THE INTERACTION OF LUCIFERASE, FLAVIN MONO-NUCLEOTIDE AND LONG-CHAIN ALDEHYDES IN THE LIGHT REACTION CATALYZED BY PREPARATIONS OF LUMINOUS BACTERIA

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INTRODUCTION

In 1953 STREHLER[1] succeeded in producing a light reaction *in vitro*, using acetonized powders of luminous bacteria. Soon after, rapid progress was made leading to the identification of a number of substances that play a role in the light reaction[2,3,4]. As a result of these investigations it is now generally accepted that light is produced in an oxidation reaction of FMNH*. This reaction is catalyzed by the enzyme luciferase and requires the presence of a long-chain aldehyde. Although some tentative reaction schemes have been suggested by STREHLER *et al.*[5,6], the mechanism of the oxidation reaction of FMNH and the role of the aldehyde in this reaction have not yet been elucidated.

For our investigations in this field, we had at our disposal a dark strain of *Photobacterium splendidum*. Evidence was obtained that in this strain some alteration in the luciferase molecule had taken place and thus this organism might be a useful tool in investigating the light reaction. For comparison a brightly luminescent strain of *Photobacterium phosphoreum* was used. The present study deals with some experiments with these two strains, which led to a hypothesis concerning the action of luciferase in the light reaction.

MATERIAL AND METHODS

The bacteria were cultivated in a liquid medium, containing 1% peptone ("Uclaf" for *Ph. splendidum*, "Uclaf" or "Difco Bacto" for *Ph. phosphoreum*, the "Difco" giving better growth), 3% NaCl and 0.3% glycerol. The pH was adjusted to 7.3–7.5. The cultures were aerated under aseptic conditions. After about 44 hours the bacteria were harvested, centrifuged down, washed with 3% NaCl, and then lysed with glass-distilled water (about 20 ml/g wet weight) in the refrigerator[3]. Following the method of MCELROY *et al.*[3] the cell debris were removed by centrifugation after 1–2 hours the proteins were precipitated with HCl, final pH 4.5, redissolved in 0.1 *M* phosphate buffer, pH 7.0, and fractionated with $(NH_4)_2SO_4$. The precipitates were dissolved in 0.1 *M* phosphate buffer, pH 7.0. The highest luciferase activity of both strains was obtained in the fractions containing about 3–4 g and 4–5 g $(NH_4)_2SO_4$/10 ml. In the preparations of *Ph. phosphoreum* these fractions often contained a low DPNH-oxidase activity or none (*cf.* ref.[7]). Thus a DPNH-oxidase preparation had to be added in order to catalyze the formation of FMNH from DPNH and FMN.

* Abbreviations: FMN: flavin mono-nucleotide; FMNH: reduced flavin mono-nucleotide; DPN: diphosphopyridine nucleotide; DPNH: reduced diphosphopyridine nucleotide.

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A preparation of *Escherichia coli*, namely the fraction C of BILLEN AND VOLKIN[8], was used as a source of DPNH oxidase. The DPNH-oxidase activity of the preparations was measured by the determination of the decrease of absorption of DPNH at 340 mμ in a spectrophotometer, using the same reaction mixture as in the light reaction.

The light reaction was produced by adding 0.5 mg DPNH, dissolved in 0.5–1.0 ml 0.1 *M* phosphate buffer, pH 7.0, 0.2 ml FMN solution (0.001 %), and 1 ml saturated decanal ($C_9H_{19}CHO$ solution in distilled water to a small amount (0.02–0.1 ml) of the luciferase preparation. In some experiments the decanal was dissolved in 0.1 *M* phosphate buffer, in order to maintain the desired pH. In qualitative experiments the light reaction was observed with the dark-adapted eye; in quantitative experiments the light was measured by means of a quantum counter. The quantum counter, which operated with a liquid–air-cooled photomultiplier, was designed to measure simultaneously light and dark current. For a description of the apparatus *cf.* SMIT *et al*[9].

