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THE DIFFUSION CONSTANT OF *CYPRIDINA* LUCIFERASE

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SUMMARY

The diffusion constant, D, of *Cypridina* luciferase was measured by the porous disk method of NORTHROP AND ANSON[3], and a value of $7.4 \cdot 10^{-7}$ cm^2/sec was found. This would indicate an upper limit of about 80,000 for the molecular weight of this enzyme.

INTRODUCTION

Cypridina luciferase is the enzyme which catalyzes the blue luminescent reaction of the ostracod crustacean, *Cypridina hilgendorfii*. The animal ejects the enzyme and its substrate, luciferin, into the surrounding sea water where, in the presence of dissolved oxygen, the luminescence occurs[1].

The purpose of this paper is to record the diffusion constant of this enzyme and to describe the measurements upon which it is based. Because the luminescent reaction in *Cypridina* is first order[2] and requires no other components than luciferase, luciferin, water and oxygen, the activity, and hence the concentration, of luciferase can be determined from the reaction velocity constant with a high degree of precision, even in the presence of protein and other impurities. Consequently, NORTHROP AND ANSON's method[3] of measuring the diffusion constant by observing the rate of passage through a porous disk is particularly applicable and yields data of a high order of reliability. It is, indeed, the only method applicable at the present time since *Cypridina* luciferase is not available in any quantity or in a pure form.

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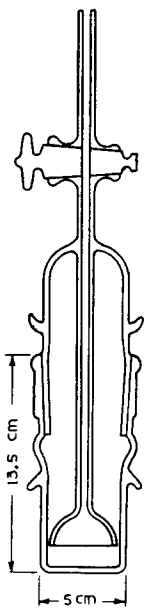
EXPERIMENTAL METHODS AND PROCEDURES

The luciferase solutions used were prepared by grinding about 15 g of the dried animals by mortar and pestle and extracting the resulting powder for 24 h with benzene in a Sohxlet apparatus to remove lipids. The powder was then freed of benzene by evaporation *in vacuo* and extracted with absolute methanol for 24 h. This removed certain impurities, including luciferin. The powder was again dried and finally extracted with 0.1 *M* phosphate buffer of pH 6.8 and centrifuged. The resulting supernatant solution was dialyzed at 2°, against several changes of the phosphate buffer. The solution was saturated with toluene to discourage growth of microorganisms.

Luciferase solutions obtained in this way had a high activity and were usually used without appreciable dilution as the starting inner solution in the NORTHROP AND ANSON diffusion apparatus. The outer solution was the same phosphate buffer mentioned above.

The diffusion apparatus itself was basically the same as that described by NORTHROP AND ANSON[3] except that it incorporated interchangeable, standard ground glass joints which imparted rigidity and prevented any evaporation during an experiment. Fig. 1 accurately represents a longitudinal cross section of the diffusion apparatus.



Two kinds of diffusion disks were used. One was of alundum, obtained from the Scientific Glass Apparatus Co. (Bloomfield, N.J.), designated R.A. 225, with a pore diameter of 8–10 μ according to the manufacturer. We ground this to a diameter of 44.5 mm and a thickness of about 1 mm. The other disk was of sintered pyrex glass, obtained from the same supplier, already ground to a diameter of 44.5 mm and a thickness of 1 mm. The disks were attached to their respective vessels with DeKhotinsky cement.

Fig. 1. Longitudinal section of the diffusion vessels, drawn to scale as indicated. They were of pyrex glass and utilized 55/50 standard taper joints supplied with glass hooks. The diffusion disks, of alundum or sintered pyrex glass, had a diameter of 44.5 mm and were 1 mm thick. They were attached to the funnel-like inner component with De Khotinsky cement. Clamps for mounting and leveling and water bath not shown. Volumes of inside and outside solutions each about 30 ml.

Preliminary experiments showed that it was essential to de-gas the diffusion disks in order that the pores should be completely free of air spaces. This was accomplished by immersing the vessels with disks attached in water in a closed container. The pressure in the container was then reduced causing air trapped in the pores of the disk to expand and escape. Upon then restoring atmospheric pressure in the container, water moved into the spaces in the disk previously filled with air. This procedure was repeated several times and the diffusion cells with disks attached were then always kept immersed in water when not in use.

The volumes of the inner vessels upon which the disks were sealed were about

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30 ml. The outside solutions had approximately this same volume when the outer vessels were in position and were so filled that the disks were in contact with the surface of the outside solution. The volumes of individual inner vessels and solutions in individual outer vessels were of course known exactly by weighing the amounts of distilled water needed to fill the various vessels under experimental conditions.

