Isolation and Properties of *Renilla reniformis* Luciferase, a Low Molecular Weight Energy Conversion Enzyme*

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ABSTRACT: From the sea pansy (*Renilla reniformis*) we have isolated in highly purified form a low molecular weight energy conversion enzyme and have studied a number of its chemical and physical properties. This enzyme, *Renilla* luciferase, catalyzes the bioluminescent oxidation of *Renilla* luciferin to produce visible light with an *in vitro* emission maximum of 475 nm. The evidence suggests the existence of a single subunit of luciferase which has an approximate molecular weight of 12,000. Each subunit contains one sulfhydryl group, one disulfide linkage, and two glucosamine residues. The native enzyme exists as an equilibrium mixture between this subunit and higher molecular weight species. From Sephadex G-75 data the active enzymatic species appears to be either a dimer or trimer of the luciferase subunit.

It has recently been established that two enzymes are operative in the pathway leading to light emission in the sea pansy, *Renilla reniformis* (Cormier *et al.*, 1970; Karkhanis and Cormier, 1970). The two reactions involved may be illustrated as follows

$$\text{luciferyl sulfate} + \text{3',5'-diphosphoadenosine} \underset{}{\overset{\text{luciferin sulfokinase; } Ca^{2+}}{\rightleftharpoons}} \text{luciferin} + \text{PAPS} \quad (1)$$

$$\text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{light } (\lambda_{max}\ 475\ \text{nm}) + \text{products} \quad (2)$$

In reaction 1 luciferin sulfokinase (3'-phosphoadenylyl sulfate:luciferin sulfotransferase) controls the level of luciferin available for bioluminescence. In reaction 2 luciferase catalyzes the bioluminescent oxidation of luciferin to produce a greenish blue luminescence (λ_{max} 475 nm).

We wish to report the isolation of luciferase and some of its chemical and physical properties.

Materials and Methods

The following materials were purchased from the suppliers indicated: cellulose phosphate powder (Reeve Angel); Sephadex G-75, 40–120 μ, L-asparagine and guanidine hydrochloride (Sigma); Blue Dextran 2000 (Pharmacia); acrylamide, *N,N'*-methylenebisacrylamide, riboflavin, *N,N,N',N'*-tetramethylenediamine, and bromophenol blue (Eastman); 2-hydroxy-5-nitrobenzyl bromide and glyceraldehyde phosphate dehydrogenase (California Biochemicals), sodium dodecyl sulfate (Mann); Amido Black (Allied Chemicals); coomassie blue (Colab Laboratories); urea (Fisher Scientific); lysozyme, trypsin, and bovine serum albumin (Sigma); ribonuclease (Worthington); β-lactoglobulin (Pentex); carbonic anhydrase was a gift from Dr. R. B. Ashworth of this department.

Prior to use acrylamide was recrystallized by the procedure of Brewer and Ashworth (1969). Guanidine hydrochloride was purified by filtration through acid-washed charcoal and recrystallized from methanol. Iodoacetic acid was twice recrystallized from petroleum ether (bp 30–60°).

Protein and Nitrogen Determination. Protein was determined by the biuret reaction (Gornall *et al.*, 1949) using crystalline BSA[1] as a standard. With the purified enzyme, protein was also determined from its absorbance at 280 nm and from amino acid analysis.

Protein nitrogen was determined by use of a microKjeldahl method (Fleck and Munro, 1964).

Spectral Analyses. Absorption spectra were measured on a Cary Model 14 recording spectrophotometer at room temperature. Fluorescence measurements were made with a constant intensity spectrofluorimeter described previously (Cormier and Prichard, 1968). Measurements were made at resolutions between 3 and 6 nm.

Electrophoretic Methods. Disc gel electrophoresis on 7.5% polyacrylamide gels was performed according to the method of Davis (1964) and the running pH was 9.6. The procedure of Hedrick and Smith (1968) was used where the running pH was 8.5. Electrophoresis was carried out at 4 mA/tube at room temperature and all gels were polymerized with riboflavin (Brewer, 1967). In some cases gels were stained with coomassie blue (Chrambach *et al.*, 1967). In the case of urea containing gels all gel solutions were adjusted so that the final concentration of urea was 8 M.

Molecular weight determinations on polyacrylamide gels containing SDS were performed according to the method of Weber and Osborn (1969). Gel concentrations used were 11% and the current was adjusted to 8 mA/tube for 6 hr at room temperature. Gels were stained with coomassie blue. Proteins used were dissolved in 10 mM sodium phosphate (pH 7.0), containing 0.1% SDS and 0.1% β-ME and incubated overnight prior to use. For luciferase, urea was included in the above buffer at a final concentration of 8 M.

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[1] Abbreviations used are: bovine serum albumin, BSA; sodium dodecyl sulfate, SDS; β-mercaptoethanol, β-ME.

Electrophoresis on cellulose polyacetate strips (Sepraphore III) was carried out at pH 7.0, 7.5, and 8.0 in a buffer consisting of 10 mM potassium phosphate, 10 mM β-ME, 2.2 mM EDTA, and 50 mM NaCl. Luciferase (80 μg) was applied to the strips and the current adjusted to 5 mA/strip for 60 min. The method used was that of Grunbaum and Collins (1963) and Grunbaum *et al.* (1963). The proteins were stained with ponceau S and the stained strips scanned with a densitometer.

Ultracentrifugal Studies. Sedimentation velocity experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with schlieren and Raleigh optics. The high-speed method of Yphantis (1964) was employed for sedimentation equilibrium studies and in all cases a temperature of 4° was maintained.

Luciferase was dialyzed for 18 hr prior to analysis against 100 mM potassium phosphate (pH 7.6) containing 1.4 mM β-ME and 2.2 mM EDTA. During sedimentation equilibrium a rotor speed of 44,000 rpm was maintained. Columns 3 mm long were used and interference patterns were recorded on Kodak Type II-G plates. The developed plates were read on a Nikon Model 6C Shadowgraph microcomparator. The fringe displacement in microns (y) was measured as a function of the radial distance.

Sedimentation coefficients were measured as a function of protein concentration at a rotor speed of 60,000 rpm. Photographs of the schlieren patterns were taken at 16-min intervals on Kodak metallographic plates. Sedimentation coefficients, $s_{20,w}$, were calculated according to the method of Schachman (1957).

The extinction coefficient of solutions of native luciferase of known absorbancy at 280 nm was determined by measurement of fringe displacements across a boundary as described by Babul and Stellwagen (1968). This determination was made at luciferase concentrations of about 2 mg/ml in 100 mM phosphate (pH 7.5) containing 1.4 mM β-ME and 2.2 mM EDTA. Luciferase was dialyzed overnight against this buffer prior to analysis.