1 % Butanal and saturated cinnamic aldehyde solutions were prepared in 0.1 *M* phosphate buffer, pH 7.0.

Palmitate solutions were prepared by adding 0.01 ml palmitic acid chloride to 10 ml 0.1 *M* phosphate buffer of pH 7.0, shaking the mixture, and removing the precipitated fatty acid by filtration.

In the experiments, 1 ml of the aldehyde or palmitate solution was present in 2.5–3.0 ml reaction mixture.

Electrophoresis experiments were made at about 5° on 4 cm strips of Whatman No. 4 filter paper in 0.05 *M* phosphate buffer, pH 7.0 or 8.0, duration 18 hours, strength of current 2–3 mA, voltage 240 V.

RESULTS AND CONCLUSIONS

Addition of DPNH, FMN, and decanal, to an enzyme preparation of *Ph. phosphoreum* resulted in a clear light reaction (Fig. 1). First it was ascertained that the dark strain of *Ph. splendidum* was not an aldehyde-free mutant of the normal strain as described by ROGERS AND MCELROY[10]. This was proved by experiments showing that after addition of decanal to a suspension of the bacteria in 3% NaCl no light reaction resulted. Moreover, growing the bacteria in a medium containing decanal did not produce a luminescent culture. Next, an attempt was made to obtain a light reaction with a "luciferase" preparation from this bacterium. It appeared that the enzyme preparation was capable of light emission upon addition of DPNH, FMN, and decanal, but both intensity and time course of the reaction were dependent on the sequence

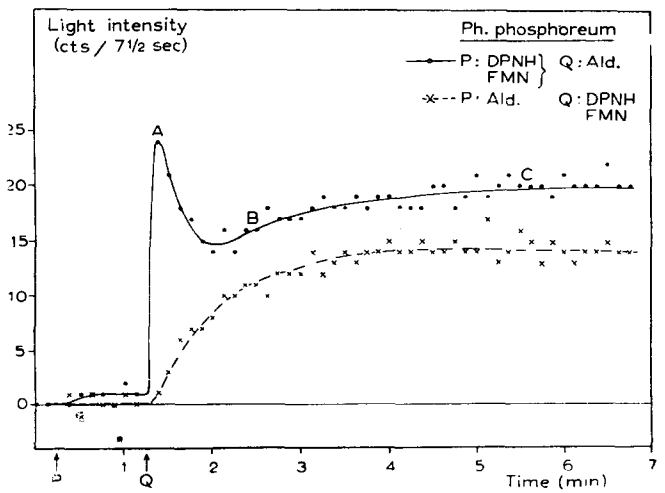

Fig. 1. Light reaction in a *Ph. phosphoreum* luciferase preparation and the influence of the sequence in which the reagents are added.

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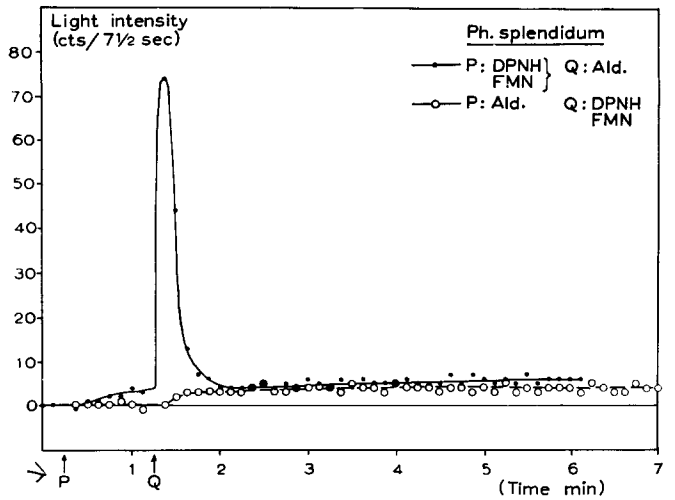

