ponents depended on the number of logarithmic cycles through which the curve was carried. Thus when the curve of Fig. 1 was fabricated on beyond three cycles, the following components were derived by backward projection: (y intercept : halftime)—four cycles, 40 : 7 and 40 : 4; five cycles, 28 : 7 and 52 : 4; and six cycles, 22 : 8 and 58 : 5.

Sometimes two separate processes may be expected a priori, as when a tracer molecule is eliminated from both intracellular and extracellular fluids, but contrary to expectation, the actual system may be a bimodal distribution of components because of variance within the two apparently separate categories. For example, the rates of elimination of the tracer molecule from some subunits which would be classed as extracellular fluid on anatomic or physiologic grounds may overlap rates of elimination from some subunits of the intracellular fluid.

If it is assumed that the experimental material being studied will exhibit variance to some degree (as is almost always the case in biology), a curve on a semilog plot may result from operation of one of four types of systems: (i) a single-mode distribution of exponential processes, of which Figs. 1 and 2 are examples; (ii) a multi-mode distribution with so little separation between modes that components derived by backward projection are not identified with the modes; (iii) a multi-mode distribution with enough separation of function that backward projection correctly separates the modes—that is, the modes operate as isolated components; (iv) a complex of process types—for example, kidney excretion of certain substances may sometimes be due to exponential processes acting simultaneously with linear, "active transport" processes.

The concavity of a semilog curve from a single-mode distribution of exponential processes is determined by the relative magnitude of the standard deviation (SD) and the mean of the distribution; the larger the SD/mean ratio, the greater the curvature (for example, the curve in Fig. 2 is more concave than the curve in Fig. 1). Conversely, the smaller the SD/mean ratio, the more nearly the composite curve resembles a single exponential process; if the histogram of Fig. 1 is moved to the right to increase the mean value without altering the SD, the resulting curve on semilog paper becomes straighter and straighter.

When backward projection is applied to the almost-straight curves from distributions with small SD/mean ratios, they tend to separate so that the largest derived component has a halftime approximating the mean halftime of the distribution, but curves which are concave tend to give two or three components of similar magnitude unless the data are carried through many log cycles. Therefore, the results of backward projection will be most misleading if the exponential processes responsible for the curve are distributed with a large SD/mean ratio; the derived components suggest two or three isolated processes of similar magnitude, where in fact there is only a single type of process, but the subunits of the system vary in their rate of operation.

HUGH D. VAN LIEW
Department of Physiology, Stanford University School of Medicine, Stanford, California

21 August 1962

Chemiluminescence of Firefly Luciferin without Enzyme

Abstract. We have been able to produce chemiluminescence in firefly luciferin without utilizing the enzyme luciferase. Following the analogous mechanism of the chemiluminescence of luminol in the organic solvent dimethyl sulfoxide, we have prepared synthetically the methyl ester of luciferin, the phosphate ester of luciferin and luciferyl adenylate by condensation in dimethyl sulfoxide with Khorana's reagent, dicyclohexylcarbodiimide and diazomethane, phosphoric acid, and adenylic acid respectively. These compounds in dimethyl sulfoxide in the presence of base emit a bright chemiluminescence. Like in vitro enzymatic bioluminescence, the luciferyl adenylate chemiluminescence emission spectrum is dependent upon *p*H.

The biochemical steps leading to the enzyme-catalyzed emission of light by firefly luciferin have already been described (*1*). Adenosine triphosphate (ATP) is required to form the "active luciferin-enzyme complex" (E·LH_2-AMP), which can then react with molecular oxygen resulting in the emission of a yellow-green band with a peak at 562 mμ. Rhodes and McElroy (*2*) have also shown that synthetically produced luciferyl adenylate (LH_2-AMP) reacts with the enzyme luciferase to produce light in the absence of ATP. We have now been able to demonstrate the nonenzymatic chemiluminescence of LH_2-AMP as well as

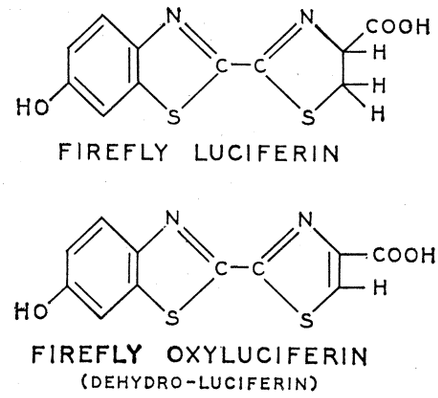

Fig. 1. Chemical structures of firefly luciferin and dehydroluciferin.

that of the phosphate and methyl esters of luciferin (*3*).

