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INVESTIGATIONS ON THE IDENTITY OF THE LIGHT-EMITTING MOLECULE IN *PHOTOBACTERIUM PHOSPHOREUM*

WILLEMKE TERPSTRA

Biophysical Research Group, Physical Institute, State University, Utrecht (The Netherlands)

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SUMMARY

1. Filtration of a bacterial lysate over Sephadex G-25 results in either a total or partial inactivation of the luciferase.

2. The filtrate contains a non-protein fraction that stimulates the light reaction *in vitro*, evoked by addition of $FMNH_2$ to a diluted, palmital-containing, luciferase preparation. Besides, a fraction inhibiting the light reaction is found.

3. None of the fractions of the Sephadex filtrate shows a clear fluorescence peak at the wavelength of maximum luminescence (about 470 mμ).

4. Addition of $FMNH_2$ to a bacterial enzyme preparation results in the formation of some compound which is transformed by irradiation with ultraviolet light (366 mμ) into a fluorescent substance with a fluorescence maximum around 470 mμ. The time during which the first compound is formed is of the same order of magnitude as the time of duration of the light reaction in the complete reaction mixture. Its formation is not enzymic.

5. The compound is suggested to be a precursor of the light-emitting molecule in bacterial luminescence.

INTRODUCTION

Bacterial light emission has a maximum at about 470 mμ, while the fluorescence maximum of FMN occurs at 530 mμ[1,2]. Therefore, FMN is very unlikely to be the light-emitting molecule in bacterial luminescence. In a previous article[2] we reported that a factor, activating luminescence *in vitro*, is present in bacterial extracts. These extracts showed fluorescence in the 470-mμ region. The aim of the present investigation was to find out whether (a) the luminescence-activating factor shows a fluorescence maximum at 470 mμ and (b) the light-emitting molecule is identical with the luminescence-activating factor.

METHODS

Photobacterium phosphoreum was cultivated as described in a previous article[2]. The enzyme preparations were made following the method of McELROY *et al.*[3]. The preparations are indicated as follows: "Lysate": the supernatant of bacterial lysate

centrifuged for 40 min at 78 000 × *g*; "HCl-precipitate": the lysate proteins precipitated by acidifying with $5 \cdot 10^{-3}$ N HCl down to pH about 4.3 and redissolved in 0.1 M phosphate buffer (pH 7.0) (4 ml per 25 ml lysate); "solution U": the supernatant after precipitation of proteins with 0.1 M HCl (final pH about 4.3), neutralized with 0.1 N NaOH and buffered with one tenth of its volume 1 M phosphate buffer (pH 7.0).

The light reaction *in vitro* was initiated by injecting $FMNH_2$ into an enzyme solution in 0.1 M phosphate buffer, containing palmital. Exact quantities of the components are given with the individual experiments. The reaction proceeded in a cooled cuvette at about 17°. The emitted light was measured in a quantum-counting apparatus using a RCA 931-A photomultiplier.

$FMNH_2$ solutions were prepared by reducing an aqueous solution of FMN with H_2, using palladium black as a catalyst. The solution could be stored in the dark for at least some hours under liquid paraffin.

Paper chromatography was performed with Whatman No. 4 filterpaper, using the ascending as well as the descending method. The chromatograms were run with *n*-butanol–ethanol–water (30:10:30, v/v) as a solvent. Thin-layer chromatography[4] was performed on glass plates of 5 × 20 cm, covered with a 1-mm layer of silicagel, and using the same solvent as in the paper chromatography experiments. In both cases the solution to be chromatographed was concentrated by means of freeze-drying.

Gel filtration[5] was done on Sephadex G-25 from Pharmacia, Uppsala, in 0.1 M phosphate buffer (pH 7.0). The length of the column was about 40 cm, the diameter 5 cm. The preparation to be filtered was buffered with 1 M phosphate buffer up to a final buffer concentration of 0.1 M. Filtration was carried out at room temperature. The filtrate was collected in 50-ml fractions; collection of the fractions was started as soon as the sample was brought upon the Sephadex. The filtration rate was about 3.5 ml/min.