Fig. 2. Light reaction in a *Ph. splendidum* luciferase preparation and the influence of the sequence in which the reagents are added.

in which the various components were added. Addition of the aldehyde one minute after adding the DPNH and FMN yielded a bright flash of light, followed by a constant light emission at a very low level (Fig. 2). If the aldehyde was added first and the DPNH and FMN one minute afterwards, the flash was absent; only a very low prolonged light emission occurred. In *Ph. phosphoreum* preparations there was also an indication of the presence of a light flash when the aldehyde was added last, but here the constant light emission was about as high as the first "flash" (Figs. 1, 3 and 5). The constant light emission at this relatively high level could also be obtained if the aldehyde was added first. However, in part of the experiments the level was somewhat lower (Fig. 1).

From the light reaction with *Ph. splendidum* preparations it was concluded that the enzyme of this bacterium is capable of catalyzing the light reaction. For some reason the light reaction cannot be maintained at a high level after the addition of aldehyde. Addition of one of the components (DPNH, FMN, decanal) to the reaction mixture did not increase the light emission; this indicates that the low light emission is not due to a scarcity of these components.

A number of possibilities was investigated that might explain the light reaction in *Ph. splendidum* preparations.

(a) In the dark strain of *Ph. splendidum* a substance that is necessary for maintaining the light reaction at a high level may be absent. In that case the missing substance should be present in *Ph. phosphoreum* preparations. We tried to separate such a substance from the luciferase of *Ph. phosphoreum* in two ways:

1. By heating the luciferase preparations at 60° and 80° for 2.5–10 min. On the assumption that the unknown substance is thermostable, this was done in order to destroy the enzyme selectively. However, addition of such heated preparations, not capable of catalyzing the light reaction themselves, to preparations of *Ph. splendidum* did not result in an increase of the constant light emission of the latter.

2. By paper electrophoresis of *Ph. phosphoreum* preparations. We did not succeed

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in finding a zone on the paper strips, that was incapable of catalyzing the light reaction on its own, but capable of increasing the low light emission in *Ph. splendidum* preparations.

(b) The flash of light in *Ph. splendidum* preparations, observed when the aldehyde is added last, might be explained by assuming that in some way or another the aldehyde inhibits the transfer of H from DPNH to FMN, thus limiting the amount of available FMNH. However, the oxidation of DPNH with FMN as a H-acceptor and a *Ph. splendidum* preparation as a catalyst, measured by observing the decrease of the absorption at 340 mμ, is not influenced by addition of decanal.

(c) As it was observed that the DPNH-oxidase activity was very high, compared with that in *Ph. phosphoreum* preparations, the flash reaction, produced in *Ph. splendidum* preparations if aldehyde is added finally, might be due to a low value of the quotient: $\frac{\text{quantity of luciferase}}{\text{quantity of DPNH-oxidase}}$ in these preparations. In order to check this possibility, we made dilutions of a *Ph. phosphoreum* preparation and added such amounts of a DPNH-oxidase preparation from *E. coli* that the resulting activity was about the same as that from *Ph. splendidum* preparations. No flash reaction could be obtained after addition of DPNH, FMN and, after one minute, decanal.

(d) The strong influence of the sequence in which the components are added to *Ph. splendidum* preparations might be explained by assuming that competition occurs between FMNH and the aldehyde with regard to a site on the enzyme molecule, the aldehyde driving the FMNH from its place. It was expected that, if this were the case, a lowering of the concentration of the aldehyde might give the FMNH a better "chance" in the competition, resulting in a higher constant light emission. This indeed proved to be the case (Fig. 3). Furthermore, if the aldehyde concentration was lowered a hundred times, the flash of light was totally or nearly absent.

