the extract was 8.2 with BAEE as substrate, identical to the *p*H optimum for crystalline bovine trypsin (Fig. 1A).

Among the proteolytic enzymes found in nature, trypsin is unique in that it is sensitive to a number of naturally occurring inhibitors. Accordingly, the sperm head extract was tested for sensitivity to two of these inhibitors and compared with crystalline bovine trypsin and crystalline papaya papain, a plant enzyme also active on this substrate. Soybean trypsin inhibitor (10 μg/ml) depressed the enzymic activity of both trypsin and sperm head extract to 10 to 20 percent of the maximum rate when no inhibitor was present (Fig. 1B). Papain was inhibited only slightly by this concentration of inhibitor. Lima bean trpsin inhibitor (Fig. 1C) (50 μg/ml) also depressed the activity of both trypsin and sperm extract to 10 percent of the maximum rate. However, the activity of crystalline papain was increased in the presence of lima bean trypsin inhibitor.

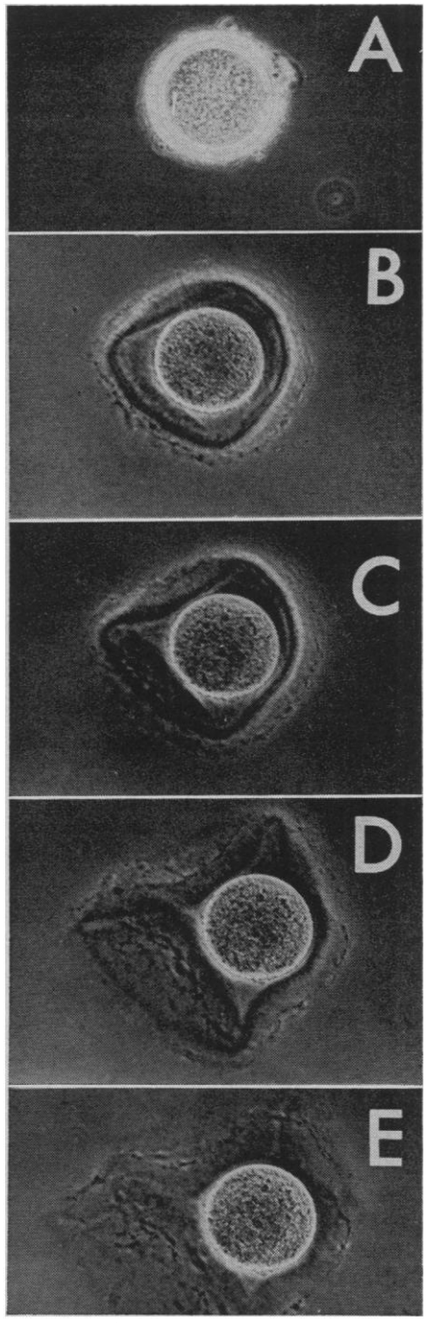

Fig. 2. Dissolution of the zona pellucida by sperm head extract at 37.5°C; (A) 0 time; (B) 20 minutes; (C) 40 minutes; (D) 60 minutes; (E) 90 minutes.

The dissolution of the zona pellucida by the extract concentrated by lyophilization is shown in Fig. 2. Complete dissolution of the zona occurred within 1.5 hours at 37.5°C, but the vitellus was not visibly affected by a 25-hour incubation in the concentrated extract. Addition of soybean trypsin inhibitor (300 μg/ml) or lima bean trypsin inhibitor (300 μg/ml) to this extract completely inhibited dissolution. Thus, the trypsin component of this extract is the more important enzyme in the dissolution process. This is supported by the fact that crystalline hyaluronidase (375 unit/ml) causes no visible dissolution of the zona pellucida even after a 24-hour incubation at 37.5°C, but crystalline trypsin (5500 unit/ml) completely dissolves the zona in 10 minutes at this temperature.

These data indicate that penetration of the zona pellucida by the spermatozoon is an enzymic process involving mainly an acrosomal enzyme similar to trypsin. The hyaluronidase might supplement this dissolution process, or its physiologic function may be to thin the cervical mucus or disperse the hyaluronic acid matrix of the cumulus oophorus of undenuded ova.