Fluorescence was excited by irradiating the sample with a blue Philips Philora lamp (mainly 366 mμ) placed behind a 1-cm 12% $CuSO_4$ filter. Fluorescence was analyzed by a Bausch and Lomb grating monochromator and measured with a RCA IP-21 photomultiplier, connected, via an amplifier, to a recorder.

RESULTS AND CONCLUSIONS

Paper chromatography and thin-layer chromatography on silicagel of solution U preparations, concentrated by means of freeze-drying, yielded about the same results upon irradiation with an ultraviolet lamp (366 mμ): a blue fluorescing zone at, or just below, the starting place, next a non-fluorescing zone and, finally, a yellow-green fluorescing zone, presumably due to flavins. The separation on silicagel was better than on paper. After drying in a N_2 current, the different zones of paper and silicagel, respectively, were extracted with 0.1 M phosphate buffer for about 30 min in the refrigerator. The resulting extracts were tested for their activating action on the light emission of a diluted enzyme preparation, containing palmital, upon addition of $FMNH_2$ (*cf.* ref. 2). Although in a number of experiments we got an indication that two activating substances were present, namely one of them in the blue fluorescing zone and another one in a zone in the neighbourhood of the yellow-green fluorescing part, no definite conclusions could be drawn. This was due to the fact that, in both cases, a considerable inhibition of the light reaction was measured in control experi-

ments in which runs were made with solvents only. In this way, very variable results were obtained. Washing of the paper or silicagel did not yield any improvement.

Gel filtration on Sephadex offered a more promising possibility in separating activating substances from the bacterial preparations. An obvious advantage is the omission of organic solvents that might cause inhibition of the light reaction even in the low quantities possibly left after evaporation.

It appeared that filtrates of buffer over Sephadex G-25, even after thorough washing of the Sephadex, inhibit the light reaction to some degree, as compared to the reaction in the original buffer solution. However, the effects on the light reaction of a number of fractions obtained after filtration of a bacterial lysate was considerably higher than those of the fractions obtained from filtering a buffer solution.

It may be mentioned here, that the measurement of the activating action upon the light reaction by solution U, as described in ref. 2, gradually became more difficult. This is probably due to an excess of activating factor U present in the diluted lysate, which is used for the test. As an enzyme source for the light reaction we therefore used a HCl-precipitate from a 1:1 diluted bacterial lysate, instead of the lysate itself.

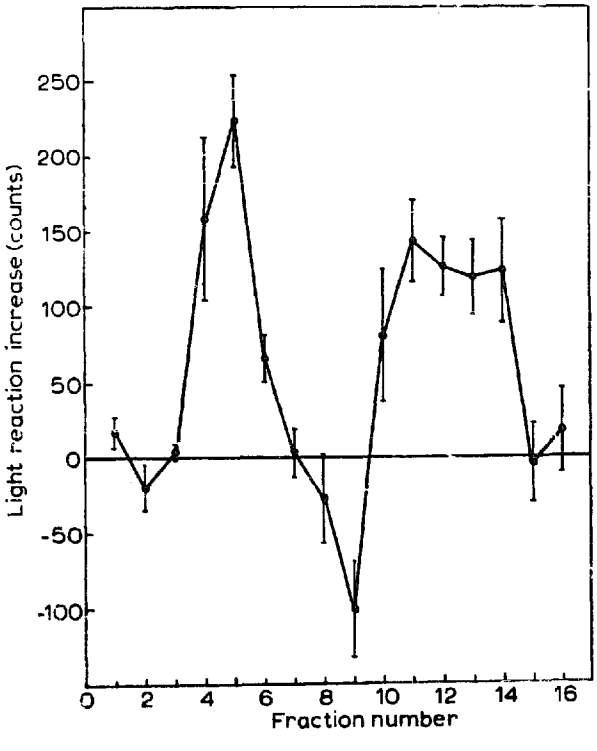

Fig. 1. The effect of Sephadex filtrate fractions from bacterial lysate on the light reaction. Mean of seven series of experiments. Vertical lines denote standard errors of the mean. Bacterial lysate filtrated: 25 ml. Fraction volume: 50 ml.