In *Ph. phosphoreum* preparations lowering the aldehyde concentration only resulted in a diminished light emission (Fig. 4). This indicates that the aldehyde is becoming a limiting factor in this case, *cf.* ref.[11,12].

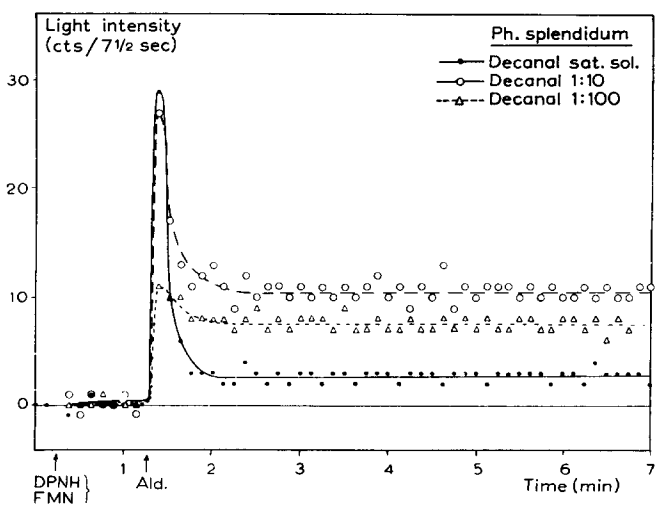

Fig. 3. Influence of the decanal concentration on the light reaction catalyzed by a *Ph. splendidum* luciferase preparation.

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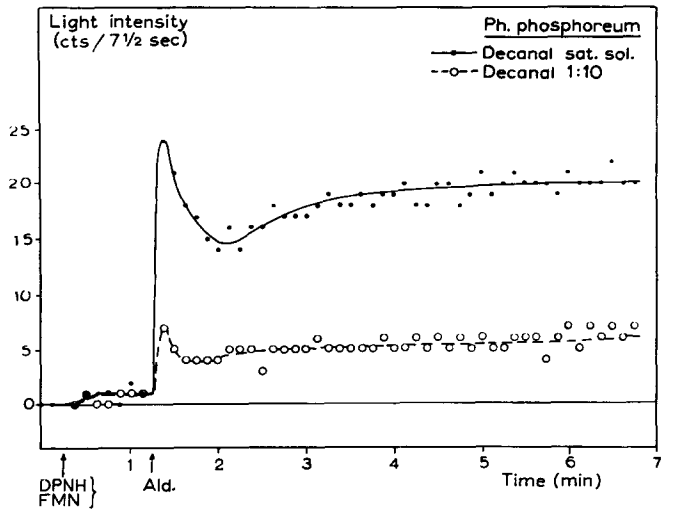

Fig. 4. Influence of the decanal concentration on the light reaction catalyzed by a *Ph. phosphoreum* luciferase preparation.

It may be remarked here that the light emission obtainable with *Ph. phosphoreum* enzyme and high aldehyde concentration is much higher than that obtainable in comparable amounts (*i.e.* prepared from comparable amounts of bacteria) of *Ph. splendidum* enzyme at low aldehyde concentrations.

Having thus obtained a support for the competition hypothesis (d), we tried to find out how the aldehyde could occupy the site (called *X*) on the enzyme from *Ph. splendidum* that is necessary for binding the FMNH. It seemed probable that this took place via the active group of the aldehyde, the carbonyl group. If this supposition were true, another aldehyde, not active in the light reaction proper, ought to be an inhibitor to this light reaction by occupying the sites that would otherwise be occupied by FMNH. We checked the influence of butanal and cinnamic aldehyde. In *Ph. splendidum* preparations as well as in *Ph. phosphoreum* preparations these substances inhibited the light reaction (Fig. 5, 6).