Gel Filtration Studies. A column of Sephadex G-75 (83 × 1.7 cm) was equilibrated at 4° with 100 mM potassium phosphate buffer (pH 7.5) containing 1.4 mM β-ME and 2.2 mM EDTA. A 1-ml volume containing 10 mg of protein was applied to the column and fractions were collected at the rate of 8 ml/hr. The Stokes radius, a, of luciferase was determined on such columns by the method of Ackers (1964). The column was calibrated with proteins of known molecular weights or Stokes radii (Andrews, 1964; Ackers, 1964, 1967). Blue Dextran 2000 was used to determine the void volume. The total bed volume was determined directly with water. The elution pattern of each protein was determined from absorbancy measurements at 280 nm on a Zeiss Model PM QII spectrophotometer. From the determined value of the Stokes radius, the diffusion coefficient, $D_{20,w}$, was calculated by use of the Stokes–Einstein equation (Gosting, 1956).

When Sephadex G-75 chromatography was carried out in the presence of urea, the column (45 × 2 cm) was equilibrated for 8 M urea containing 100 mM β-ME and 10 mM EDTA (pH 6.0). Prior to column application each protein was dissolved in the above urea solution, adjusted to pH 8.5, and kept overnight. Chromatography was done at room temperature and fractions were collected at a flow rate of 6 ml/hr. The total volume was determined from the elution position of DNP-alanine. The elution positions of the applied proteins were determined by a turbidometric method (Davison, 1968; Fish *et al.*, 1969; Layne, 1957).

For purposes of rechromatography of peak tubes a column (12 × 1.2 cm) of Sephadex G-75 was equilibrated with 100 mM potassium phosphate (pH 7.5) containing 1.4 mM β-ME and 2.2 mM EDTA.

Amino Acid Analysis. Amino acid analyses were performed with a Spinco Model 120B automatic amino acid analyzer (Spackman *et al.*, 1958). To prepare acid hydrolysates 2-mg aliquots of luciferase were heated at 110° in 6 N HCl in vacuum-sealed tubes for 24, 48, and 72 hr as described by Moore and Stein (1963).

The total half-cystine content of luciferase was determined as cysteic acid by analysis of acid hydrolysates of the performic acid oxidized enzyme (Moore, 1963) and as *S*-carboxymethylcysteine in hydrolysates of *S*-carboxymethylluciferase prepared according to the method of Crestfield *et al.* (1963). The procedure of Crestfield *et al.* (1963) was slightly modified as follows. Luciferase (2 mg) was added to 0.8 ml of 100 mM potassium phosphate buffer (pH 7.5), which contained 1.4 mM β-ME, 2.2 mM EDTA, and 10 M urea. The pH of the solution was increased to about 8.5 by the addition of 0.1 ml of 25% NaOH. The solution was flushed with N_2 for 15 min, 1.43 mmoles of β-ME was added, and the tube was tightly stoppered. After 24 hr 2.2 mmoles of twice-recrystallized iodoacetic acid was added after being dissolved in a minimal volume of 25% NaOH. The pH was raised to 8.5 by the addition of 25% NaOH. After 15 min in the dark the solution was dialyzed for 18 hr against five changes of glass-distilled water (1-l. volumes). The carboxylmethylated protein was acid hydrolyzed as indicated above and analyzed for carboxylmethylated cysteine.

The sulfhydryl content of luciferase was determined by reaction of the enzyme with 5,5′-dithiobis(2-nitrobenzoate) in the presence and absence of 8 M urea as described by Ellman (1959). Absorbance changes at 412 nm were recorded and the cysteine content was calculated by using 13,600 as the molar extinction coefficient for the thionitrobenzoate (Ellman, 1959).

Tryptophan was determined spectrophotometrically by the method of Bencze and Schmid (1957) and by a colorimetric method previously described (Barman and Koshland, 1967; Koshland *et al.*, 1964). The colorimetric method was modified as follows: 2 mg of luciferase was dissolved in 1 ml of 8 M urea and allowed to stand for 18 hr. To this solution was added 5 mg of 2-hydroxy-5-nitrobenzyl bromide and the mixture stirred for 30 min. Such additions of reagent were repeated twice and in each case stirred for 30 min as outlined above. Not all of the reagent was soluble under these conditions but that which dissolved was in sufficient excess for labeling all of the tryptophan residues. About 0.1 ml of 25% NaOH was added to dissolve the excess reagent and the mixture was centrifuged for 10 min at 2800g. The supernatant was applied to a 1 × 20 cm Sephadex G-25 column which had been equilibrated with 0.1 M $(NH_4)_2CO_3$ containing 8 M urea. The yellow protein band which moves in the front was collected and the protein content determined by amino acid analysis. From the optical density at 410 nm the number of tryptophan residues in luciferase were determined by using an extinction coefficient of 18,000 M^{-1} cm^{-1} (Barman and Koshland, 1967).

Glucosamine was detected qualitatively from amino acid analysis. Additional sugar determinations were made from samples prepared by hydrolyzing 0.2 μmole of luciferase in 3 ml of 2 N HCl for 14 hr at 110° and a similar sample in 1 N HCl for 7 hr at 110°. The HCl was removed by passing the hydrolysates through Dowex 1 acetate and flashing to dryness. Qualitative sugar determinations were made by paper