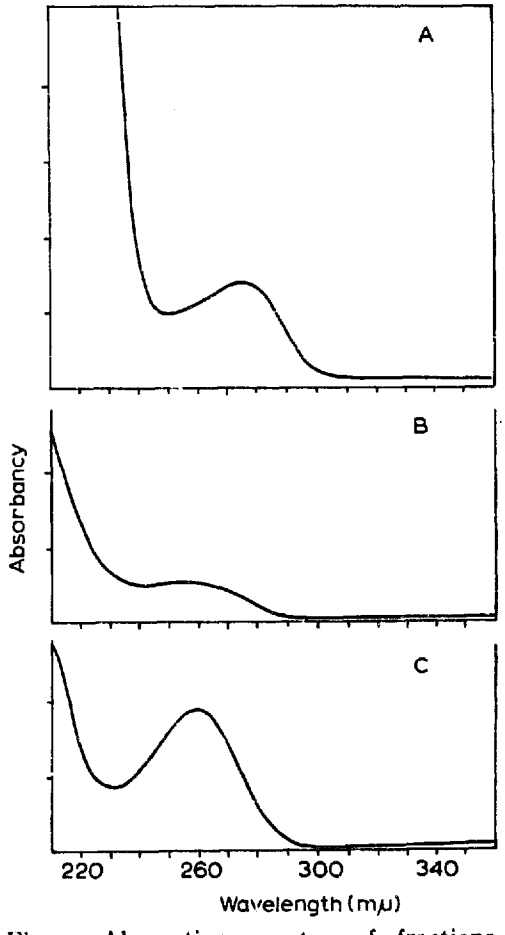

Fig. 2. Absorption spectra of fractions influencing the light reaction. A, activating protein. B, inhibiting fraction. C, activating non-protein fraction.

Washing liquid: 0.1 M phosphate buffer (pH 7.0). Reaction mixture of the test reaction: Filtrate fractions, 2.25 ml; HCl-precipitate, 0.005–0.02 ml; palmital, saturated solution in methanol, 0.02 ml; $FMNH_2$ (from $1.1 \cdot 10^{-4}$ M FMN), 0.25 ml

In order to obtain the possibly highest concentration of factor U, bacterial lysate, instead of solution U, was filtered through Sephadex. Contrary to what happens in the chromatography experiments the protein can be expected not to interfere with the separation procedure, as it will be filtered out in the first fractions. Besides, it is possible to check the activity of the luciferase, purified in this way.

Although there were some differences in the individual experiments, the graph of activity *versus* fraction number of the first 16 fractions obtained from Sephadex filtration all showed the following features (Fig. 1): (a) A peak in the activating action upon the light reaction (Fractions 4 and 5). The absorption spectra of these fractions show a protein maximum at about 275 mμ (Fig. 2A). (b) A minimum, representing an inhibiting effect of one or two fractions upon the light reaction (Fraction 9 in Fig. 1). This fraction generally shows the absorption spectrum represented in Fig. 2B. (c) A second peak in the activating action upon the light reaction, generally comprising a number of fractions. The absorption spectrum in this region is shown in Fig. 2C. It resembles the spectrum of nucleic acids. It should be emphasized that this absorption spectrum should not necessarily be that of the activating substance, it may be an accompanying substance of about the same molecular size. This possibility is indicated by the fact that no clear correlation was found between the height of the absorption peak and the activity in the light reaction.

In some experiments indications were found for another activating region in higher numbered fractions. These results, however, were not reproducible and, therefore, will be left out of consideration here.

The results of the light-reaction measurements show, for known (ref. 2) and unknown reasons, a considerable scattering. Therefore, all fractions were tested four times and the mean of these four measurements was taken. As a reference the mean effect of the first three fractions collected, not yet containing the first components of the lysate, was used. Only those effects that were observed in all series of experiments are considered here.

The activating and inhibiting action of the fractions has been given in absolute units (number of counts of the counting apparatus) and not as an increase or decrease in percents of the control light reaction. It appeared, namely, that the absolute increase, caused by the activating fraction, was relatively independent of the initial light reaction, whereas the increase, expressed in percents of the initial reaction, decreased as the height of the initial reaction increased.

The enzymic activity in the light reaction of the protein fractions (4 and 5 in Fig. 1), that is: the ability to evoke a light reaction upon addition of palmital and $FMNH_2$ but without added active enzyme, is nearly always zero. In other words, the luciferase activity of the protein fractions of the lysate is lost. This activity could not be restored by adding other fractions of the Sephadex filtrate. A bacterial lysate, stored at room temperature during the time needed for Sephadex filtration (about 5 h) remained active. Therefore, the inactivation is not due to raised temperature.