The diffusion experiments were run in a cold room whose air temperature normally fluctuated between 1.6 and 2.1°. By keeping the diffusion apparatus immersed in a large volume of water, occasional larger fluctuations, as when the door was opened, were eliminated. The low temperature was necessary in order to minimize heat inactivation of the luciferase during the several days required for a complete diffusion run.

The beginning of an experiment involved rinsing the disk and inner vessel by carefully sucking the luciferase solution up through the porous disk to a point above the stopcock, then expelling it and refilling (see Fig. 1). The stopcock was then closed and the outer vessel was connected, having previously been filled with the proper volume of phosphate buffer so that the outer surface of the porous disk was just covered. The experimental set-up was so arranged that the disk was perfectly level. The water bath, at the temperature of the cold room, was then raised so that the diffusion apparatus was immersed in it. A sample of the original inner luciferase solution was always kept for analysis of the initial enzyme concentration.

After diffusion had gone on for the desired interval, the outside solution was removed and stored for subsequent analysis and the outer vessel refilled with fresh buffer and quickly replaced. As many samples of diffusate were collected as was necessary to give results which would define the "steady state" of the diffusion system; *i.e.*, when the quantity of luciferase diffusing through the disk was constant for equal time intervals. At the end of the experiment a sample of the luciferase solution remaining within the diffusion cell was also taken for determination of its final concentration.

A photoelectric light integrating method utilizing a graphic recorder was used to assay luciferase activity in the samples in order to determine luciferase concentration. This method was based in principle upon that used by ANDERSON[4]. The particulars of the assay were essentially the same as described by CHASE[5].

ANALYSIS OF THE DATA

In the paper by NORTHROP AND ANSON[3], the diffusion constant (D) is given by the following equation:

$$D = \frac{h}{A} \cdot \frac{Q_{ml}}{t}$$

where h is the effective distance through which the solute diffuses, A the effective area of the pores of the disk, and Q_{ml} the number of ml of the initial concentrated inner solution that would contain the amount of substance diffused in time, t. The value of h/A differs for each disk but is constant for any particular one, regardless of the material which diffuses through it. This ratio, h/A, is therefore the "disk constant", K, and may be expressed by the following relation:

$$K = \frac{h}{A} = \frac{Dt}{Q_{ml}}$$

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Since, in this method, the dimensions of the pores of the disks are not known, it is necessary to evaluate K for each disk with a substance of known diffusion constant. Sodium chloride in 2 M concentration was used in the present case, and its rate of diffusion through the disk was measured under the same conditions as existed during the experiments with the luciferase, except that the samples of diffusate were collected more frequently. Chloride concentrations were determined by titration with silver nitrate in the usual way.

With the constant for a particular porous disk known, and once the rate of diffusion of luciferase at "steady state" had been established, the diffusion constant of the enzyme could be calculated.

The radius of the luciferase molecule may be estimated from the diffusion constant by means of EINSTEIN's[6] equation,

$$r = RT/D6\pi\eta N,$$

assuming the molecule to be spherical and to lack water of hydration.

The molecular weight may be estimated from the radius by means of the expression,

$$M = 4\pi r^3 dN/3,$$

assuming 1.33 as the specific gravity, d, of luciferase.

Because proteins in general are non-spherical and hydrated, EINSTEIN's equation probably does not yield the true value of the radius, and the resulting figure for the molecular weight is a maximal one. The value of the diffusion constant itself is, however, unequivocal.

Since it was necessary to determine the diffusion constant at a temperature of about 2°, with diffusion into phosphate buffer, the corresponding values were calculated for standard conditions; *i.e.*, pure water at 20°. The method of referring diffusion constants to standard conditions is described in GREENBERG[7].

RESULTS

After a purely exploratory run to establish conditions, five diffusion experiments were performed, three with the alundum and two with the sintered pyrex glass disk. Special experiments of long duration showed a regular activity loss of about 15 % as the enzyme passed through both kinds of disks. This probably is caused by surface inactivation of some kind. Such inactivation has been observed before (A. M. CHASE, unpublished experiments), when solutions of the enzyme have been in contact with powdered glass, for example. The apparent rate of diffusion was of course, corrected for this activity loss.