The structure of firefly luciferin (*4*) is shown in Fig. 1. We are reasonably certain on the basis of synthesis of other analogs of luciferin (LH_2) that LH_2-AMP is formed by the condensation of adenylic acid (AMP) at the carboxy group. By analogy with the chemiluminescent oxidation of luminol (*5*) the abstraction of one of the hydrogen atoms in the 1- or 2-position in basic solution could then permit oxygen attack which results in an excited state of the product molecule. Since LH_2-AMP is extremely labile in aqueous alkaline solution we have worked primarily in the strongly hydrogen-bonding organic solvent, dimethyl sulfoxide. This solvent is also extremely efficient for the chemiluminescent reaction of luminol.

Firefly luciferin was condensed with adenylic acid, metaphosphoric acid or diazomethane in dry pyridine with dicyclohexylcarbodiimide, according to the method of Khorana (*6*). We have since found that the yields are considerably higher if the reaction is performed in dimethyl sulfoxide. An aliquot was delivered to 2 ml of dimethyl sulfoxide in a 10- by 75-mm test tube mounted in front of a 1P21 phototube. Chemiluminescence was obtained upon addition of a solid pellet

Table 1. Relative peak chemiluminescence intensity.

Compound	Intensity
Luciferyl adenylate (LH_2-AMP)	100
Luciferyl inosinate (LH_2-IMP)	4
Luciferin (LH_2)	1
Dehydroluciferyl adenylate (L-AMP)	3
Dehydroluciferin (L)	1
Dimethyl sulfoxide alone	1
Adenylic acid (AMP)	1
Inosinic acid (IMP)	1

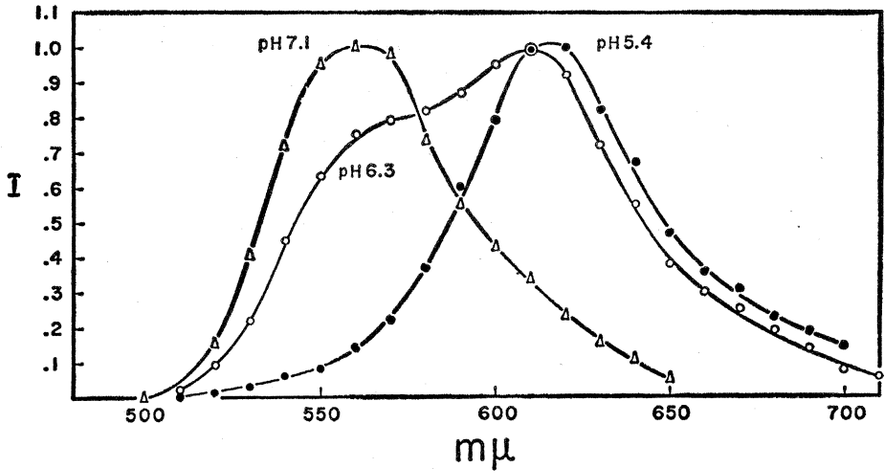

Fig. 2. Variation with *p*H of the in vitro enzymatic light emission of firefly luciferin.

of KOH, by the addition of a droplet of 10*M* NaOH, or by the addition of a drop of tertiary butyl alcohol solution in which a small amount of potassium metal had been dissolved so that tertiary butoxide ions were formed. The initial experiments were performed with either the KOH pellet or the NaOH droplet. Table 1 shows peak chemiluminescence intensities for both LH_2-AMP and controls in the experiment. Only in the case of LH_2-AMP chemiluminescence was there an immediate flash followed by a rapid decay. The other weak light emissions were presumably due to a reaction in dimethyl sulfoxide alone and possibly an associated sensitized fluorescence.

Except in the cases of the esters of luciferin (LH_2) the observed chemiluminescence was of too low an intensity to estimate color. In the case of LH_2-AMP, as a drop of 10*M* NaOH was added to the test tube there was a yellow-green glow surrounding the drop as it sank to the bottom of the tube. If the tube was then shaken vigorously there appeared to the dark-adapted eye a brilliant red emission over the entire solution volume. This disappeared in 1 to 2 seconds, and there remained the very low intensity steady emission characteristic of the NaOH and dimethyl sulfoxide reaction alone. This yellow-green light emission from LH_2-AMP was seen most clearly upon the initial introduction of the NaOH droplet. Upon successive additions of NaOH the chemiluminescence emission became more orange until only a red glow was visible. These results are consistent with the appearance of the red flash when either the NaOH or KOH was shaken vigorously in the solution and also with the red flash which was observed when the strongly basic tertiary butoxide ions were added to the solution; they show that the color of the chemiluminescence is strongly dependent on *p*H. This is analogous with the enzymatic dependence upon *p*H for color emission previously reported (*7*) and shown in Fig. 2. When the technique for the measurement of transient spectra was used (*8*), the red emission spectrum in strongly basic dimethyl sulfoxide was obtained. This is shown in Fig. 3. The peak emission at 6255 Å differs from the 6140-Å peak in the enzymatic reaction. However, this was not unexpected, since in the case of luminol there was also a red shift in chemiluminescence emission from 4300 Å in aqueous solution to 4800 Å in dimethyl sulfoxide.