In order to check whether any of the lysate fractions separated by Sephadex filtration contained a substance with a fluorescence peak at 470 mμ, the wavelength of maximum light emission in luminescence, we studied the fluorescence spectra of the different fractions. Apart from a yellow-green fluorescence with a maximum at about 530 mμ, probably due to flavins, in the fractions around number 15 (Fig. 1), no significant fluorescence could be measured. In order to obtain more concentrated

solutions, three successive fractions (together 150 ml) were freeze-dried and the residue was dissolved in about 3 ml distilled water. Crystallizing buffer salts were discarded. The fluorescence spectrum of Fractions 4+5+6 (active protein), 7+8+9 (inhibiting substance), 11 + 12 + 13 (active non-protein) and 15 + 16 + 17 (no significant activity) were compared with that of Fractions 1 + 2 + 3 (control). All preparations showed more or less fluorescence around 470 mμ, but with none of them a clear peak was found. The fluorescence spectra of the combined fractions of such an experiment are given in Fig. 3. From these results we decided that no clear correlation exists between the activating action upon the light reaction and the fluorescence at about 470 mμ.

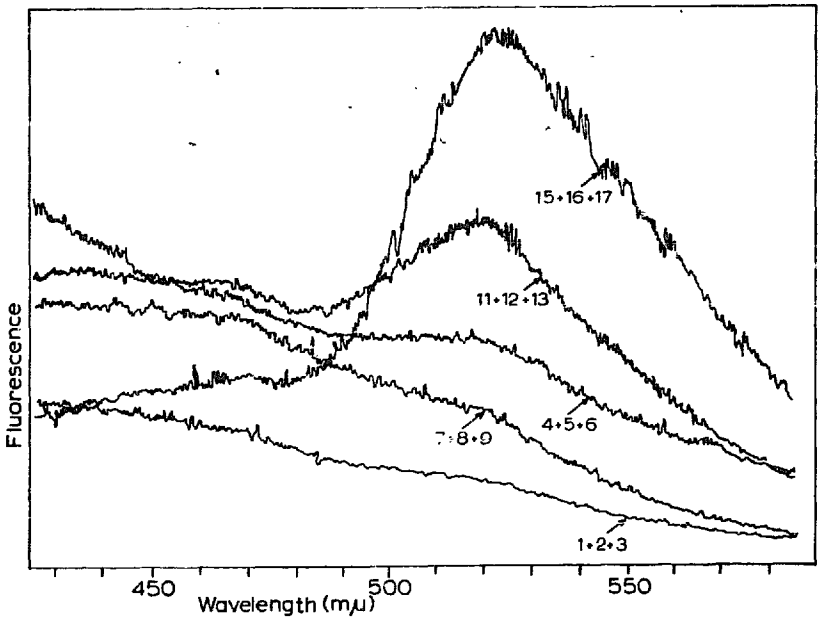

Fig. 3. Fluorescence spectra of combined fractions of a Sephadex filtrate from bacterial lysate Fractions 1 + 2 + 3, control. Fractions 4 + 5 + 6, activating protein fractions. Fractions 7 + 8 + 9, inhibiting fractions. Fractions 11 + 12 + 13, activating non-protein fractions. Fractions 15 + 16 + 17, fractions without significant activity.

In bacterial lysate no substance could be detected which shows a clear fluorescence maximum around 470 mμ and at the same time is active in the light reaction. However, it may be that the substance fluorescing at 470 mμ is only formed during the light reaction. In order to investigate this possibility the time course of fluorescence at 470 mμ during the light reaction *in vitro* was followed. If some fluorescent substance is formed, an increase in the fluorescence intensity might be expected. Indeed we found, in addition to an immediate increase, due to FMN formation, a gradual fluorescence increase during about 1 min upon addition of $FMNH_2$ to a solution of HCl-precipitate. The latter increase did not occur upon addition of FMN instead of $FMNH_2$. The effect proved to be independent of the presence of palmital in the reaction mixture. The time course of 470-mμ fluorescence of a HCl-precipitate upon addition of $FMNH_2$ is shown in Fig. 4.