The question now arises why the competition between FMNH and the long-chain aldehyde is much clearer in *Ph. splendidum* extracts than in *Ph. phosphoreum* extracts. We supposed that in the latter the reaction of the aldehyde with the *X*-sites on the enzyme is inhibited in some way or another, *e.g.* by the aldehyde being linked to other sites on the enzyme. This probably would take place via the long hydrocarbon chain, since the aldehyde group is supposed to be used in the light reaction[5]. We might represent this as illustrated in Fig. 7. The supposition could be checked by studying the influence of another compound with a long hydrocarbon chain but without an aldehyde group. This compound should take the place of the aldehyde on the enzyme but not its function. We checked the influence of palmitate. This substance indeed proved to inhibit the light reaction, in *Ph. phosphoreum* extracts as well as in *Ph. splendidum* extracts (Figs. 5, 6). It should be noted that the influence of the palmitate on the intensity of the flash of light in *Ph. splendidum* preparations is not as pronounced as it is upon light emission in *Ph. phosphoreum* preparations. This will be explained in the discussion.

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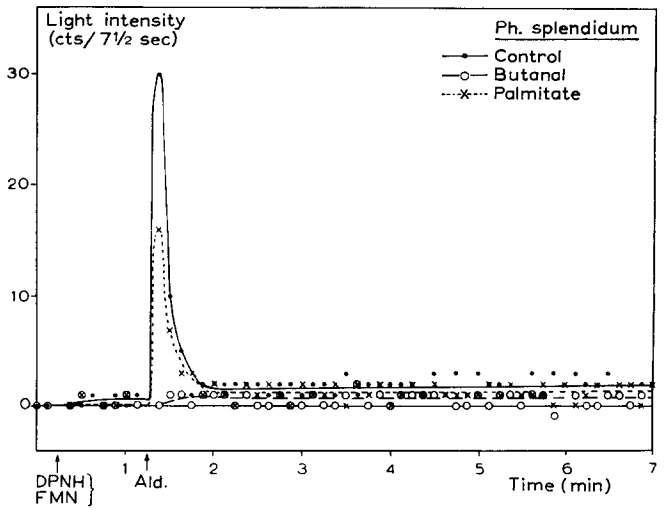

Fig. 5. Influence of butanal (about 1 %) and palmitate (satd. soln. in 0.1 *M* phosphate buffer pH 7.0) on the light reaction catalyzed by *Ph. phosphoreum* luciferase preparations.

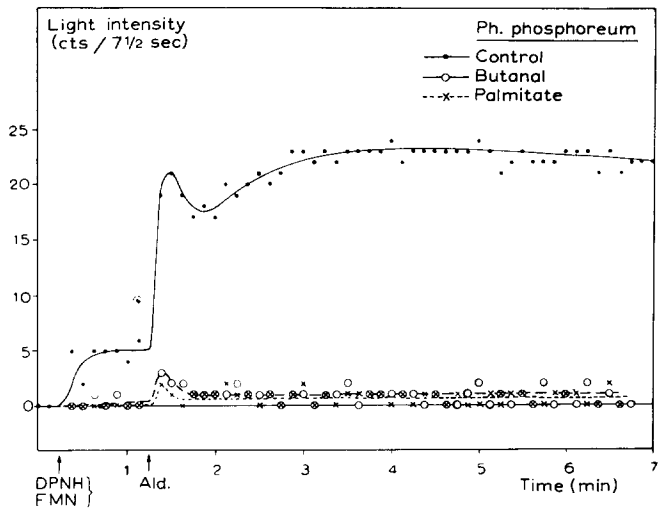

Fig. 6. Influence of butanal (about 1 %) and palmitate (satd. soln. in 0.1 *M* phosphate buffer, pH 7.0) on the light reaction catalyzed by *Ph. splendidum* luciferase preparations.

Obviously the competition between FMNH and the long-chain aldehyde is prevented to a large extent in *Ph. phosphoreum* preparations. We have to assume, therefore, the presence of a large number of aldehyde-binding groups on the *Ph. phosphoreum* enzyme, while the number of these groups on the *Ph. splendidum* enzyme should be much less. In Fig. 7 a tentative scheme of the luciferase enzyme in both kinds of bacteria is presented.

