Biophysics. — *The effect of radiation on light emission by luminous bacteria.* **II.** By A. J. KLUYVER, G. J. M. VAN DER KERK and A. VAN DER BURG.

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5. *The photochemical action in the ultraviolet region of the spectrum.*

The exposition as given in the foregoing has been incomplete as to one important point. Until now we have accepted the idea that the photochemical action investigated was fully reversible. The reversibility as observed in the initial experiments has indeed been a strong argument in favour of the view that the action of the light was confined to some compound directly involved in the light emitting process and did not result in a general disorganisation of the cell.

On the other hand, however, it is well known that ultraviolet radiation has a pronounced lethal effect on all living cells, and therefore it would have been surprising, if such an effect would not also have been manifest in our investigation.

As a matter of fact we soon obtained conclusive evidence for the lethal action of the ultraviolet part of the spectrum also in our experiments, and in this connection we wish to draw the attention of the reader to a curious phenomenon which is observed on studying the recovery of the light emission after the irradiation, and which is clearly shown in Fig. 1 under *C* and *D*.

In this figure under *C* the same part of the luminescent culture as is given under *A* is photographed two hours after the irradiation. It will be seen that in the visible region of the spectrum the inhibition has already largely disappeared, or in other words here the recovery has already made good progress. In the ultraviolet region, however, we are struck by the remarkable phenomenon — already described by GERRETSEN[1]) in 1920 — that the brightness surpasses that of the non-irradiated part of the culture. The reproduction under *D* which brings a photograph taken 6 hours after the irradiation shows that at that time the ultraviolet region has become quite dark, in sharp contrast to what holds for the region of the visible rays where the recovery of the light emission is now quite complete. It is clear that at this time the bacteria in the ultraviolet region have died.

It will not need elucidation that at first sight these observations seem to invalidate the conclusions we have drawn from the experimental results obtained in the ultraviolet region of the spectrum. A closer study of the recovery process of the light emission did, however, show that this was not the case.

In order to study the recovery process a bacterial culture was irradiated during two minutes, using the quartz spectrograph. Then photographs were taken at various moments, *viz.* immediately after the irradiation, and 4, 8, 15, 30, 60 and 120 minutes later. At definite spots in the irradiation spectrum, to wit for 10 wave-lengths in the visible and near ultraviolet region, and for 4 wave-lengths in the ultraviolet region below 300 m μ, the relative intensities of the bacterial light were determined in the usual way, and from these data the course of the recovery of the photochemical effect with time could be computed.

The results are collected in Table II in which the photochemical effects obtained are expressed in an arbitrary measure, for each wave-length taking the effect immediately after the irradiation to be 100.

From Table II it is clear that in the first stages, almost up to a recovery time of 30 minutes, the average figures for the course of the recovery in the visible and near ultra-

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1) F. C. GERRETSEN, Zentralbl. f. Bakt. II, **52**, 353 (1920).

violet range of the spectrum agree very satisfactorily with the same figures in the short wave ultraviolet range. It is only after that time that in the latter case a quick rise in luminescence takes place leading to the phenomenon earlier described by GERRETSEN.

The results seem to justify the conclusion that the primary photochemical effect, as measured by us immediately after the short time of irradiation, is due to the same cause both in the short wave ultraviolet and in the longer wave range of the spectrum. Apparently the effect, which we may call after its discoverer the GERRETSEN-phenomenon, manifests itself only in the long run and, therefore, does not interfere with our determination of the absorption spectrum.

TABLE II.

Course of the recovery process from photochemical inhibition of the light emission. (For each wave-length the photochemical effect has been expressed in percentage of the effect immediately after the irradiation).