The fluorescence increase at 470 mμ appeared to occur only upon irradiation with the ultraviolet light (mainly 366 mμ) used for exciting fluorescence. This was deduced from experiments in which $FMNH_2$ was added to the enzyme preparation

in the dark and fluorescence was measured after 0.5–4 min, *i.e.* when the light reaction would practically have ceased. After the dark period fluorescence has not reached the height as would be expected from the experiments represented in Fig. 4, but starts to increase as soon as the sample is irradiated.

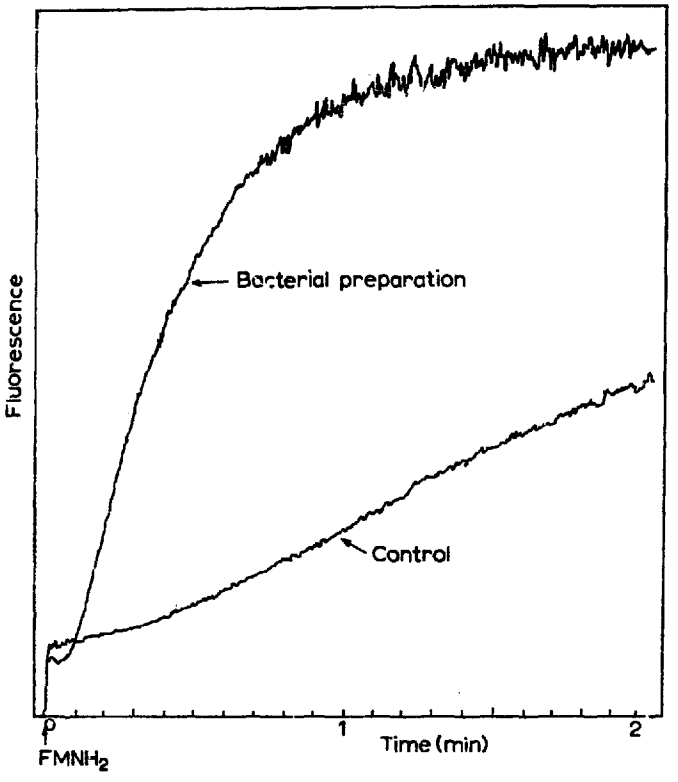

Fig. 4. The increase of fluorescence at 470 mμ upon addition of $FMNH_2$ to a bacterial enzyme preparation. Reaction mixtures: "Bacterial preparation": 3.0 ml 0.1 M phosphate buffer (pH 7.0) plus 0.1 ml HCl-precipitate. "Control": 3.1 ml phosphate buffer. At zero time 0.2 ml $FMNH_2$ (from $1.1 \cdot 10^{-4}$ M FMN) is added.

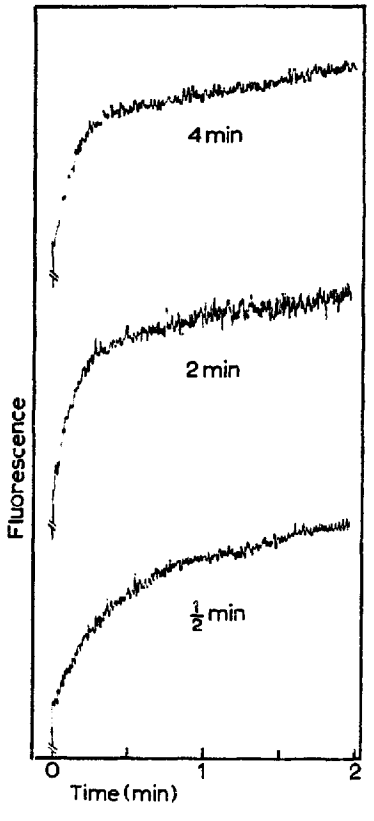

Fig. 5. The time course of fluorescence increase at 470 mμ after different dark periods. Reaction mixture: 3.0 ml 0.1 M phosphate buffer (pH 7.0) plus 0.1 ml HCl-precipitate. At zero time 0.2 ml $FMNH_2$ (from $1.1 \cdot 10^{-4}$ M FMN) is added. Measurement after dark periods of ½, 2 and 4 min.