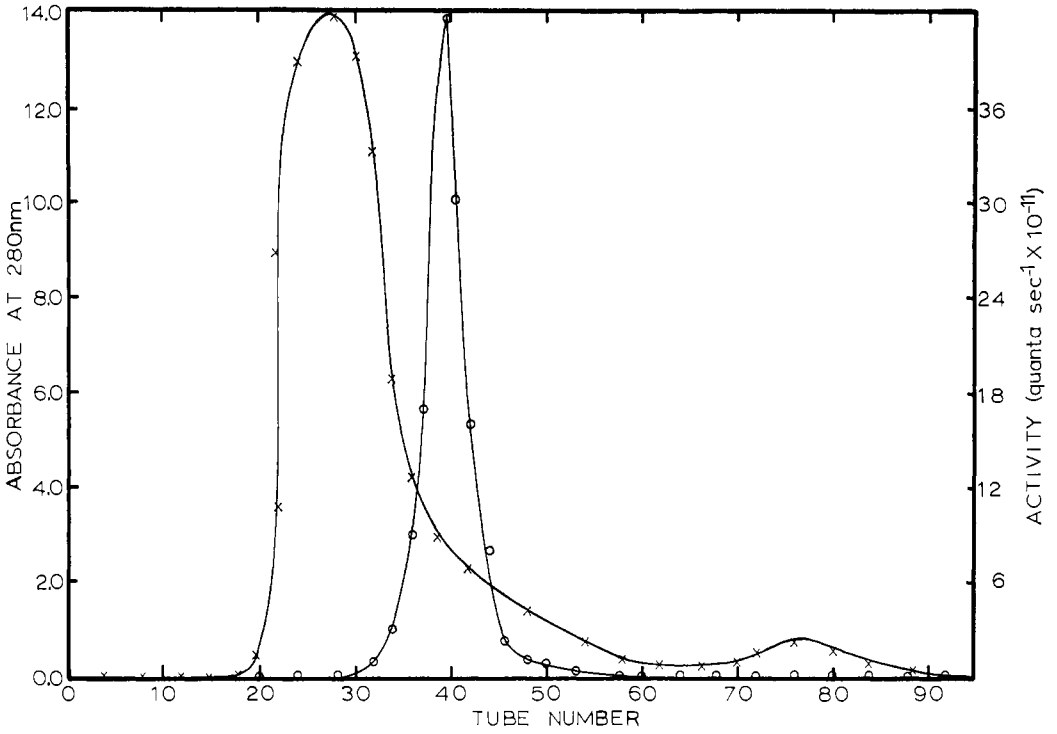

FIGURE 1: Sephadex G-75 chromatography of partially purified luciferase. The column (60 × 4 cm) was equilibrated with 100 mM potassium phosphate (pH 7.5) containing 1.4 mM β-ME and 2.2 mM EDTA and the protein eluted with this same buffer at 4°. Fractions (10 ml) were collected at the rate of 150 ml/hr. (×-×) Absorbance at 280 nm; (○-○) luciferase activity.

chromatography using butanol–ethanol–water (52:32:16, v/v) as a solvent system (Putman, 1957). Sugars were located by passing the dried paper through solutions of silver nitrate and alcoholic sodium hydroxide (Trevelyan *et al.*, 1950). Quantitative determinations for glucosamine were made by converting into *N*-acetylglucosamine (Roseman and Daffner, 1956) and performing a colorimetric assay as previously described (Reissig *et al.*, 1955).

Enzyme Assays. The assay mixture for the determination of luciferase contained 50 mM potassium phosphate (pH 7.5) plus luciferase in a volume of 1 ml. Into this solution 0.2 ml of a luciferin solution (pH 7.5) was injected and the light intensity at the flash peak was recorded. The amount of luciferin used was sufficient to saturate the enzyme. For most assays reported here partially purified preparations of luciferyl sulfate were used and this was converted into luciferin by a previously described procedure (Cormier and Hori, 1964). Because these partially purified preparations contained some inhibitory material we also checked the crude and highly purified luciferase preparations with pure luciferin (Cormier *et al.*, 1970) to obtain accurate values for the absolute specific activity of the preparations. Partially purified luciferin generally gave about one-third the specific activity of that of the pure substrate. A unit of luciferase is defined as that amount of enzyme that produced 10^{11} quanta sec^{-1} at the flash peak using partially purified luciferin as the substrate. Specific activity is defined as units per milligram of protein. Relative units were converted into quanta sec^{-1} by the use of light standards as described by Hastings and Weber (1963).

The presence of luciferin sulfokinase (Cormier *et al.*, 1970) in luciferase preparations was detected by using luciferyl sulfate as the substrate in an assay system previously described (Cormier, 1962).

Purification of Luciferase. Acetone powder (1000 g) (Kreiss, 1967) was extracted for 2 hr with 3 l. of 100 mM potassium phosphate (pH 7.5) containing 1.4 mM β-ME and 2.2 mM EDTA. The mixture was centrifuged at 27,300*g* for 30 min and the residue was discarded. All operations were carried out at 4° (step 1).

Solid ammonium sulfate was slowly added with stirring to the supernatant until 49% saturation (w/v) was obtained. This mixture was allowed to equilibrate for 1 hr and was centrifuged at 24,000*g* for 15 min. The supernatant contained most of the luciferase and 5–10% of the original luciferin sulfokinase activity. Solid ammonium sulfate was added as above to the supernatant to give 77% saturation (w/v). The precipitate was dissolved in the buffer described above to a final protein concentraion of about 20 mg/ml (step 2).

The luciferase solution from step 2 was dialyzed overnight against 10 volumes of 10 mM potassium phosphate (pH 7.5) containing 1.4 mM β-ME and 2.2 mM EDTA. During the dialysis a large inactive precipitate forms which represents about half of the total protein. This precipitate was removed by centrifugation at 39,00*g* for 30 min (step 3).

The supernatant from step 3 was applied to a G-75 Sephadex column equilibrated with the buffer described in step 1. Conditions of operation of the column are given in the legend to Figure 1. Figure 1 shows that luciferase is somewhat retarded on G-75 while the bulk of the protein is excluded in the void volume. This step results in an approximately 5-fold purification. The active fractions were pooled and the luciferase precipitated with solid ammonium sulfate to a final saturation value of 77% (w/v). The precipitate was collected by centrifugation as above and dialyzed against ten volumes of the buffer described in step 3. The dialysis was carried out for 48 hr with four changes of buffer (step 4).

The dialyzed enzyme from step 4 was centrifuged for 30 min at 39,100*g* and the supernatant applied to a cellulose

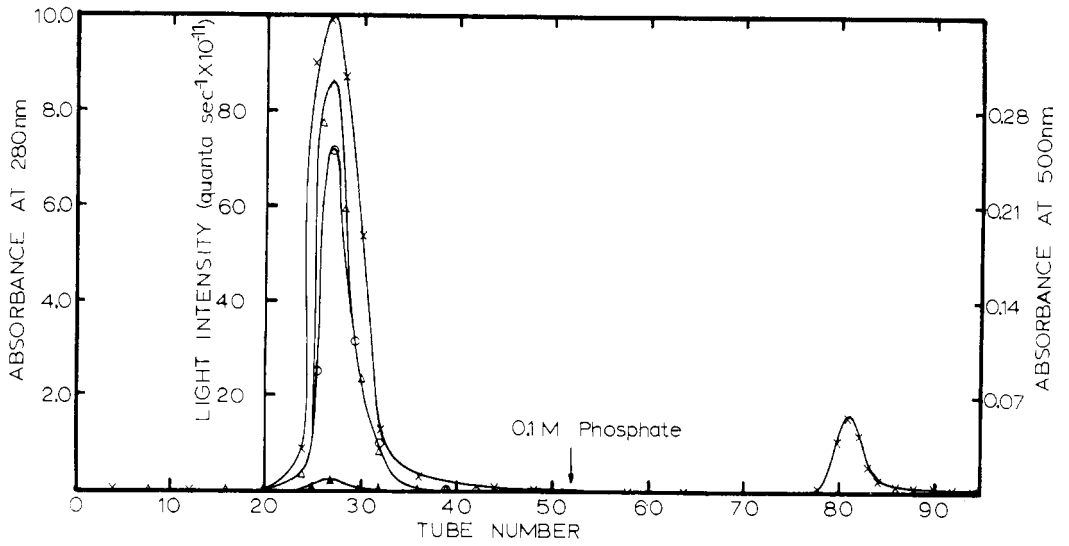

FIGURE 2: Cellulose phosphate chromatography of partially purified luciferase. Fractions (3.8 ml) were collected at a flow rate of 114 ml/hr. Other details are given in the Methods. (×-×) Absorbance at 280 nm; (△-△) absorbance at 500 nm; (○-○) luciferase activity; (▲-▲) sulfokinase activity.