Wave-length in mμ	Time after irradiation in minutes						
	0	4	8	15	30	60	120
436	100	92	82[5]	71	51	29[5]	11[5]
420	100	91	82[5]	68[5]	49[5]	26	10[5]
405	100	90	81	69	51	29[5]	12
379	100	92	80	66	50[5]	25	12[5]
366	100	89	81[5]	68	49[5]	24[5]	11
334	100	87	71[5]	66	49	—	—
328	100	93[5]	78[5]	58	50[5]	—	—
313	100	82[5]	70[5]	58	43	—	—
307	100	87[5]	76[5]	64	53	—	—
302	100	83[5]	75	58[5]	45	—	—
Average in region >300	100	89	78	65	49	27	11[5]
297	100	83	77[5]	65[5]	46[5]	<0	<0
292	100	89[5]	77	70	55	<0	<0
289	100	87	76[5]	73[5]	62[5]	<0	<0
280	100	90[5]	84	—	—	<0	<0
Average in region <300	100	87[5]	79	70	55	<0	<0

<0 = increased intensity as compared with non-irradiated zone.

6. *Some remarks regarding the significance of the results obtained.*

The reversible inhibition of bacterial luminescence, as observed in our experiments, leaves practically no doubt that in this phenomenon we are dealing with a photochemical conversion of one of the components of the light emitting system present in the bacteria. It remains, however, questionable whether this conversion is due to a direct absorption of the light quanta by the component in question or whether these quanta are absorbed by some other compound which acts as a *photosensitizer* in the said conversion, thus giving rise to a so-called photochemical reaction of the 2nd type. In the latter case the absorption spectrum determined might relate to a compound which is quite foreign to the light emitting system.

Recent experiences of HARVEY and his school seem indeed to speak in favour of the latter idea. In the Introduction mention has already been made of the fact that in 1925 HARVEY published his observations on the light inhibition of the luminescence of

Cypridina hilgendorfii. In the same publication experiments were reported in which crude luciferin and luciferase solutions were also submitted to irradiation and which clearly showed that only the luciferin was affected by the light. Moreover, it was found that for the conversion of the luciferin the presence of oxygen was indispensable. Recently, however, HARVEY's collaborators CHASE and GIESE [1]) have repeated these experiments, this time working with a luciferin preparation highly purified according to the prescription given by ANDERSON [2]). They now found that contrary to HARVEY's earlier observation the purified luciferin preparation was quite stable in light of wave-lengths longer than 300 m μ. In the short wave ultraviolet (230—280 m μ) luciferin was quickly destroyed, but this destruction differed from that described by HARVEY in its being independent of the presence of oxygen. Besides they found that by adding fluorescent dyes, like eosin, fluorescein or riboflavin, to the solution luciferin could be sensitized to visible light and for this photochemical reaction oxygen proved to be essential. They conclude, therefore, that the photochemical luciferin decomposition observed by HARVEY in his earlier experiments must have been due to the presence of a photosensitizer in the crude extracts, and remark that riboflavin may well have acted as such.

With a view to these results it appeared quite possible that also in the photochemical effect studied by us a photosensitizer would play a role, the more so since ROTTIER [3]) has shortly ago proved that *Ph. phosphoreum* is characterized by a comparatively high content of riboflavin.

However, if riboflavin would have acted as a photosensitizer in our experiments, the absorption spectrum obtained should have been that of riboflavin, and even a superficial comparison of both spectra suffices to reject this idea.

We have, therefore, carefully examined the possibility that our absorption spectrum could be identified with that of some carotenoid, since representatives of this group of compounds have also been encountered as a photosensitizer in physiological processes, for instance in the phototropic reaction of the sporangiophores of *Pilobolus* [4]). But also this hypothesis had to be rejected, since the general character of all known carotenoid spectra differs markedly from that of the absorption spectrum obtained by us.

Now it can, of course, not quite be excluded that in the photochemical effect on the light emission of luminous bacteria some still unknown compound acts as a photosensitizer. However, several arguments are in favour of the idea that in our case we are dealing with a direct photochemical conversion of a component of the light emitting system. So for instance it is difficult to see this conversion as a physiological effect for which a special perception apparatus has been built up in the cell, as may well be the case in the phototropic response of the sporangiophores of *Pilobolus* and related fungi. And the idea that some photosensitizer incidentally present in the cell is responsible for the effect observed can scarcely be reconciled with some additional observations made by us, *viz.*, that the light emission by *Ph. splendidum* and *Ph. Fischeri*, species which widely differ from *Ph. phosphoreum* in several respects, is also inhibited by radiation and that the inactivation spectra in all these cases are closely related.