A nearly horizontal fluorescence level is reached sooner after a preceding dark period than in the absence of such a period (Fig. 5). From this we conclude that at least two reactions are taking place, namely formation of a non-fluorescing intermediate in the dark and subsequent formation of a fluorescent substance upon irradiation with ultraviolet light. No significant decrease of the time needed for reaching the nearly horizontal fluorescence level was obtained upon extending the dark period above 1 or 2 min. Therefore, under the experimental conditions used, the dark reaction takes about this time. Under similar conditions the light reaction is measurable about the same time. The reaction upon ultraviolet irradiation, measured after a relatively long dark period, takes about 0.25 min (Fig. 5).

The formation of the fluorescent substance as well as the effect of preincubation in the dark also takes place upon addition of $FMNH_2$ to a heated (30 min at 100°) enzyme preparation. Therefore, these reactions are not enzymic.

The fluorescence of the control without enzyme preparation appears to increase

during the measuring time (Fig. 4). However, the increase is much slower than that with enzyme preparation and shows a different time course. It may be due to the formation of an FMN derivative, probably lumichrome (*cf.* ref. 7), caused by irradiation with the ultraviolet light used for exciting fluorescence, for after a dark period the fluorescence of the control has not increased.

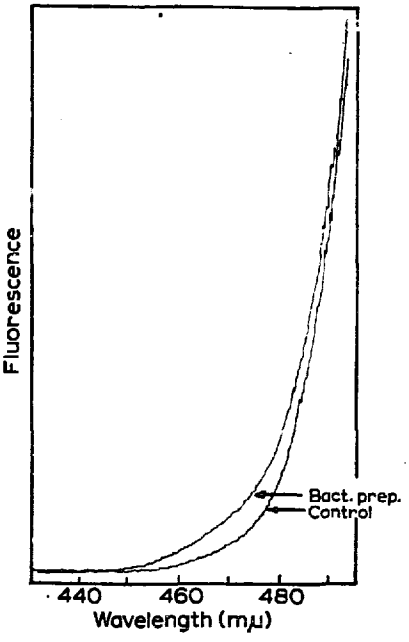

Fig. 6. Determination of the fluorescence maximum of the substance formed upon ultraviolet irradiation after addition of $FMNH_2$ to a bacterial enzyme preparation. Fluorescence spectra of 2.9 ml buffer (0.1 M phosphate, pH 7.0) plus 0.2 ml HCl-precipitate 1 min after addition of 0.2 ml $FMNH_2$ (from $1.1 \cdot 10^{-4}$ M FMN), and 3.1 ml buffer, 1 min after addition of 0.2 ml $FMNH_2$.

An increase in fluorescence upon ultraviolet irradiation during 1 min after adding $FMNH_2$ to a luciferase preparation was not perceptible at 525 mμ, near the fluorescence maximum of FMN. The effect at 470 mμ thus could not be ascribed to the formation of more FMN from $FMNH_2$ in the presence of enzyme preparation than in the control. We therefore attempted to establish a fluorescence spectrum of the substance formed. This was done by comparison of the fluorescence spectrum of enzyme preparation plus $FMNH_2$ with that of buffer plus $FMNH_2$, both after 1 min in the dark (Fig. 6). The difference between these two spectra may be expected to yield the fluorescence spectrum of the substance formed in addition to that of the enzyme preparation. The latter, however, is negligible under our measuring conditions. The dark incubation period was chosen to ensure the presence of the mentioned intermediate in sufficient amounts, and thus a rapid formation of the fluorescent compound upon illumination, while complications due to the formation of a fluorescing substance upon illumination of FMN formed from $FMNH_2$ were avoided as much as possible. Establishment of a spectrum took 1¼ min. The determination of the difference spectrum is difficult at wavelengths longer than about 480 mμ, as the FMN fluorescence spectrum is very steep in this region. Therefore, spectra were selected that show practically no difference at long wavelength (490–500 mμ). In this case a clear divergence shows up in the region between 460 and 480 mμ (Fig. 6). The resulting difference spectrum of a similar experiment is shown in Fig. 8.