TABLE I: Summary of Purification Procedure for *Renilla* Luciferase.[a]

Step	Total Vol (ml)	Total Soluble Protein (mg)	Total Units	Sp Act. (Units/mg of Protein)	Recov (%)	Purifn
I	2400	14000	3000	0.21	100	1.0
II	100	3400	2780	0.82	93	3.9
III	130	2000	2700	1.4	90	6.5
IV	150	280	2000	7.2	67	34.0
V	70	250	1820	7.3	61	35.0
VI	8	80	800	10.0	27	48.0

[a] Details on individual steps are given in Methods. A unit of luciferase is defined as that amount of enzyme that produces 10^{11} quanta sec^{-1} at the flash peak using partially purified luciferin as the substrate.

phosphate column (17 × 3.2 cm) equilibrated with the buffer described in step 3. Conditions of operation of the column are listed in the legend to Figure 2. About 10% of the total protein was retained on the column while luciferase came through unretarded (step 5).

The active fractions from step 5 generally showed a trace of luciferin sulfokinase activity. This could be removed by repeating steps 2 and 3 (step 6).

Results

Purification of Luciferase. Table I summarizes the purification procedure for luciferase. The enzyme has been purified approximately 48-fold with an overall yield of 27%. Further purification was not achieved on DEAE-cellulose or on a number of other anion and cation exchangers and the enzyme appears to be homogeneous by a variety of criteria examined as outlined below. The specific activity of the purified enzyme was found to be 3.4×10^{12} quanta sec^{-1} mg^{-1} using pure luciferin as the substrate. This is to be compared to a value of 1.0×10^{12} quanta sec^{-1} mg^{-1} found with partially purified luciferin.

Behavior on G-75 Sephadex and Spectral Properties. When purified luciferase is rechromatographed on G-75 sephadex an elution profile like that in Figure 3 is obtained. The specific activity of the fractions are enclosed in parentheses.[2] Note that at low protein concentrations the specific activity decreases whereas at higher protein concentrations the specific activity is rather constant. This effect with *Renilla* luciferase was first noted by Kreiss (1967). He showed that at concentrations below about 0.3 mg/ml the specific activity dropped sharply and that this effect was reversible.

Luciferase exhibits a visible absorption as well as a typical protein absorption as shown in Figure 4. In the ultraviolet the absorption maximum lies at 279 nm whereas in the visible there exists a major band at 500 nm and a shoulder at 470 nm. As seen in Figure 3 this 500-nm absorption appears to follow luciferase activity. If luciferase is chromatographed on Sephadex G-75 in the presence of 8 M urea, the 500-nm absorption again follows the protein profile suggesting that it is bound to the protein. However the available evidence argues against a requirement for the visible chromaphore for luciferase activity. For example, if one uses a monomeric molecular

[2] The specific activities of the major fractions are higher by a factor of 2 over that reported in Table I. This difference reflects differing amounts of inhibitors present in different preparations of partially purified luciferin.

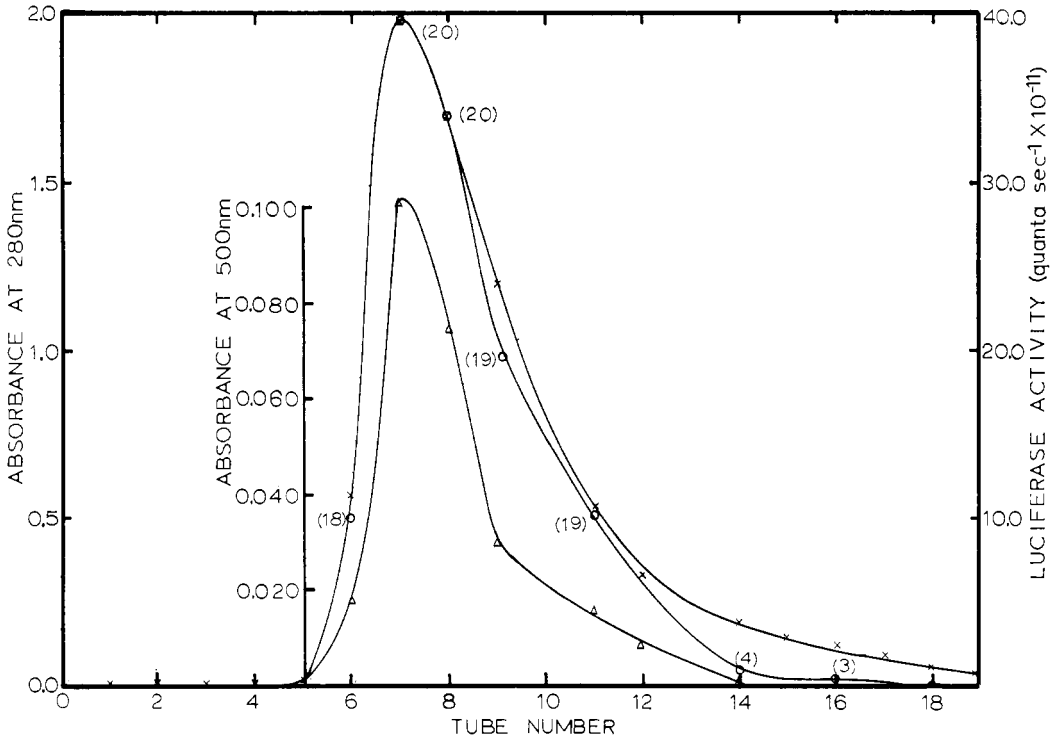

FIGURE 3: Sephadex G-75 chromatography of pure luciferase. The column (12 × 1.2 cm) was equilibrated with, and the enzyme eluted with, the same buffer listed in the legend to Figure 1. The enzyme solution (0.7 ml) was placed on the column and 0.5-ml fractions were collected at a rateof 60 ml/hr. (×-×) Absorbance at 280 nm; (△-△) absorbance at 500 nm; (○-○) luciferase activity.