The most weighty consideration that the absorption spectrum in question is not that of a photosensitizer, but is directly related to some component of the light emitting system is, however, to be found in the results of an as yet unpublished investigation made by A. VAN DER BURG. In this study it has been shown that — contrary to the earlier observations of EYMERS and VAN SCHOUWENBURG [5]) — there exists a slight but un-

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[1]) A. M. CHASE and A. C. GIESE, Journ. Cell. and Comp. Physiol. **16**, 323 (1940).

[2]) R. S. ANDERSON, Journ. Gen. Physiol. **19**, 301 (1935).

[3]) P. B. ROTTIER, Fluorometrische en spectrophotometrische bepaling van lactoflavine in micro-organismen. Diss. Delft (1942).

[4]) E. BÜNNING, Planta **26**, 719 (1937); Ibid. **27**, 148 (1937).

[5]) J. G. EYMERS and K. L. VAN SCHOUWENBURG, Enzymologia **3**, 235 (1937).

mistakable difference between the spectra of the light emitted by *Ph. phosphoreum* and by *Ph. splendidum*, in so far that the spectrum of the first named species is characterized by a much steeper descent to the side of the short wave-lengths and therefore extends less far in the violet. This result is remarkable, because we have established that the inactivation spectrum of *Ph. phosphoreum* shows a somewhat similar deviation from the otherwise closely related spectrum of *Ph. splendidum* in so far as it extends farther to the side of the longer wave-lengths. Without entering here into a discussion of the interrelationship between emission and absorption spectra, it may be remarked that it can scarcely be incidental that analogous differences exist between the inactivation and emission spectra of both species. This only seems conceivable, if the compound, to which the absorption spectrum is related, is either identical with, or at least closely related to the compound ultimately responsible for the light emission.

Once accepting this idea, it is tempting to make a few suggestions both regarding the role of the compound of which we indirectly determined the absorption spectrum in the mechanism of light emission, and regarding its chemical nature.

Since in the present stage of our investigation these considerations necessarily bear a speculative character, we shall restrict ourselves to a few remarks.

Accepting a close relationship between the mechanism of luminescence both in *Cypridina* and in luminous bacteria, VAN DER KERK [1]) has arrived at the conclusion that this mechanism can be best represented by the following equations:

$$XH_2 + L \rightleftarrows X + LH_2 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

$$LH_2 + A \rightleftarrows A \,.\, LH_2 . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

$$A \,.\, LH_2 + 1/2\, 0_2 \rightarrow A \,.\, LH_2 \,.\, 0 \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

$$A \,.\, LH_2 \,.\, 0 \rightarrow A \,.\, L_1^* + H_2 0 \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

$$A \,.\, L_1^* \rightarrow A \,.\, L_1 + h\nu . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (5)$$

$$A \,.\, L_1 \rightleftarrows A + L_1 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (6)$$

In these equations LH_2 = luciferin, L = dehydroluciferin, L_1 = product of the irreversible (light emitting) oxidation of luciferin, A = luciferase, and * indicates: excited state of a molecule.

Now the results of the investigation of CHASE and GIESE, earlier discussed in this chapter, practically exclude the possibility that either luciferin or luciferase is the photosensitive molecule responsible for the irradiation inhibition observed in our experiments. Maintaining the idea of a close relationship between this molecule and that responsible for the light emission one is, therefore, brought to an identification of our compound with dehydroluciferin (L).

In this connection we wish to point out that the purely chemical investigations of ANDERSON on the chemical nature of luciferin have made it very likely that this compound has the character of a polyphenol, a result which makes it most probable that dehydroluciferin will be some quinone.

Now a fairly recent note by CHAKRAVORLY and BALLENTINE [2]) has brought a most

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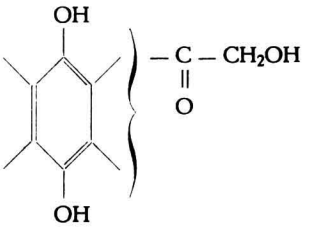
[1]) For the documentation of this scheme we must again refer to the doctorate thesis by VAN DER KERK. The scheme is an elaboration of earlier proposals made by E. N. HARVEY (Erg. d. Enzymforschung **4**, 365 (1935)), by F. H. JOHNSON (Enzymologia **7**, 72 (1939)), by F. H. JOHNSON, K. L. VAN SCHOUWENBURG and A. VAN DER BURG (Enzymologia **7**, 195 (1939)) and by B. CHANCE, E. N. HARVEY, F. H. JOHNSON and G. MILLIKAN (Journ. Cell. and Comp. Physiol. **15**, 195 (1940)).