In another series of experiments we determined, at a suitable voltage of the photomultiplier, the fluorescence increase in per cent of the initial value at a number of wavelengths between 440 and 500 mμ as a result of 2 min ultraviolet (366 mμ) illumination after $FMNH_2$ addition to HCl-precipitate. The values were corrected for the effect obtained with buffer instead of enzyme solution at the same voltage (*cf.* Fig. 4). The percentages thus found were then multiplied with the relative fluorescence intensities of buffer plus $FMNH_2$ at the different wavelengths, taken from a curve as depicted in Fig. 6. The resulting numbers, plotted against wavelength, form an approximate spectrum of the fluorescence increase effect. Again a maximum was found between 460 and 480 mμ (Fig. 7).

From these two kinds of experiments, described above, we conclude that the compound formed upon irradiation of an enzyme solution after addition of $FMNH_2$ has a fluorescence maximum in the region of 460–480 mμ, *i.e.* in the region of bacterial luminescence. No further conclusions can be drawn, as yet, about the exact shape of the spectrum.

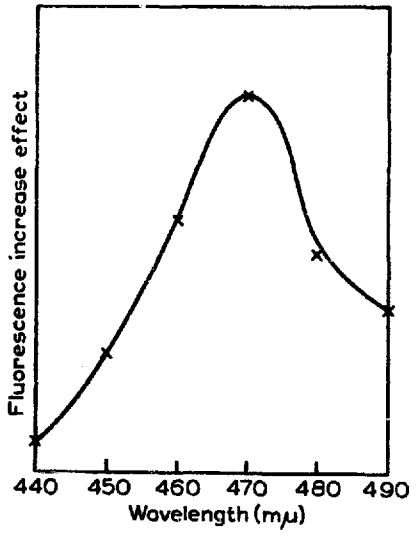

Fig. 7. The relative intensity of the fluorescence increase effect upon ultraviolet irradiation (366 mμ) measured at different wavelengths. Reaction mixtures: 3.0 ml 0.1 M phosphate buffer (pH 7.0) plus 0.1 ml HCl-precipitate. Control: 3.1 ml buffer. At zero time 0.25 ml $FMNH_2$ (from $1.1 \cdot 10^{-4}$ M FMN) is added. Measurement of fluorescence after 0 and 2 min. Further explanation: see text.

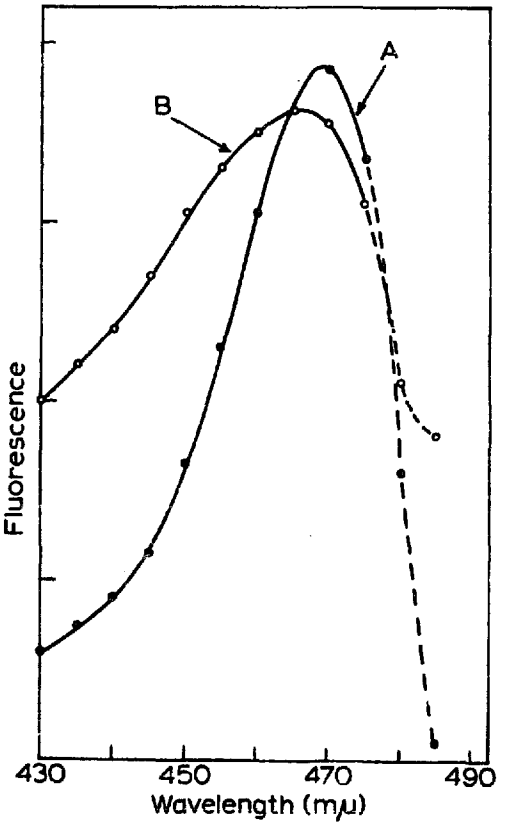

Fig. 8. Fluorescence spectra of the compound formed upon ultraviolet irradiation after addition of $FMNH_2$ to bacterial preparation (A) and of an ultraviolet-irradiation derivative of FMN (B). A, difference between the fluorescence spectra of (a) 0.1 ml HCl-precipitate and 3.0 ml phosphate buffer (0.1 M, pH 7.0), 1 min after addition of 0.2 ml $FMNH_2$ (from $1.1 \cdot 10^{-4}$ M FMN). (b) 0.1 ml HCl-precipitate and 3.0 ml buffer, 1 min after addition of 0.2 ml $1.1 \cdot 10^{-4}$ M FMN. B, difference between the fluorescence spectra of (a) 3.1 ml buffer and 0.2 ml $FMNH_2$, irradiated during 5 min with the fluorescence-exciting light (mainly 366 mμ). (b) the same solution not irradiated.