The unique character of the LH_2-AMP compound was shown by the

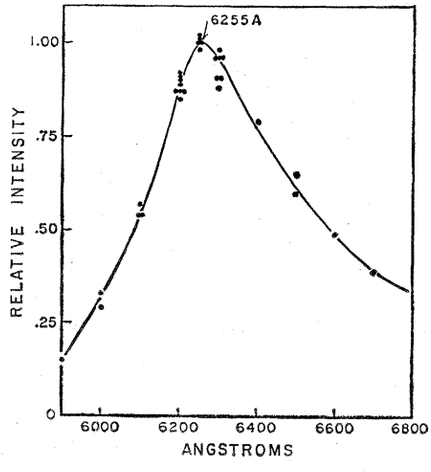

Fig. 3. Emission of spectrum of LH_2-AMP chemiluminescence in dimethyl sulfoxide upon addition of strong base.

fact that the methyl ester of luciferin, LH_2-CH_3, gave *only* a yellow-green chemiluminescence and the phosphate ester, LH_2-PO_4, gave *only* a red chemiluminescence under the same experimental conditions which caused LH_2-AMP emission to shift from yellow-green to red. Thus, the oxidized product of LH_2-AMP can exist in either of two fluorescent excited species, dependent on *p*H.

In aqueous solutions basic enough to ionize the LH_2-AMP derivative at the 1- or 2-hydrogen atoms the compound hydrolyzes immediately to form free LH_2 and free AMP. However, aqueous chemiluminescence of LH_2-AMP was observed under the following experimental conditions. An aqueous solution of LH_2-AMP at *p*H 5.5 was added to 2 ml of water plus 0.1 ml of a 3 percent H_2O_2 solution in a small test tube. A drop of 5*M* NaOH containing 0.01 percent OsO_4 was then permitted to run down the inside wall of the small tube. Light emission could be seen by the dark-adapted eye. When the tube was shaken, a brighter flash was visible. The brightness of the flash even on this level was just on the borderline of unambiguous color estimation, and appeared green. This could place the true peak in the yellow-green band, if one takes into account the blue shift of the Purkinje effect. Under identical conditions no light at all was observed for LH_2 alone.

The fact that LH_2 alone gave no aqueous chemiluminescence shows that the observed light emission of LH_2-AMP was not a sensitized fluorescence. The addition of OsO_4 to catalyze the product of a high concentration of OH radicals in the same volume, and at the same instant as the solution surrounding the drop of NaOH was made strongly basic, insured that a small fraction of the LH_2-AMP molecules could be ionized before they were hydrolyzed. They could then react with OH radicals to produce chemiluminescence, exactly analogous to the aqueous chemiluminescence of luminol. This indicates that an essential role of the enzyme in the oxidation of firefly luciferin is to permit, by virtue of the binding of the LH_2-AMP, the removal of a proton and the subsequent attack by oxygen. On the other hand, in the absence of enzyme the chemical environment would have to be so basic that LH_2-AMP would hydrolyze before oxygen attack could occur. By reacting

the LH_2-AMP in dimethyl sulfide we have inhibited hydrolysis by chemical means even at high *p*H values, effectively accomplishing what the enzyme can do at neutral *p*H. As might be expected D-LH_2-AMP and L-LH_2-AMP do not show the stereospecificity in nonenyzmatic chemiluminescence which was previously reported for the enzymatic reaction (*9*).

H. H. SELIGER
W. D. MCELROY
McCollum-Pratt Institute and
Department of Biology,
Johns Hopkins University,
Baltimore, Maryland

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21 June 1962

Evoked Responses to Clicks and Electroencephalographic Stages of Sleep in Man

Abstract. The form of the average evoked response to clicks is highly correlated with the background electroencephalogram. However, the response during the emergent low-voltage "dreaming" stage is different from that seen during the low-voltage phase at the beginning of sleep. The results provide additional evidence that the emergent low-voltage stage is a neurophysiologically unique phase.

