[13] Yphantis, D. A., *Biochemistry*, **3**, 297 (1964). We are indebted to Dr. Vern Schumaker for the determination of the molecular weights by this method.

[14] Arnaud, C., C. Anast, and A. Tenenhouse, in preparation.

[15] Rasmussen, H., C. Arnaud, and C. Hawker, *Science*, **144**, 1019 (1964).

[16] Moore, S., personal communication. We are indebted to Dr. Moore for several helpful discussions during the course of this work.

---

*CRYSTALLINE BACTERIAL LUCIFERASE FROM PHOTOBACTERIUM FISCHERI**

BY SEISHI KUWABARA, MILTON J. CORMIER, LEON S. DURE, PAUL KREISS, AND PETER PFUDERER

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF GEORGIA, ATHENS, AND BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY

Communicated by W. D. McElroy, February 17, 1965

Bacterial luminescence has been shown to involve $FMNH_2$, a straight-chain fatty aldehyde, O_2, and bacterial luciferase.[1-3] Progress has also been made recently on the nature of some of the intermediates involved in the bioluminescent oxidation of $FMNH_2$ by bacterial luciferase.[4]

We wish to report the crystallization of bacterial luciferase by methods which result in high yields of the enzyme from starting material that can be stored for years without loss of activity. Evidence for the existence of active subunits and some properties of the enzyme are also reported.

Methods.—Photobacterium fischeri, American Type Culture Collection no. 7744, was routinely cultured at 25°C in a medium containing the following amounts of components per liter: 30 gm NaCl, 10 gm $Na_2HPO_4 \cdot 7H_2O$, 12 gm KH_2PO_4, 8 gm nutrient broth, and 3 ml glycerol. The resulting medium was brought to pH 7.2 by the addition of NaOH. Cells were grown in a 40-liter Biogen culture apparatus, and acetone powders were prepared from the cells as previously described.[5] The acetone powder may be stored for years at −20°C without loss of activity.

Preparation of crude extract and purification of luciferase: The following is a brief outline of the purification procedure, the details of which will be published elsewhere. Routinely, 200 gm of acetone powder were extracted with 3500 ml of 0.05 *M* Na_2HPO_4-KH_2PO_4 buffer, pH 7.0, which contained 0.001 *M* mercaptoethanol (phosphate-MSH). The extraction was carried out by grinding with sand in a mortar at 2–4°C. The extract was centrifuged at 14,000 × *g* for 30 min and the supernatant referred to as "crude extract." Purification of crude extract involved addition of streptomycin sulfate to a final concentration of 1.75% (w/v) followed by centrifugation at 14,000 × *g* to remove nucleic acids. The supernatant was incubated for 30 min at 25°C in the presence of 20 mg each of DNAase and RNAase. The protein was precipitated with ammonium sulfate (0–80% saturation)[6] and dissolved in phosphate-MSH. When this enzyme solution was then fractionated with saturated ammonium sulfate, pH 6.6, the bulk of luciferase activity appeared in the 45–65% saturation fraction. The stock solution of saturated ammonium sulfate was prepared at 2–4°C. The 0–45 and 65–80 fractions were recombined and refractionated, and the 45–65 fraction thus obtained was combined with the original 45–65 fraction. The combined 45–65 fractions were dissolved in, and dialyzed overnight against 0.01 *M* Tris-HCl buffer, pH 7.3, which contained 0.005 *M* magnesium acetate, 0.001 *M* EDTA (ethylene diamine tetraacetic acid), and 0.001 *M* mercaptoethanol (Tris-MSH). The total volume was 300 ml. This dialyzed enzyme was chromatographed on a 3.5 × 50-cm DEAE-cellulose column. Elution of luciferase from the column was achieved by use of a linear gradient consisting of Tris-MSH in the mixing chamber and an equal volume of the same buffer containing 1 *M* NaCl in the reservoir. Ten-ml fractions

were collected every 10 min. After concentration of the eluted luciferase (fractions 37–64 by precipitation with ammonium sulfate (0–80% saturation), crystallization was achieved by dissolving the precipitate in, and dialyzing against 10% (w/v) ammonium sulfate, pH 7.3, which contained 0.001 *M* EDTA and 0.001 *M* MSH. This ammonium sulfate concentration was increased by 1% every 12 hr. Most of the activity crystallized out when the solution was 40–50% saturated ammonium sulfate. The crystals were collected by centrifugation and dissolved in 10% ammonium sulfate, pH 7.3, which contained 0.001 *M* EDTA and 0.001 *M* mercaptoethanol. In this condition, and at a concentration of 25 mg/ml, luciferase is stable for months at 2–4°C. Table 1 is a summary of these purification steps.