An essential point in this hypothesis is that the aldehyde gets bound to the luciferase. In order to test this, a mixture of 0.1 ml *Ph. phosphoreum* enzyme preparation together with 2 ml decanal solution was dialyzed and the concentration of the aldehyde solution diffused through the membrane (A) was compared with that from

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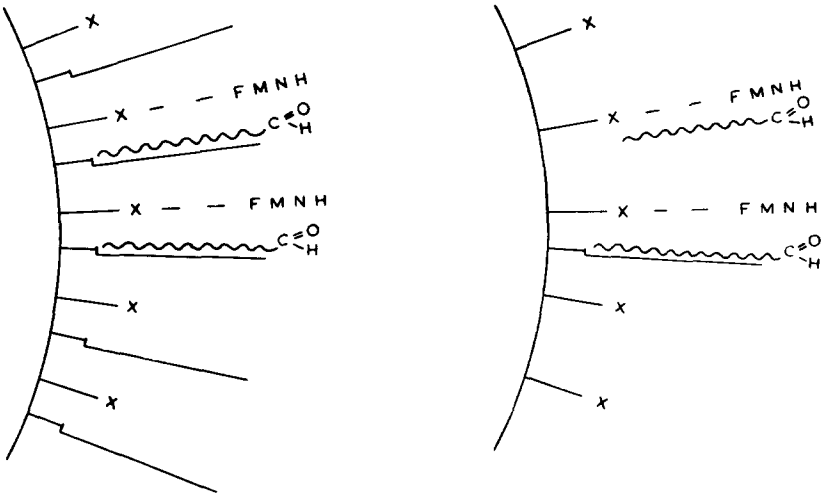

Fig. 7. Schematic diagram of the luciferase enzymes in *Ph. phosphoreum* and a dark strain of *Ph. splendidum*. ——X: Sites to be occupied by FMNH. ——⌊——: Specific aldehyde-attracting groups. ~~~~C(=O)H: A long-chain aldehyde.

a control, dialyzed without the enzyme (B). Dialysis was carried out in a refrigerator. It was expected that the concentration of the dialyzed aldehyde solution A would be lower than that of solution B. We checked this by estimating the relative aldehyde concentration of these solutions from their influence on light emission in our preparations. It may be recalled that lowering the concentration of the aldehyde reduces the steady-state light emission in *Ph. phosphoreum* preparations and increases that in *Ph. splendidum* preparations. The results of the experiments, presented in Table I, fit in very well with our supposition.

TABLE I

EFFECT OF *Photobacterium phosphoreum* LUCIFERASE PREPARATIONS ON THE CONCENTRATION OF FREE ALDEHYDE IN A DECANAL SOLUTION

Expt. No.	*Substances in dialysis membrane*	*Enzyme prep. used for testing dialysate*	*Steady state light emission counts/min*
1.	Decanal, satd. soln. (B)	*Ph. splendidum*	56
	Decanal, satd. soln. *Ph. phosph.* enzyme preparation (A)	*Ph. splendidum*	255
2.	Decanal, satd. soln. (B)	*Ph. phosphoreum*	132
	Decanal, satd. soln. *Ph. phosph.* enzyme preparation (A)	*Ph. phosphoreum*	56

Decanal was dissolved in 0.1 *M* phosphate buffer, pH 7.0. Dialysis of 2 ml decanal soln. with (A) or without (B) 0.1 ml *Ph. phosphoreum* luciferase preparation during about 4 hours. Temp. about 5°. In the test 0.7–0.9 ml dialysate was added to 1.3–1.5 ml reaction mixture, containing the enzyme preparation, DPNH, and FMN.

DISCUSSION

An attempt will be made to explain the shapes of the light curves from Figs. 1–4 in terms of the enzyme schemes of Fig. 7.