When recorded from the human scalp, the amplitude of specific electrical events evoked in the brain by sensory stimuli is usually smaller than that of the complex background activity. Since Dawson (*1*) described an electronic averaging technique for discriminating these signals from background noise, several such methods have been developed which are based on the assumption that the evoked electrical response bears some definite time relation to the stimulus (*2*). The present study used a digital system to average evoked responses to clicks both during waking and sleep in human subjects.

As a human subject sleeps, electroencephalographic (EEG) potentials shift continuously through several characteristic patterns. At the onset of sleep a low-voltage pattern with irregular frequency is seen, stage 1 (*3*). There is then a progression through stages 2 and 3 which is characterized by 14-cy/sec spindling and slower activity, to stage 4 where trains of delta waves dominate the record. Stage 4 gives way to stages 3 and 2, and then a low-voltage, irregular pattern appears which is similar to the pattern at the beginning of sleep. Rapid eye movements accompany this emergent low-voltage phase, however, and if awakened from this stage, subjects are likely to report dreaming (*3*). The low-voltage pattern at the onset of sleep is usually not accompanied by rapid eye movements or dream recall (*3*). During a normal night of sleep the cycle described above is repeated about every 90 to 120 minutes.

Generally, thresholds for arousal are lowest during low-voltage and highest during high-voltage stages, but classification of the emergent irregular, low-amplitude phase is controversial. Dement and Kleitman found that the threshold for awakening was considerably higher in this phase than at the beginning of sleep (*3*), and Jouvet and others have shown that in the cat a similar low-voltage pattern of sleep is associated with raised thresholds for arousal (*4*). To distinguish this emergent low-voltage phase from the similar pattern at the beginning of sleep, we will call it stage 1_{rem}.

For this study three young adult male subjects slept in an electrically shielded chamber where the ambient noise level was constant at approximately 60 decibels (db), relative to 0.0002 dyne/cm^2. The click stimulus, with an intensity of 85 db, was the amplified gate output pulse from a type 162 Tektronix wave-form generator which was fed to an 8-inch cone speaker located about 1 foot above the subject's head. A click was presented every 2 seconds throughout the night. Potentials recorded from bipolar electrodes which were placed on the vertex at c_z and in the left occipital region at o_1 were amplified and recorded on magnetic tape (*5*). For each stimulus the recorded activity was sampled every 6 msec through a period of 1536 msec from stimulus onset. The voltage of each of these 256 ordinates was fed to a Packard Bell 250 general-purpose digital computer which was programmed to compute the mean on each ordinate after the data from 100 stimuli were received. These averages of the individual ordinates were graphically displayed by an *x-y* plotter.

Fig. 1 shows typical samples from the computed record of one subject. An upward deflection represents positivity at c_z. Note that several prominent components of the response are labeled,

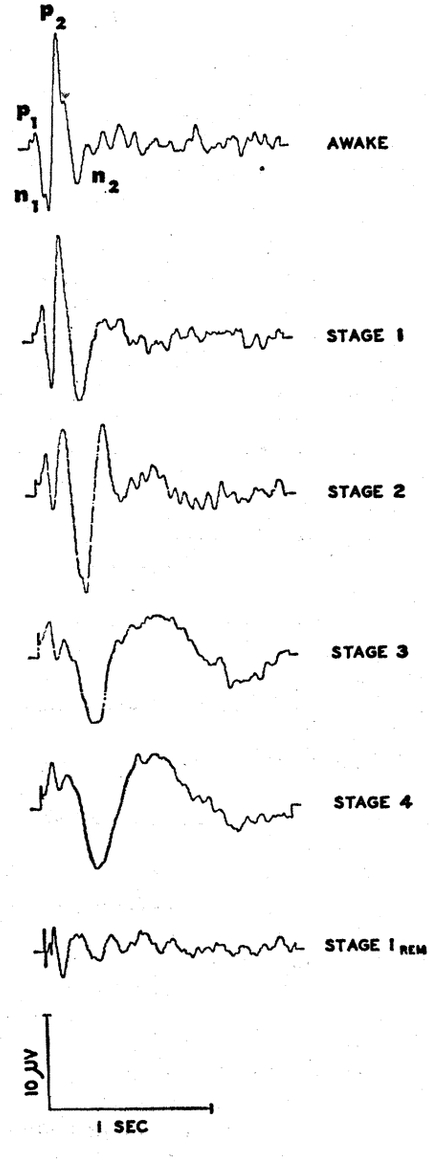

Fig. 1. Average responses to clicks as a function of the EEG stage of sleep (subject B.D.). Upward deflection denotes positivity at c_z.