It was mentioned above, that irradiation of FMN formed by addition of $FMNH_2$ to 0.1 M phosphate buffer caused an increase in fluorescence at 470 mμ. In order to check the possibility that the irradiation derivative of the substance formed in the reaction from enzyme preparation and $FMNH_2$ is identical with the substance formed during irradiation of oxidized $FMNH_2$, the fluorescence spectrum of $FMNH_2$ plus buffer, irradiated during 5 min with the fluorescence provoking light, was compared with that of the same, but non-irradiated, solution. The resulting difference spectrum resembled somewhat the spectrum of the substance formed in the presence of the enzyme preparation, but was not identical with it: the fluorescence maximum occurs at a shorter wavelength and there is a less steep decline of the curve at the short wavelength side (Fig. 8). The experiment indicates, however, that a FMN derivative with a fluorescence peak lower than 530 mμ may relatively easily be formed.

DISCUSSION

The gel-filtration experiments indicate the occurrence of two substances, activating the light reaction, namely a protein and a non-protein. The action of the protein may be a non-specific one, as the enzyme preparation, used for testing in these experiments (a HCl-precipitate of diluted bacterial lysate) appears to be activated by addition of albumin as well (*cf.* also ref. 8). The non-protein activating substance is probably the one causing the activating action of solution U, as was described in a previous paper[2]. The inactivation of luciferase upon filtration through a Sephadex column may serve as a further indication (*cf.* ref. 2) that a readily dissociable cofactor is bound to the active enzyme. The fact that recombination of the active non-protein fraction with the protein fractions does not result in an active luciferase fits in with our previous finding that the enzyme without activator is very labile.

TOTTER AND CORMIER[9] subjected a luciferase preparation to electrophoresis on a starch column. The luciferase remained active and was identified by its flavin-like fluorescence. In our case, flavin as well as some activator dissociated from the enzyme during gel filtration. This may indicate that both substances are weakly bound by non-polar groups to the enzyme. Indications for such binding of FMN had already been found in previous experiments[6].

Our fluorescence experiments indicate that a fluorescing substance is formed as soon as an enzyme preparation, to which $FMNH_2$ has been added, is irradiated. Experiments with different dark periods prior to irradiation indicate that some precursor is formed in the dark, which subsequently is converted by ultraviolet irradiation into a fluorescent compound. TETHER AND TURNBULL[10] found that FMN in an excited state may function as an oxidant provided a hydrogen donor is present. In the light of their experiments it is tempting to ascribe the influence of ultraviolet light in our experiments to the oxidation of the precursor formed in the dark, leading to the formation of a fluorescent substance. The precursor could be formed by reaction of $FMNH_2$ or $FMNH^{\cdot}$ with a thermostable compound C present in the enzyme preparation. Both reactions can be written as follows:

$$FMNH^{\cdot} + C \longrightarrow FMNHC \text{ (precursor)}$$

$$2\ FMNHC + FMN \xrightarrow{h\nu} 2\ FMNC + FMNH_2$$

FMNC is supposed to fluoresce in the 460–480-mμ region.

In the light reaction a substance with a luminescence maximum at about 470 mμ is present during the reaction. No measurable amount of the fluorescing compound could be detected after completion of the light reaction, but from our fluorescence experiments it is evident that the precursor fluorescing upon ultraviolet irradiation is still present. It may well be that a very small fraction of the precursor is oxidized by O_2 during the light reaction in the presence of luciferase and palmital. The luminescence should be due, then, to the oxidation product of the precursor, which may be identical with the assumed oxidation product formed by ultraviolet irradiation.

The identity of compound C and of the precursor is not yet elucidated. As to the precursor, this may be (a) a derivative of the compound C present in the enzyme preparation, formed by reaction with $FMNH_2$; (b) a derivative of FMN, formed by reaction of $FMNH_2$ with compound C, or an addition product of the two components.

Considering that a derivative of FMN, fluorescing in the 470-mμ region is readily formed upon illumination, the second possibility seems, so far, the most probable one.

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