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1. *Ph. phosphoreum*

Addition of DPNH and FMN to the enzyme preparations containing DPNH-oxidase will result in the formation of FMNH, which is assumed to occupy the *X*-sites on the enzyme. On addition of aldehyde, which for a large part becomes attached to the specific aldehyde-binding groups, the FMNH groups can react immediately with the aldehyde, resulting in a flash of light (part A of the curve). Now, according to TOTTER AND CORMIER[15] there is a competition between FMN and FMNH for the sites on the enzyme, with a many fold lower dissociation constant for FMNH. Thus the oxidized FMN may diffuse from its places, which are reoccupied by FMNH as soon as a sufficient quantity of this substance has been formed under the influence of the DPNH oxidase to compete successfully with the remaining free aldehyde (part B). It may be recalled that our results led to the assumption that FMNH and aldehyde compete in occupying the *X*-sites. The bound FMNH reacts again with aldehyde and so on. The steady-state light reaction (part C) represents the equilibrium state. At a given concentration of the luciferase and a sufficient supply of DPNH and FMN, the intensity of the steady-state light emission will depend on the ratio $\frac{\text{FMNH concentration}}{\text{FMN concentration}}$ (the concentration of DPNH oxidase and the autoxidation rate of FMNH) and the aldehyde concentration. Lowering the aldehyde concentration will leave more aldehyde-binding groups unoccupied and thus result in a lower light emission.

If the aldehyde is added before the addition of DPNH and FMN, the first part of the curve (A), representing the light reaction of FMNH groups already attached to the enzyme, will be absent.

2. *Ph. splendidum*

Addition of DPNH and FMN to an enzyme preparation containing DPNH oxidase will result here also in the occupation of the *X*-sites by FMNH. On addition of the aldehyde this substance can be attached to aldehyde-catching groups only for a minor part, but it is supposed that the light reaction also occurs between bound FMNH and free aldehyde in the manner illustrated in Fig. 7. This supposition may explain why the palmitate is not very active in inhibiting the first flash of light (A). After the first light reaction (the flash reaction A in Figs. 2 and 3) the oxidized FMN diffuses from its place, and there is then competition between the newly-formed FMNH and the remaining aldehyde. Since only a small part of the aldehyde is "bound" to the enzyme, the concentration of the free aldehyde will be relatively high. This results in the occupation of a fairly large proportion of the *X*-sites by aldehyde. In this way it can be understood why the steady-state reaction (C) is at a low level. Lowering the concentration of the aldehyde diminishes the chance for an *X*-group adjacent to an aldehyde-binding group, to be occupied by the aldehyde, thus increasing the light emission. Lowering the concentration of the aldehyde still further will render the aldehyde a limiting factor (Fig. 3); then the situation resembles that with the *Ph. phosphoreum* enzyme.

Addition of the aldehyde to the enzyme preparation before DPNH and FMN yields about the same ultimate reaction, but, just as in *Ph. phosphoreum* preparations, the first flash of light cannot take place.

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It may be asked why a limited number of aldehyde-binding groups is still proposed in the hypothetical picture of the *Ph. splendidum* enzyme. This has been done because the steady-state light emission at low aldehyde concentrations is strongly inhibited by palmitate. We have to assume, therefore, that this steady-state reaction occurs in the normal way, just as in *Ph. phosphoreum* preparations.

The finding that palmitate inhibits the light reaction may be considered as an argument in favour of STREHLER'S view[5] that the aldehyde is not consumed in the light reaction. For if this were the case, we should expect a gradual decline in the light emission, owing to the formation of inhibiting long-chain compounds.