Assay methods consisted of injecting catalytically reduced FMN into an otherwise complete system.[1] Final concentrations were 1×10^{-2} *M* mercaptoethanol; 3.9×10^{-4} *M* $FMNH_2$; 1×10^{-2} *M* Na_2HPO_4-KH_2PO_4 buffer, pH 7.3; 2×10^{-5} *M* dodecyl aldehyde; 2.4 mg bovine serum albumin; and 0.05 ml of enzyme in a total volume of 1.23 ml.

Absorption spectra were determined on a Cary model 14 recording spectrophotometer. Electrophoretic analyses were performed using a model 38A electrophoresis apparatus made by the Perkin-Elmer Corporation. A Beckman model E analytical ultracentrifuge was utilized in the determination of sedimentation constants and sedimentation equilibrium.

Results and Comments.—Purification and yield of luciferase: As shown in Table 1, the crystalline enzyme represents 1.7 per cent of the total soluble protein in the crude material. This value is a minimum one since 20–30 per cent of the total units appear in other fractions during purification. Thus the theoretical recovery and purification are slightly exceeded—indicative of partial inhibition by substances in the crude extract. The over-all recovery is probably in the range of 70–80 per cent, and the yields are routinely high. Crystalline luciferase is shown in Figure 1. The crystals appear rhombohedral.

Absorption spectra: As illustrated in Figure 2, absorption in the ultraviolet region of the spectrum at pH 7.5 indicates an absorption maximum at 277 mμ and that the preparation is essentially devoid of nucleic acids. Although not seen here because of enzyme dilution, there is an absorption shoulder at 315 mμ. If the pH is adjusted to 12.5, the absorption maximum shifts from 277 to 288 mμ, which is indicative of considerable amounts of tyrosine in the enzyme.[7] In the visible region of the spectrum the only noticeable absorption is a shoulder located at 415 mμ.

Homogeneity studies: (1) *Ultracentrifuge analysis:* Sedimentation studies on luciferase were complicated by the existence of a monomer-polymer equilibrium. Three different crystalline luciferase preparations, from different cell batches, were used for these studies and labeled I, II, and III. All three preparations studied could be broken down to a monomer of $s_{20,w}$ of 2–2.5S. All the sedimentation

TABLE 1

SUMMARY OF PURIFICATION AND CRYSTALLIZATION OF BACTERIAL LUCIFERASE

Fraction	Total light units	Total soluble protein (mg)	Units per mg protein	Recovery, %*	Purification
Crude	1,840,000	69,000	27	100	1
Streptomycin, DNAase, and RNAase treatments and precipitation with $(NH_4)_2SO_4$ (0–80% fraction) 45–65% $(NH_4)_2SO_4$	2,093,000	9,600	218	114	8
DEAE chromatography	2,000,000	4,750	421	109	16
1st crystallization	1,935,000	1,280	1,510	105	56
2nd crystallization	1,890,000	1,170	1,620	103	60

* These recovery values are based on total light units. As indicated in the text the over-all recovery is probably 70–80%.