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4 May 1968

Bioluminescence: pH Activity Profiles of Related Luciferase Fractions

Abstract. *The 27,000g supernatant from crude extracts of the dinoflagellate* Gonyaulax *can be fractionated into two components having luciferase activity that differ both in molecular weight (35,000 and 150,000) and in* p*H activity profile. The smaller component has activity over a broad range from* p*H 5 to 9, while the larger one is active only in the acid region. This clarifies the previous ambiguity in the literature regarding the optimum* p*H for the assay of luciferase.*

Gonyaulax polyedra is one of the bioluminescent marine dinoflagellates. These are unicellular algae in which light emission commonly occurs as a brief (0.2-second) bright (10^8- to 10^{10}-photon) flash of light evoked by mechanical stimulation (*1*). Biochemical studies with *Gonyaulax* have shown that bioluminescence may be obtained in vitro from two distinctly different systems—one being soluble (*2*) and the other particulate (*3*).

The particulate system involves a large particle (molecular weight, $> 10^9$), termed the scintillon, which has the potential for bioluminescence when isolated and kept at *p*H 8.2. Activity occurs specifically when the *p*H is lowered to about 5.7, as a brief flash with a duration of about 0.2 second. The only additional factor required is oxygen.

The soluble system was first characterized (*2*) as the supernatant from a cell homogenate that had been centrifuged at 36,000*g* for 10 minutes. The activity was shown to involve a dialyzable, heat-stable component (*Gonyaulax* luciferin), a heat-labile protein (*Gonyaulax* luciferase), oxygen, and a high concentration of salt. The luminescence in this system is relatively long-lived, having a half-life of about 10 minutes at concentrations normally used in partially purified preparations (*4, 5*).

Using Sephadex gel filtration, we have now resolved the luciferase of the soluble system into two components that have molecular weights of approximately 150,000 and 35,000. The component with the higher molecular weight has a narrow *p*H activity profile similar to that described by Hastings and Sweeney (*2*), while the one with the lower molecular weight is active over a wider range from *p*H 6.0 to *p*H

9.0. This clarifies the previous ambiguity in the literature (*5*) regarding the optimum *p*H for the assay of luciferase.

Extracts were prepared by modifications of the method of Bode and Hastings (*6*). Cells were grown at 21°C under conditions of alternating periods of light and dark of 12 hours each and were harvested by filtration between the 2nd and 3rd hour of the light period. The cells from 15 liters of culture (5×10^6 cells per liter) were extracted twice at 23°C with a hand-operated homogenizer (Fisher 11-504-200) in 30 ml of tris-HCl buffer ($5 \times 10^{-3}M$), *p*H 7.7, with 5×10^{-3} dithiothreitol (DTT). The extract was chilled and centrifuged at 27,000*g* for 10 minutes at 2°C. The supernatant provided a crude source of the soluble luciferase activity (optical density, 280 nm, 10.7; 260 nm, 14.6). The in vitro yield from this preparation is approximately 10^7 quanta per cell. Crude luciferin was prepared in quantity by boiling a fresh harvest of cells for 2 minutes and was stored at −80°C until needed.

Assays for luciferase and luciferin were performed by measuring the light emission during the reaction at 23°C. In the reaction mixture described below, luciferin was added after luciferase so that the light intensity (expressed in quanta per second) was measured both with and without luciferin. Measurements were made with a photomultiplier calibrated in absolute units of photons per second (*7*). If we assume that the reaction liberates photons in proportion to the number of substrate molecules utilized, the intensity reveals the instantaneous rate of the reaction. For a fixed concentration of substrate and rate-limiting concentrations of luciferase the initial rate will be a measure of the luciferase concentration.

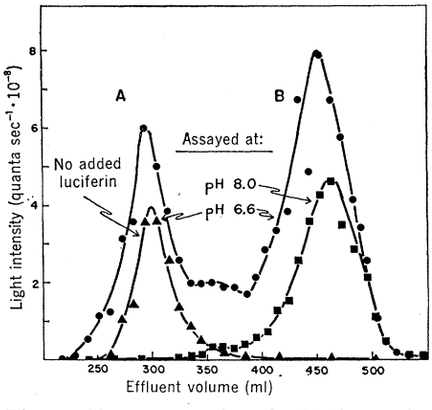

Fig. 1. Chromatography of 27,000*g* crude supernatant on Sephadex G-100. The eluting buffer was $5 \times 10^{-3}M$ tris and $10^{-3}M$ DTT adjusted to *p*H 8.0 with HCl at 4°C, equilibrated with N_2. ●, Assayed at *p*H 6.6 with luciferin added; ■, assayed at *p*H 8.0 with luciferin added; and ▲, assayed at *p*H 6.6 with no luciferin added.