Another question that arises is why no flash reaction is obtained in *Ph. phosphoreum* preparations to which palmitate has been added, since this inhibited enzyme is comparable to the enzyme in *Ph. splendidum*. To explain this, we assume that the aldehyde has to take a special spatial position for producing the light reaction with FMNH (Fig. 7), this position not being possible if the aldehyde-attracting groups together with palmitate are present. We might tentatively suggest that one function of the aldehyde-binding groups is the arranging of the aldehyde in the right spatial position. The nature of these groups is fully unknown; they might be lipoic groups. It might be worth while to investigate whether luciferase is a lipoprotein, *cf.* ref.[13,14].

What are the groups *X* on the enzyme that are occupied by the FMNH and the aldehyde? It has been found[3,15], that the light reaction in *Achromobacter fischeri* preparations is strongly inhibited by *p*-chloromercuribenzoate, a sulfhydryl inhibitor. This was interpreted to mean that luciferase is a sulfhydryl enzyme. One might easily imagine that the aldehyde reacts with these sulfhydryl groups, forming a "mercaptal". In this case, we have to assume that the FMNH likewise becomes linked to a sulfhydryl group in the enzyme. However, THEORELL AND NYGAARD[16] presented evidence that the FMNH in the "Old Yellow Enzyme" is attached to the protein by means of a linkage between the phosphate of the FMNH and primary amino groups of the protein. If this also holds for the luciferase-FMNH compound we have to assume a reaction of the aldehyde with these NH_2 groups.

In conclusion, we may state that the proposed structures of the luciferase enzymes of *Ph. phosphoreum* and *Ph. splendidum*, as represented in Fig. 7, may tally with the experimental facts. This does not mean that the diagrams actually do represent a correct conception of the enzymes. Since no direct proof is available we must consider them, for the time being, as hypothetical. In any case, we are well aware of the fact that they do not give a real explanation of the light reaction; this explanation will depend on future research.

SUMMARY

The light reaction catalyzed by a luciferase preparation of a dark strain of *Photobacterium splendidum*, probably containing a slightly altered luciferase molecule, was compared with the light reaction catalyzed by a similar preparation of a brightly luminescent strain of *Photobacterium phosphoreum*. Experiments concerning the influence of the aldehyde concentration and the inhibition of the light reaction by short-chain aldehydes and palmitate indicated that the normal luciferase molecule contains certain groups, necessary for "binding" the long-chain aldehyde that is required in the light reaction. These groups are supposed to be partly missing in the enzyme of the dark strain of *Ph. splendidum*. A tentative scheme of the luciferase molecules in the dark and luminescent strains used is presented; the shape of the curves light intensity *versus* time is explained using these schemes.

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THE INFLUENCE OF ADRENALECTOMY AND OF CORTISONE TREATMENT ON ARGINASE AND ESTERASE ACTIVITIES IN LIVER TISSUE

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INTRODUCTION

The effect of hormones on enzymic reactions within the cell represents one of the burning problems of modern biochemistry. While hitherto it was possible in only a few cases to establish an effect of a hormone, added *in vitro* to enzyme preparations, an increasing number of cases has been reported in recent years in which changes in the activity of extracted enzyme systems have been observed after the animals had received hormone treatment; in particular, treatment with adrenal corticoids has been found to be effective in this respect. In slice experiments, the glucose-6-phosphatase level in the liver of animals treated with 17-hydroxycorticosterone was seen to be increased (ASHMORE, HASTINGS, NESBETT AND RENOLD[1]). In other experiments the treatment of rats with cortisone had a considerable effect on the enzymes concerned with the metabolism of tryptophan (BROWN AND BERG[2], KNOX AND AUERBACH[3]). Conversely, the effect of an enzyme (phosphorylase) on the synthesis and the release of corticosteroids has also been claimed (HAYNES AND BERTHET[4]). Adrenalectomy decreases the activities of many, but not of all, enzymes; the levels of succinic dehydrogenase and of various nucleases, for instance, tend to rise after adrenalectomy (STEVENS AND REID[5]), while that of catalase is unaffected (TROOP AND STANLEY[6]).

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