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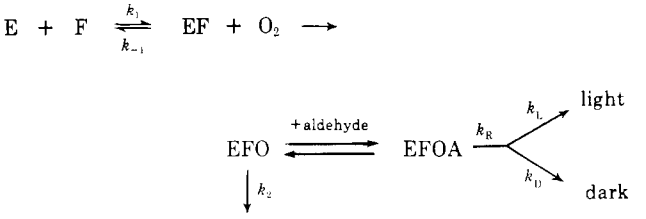
Flavine Specificity of Enzyme–Substrate Intermediates in the Bacterial Bioluminescent Reaction. Structural Requirements of the Flavine Side Chain†

E. A. Meighen* and R. E. MacKenzie

ABSTRACT: The specificity of bacterial luciferase for the side chain of FMN was investigated with the use of flavine analogs. The charge, length, and secondary hydroxyl groups of the flavine side chain affected the stability of the intermediates (enzyme–flavine (EF), enzyme–flavine–oxygen (EFO), and enzyme–flavine–oxygen–aldehyde (EFOA and EFOA′) in the bioluminescent reaction. A negative charge on the side chain was necessary not only for tight binding but good activity and could be supplied either by carboxyl or phosphate groups. This charge had to be located at least 8.4 Å from the N-10 position of the flavine ring. Secondary hydroxyl groups had no effect on flavine binding or activity (initial light intensity I_0) but were implicated in aldehyde binding. The importance of the negative charge in the bioluminescent reaction was further demonstrated by a large stimulation of the activity of neutral flavines by inorganic anions. However, anions had no effect on the binding of neutral flavines. The dissociation constant for inorganic phosphate, measured by stimulation of the activity of the neutral flavines, was the same as that obtained by competitive inhibition with $FMNH_2$, thus demonstrating a specific site for phosphate. The results indicate that the phosphate of FMN binds in a subsite on the enzyme such that it stabilizes the enzyme–flavine intermediates in the bioluminescent reaction.

Bacterial luciferase catalyzes the emission of light "*in vitro*" in the presence of reduced flavine mononucleotide ($FMNH_2$),[1] $^1O_2$, and a long-chain aliphatic aldehyde (Hastings, 1968). A number of intermediates in the bioluminescent reaction have been demonstrated by Hastings and Gibson (1963). A scheme incorporating such intermediates is



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[1] Abbreviations used are: FMN and $FMNH_2$, oxidized and reduced forms of flavine mononucleotide; isoFMN, 6,7-dimethyl-10-(1′-D-ribityl 5′-phosphate)isoalloxazine; 3-MeFMN, 3,7,8-trimethyl-10-(1′-D-ribityl 5′-phosphate)isoalloxazine; EF, enzyme–flavine; EFO, enzyme–flavine–oxygen; EFOA and EFOA′, enzyme–flavine–oxygen–aldehyde. Trivial names were used for compounds of the following series: ω-carboxyethyl-, ω-carboxypentylflavine, 7,8-dimethyl-10-(ω-carboxyalkyl)isoalloxazines; ω-hydroxyethyl-, ω-hydroxypropyl-, ω-hydroxybutyl-, ω-hydroxypentyl-, ω-hydroxyhexylflavine, 7,8-dimethyl-10-(ω-hydroxyalkyl)isoalloxazines; ω-phosphopropyl-, ω-phosphobutyl-, ω-phosphopentyl-, ω-phosphohexylflavine, 7,8-dimethyl-10-(ω-phosphoalkyl)isoalloxazines. For simplicity, flavines are generally referred to in the text as the oxidized forms, but it must be emphasized that it is the reduced forms that are active with luciferase.

In this reaction scheme, a reduced flavine molecule binds to luciferase to form an enzyme–reduced flavine complex with a dissociation constant, K_d, equal to k_{-1}/k_1 (Meighen and Hastings, 1971). The EF complex can then interact with O_2 to form a stable enzyme–flavine–oxygen complex. In the absence of aliphatic aldehyde, the EFO complex decays exponentially at a rate given by k_2. However, in the presence of a long-chain aldehyde, another stable intermediate, an enzyme–flavine–oxygen–aldehyde complex (EFOA) is formed. The oxidation states of flavine and oxygen in the EFO and EFOA complexes are unknown. The EFOA intermediate decays with the emission of light in a rate-limiting step, k_R. Since the quantum yield per mole of EFOA is much less than unity (Hastings *et al.*, 1965) and depends on the type of aldehyde (Hastings *et al.*, 1969) and flavine involved in the bioluminescent reaction, this intermediate must decay through at least two pathways,[2] a light pathway with a rate given by k_L, and a dark pathway with a rate given by k_D. The relationship between k_R, k_D, and k_L is not a simple one. For example, the dark reaction could occur as part of and/or subsequent to the rate-limiting step, as shown in

$$\longrightarrow \text{EFOA} \xrightarrow{k_m} \text{EFOA}' \xrightarrow{k_o} \text{light}$$
$$\qquad\quad \downarrow k_n \qquad\quad \downarrow k_p$$
$$\qquad\quad \text{dark} \qquad\quad \text{dark}$$

Thus EFOA would decay with a rate-limiting step, k_R, given above by $k_m + k_n$. The pathway with a rate constant k_m results in a new intermediate, EFOA′, which decays by both light and dark pathways. Thus the overall rate constant for the light pathway, k_L, would be $k_m k_o/k_o + k_p$), and k_D would be equal to $k_n + k_m k_p/(k_o + k_p)$.