The complete reaction mixture (2 ml) contained a final concentration of 0.1*M* tris (hydroxymethyl) amino methane-maleate-NaOH buffer at the specified *p*H; 0.38 g of bovine serum albumin per liter; 1*M* ammonium sulfate; $2.5 \times 10^{-4}M$ ethylenediaminetetraacetate; 0.1 ml crude luciferin; and 0.1 ml luciferase extract.

In the experiment depicted in Fig. 1, 22 ml of the cell extract described above was applied to a Sephadex G-100 column (80 by 4 cm). Fractions of 10 ml each were assayed at *p*H 6.6 and *p*H 8.0.

The experiment illustrates two separate findings. First, the 27,000*g* supernatant contains two different bioluminescent activities that are separable on the basis of molecular size. This is shown by assaying each fraction at *p*H 6.6. Second, only the entity of lower molecular weight has substantial activity at *p*H 8.0. Most of the 280 nm absorbance is eluted in the early fractions (225 to 300 ml). This absorbance arises from the bulk protein of the cell and is not interpreted as a measure of luciferase concentration.

The separation of the two components was also achieved by centrifugation. The resulting sedimentation pattern indicated a molecular weight distribution similar to that obtained in Fig. 1 with Sephadex. The heavier component was estimated to be 7*S* to 8*S* (150,000 to 200,000 molecular weight) by comparison with the internal marker, histidase, and bovine serum albumin run in a separate gradient (*8*). The molecular weight of the lighter component was similarly estimated to be about 35,000. Both of these values were corroborated by estimates obtained from Sephadex gel filtration with the use of markers according to the method of Andrews (*9*) (see Fig. 3).

Several details should be noted. A and B refer, respectively, to the Sephadex fractions having high and low molecular weights.

1) The relative amounts of these two fractions in a given crude extract appear to be dependent on the conditions of extraction. Data from ten extractions indicate that most of the activity is obtained in A at a higher concentration of buffer ($5 \times 10^{-2}M$), whereas a preponderance of the B activity is found when the extraction is carried out in $5 \times 10^{-3}M$ tris. The