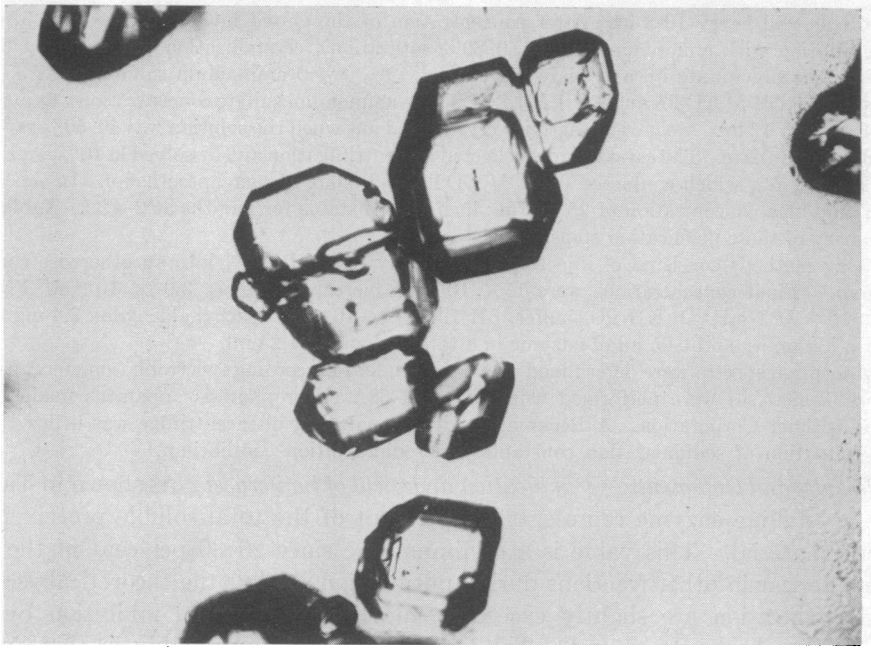

FIG. 1.—Crystalline bacterial luciferase. ×100.

studies, unless otherwise specified, were run in 10 per cent ammonium sulfate, pH 7.3; 0.001 *M* in EDTA and mercaptoethanol.

The sedimentation patterns of preparations I and II of luciferase are shown in Figure 3, and the concentration dependence is shown in Figure 4. The main peak of luciferase II had an $s_{20,w}$ of 4.3 at infinite dilution, while the extrapolated value for luciferase I would have been 4.9 (two points), but on standing, the luciferase I preparation reverted to a 4S species similar to luciferase II (see Fig. 4; the points between 2 and 3 mg/ml were run last). The slow peaks of luciferase I and II had $s_{20,w}$'s of 2.2 and 1.9, respectively, at 5 mg/ml concentration. The amount of this slow species increased with the age of the preparation. Preparation I was 85 per cent 4S, 15 per cent 2S, while preparation II was 79 per cent 4S and 21 per cent 2S according to the area under the peaks.

The concentration dependence of the sedimentation coefficient of luciferase III is shown in Figure 5. Species initially having sedimentation rates of 5 and 3S break down to a 2S component at low concentrations, with the appearance of a 4S species at intermediate concentrations. A positive slope such as this in an *s* versus *c* plot is characteristic of a monomer-polymer equilibrium, and in general, the sedimentation coefficient of the monomer is near its true value, while those of the polymers approach their true values at high concentrations.[8]

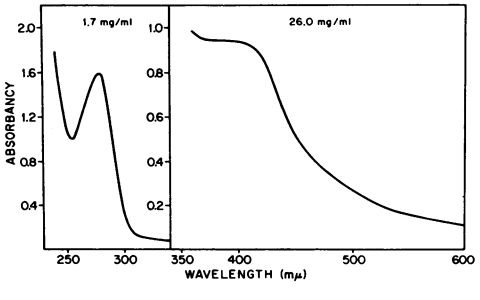

FIG. 2.—Absorption spectrum of a luciferase solution.