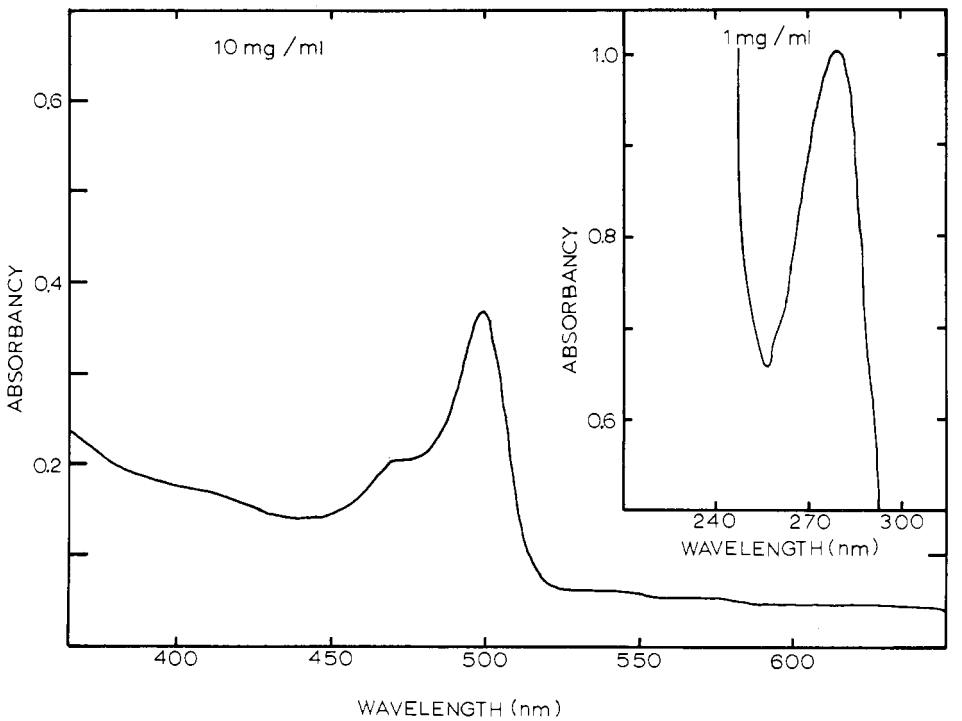

FIGURE 4: Absorption spectrum of luciferase. Luciferase was dissolved in the buffer listed in the legend to Figure 1 at the concentrations indicated and its absorption monitored on a Cary Model 14 recording spectrophotometer.

weight of 12,000 for luciferase (see text below) and assumes a 1:1 molar ratio of chromaphore per monomer then one can calculate a molar extinction coefficient for the chromophore. This calculation gives a value of about 400 which would seem unrealistically low. Further, individual batches of enzyme have been found to vary in their chromaphore content by a factor of 4 without lowering their specific activity. In fact preparations with less chromaphore have slightly higher specific activities. It appears that only a small fraction of luciferase has this visible chromaphore attached to it. This

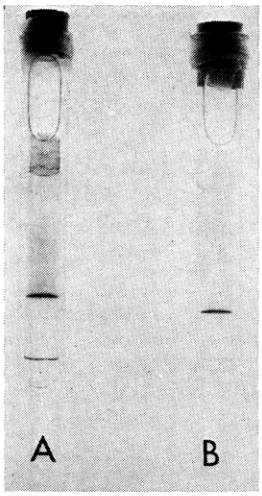

FIGURE 5: Disc gel electrophoresis of luciferase at pH 8.5 in the presence and absence of 8 M urea. Luciferase (60 μg) was applied to each gel. See Methods for details. (A) Without urea; (B) in presence of 8 M urea.

material may represent a bioluminescent oxidation product attached to the enzyme. In this regard it is of interest to note that the chromaphore is highly fluorescent. Its fluorescence excitation coincides with its visible absorption and its fluorescence emission emission maximum lies at 510 nm when excited at either 470 or 500 nm. This emission maximum is the same as that observed in the *in vivo* bioluminescence emission.

Electrophoretic Behavior of Luciferase. As seen from Figure 5 disc gel electrophoresis of luciferase on 7.5% gels at pH 8.5 shows a single disc moving toward the anode followed by a smear. Luciferase activity resides in the midportion of the smear and not in the major band. As indicated from the data below this may be because native luciferase is being dissociated into an inactive and lower molecular weight form during the experiments. This smear was independent of gel concentration, temperature of electrophoresis, current, and protein concentration. When disc gel electrophoresis was carried out in 8 M urea only a single disc was observed and the smear disappeared suggesting that it arose from luciferase itself. The smear could also be considerably reduced by shortening the height of the spacer gel. This may be related to the fact that luciferase is denatured at the pH of the spacer gel which is 5.3.

Electrophoresis on cellulose acetate membranes was carried out at pH 7.0, 7.5, and 8.0. In all cases densitometer scans of the stained membranes showed one major band corresponding to the activity preceeded by an inactive minor band which constituted about 2% of the total protein as judged by intergrating under the curves. A densitometer profile of such a stained membrane is illustrated by Figure 6.

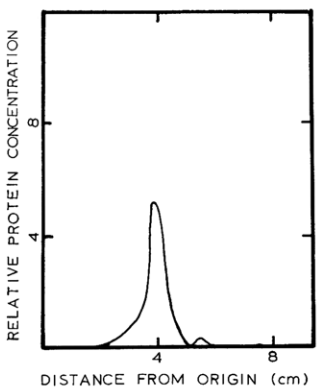

FIGURE 6: Cellulose acetate strip electrophoresis of luciferase at pH 7.5. The anode is located to the right of zero. See Methods for details.

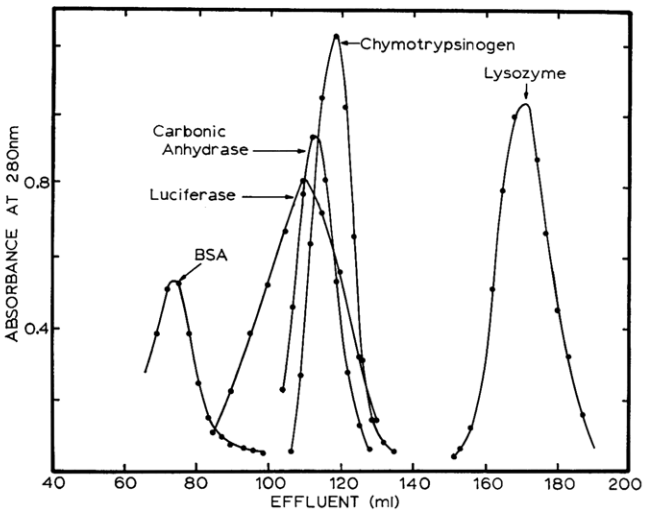

FIGURE 7: Stokes radius determination on luciferase by Sephadex G-75 chromatography. The column (83 × 1.7 cm) was equilibrated with, and the proteins eluted with, the same buffer listed in the legend to Figure 1. A 1-ml solution of each protein was applied to the column and 2.2-ml fractions were collected at the rate of 8 ml/hr. Elution positions were determined by measuring absorbancy at 280 nm. In the case of luciferase, activity was also followed (see text).