It would be prohibitive and serve no useful purpose to present all the data involved in the various experiments. In order, however, to show the time intervals used and the order of precision of the measurements, the actual figures are presented, in Table I, for one of the diffusion runs with the apparatus equipped with the sintered glass disk. Duplicate determinations of the luciferase concentration (proportional to the first order reaction velocity constant of the luminescent reaction) were made on each diffusate, as well as on the concentrated inner solution before and after the run. As the last column of Table I indicates, a steady state is achieved after the initial

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TABLE I

NUMERICAL DATA FROM A TYPICAL DIFFUSION EXPERIMENT TO ILLUSTRATE DETAILS AND THE ORDER OF PRECISION OF THE MEASUREMENTS

D calculated from these data is $3.36 \cdot 10^{-7}$ cm²/sec, for 1.8° and phosphate buffer. Correction for 15 % activity loss during passage through disk pores yields $D = 3.94 \cdot 10^{-7}$ cm²/sec. K for the disk used = 0.0465. Volume of concentrated inner solution = 33.3 ml. Therefore, 0.333 ml is the value for Q_{ml} in equation of NORTHROP AND ANSON.

Sample	*Dilution factor for assaying L'ase activ.*	*Relative first order reaction rate const.*	*Relative amount of L'ase present**	*Diffusion interval h*	*% of original amount of L'ase diffused***	*Time for 1 % to diffuse sec*
Conc. inner soln., start	0.005 0.005	1.39 1.39	278.0 278.0	—	—	—
Diffusate, No. 1	0.406 0.406	1.355 1.36	3.34 3.35	7.6	1.203	22680
Diffusate, No. 2	0.384 0.448	0.88 0.965	2.29 2.15	10.2	0.799	46080
Diffusate, No. 3	0.533 0.533	1.18 1.22	2.21 2.29	10.0	0.809	44640
Diffusate, No. 4	0.450 0.450	0.825 0.855	1.83 1.90	9.0	0.671	48240
Diffusate, No. 5	0.491 0.491	1.295 1.25	2.64 2.55	13.0	0.933	50040
Diffusate, No. 6	0.376 0.376	0.985 0.93	2.62 2.47	13.1	0.915	51480
Diffusate, No. 7	0.528 0.528	1.11 1.125	2.10 2.13	11.1	0.761	52560
Conc. inner soln., end	0.0064 0.0064	1.685 1.665	263.0 260.0	—	—	—

* Relative luciferase concentration (*i.e.*, relative first order reaction rate constant) divided by dilution factor.

** Average of duplicate determinations.

TABLE II

CALCULATED VALUES OF THE DIFFUSION CONSTANT (D) OF *Cypridina* LUCIFERASE, BASED UPON THE RESULTS OF THE DIFFUSION EXPERIMENTS

Expt. No.	*Kind of disk*	*Exptl. temp.* (° C)	*D for diffusion under exptl. conditions* (cm²/sec)	*D referred to st'd. conditions of pure water at 20°* (cm²/sec)
1	Alundum	1.9	$3.10 \cdot 10^{-7}$	$5.78 \cdot 10^{-7}$
2	Alundum	1.9	$3.55 \cdot 10^{-7}$	$6.62 \cdot 10^{-7}$
3	Glass	1.8	$3.98 \cdot 10^{-7}$	$7.46 \cdot 10^{-7}$
4	Alundum	1.8	$3.71 \cdot 10^{-7}$	$6.94 \cdot 10^{-7}$
5	Glass	1.8	$3.94 \cdot 10^{-7}$	$7.36 \cdot 10^{-7}$

8 h or so and the rate of diffusion is then relatively constant for three collection intervals. Samples 2, 3 and 4 were therefore used to provide the data from which the diffusion constant for this particular experiment was calculated.

The other experiment involving the sintered glass disk gave practically identical results. The experiments with the alundum disk were less regular and suffered from difficulties of one sort or another, such as clogging of the disk pores. The data obtained with the alundum disk were therefore not averaged with the other data in calculating the diffusion constant, although the values from all the experiments are presented individually for comparison.

The diffusion constants obtained from the five experiments are given in Table II. If luciferase is assumed to be a completely spherical molecule lacking water of hydration, its radius and molecular weight can be calculated using the last two equations, above. The experiments with the sintered pyrex glass disks yield values for the radius and molecular weight of $2.88 \cdot 10^{-7}$ cm and 80,000, respectively. The last figure would therefore represent the upper limit for the molecular weight of *Cypridina* luciferase.

When and if this enzyme ultimately becomes available in sufficient quantity in crystalline form, an unequivocal value of its sedimentation constant can be obtained and, in conjunction with the present value of the diffusion constant the true molecular weight* can be calculated.

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* The present value of the diffusion constant, in conjunction with an earlier determined value of $3.4 \cdot 10^{-13}$ sec for the sedimentation constant from ultracentrifuge studies of partially purified *Cypridina* luciferase solutions[8], yields a value of about 45,000 for the molecular weight.