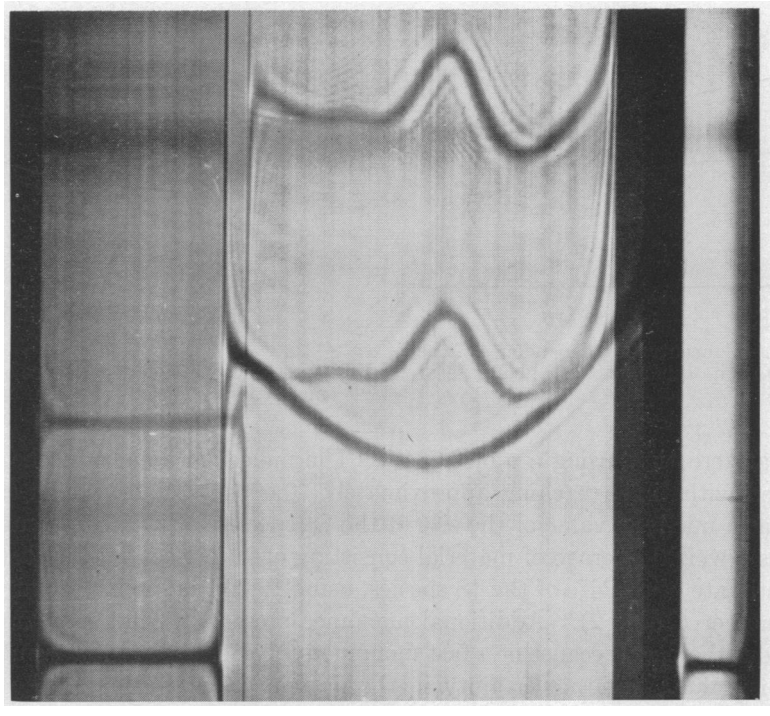

FIG. 3.—Sedimentation pattern of crystalline luciferase I and II. The upper figure is luciferase II and the lower is luciferase I. A solvent baseline from a separate run (at the same time, bar angle, and speed) has been superimposed on the lower photograph. The picture was taken after 154 min of sedimentation of 59,780 rpm. The bar angle was 60°, the temperature 5.9°C, solvent 10% $(NH_4)_2SO_4$·pH 7.3, made 0.05 *M* in β-mercaptoethanol and 0.001 *M* in EDTA. Concentrations of luciferase I and II were 4.8 and 5.0 mg/ml, respectively. The direction of sedimentation is from left to right.

Evidence for the existence of the 4S peak as a true species in the luciferase III preparation rests on the sedimentation of preparations I and II where it was also found, and on the sucrose gradient analysis below, where it is shown to have a unique enzyme activity.

Sucrose gradient sedimentation of this luciferase III preparation (3–10% linear gradient) showed protein peaks corresponding to $s_{20,w}$'s of 1.8, 4.3, and 5.2S. Both the 4.3 and 5.2S components were broken down to the 1.8S component on aging. Luciferase activity appeared in all three peaks with the highest specific activity present in the 4.3S peak, while DPNH oxidase activity was present only in the 5.2 and 1.8S peaks. Thus the monomer still has luciferase activity.

Ultracentrifugational analysis employing two short-column (2.6 and 1.3 mm) equilibrium runs at 20,410 rpm were made on the luciferase II preparation, which consisted initially of 79 per cent 4S and 21 per cent 2S by analysis of the area under the schlieren peaks, corrected for the Johnson-Ogston effect and radial dilution but not for any abnormality introduced by the monomer-polymer equilibrium. A high-molecular-weight aggregate accumulated in the bottom of the cell and its concentration increased with time, as observed by Riley[9] with luciferase. Because of the continual aggregation, equilibrium throughout the cell was not reached during the run. An analysis of the material at the meniscus gave 58,000 for the molecular

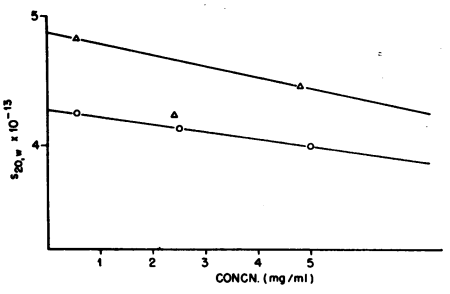

FIG. 4.—Concentration dependence of the sedimentation coefficient of luciferase I and II.

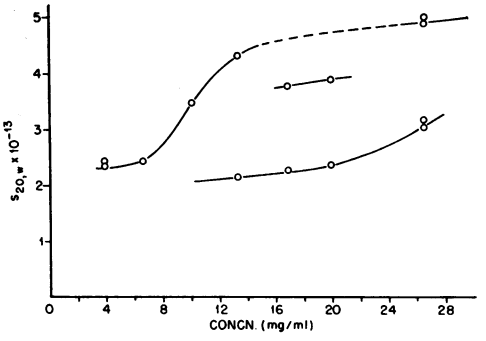

FIG. 5.—Concentration dependence of the sedimentation coefficient of luciferase III.

weight at zero time using a $\bar{v}$ of 0.734.[10] The molecular weight at the meniscus decreased with time, reaching approximately 30,000 at 3.5 hr, which can be regarded as a maximal value of the size of the 2S species. Similarly, assuming that 58,000 is a weight average of material consisting of 79 per cent 4S, we have 68,000 as an estimate of the size of the 4S species, using 19,000 as the molecular weight of the monomer (Table 2). Additional monomer present but not measured by the area under the peak because of a fast monomer-polymer equilibrium would tend to raise this value.[8] The uncertainty in the protein concentration at the meniscus is reflected in an uncertainty of about 20 per cent in the molecular weights. The apparent molecular weight of the aggregate was greater than 100,000, and consequently its concentration at the meniscus was negligible at later times.