Determination of Stokes Radius. Figure 7 presents the result of an experiment designed to determine the Stokes radius of luciferase. The elution position of luciferase on Sephadex G-75 was compared to that of a number of proteins of known molecular weight or Stokes radius. It can be seen that the elution position of luciferase is near that of carbonic anhydrase. The luciferase activity (not shown) and absorbance at 280 nm essentially coincide as shown above. These data suggest a molecular weight in the vicinity of 34,000 and a Stokes radius of 27 Å. It should also be noted that the elution profile of luciferase is broad compared to that of the markers on G-75.

When the experiment illustrated in Figure 7 is performed in the presence of 8 M urea luciferase appears at an elution position near that of lysozyme and the elution profile is no longer broadened. Under these conditions the estimated molecular weight of luciferase, determined from a plot of the logarithm of the molecular weight of the proteins against their distribution coefficients, is about 12,000.

Ultracentrifugal Studies. During sedimentation velocity studies of luciferase it was observed that the enzyme sediments as a single component and that the schlieren pattern was symmetrical but broadened with time. Figure 8 shows the type of sedimentation pattern observed with luiciferase as a function of time. Figure 9 illustrates that there is little concentration dependence on the sedimentation coefficient, $s_{20,w}$. A linear extrapolation to zero protein concentration yields an $s^0_{20,w}$ of 1.57 S.

Sedimentation equilibrium data for luciferase are presented in Figure 10 in which the logarithm of the fringe displacement in microns is plotted against the square of the distance from the center of rotation. As shown in Figure 10 luciferase

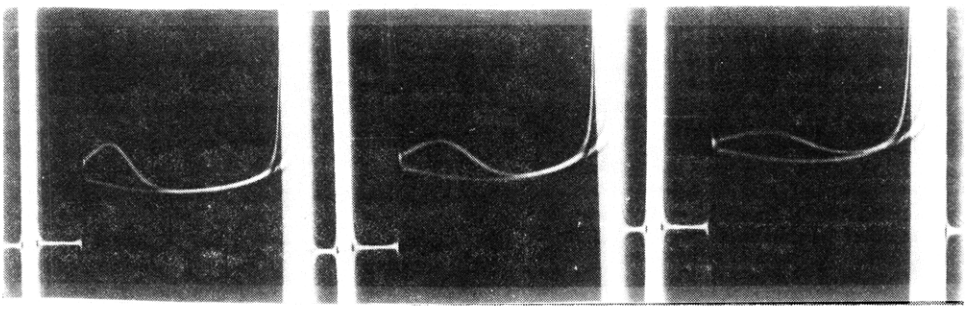

FIGURE 8: Sedimentation pattern of *Renilla* luciferase. The photographs shown were taken at 81, 129, and 177 min after reaching a speed of 60,000 rpm. The enzyme was dissolved in the buffer given in the legend to Figure 1 at a concentration of 10 mg/ml. The bar angle was 60°, the temperature 4° and sedimentation was from left to right.

represents a heterogeneous mixture of components in a buffer in which the enzyme maintains its activity. The lowest weight-average molecular weight observed was 12,600 corresponding to the subunit molecular weight of luciferase (see below). The value for the apparent partial specific volume, $\bar{V}$, used in this calculation was obtained from amino acid composition by the method of Cohn and Edsall (1943) and found to be 0.732.

Molecular Weight Determination by SDS Acrylamide Electrophoresis. Figures 11 and 12 present data designed to determine the molecular weight of the subunit of luciferase. The method of Weber and Osborn (1969) was used. As shown in Figure 12 a single band was observed during electrophoresis of luciferase on acrylamide containing 0.1% SDS suggesting the existance of a single subunit. Using various protein markers shown in Figure 11 the molecular weight of the luciferase subunit by this method was found to be about 12,000.

Amino Acid Composition. The amino acid composition of luciferase is presented in Table II. Values for those amino acids stable to hydrolysis in 6 N HCl did not show a significant change between 72- and 96-hr hydrolysis times. From amino acid composition data the calculated nitrogen content of luciferase is 15.0% based upon a molecular weight of 12,253. The experimental value, as determined from a microKjeldahl method, was 15.3%. The analysis shows a predominance of lysine, aspartic, and glutamic acid residues whereas those of histidine, methionine, arginine, and tryptophan are low in number. The calculated number of residues per mole of luciferase is based upon the subunit size of the enzyme as determined by SDS acrylamide electrophoresis and gel filtration in 8 M urea. On this basis the calculated molecular weight based on amino acid composition is 12,253.

A spectrophotometric method for tryptophan (Bencze and Schmid, 1957) gave a value of 1.7 residues/mole of enzyme. A colorimetric method for this amino acid described by Barman and Koshland (1967) gave a value of 1.8 residues/mole of enzyme.

Titration of luciferase with 5,5′-dithiobis(2-nitrobenzoate) in the presence of 8 M urea gave a value of 0.9 residue of sulfhydryl/luciferase subunit. In the absence of urea about 25% of the total sulfhydryl residues reacted immediately with no further reaction occurring at the end of 60 min at 25°. The total half-cystine content was determined as described in the Methods. Performic acid oxidation (Moore, 1963) gave a half-cystine content of 2.8 residues per subunit of luciferase while the half-cystine content determined by carboxymethylation (Crestfield *et al.*, 1963) resulted in a value of 2.7 residues. Thus it appears that luciferase contains one sulfhydryl group and one disulfide bond per subunit.

It is of interest to note that when luciferase was examined

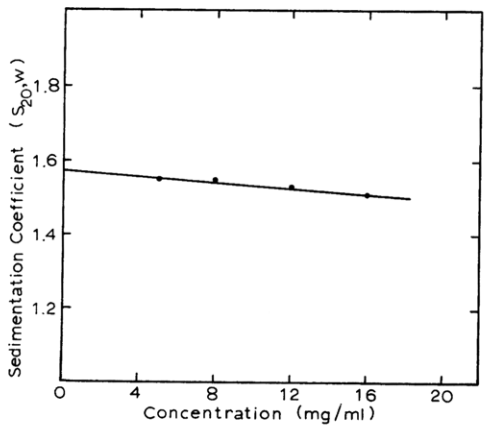

FIGURE 9: Sedimentation coefficient as a function of luciferase concentration. Sedimentation velocity experiments were performed in the buffer listed in the legend to Figure 1. Other conditions are given in the Methods.

