to the cells; in contrast, extensive methemoglobin formation and oxidation of reduced glutathione (GSH) may be noted when the addition of peroxide is preceded by the addition of a catalase inhibitor such as azide. However, the toxic effects of very low concentrations of hydrogen peroxide generated within cells cannot be judged from experiments such as that quoted above, namely, experiments in which much higher concentrations of peroxide are added directly to erythrocytes. For example, it has been demonstrated (*2*) that at low concentrations of hydrogen peroxide, the usual decomposition to water and oxygen, by catalase, yields way to a peroxidatic coupling of peroxide to various hydrogen donor molecules. In this regard, the erythrocyte enzyme glutathione peroxidase, recently reported by Mills (*3*), and which is capable of detoxifying hydrogen peroxide by coupling it to reduced glutathione, is of particular interest.

The primary importance of erythrocyte GSH levels in drug-induced hemolytic anemia is apparent from previous observations in erythrocytes deficient in glucose-6-phosphate dehydrogenase, of decreased average GSH levels (*4*), and instability of GSH in the presence of hemolytic agents such as acetylphenylhydrazine (*5*). We have therefore re-evaluated the effect of low-level, steady-state concentrations of hydrogen peroxide on the GSH levels of normal and G-6-PD deficient erythrocytes.

In order to mimic the conditions under which hydrogen peroxide might be generated within cells, either from metabolic processes or from the autoxidation of various drugs or their metabolites, a hydrogen peroxide diffusion technique was used (*6*). The peroxide was added to the center well of a Warburg flask and permitted to diffuse slowly to the erythrocytes in the main compartment. A grease ring at the top of the center well prevented any cross-contamination by "creeping." The rate of diffusion of peroxide could be controlled by adjusting the amount and concentration of peroxide placed in the center well. The diffusion rate was also dependent upon the temperature, the rate of shaking, and the geometry of the flask.

Heparinized blood was obtained from a group of individuals with erythrocytes deficient in glucose-6-phosphate dehy-

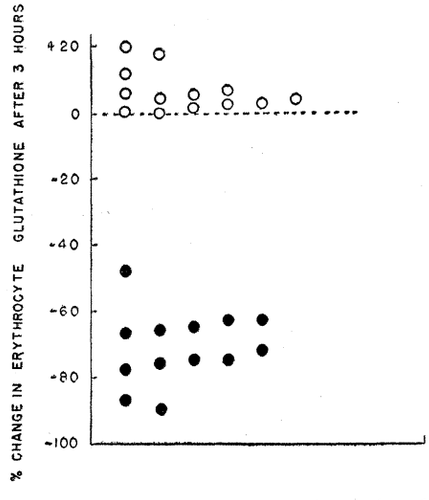

Fig. 1. Percentage change in reduced glutathione (GSH) in erythrocytes of 13 individuals with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency (solid circles) and 13 individuals with normal G-6-PD activity (open circles) after 3 hours of hydrogen peroxide diffusion. Average initial GSH values were: 50 mg/100 ml (range 31 to 68 mg/100 ml) for the G-6-PD deficient erythrocytes, and 58 mg/100 ml (range 36 to 77 mg/100 ml) for the normal erythrocytes. Included in the G-6-PD deficient group were three subjects receiving isoniazid for treatment of tuberculosis; included in the normal G-6-PD group was one individual receiving isoniazid for treatment of tuberculosis, and one recovering from hemolytic anemia that was not drug-induced.