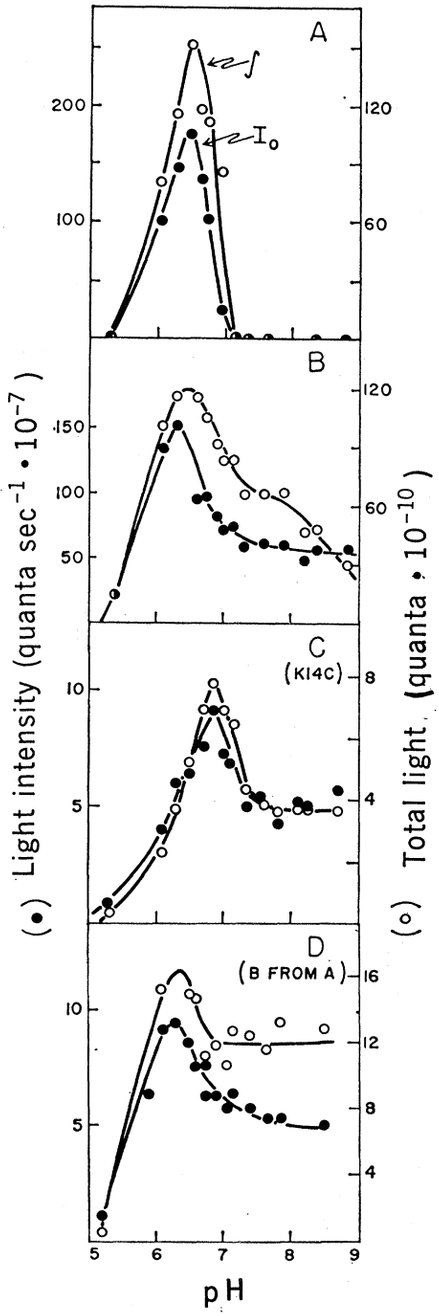

Fig. 2. Effect of *p*H on the bioluminescence of different luciferase preparations, both in terms of intensity, I_0 (quanta per second), shown at time 1 minute (●) and total light in quanta (○). Ordinate values are dependent on the specific concentrations of luciferase and luciferin used and have not been normalized with respect to one another. (A) Fraction A from Fig. 1; (B) fraction B from Fig. 1; (C) enzyme K14C purified by Bode and Hastings (*6*); and (D) fraction B after conversion from A (Fig. 3).

weaker buffer results in a lower final *p*H of the cell extract and this probably explains the difference.

2) Crude luciferase prepared at about *p*H 8 possesses some bound luciferin. Upon chromatography on Sephadex this bound luciferin is associated only with the fraction of higher molecular weight. This follows from the observation that, when assayed at *p*H 6.6, A possesses some activity without added luciferin, whereas B does not (Fig. 1, triangles). Free luciferin prepared by heating a crude extract has a molecular weight of about 400, as determined by gel filtration.

3) Although the light intensity was not measured in these experiments during the mixing of reactants, the values were obtained a few seconds later, and are believed to be a good estimate of the luciferase concentrations.

The difference in the activity of A and B as a function of *p*H (at 23°C) is shown in Fig. 2. The material used was separated in the experiment of Fig. 1 (A, 289 to 299 ml; B, 438 to 468 ml). Both the initial and integrated intensity values are plotted. The response of the A fraction (Fig. 2A) is very similar to that reported by Hastings and Sweeney (*2*) for a crude extract. The behavior of the B fraction (Fig. 2B) is very different in that it exhibits appreciable activity in the *p*H range of 7 to 9. This behavior is also exhibited (Fig. 2C) by a sample of the protein (K14C) purified by Bode and Hastings (*6*) which had been stored at −15°C during the intervening 6 years. Furthermore, a sample of that protein, when chromatographed on Sephadex, moved coincidentally with the B activity, indicating that it was identical with our material of lower molecular weight. This accounts for DeSa's success (*10*) in using the soluble assay system at *p*H 8.0, in apparent contradiction to the prediction of the *p*H curve of Hastings and Sweeney (*2*). DeSa was observing the activity of the partially purified component with a molecular weight of 35,000, while the activity described by Hastings and Sweeney corresponds to that of our entity with a molecular weight of 150,000.

The 35,000 molecular weight species can be obtained from the larger one by allowing the latter to stand at *p*H 6.0 at 4°C for 9 hours. The species with a molecular weight of 35,000 is then identified by its characteristic elution volume on Sephadex and by its *p*H activity profile (Fig. 2D).

When a sample from the pooled volume of A (Fig. 1, 267 to 330 ml) was treated in this fashion and then rechromatographed on Sephadex G-75, approximately 30 percent of the activity recovered from the Sephadex column was of the B type (Fig. 3). Because of activity and column losses this is only 8 percent of the absolute activity of the original A material. A duplicate sample kept at *p*H 8.0 for 10 hours before being rechromatographed showed no B material. There was some intermediate molecular weight material present, but it was devoid of activity at *p*H 8.0. A 100-percent conversion with no attendant loss in activity would prove unambiguously the hypothesized molecular conversion.

Fraction A is not only devoid of activity at *p*H 8; it inhibits the activity of B at *p*H 8. This is illustrated in Fig. 4, where small samples of A were added to a reaction in progress, containing B at *p*H 8. Additional experiments will be required to prove that the inhibitory action is due specifically to luciferase in the A fraction. Interpretation here is further complicated by the presence of luciferin in A. In a control in which small samples of A were added to a reaction containing B at *p*H 6.6 instead of at *p*H 8, the addition of A augmented the activity of B.

These observations explain the apparent lack of activity at *p*H 8 in crude extracts even though B may be present. In addition, the resolution of two separate luciferase components within the soluble system, each with its own characteristic *p*H profile, clarifies the previous ambiguity in the literature (*5*) regarding the optimum *p*H for the assay of luciferase. Crude extracts of A must be assayed at about *p*H 6.6, whereas purified B exhibits activity over a much broader *p*H range.