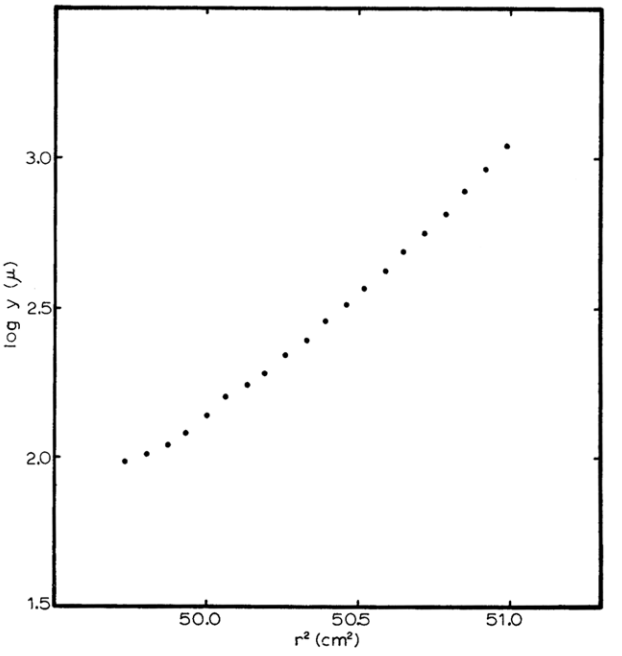

FIGURE 10: High-speed sedimentation equilibrium of luciferase. See Methods for details.

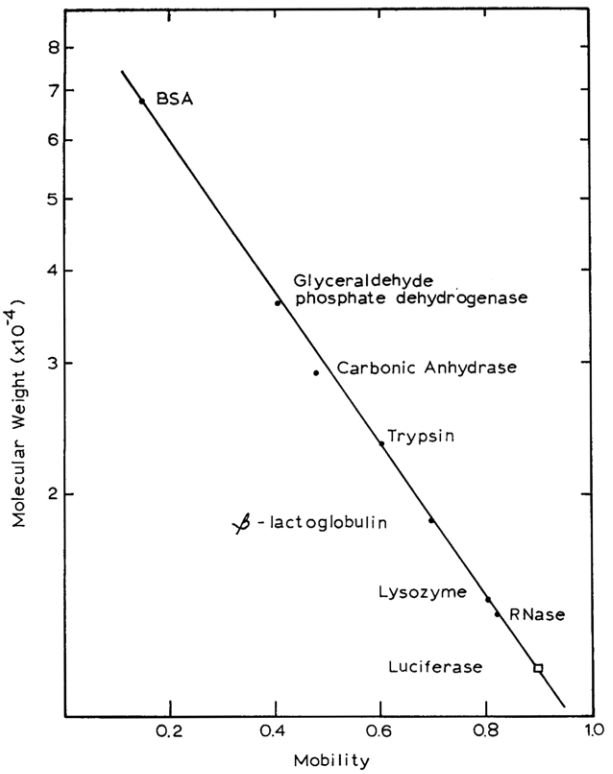

FIGURE 11: Determination of the subunit molecular weight of luciferase by SDS acrylamide gel electrophoresis. See Methods for details.

for carbohydrate content as described under Methods 2 moles of glucosamine was found per subunit.

Summary of Physicochemical Constants of Luciferase. Table III summarizes values obtained for the physicochemical constants of luciferase by methods listed in the above Methods and Results sections of the text. Note that the molecular weight of the luciferase subunit is in the range of 12,000 by four independent methods. On the other hand, the molecular weight of native luciferase, as determined from Sephadex G-75 chromatography, appears to be in the range of 34,000.

The extinction coefficient of native luciferase, $\epsilon_{280}^{0.1\%}$, was determined by measuring the concentration of luciferase from fringe displacements according to the method of Babul and Stellwagen (1968) and comparing this to the optical density at 280 nm on the Zeiss PM QII spectrophotometer. The value obtained was 1.04.

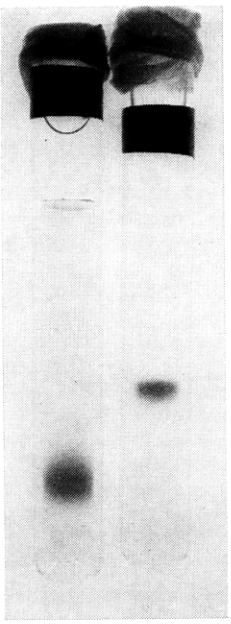

FIGURE 12: SDS acrylamide gel electrophoresis pattern of luciferase compared to that of lysozyme. In the case of luciferase (left) 50 μg of protein was used due to the fact that this protein does not stain easily; right, 20 μg of lysozyme. See Methods for details.

TABLE II: Amino Acid Composition of Luciferase.[a]

Amino Acid	Residues/Molecule[b] after Hydrolysis for 24 hr	48 hr	72 hr	Av	Nearest Integer
Lysine	6.98	7.32	7.90	7.61[c]	8
Histidine	2.0	2.0	2.0	2.0[d]	2
Arginine	3.17	3.35	3.26	3.26	3
Aspartic acid	14.18	13.18	13.38	13.58	14
Threonine	5.87	5.91	5.97	5.92[e]	6
Serine	6.30	5.84	5.77	5.97[e]	6
Glutamic acid	13.78	13.28	13.91	13.69	14
Proline	4.99	5.17	5.18	5.13	5
Glycine	7.93	7.96	7.96	7.95	8
Alanine	7.0	7.0	7.0	7.0[d]	7
Valine	5.05	6.25	6.47	6.36[c]	6
Methionine	2.14	2.21	2.19	2.18	2
Isoleucine	3.82	4.96	5.26	5.11[c]	5
Leucine	7.29	7.58	7.83	7.47	8
Tyrosine	2.62	2.91	2.93	2.83	3
Phenylalanine	3.55	3.67	3.65	3.62	4
Tryptophan				1.75[f]	2
Half-cystine				2.75[g]	3
Glucosamine				2.0[h]	2

[a] Duplicate analyses were performed at each time of hydrolysis. [b] Based on a molecular weight of 12,000 as determined by SDS acrylamide electrophoresis and gel filtration in 8 M urea. [c] The average value from 48- to 72-hr hydrolyses is presented. [d] Values are calculated relative to two residues of histidine for the basic amino acids and seven residues of alanine for the acidic and neutral amino acids. [e] Since very little destruction was observed with time the average value is presented. [f] Measured by spectrophotometric and colorimetric methods as described in the text. [g] Measured as cysteic acid after performic acid oxidation (2.8 residues/molecule) and as *S*-carboxymethylcysteine in hydrolysates of *S*-carboxymethylluciferase (2.7 residues/molecule). The average of the two determinations is presented. [h] Measured colorimetrically as described in the text.