A series of velocity sedimentation studies was conducted on luciferase II in 0.01 *M* sodium dodecyl sulfate (SDS). The detergent dissociated the enzyme into a single homogeneous species in the centrifuge. Sedimentation coefficients were measured and diffusion coefficients calculated from boundary spreading during sedimentation. These values have been combined to give a molecular weight assuming no SDS bound, and assuming all of the SDS was bound to the protein. The data are shown in Table 2. As the protein concentration decreases, the assumption that all the SDS can be regarded as bound becomes less valid. The best estimate of the molecular weight of the monomer from the data in Table 1 would be 19,000 ± 3,000.

(2) *Electrophoretic analysis:* Figure 6 shows a schlieren pattern of a 1 per cent luciferase II solution obtained in the descending limb of the Tiselius cell. This pattern is typical from pH 6.5 to 8.0 and with protein concentrations of 0.5–1 per cent. There is considerable broadening of the boundary with time, and we attribute this to a monomer-polymer equilibrium as indicated by the ultracentrifugational

TABLE 2

MOLECULAR WEIGHT OF LUCIFERASE IN 0.01 *M* SDS

Protein conc. (mg/ml)	$s_{20,w} \times 10^{13}$ (sec)	$D_{20,w} \times 10^{7}$ (cm²/sec)	*M* no SDS bound	*M** all SDS bound
14.4	2.41	11.2 ± 3	21,800	18,200
10	2.61	11.4 ± 2	21,000	18,800
5	2.64	10.5 ± 2	23,000	18,500
2.25	2.68	10.5 ± 2	23,400	15,200

* The partial specific volume of SDS was taken as 0.885; $\bar{v}_C = (X\bar{v}_{SDS} + \bar{v}_P)/(X + 1)$; the molecular weight of the protein was calculated by $M_c = M_p (1 + X)$.[10]

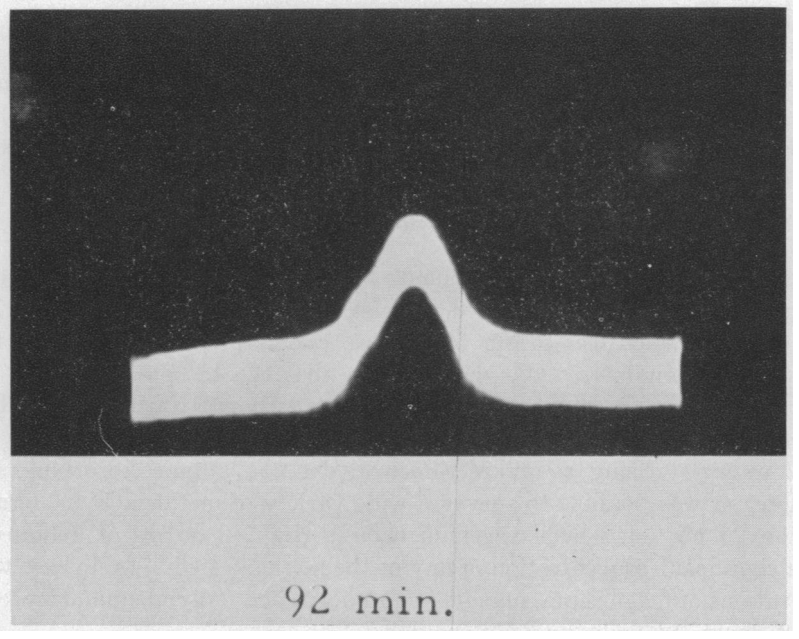

FIG. 6.—Photograph of a schlieren pattern of luciferase after electrophoresis for 92 min (descending limb of the Tiselius cell). A 1% solution of luciferase was dialyzed for 16 hr *vs.* a buffer consisting of 0.175 *M* NaCl, 0.0089 *M* Na_2HPO_4 KH_2PO_4·0.001 *M* EDTA, and 0.01 *M* MSH.

analysis. This concept is supported by the fact that after dialysis against buffers containing 0.01 *M* SDS the schlieren pattern remains sharp and as a single peak after 2 hr. Thus when we take into account the equilibrium phenomenon that exists, it appears that our luciferase preparation is probably homogeneous by electrophoretic analysis as well as in the ultracentrifuge.

The mobility at pH 7.0 was calculated to be -7.3×10^{-5} cm^2 V^{-1} sec^{-1}.