One may speculate that the distinctive *p*H activity profiles of these luciferase fractions relate to the mechanism of scintillon control, since emission of light by the scintillon is specifically evoked by a decrease in the *p*H. The hypothetical inhibitor in the A fraction could be assigned the role of an on-off switch. It might, for example, bind luciferin above *p*H 7 and not at *p*H 6.6. This is consistent with the hypothesis (*1*) that luciferase and luciferin form a part of the scintillon. The composition of A is complex, since it can bind luciferin, produce bioluminescence at *p*H 6.6, and inhibit bioluminescence at *p*H 8.0. These functions could conceivably be accommodated within a multimer-monomer protein model for a 7*S*

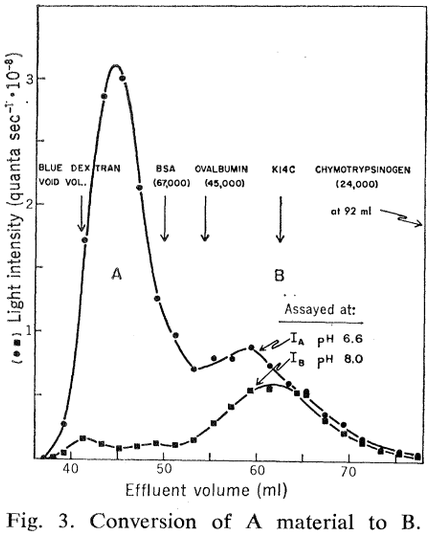

Fig. 3. Conversion of A material to B. A sample of the A fraction (Fig. 1, 267 to 330 ml) was adjusted to *p*H 6.0 and left for 9 hours upon rechromatography. As shown here, activity at *p*H 8 was found in the B region. A duplicate sample on Sephadex G-75, kept at *p*H 8.0 for 10 hours before being rechromatographed, showed no *p*H 8 activity in the B region. The eluting buffer was $5 \times 10^{-3}M$ tris and $5 \times 10^{-3}M$ DTT at *p*H 8.0 Luciferin was added in all assays. BSA, bovine serum albumin; I_A and I_B, intensities of fractions A and B.

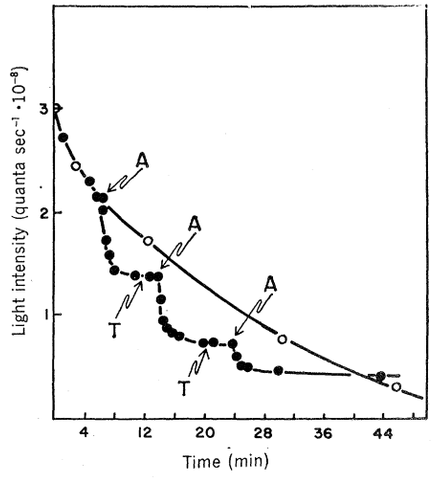

Fig. 4. Inhibition of B fraction by A fraction (both A and B are taken from Fig. 1). Two similar reactions were initiated with B and luciferin at *p*H 8. One was followed with no additions (○). To the second sample (●) 0.05-ml samples of A in $5 \times 10^{-3}M$ tris and $5 \times 10^{-3}M$ DTT at *p*H 8.0 were added at the times indicated by the arrows (A). Buffer alone, added at times indicated by the arrows (T), produced no effect. Larger samples of A (not shown) produced greater inhibition.

luciferase without invoking additional entities, but more experimental data is required to elaborate further on this model.

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Beta Thalassemia Trait: Detection at Birth

Abstract. *The synthesis of alpha, beta, and gamma chains in samples of cord blood was measured by the incorporation of leucine labeled with carbon-14 into these chains. In a newborn affected with beta thalassemia trait, the presence of one beta thalassemia gene was revealed on the 1st day of life by the lower specific radioactivity of the beta chain.*

In beta thalassemia there is a hereditary defect in the synthesis of beta chains (*1*). The disease becomes evident only after the age of 2 to 3 months, when production of gamma chains decreases and total circulating hemoglobin becomes dependent upon adequate production of beta chains (*2*). This report describes a study of hemoglobin synthesis, in a patient with beta thalassemia trait, by means of a method sufficiently sensitive to detect the disorder at birth.