[2]) P. N. CHAKRAVORLY and R. BALLENTINE, Journ. Amer. Chem. Soc. **63**, 2030 (1941).

important contribution to our knowledge of the constitution of *Cypridina*-luciferin. These authors have made it acceptable that the polyphenol nucleus bears a side chain of a ketol character which in the light emitting oxidation should be irreversibly oxidized to a carboxyl group with simultaneous production of carbon dioxide.

Assuming that in bacterial luciferin too the mentioned essential groupings are present, we can also represent it by the partial and schematic formula given by the American authors, *viz.*:



We must, however, emphasize that in this formula the para position of the phenol groups is fully hypothetical.

The question now arises in how far the absorption spectrum determined supports the above formula, and if so, whether it can add to a further identification of the molecule in question.

Although the present state of our knowledge regarding the relation between absorption spectrum and chemical constitution is still far from satisfactory, we may at once conclude that the character of the spectrum in question seems, indeed, to indicate that we are dealing with a compound containing some aromatic ring system. For it has been found that such compounds are all characterized by a pronouncedly steep maximum in the ultraviolet[1]). The general character of our spectrum shows, moreover, a strong resemblance with that of the spectra of naphthalene, anthracene and naphthacene (RADULESCU and collaborators[2])).

The same type of spectrum is also found in various 1.4-naphthoquinone derivatives[3]), whilst the spectra of corresponding compounds of the 1.2-naphthoquinone series are markedly different.

These facts strongly suggest that in dehydroluciferin we are dealing with a compound of the para quinone type, a conclusion which is in agreement with the well-known experience that all such quinones are definitely photosensitive.

The situation of the spectrum, and more especially that of the final absorption to the side of the longer wave-lengths, enables us further to make a choice between the possibilities of a naphthalene, an anthracene and a naphthacene quinone. A critical survey of the spectra of the said hydrocarbons and their derivatives, in so far as these have been determined until now, is strongly in favour of the view that our photosensitive molecule belongs to the naphthoquinone series, and that moreover the $C = O \,.\, CH_2OH$ group is directly substituted in the quinone ring. This would bring the dehydroluciferin into the physiologically important class of Vitamine K derivatives, a possibility which is the more attractive, since it has been definitely shown that several bacteria are able to synthesize such compounds[4]).

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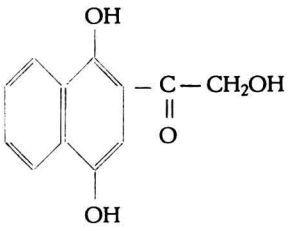
[1]) Cf. for instance: K. DIMROTH, Angew. Chemie **52**, 545 (1939).

[2]) D. RADULESCU und F. BARBULESCU, Ber. **64**, 2225 (1931); D. RADULESCU und G. OSTROGOVITCH, Ber. **64**, 2233 (1931).

[3]) L. F. FIESER, W. P. CAMPBELL and E. M. FRY, Journ. Amer. Chem. Soc. **61**, 2206 (1939).

[4]) H. DAM, J. GLAVIND, S. ORLA-JENSEN und A. D. ORLA-JENSEN, Die Naturwissenschaften **29**, 287 (1941) and Zentralbl. f. Bakt. II, **104**, 202 (1941).

The foregoing deductions would,of course, imply that luciferin should either be identical with, or closely related to a compound of the formula:



Although the hypothetical character of this conclusion is fully acknowledged, it seems worth-while to submit it to a more direct test. Accordingly one of our collaborators has made a beginning with the synthesis of several compounds in the naphthoquinone series and with the determination of their absorption spectra.

Utrecht–Delft, Biophysical Research Group, September 1942.