Properties of the enzyme: Bioluminescent oxidation of $FMNH_2$ and reduced pyridine nucleotides: Solutions of the crystalline enzyme will catalyze the bioluminescent oxidation of $FMNH_2$ in the presence of long-chain aldehydes and O_2, as has been observed with partially purified preparations.[1]

The relationship between DPNH oxidase and luciferase activities in *Ph. fischeri* is not fully understood,[11, 12] and we have made some additional observations that bear on this point.

During purification on DEAE-cellulose, two quite different FMN-dependent DPNH oxidases are removed from the column. One of these (DPNH oxidase I) is removed from the column (fractions 11–25) prior to the elution of luciferase (fractions 37–64). The other (DPNH oxidase II) appears in column fractions along with luciferase, although its removal from the column precedes that of luciferase by one to three 10-ml fractions. Thus there is slight separation of luciferase from DPNH oxidase II on DEAE, but for the most part these two enzymes seem to be closely associated. The once recrystallized enzyme will still produce light with DPNH (or TPNH) + FMN.

As mentioned previously, sucrose gradient analysis reveals 2, 4, and 5S species. The 2 and 4S species both show luciferase activity, but the specific activity of the

4S species is greater than that of the 2S species by an order of magnitude. Interestingly enough, DPNH oxidase activity appears in the 2S but not in the 4S species. Although these two activities frequently follow one another, they are sometimes displaced by one tube (3% of the gradient). This supports the idea that there are at least two monomers with a molecular weight of approximately 19,000 each (according to ultracentrifuge data), one being luciferase and the other DPNH oxidase. The relationship between these and the native enzyme is being investigated.

A luciferase preparation which is homogeneous in the centrifuge, with a sedimentation rate of 5.15S has been reported by Riley to have a molecular weight of 76,000.[9] This is probably identical to our 5S species, and is presumably formed by four 19,000 mol wt monomers. Our data suggest that the 4S species lacking DPNH oxidase activity is also composed of 4 monomers, although a trimer might be more satisfactory.

Metal content: Using a Perkin-Elmer model 214 atomic absorbing spectrophotometer, it was possible to survey a wide variety of metals at a luciferase concentration 25 mg/ml, which was high enough (by 1–2 orders of magnitude) to detect a significant concentration of any of the metals tested. In no case were the concentrations of magnesium, manganese, iron, copper, molybdenum, zinc, calcium, or cobalt above 0.1 moles/mole of enzyme.

We would like to thank Mr. Daniel R. Holladay for excellent technical assistance.

* This research was jointly supported by grants from the U.S. Atomic Energy Commission, the National Science Foundation, and the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation. Facilities provided by the Sapelo Island Research Foundation are also acknowledged. This paper is contribution no. 83 from the University of Georgia Marine Institute, Sapelo Island, Georgia.

[1] Strehler, B. L., E. N. Harvey, J. J. Chang, and M. J. Cormier, these PROCEEDINGS, **40**, 10 (1954).

[2] Cormier, M. J., and B. L. Strehler, *J. Am. Chem. Soc.*, **75**, 4864 (1953).

[3] McElroy, W. D., and H. H. Seliger, in *Advances in Enzymology*, ed. F. F. Nord (New York: Interscience, 1961), vol. 25, p. 144.

[4] Hastings, J. W., and Q. H. Gibson, *J. Biol. Chem.*, **238**, 2537 (1963).

[5] Strehler, B. L., and M. J. Cormier, *Arch. Biochem. Biophys.*, **47**, 16 (1953).

[6] Green, A. A., and W. L. Hughes, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, Inc., 1955), vol. 1, p. 76.

[7] Beaven, G. H., and E. R. Holiday, *Advan. Protein Chem.*, **7**, 320 (1952).

[8] Gilbert, G. A., *Discussions Faraday Soc.*, **20**, 68 (1955).

[9] Riley, W. H., "The *in vitro* luminescent reaction of *Photobacterium fischeri*," Ph.D. thesis, Univ. of Illinois, Urbana (1962).

[10] Hersh, R. T., and H. K. Schachman, *Virology*, **6**, 234 (1958).

[11] Totter, J. R., and M. J. Cormier, *J. Biol. Chem.*, **216**, 801 (1955).

[12] McElroy, W. D., and H. H. Seliger, in *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1961), p. 251.