The patient was the third child of parents with beta thalassemia trait. The mother had previously given birth to two children, both of whom were affected with homozygous beta thalassemia (Cooley's anemia). The baby was delivered at the end of the 37th week of pregnancy and weighed 5½ lb (2.5 kg). Routine studies of his blood at birth and thereafter at monthly intervals revealed that the initial hemoglobin concentration was at the lower limit of normal; morphology of the red cells was normal. The percentage of hemoglobin A_2 was less than 1 percent at birth and rose to 2.8 percent at 5 months of age. The clinical diagnosis of beta thalassemia trait could not be established until the child was 4 months old, when hypochromia, microcytosis, and target appearance of the red blood cells became sufficiently pronounced.

The infant's cord blood was incubated with L-leucine uniformly labeled with ^{14}C (specific activity, 200 mc/mmole) for 2 hours. Red blood cells were washed three times with isotonic saline and lysed according to the method of Lingrel and Borsook (*3*). The cord blood of four normal newborns was similarly studied. Globin was prepared from the whole red cell lysate by acid-acetone precipitation. The alpha, beta, and gamma chains were separated on carboxymethyl cellulose column (1 by 20 cm) in the presence of 8*M* urea by the method of Clegg, Naughton, and Weatherall (*4*). The absorbance (expressed as optical density) of each fraction was determined at 280 nm. Samples (1 ml) were counted in a liquid scintillation spectrometer. Figure 1 shows the separation and radioactivity of the alpha, beta, and gamma chains of cord bloods of the affected newborn and of one of four normal newborns. The identity of these chains has been established by Clegg and his co-workers and by Bank and Marks (*2*, *4*). The radioactivity under the peak of the beta chain in the affected newborn is diminished compared with the representative normal newborn, the latter study being similar in all respects to the results obtained in the three other normal newborns.

The specific activity of each chain was expressed as counts per minute divided by optical density, the fractions at and immediately around the peak for

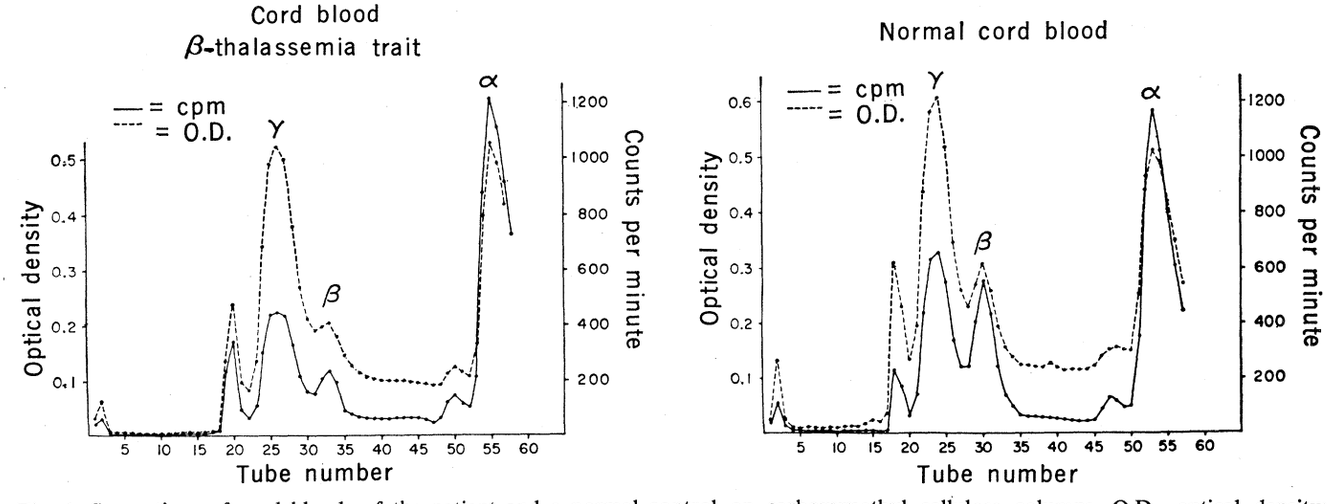

Fig. 1. Separations of cord bloods of the patient and a normal control on carboxymethyl cellulose columns. O.D., optical density; cpm, counts per minute.