Discussion

A number of observations reported here suggest that *Renilla* luciferase has been isolated in highly purified form. For example, during Sephadex G-75 chromatography the specific activity of luciferase is constant across the peak at protein concentrations of 0.3 mg/ml or greater. At concentrations lower than this the specific activity of such fractions increase

TABLE III: Summary of Physicochemical Properties of Luciferase.

1. Stokes radius, a (Å)	27.0
2. Diffusion coefficient, $D_{20,w} \times 10^7$ (cm^2 sec^{-1}) (from Stokes radius)	8.0
3. Sedimentation coefficient, $s^0_{20,w} \times 10^{13}$ (S)	1.57
4. Apparent partial specific volume, $\bar{V}_{app}$ (cm^3 g^{-1})	0.732
5. Molecular weight	
From Sephadex G-75 chromatography	34,000 ± 2000
From sedimentation equilibrium	12,600
From amino acid composition	12,253
From SDS acrylamide electrophoresis	11,700
From gel filtration in 8 M urea and MSH	12,000
6. Extinction coefficient, $\epsilon^{0.1\%}_{280}$	
For native luciferase at pH 7.5	1.04

upon concentration of the fractions. Further, disc gel electrophoresis in 8 M urea and electrophoresis at pH 7.0–8.0 on cellulose acetate also indicate a high degree of purity. The titration of 1 free sulfhydryl group per molecular weight of 12,000 is also consistent with this interpretation. It can also be argued from the data in Figure 12 that any protein contaminant in these luciferase preparations must have a subunit size identical with that of luciferase and migrate with luciferase on Sephadex G-75. This would seem to be an unlikely possibility.

From the data reported it appears that luciferase is a self-associating protein that exist in solution as a mixture of monomers, dimers, trimers, and higher molecular weight forms. Data obtained from Sephadex G-75 chromatography, sedimentation velocity, and equilibrium analysis, and determinations of the subunit molecular weight are all consistent with this view. The data do not allow us to decide whether or not an equilibrium exist between higher and lower molecular weight forms of the enzyme.

Molecular weight determinations on the subunit size of luciferase using SDS acrylamide electrophoresis gave a value of 11,700. This technique also suggested the existance of a single subunit species since only a single protein band was observed. Gel filtration studies in 8 M urea suggested a subunit molecular weight of 12,000 whereas sedimentation equilibrium experiments gave a limiting weight-average molecular weight of 12,600. These values agree well with the value obtained from amino acid composition which was 12,253.

When native luciferase is chromatographed on Sephadex G-75 an apparent molecular weight of 34,000 and a Stokes radius of 27 Å are observed. From a consideration of the subunit molecular weight of luciferase it appears that native luciferase exists as some multiple of these subunits, possibly a dimer or trimer.

Chemical analysis of luciferase suggest the presence of one sulfhydryl group, one disulfide linkage, and 2 glucosamine residues per subunit molecular weight of 12,000. Evidence obtained by Kreiss (1967) suggest that the enzyme does not contain a metal.

Because *Renilla* luciferase is a low molecular weight protein and because it is an enzyme which converts chemical energy into light energy we feel that studies on this enzyme should provide valuable information relative to the structure–function relationships of oxygenase-type enzymes generally. Furthermore since luciferase catalyzes the production of visible light it is possible to measure the levels of electronic transitions occurring during the course of the enzymatic reaction.

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Chemical and Physical Studies of *Neurospora crassa* Invertase. Molecular Weight, Amino Acid and Carbohydrate Composition, and Quaternary Structure*

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ABSTRACT: *Neurospora crassa* invertase was found to be a glycoprotein containing 11% mannose and 3% glucosamine. Its molecular weight was found to be 210,000 ± 15,600 in pH 5.0 buffer and 51,500 in 6 M guanidine hydrochloride under reducing conditions. A tetrameric structure is thus indicated. At pH 5.0 the enzyme had an $s^0_{20,w}$ = 10.5 S. Under alkaline conditions it dissociated to give a molecule with $s_{20,w}$ = 5.2 S, and as alkali concentration was increased, it was converted into a 3.8S species. Both the 10.5S and 5.2S forms occur as such in crude extracts of the organism and both are enzymatically active. The amino acid composition of the enzyme has been determined and tryptic peptide fingerprints have been prepared. These studies suggest the possibility that the enzyme may be composed of more than one type of subunit. The native enzyme was found to contain four disulfide bonds per mole.

The *Neurospora crassa* invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) catalyzes the hydrolysis of β-fructoside bonds in a variety of substrates ranging in molecular size from β-methylfructose to inulin. The chemical characterization of this enzyme is of interest because of its localization in the area between the cell wall and the cell membrane of the fungus. In addition, once fully characterized, the wild-type enzyme will serve as a standard with which to compare cross reacting proteins purified from *Neurospora* mutants lacking invertase activity (Sargent and Woodward, 1969).

Metzenberg (1963a) purified invertase from *Neurospora* and partially characterized the enzyme. His results indicated that the enzyme exists in two active multimeric forms, the larger species having an $s_{20,w}$ of 10.3 S and the smaller one an $s_{20,w}$ of 5.2 S (Metzenberg, 1964). His studies showed that the 10.3S form could be dissociated into active subunits (5.2 S) by heat or by formic acid treatment in the presence of 1 M sodium chloride.

The studies described here were undertaken with the goal of elucidating the quaternary structure of the enzyme using chemical and physical evidence. This communication reports the homogeneity of the purified enzyme, its molecular weight, amino acid composition, and disulfide content, and evidence for subunits smaller than the active subunit. Preliminary studies on the carbohydrate moiety of the enzyme are also reported.

Experimental Section

Materials. Diethylaminoethylcellulose (DEAE) and carboxymethylcellulose (CM) were obtained from Schleicher & Schuell. Glucostat reagents and L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone trypsin were obtained from Worthington Biochemical Corp., and glucose Tes-Tape from Eli Lilly. Anti-invertase antiserum was prepared by Antibodies Inc., Davis, Calif., using invertase preparations demonstrated to be homogeneous by several criteria. Sodium *p*-hydroxymercuribenzoate and Tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co., Sephadex G-100 was obtained from Pharmacia Fine Chemicals, and guanidine hydrochloride (Sequanal grade) from Pierce Chemical Co. All other chemicals were reagent grade.

Growth Conditions. N. crassa wild-type strain SF 26 (Gratzner and Sheehan, 1969) was used as the source of invertase. This strain, a derivative of the standard wild-type STA 4, was selected for increased levels of the carbohydrases amylase and invertase. Conidia from 4- to 5-day-old cultures were used to inoculate 500-ml florence flasks containing 250 ml of Vogel's (1964) citrate minimal medium to which had been added sufficient sucrose (autoclaved separately in H_2O) to achieve a final concentration of 1.5% carbon source. After growth on a shaker at 34° for 4 days, one of these cultures was then used

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