The "*in vitro*" assays for bioluminescence permit many of these rate constants to be measured directly without resorting to rapid-mixing techniques. In the assay illustrated by curve A in Figure 1, luciferase is first incubated with flavine reduced with $Na_2S_2O_4$. The reaction is initiated by injection of an aerobic aldehyde suspension which converts the EF complex to EFOA and oxidizes the free reduced flavine. The initial light intensity obtained is a measure of the amount of EF complex at any given flavine concentration and thus the dissociation constant, K_d, can be determined (Meighen and Hastings, 1971). After the injection of aldehyde, the light intensity rises rapidly to a maximum and then decays exponentially. The maximal light intensity at saturating substrate concentrations is a measure of the magnitude of the rate constant, k_L. Since the free reduced flavine is oxidized within the first second after injection of aldehyde, no additional EFOA complex is produced and therefore the exponential decay of the light intensity simply reflects turnover of the intermediate, EFOA, at a rate given by k_R.

The decay rate of the intermediate, EFO, as given by k_2, can be measured by injection of reduced flavine into an assay medium containing luciferase followed by injection of an aldehyde solution (curve B, Figure 1) (Hastings and Gibson, 1963). In this assay, very little light is obtained until the aldehyde is injected and the light rises to a maximum and then

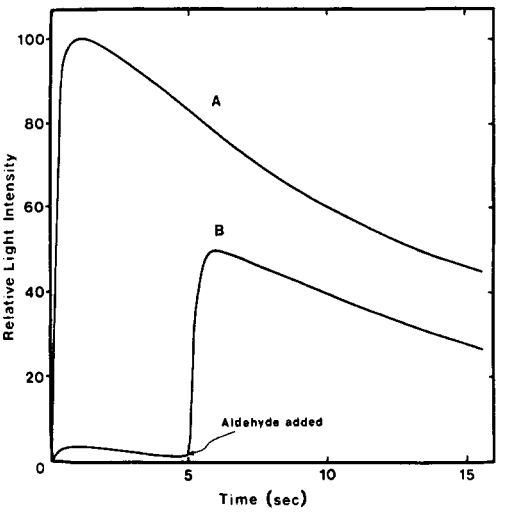

FIGURE 1: (A) The bioluminescent activity of luciferase measured by the dithionite assay. One milliliter of 0.1% dodecanal was injected at zero time into 2.0 ml of 0.01 M phosphate–0.1% bovine serum albumin (pH 7.0) containing luciferase, FMN, and a small amount of $Na_2S_2O_4$. (B) The measurement of the decay of the EFO complex by the delayed addition of aldehyde. One milliliter of catalytically reduced FMN was injected at zero time into 1.0 ml of 0.02 M phosphate–0.2% bovine serum albumin (pH 7.0) containing luciferase. After 5 sec, 1.0 ml of 0.1% dodecanal was injected.

decays exponentially. These maximal light intensities give a relative measure of the amount of EFO at the time of injection of aldehyde and thus the decay rate of EFO can be measured.

In this communication we have used flavine analogs to study various steps in the bioluminescent reaction. The effects of side-chain substituents of the 7,8-dimethylisoalloxazine ring on flavine binding, on the decay of intermediates, and on the activity[3] of luciferase have been examined. Evidence is presented for the role of the phosphate of FMN both in binding and in the production of light.

Experimental Section

Materials. Bacterial luciferase, isolated from *Photobacterium fischeri*, strain MAV, by the method of Gunsalus-Miguel *et al.* (1972), was a gift from Dr. J. W. Hastings, Biological Laboratories, Harvard University, Cambridge, Mass. The enzyme had a specific activity of 2×10^{13} quanta/sec per mg when assayed with FMN and dodecanal in the standard assay. Triton X-100 and the various aldehydes were from Eastman. Bovine serum albumin was from Pentex, and 30% platinized asbestos was obtained from the Arthur H. Thomas Co. Thin-layer chromatograms were Polygram cellulose sheets from Machery-Nagel and Co., and DEAE-cellulose (DE-23) was a product of Whatman Co. Phosphate buffers were made by mixing appropriate volumes of K_2HPO_4 and NaH_2PO_4. All other compounds were reagent grade.

Flavines. FMN and riboflavine were obtained from Sigma. Samples of isoriboflavine, 3-methylriboflavine, ω-hydroxyalkylflavines, and ω-carboxyalkylflavines (Föry *et al.*, 1968) were gifts from Dr. D. B. McCormick, Section of Biochemistry

[2] This fact distinguishes the bacterial system kinetically from most other enzymatic reactions in which a mole of product is produced per mole of ES complex that turns over. The two pathways may be the decay of an excited intermediate *via* nonradiative and light-emitting pathways. However, the mechanism of the bioluminescent reaction is not fully understood and in fact there may exist two or more chemical pathways of decomposition of EFOA.

[3] The activity of luciferase refers to the initial maximal light intensity (I_0) in quanta per second and not to quantum yield (total light emitted). This definition is consistent with the general usage of the term activity with other enzymes to indicate product produced per unit time.

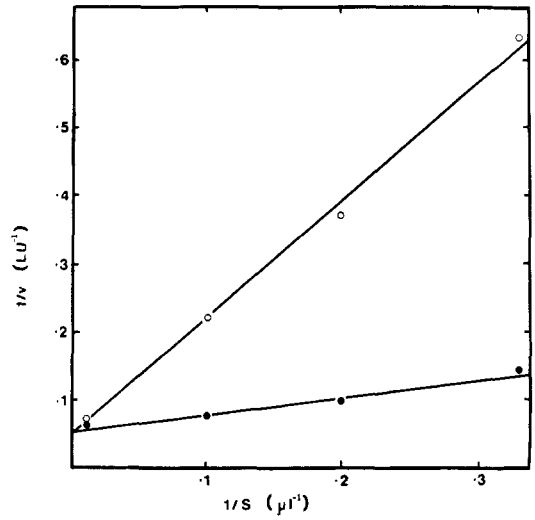

FIGURE 2: Effect of the structure of the flavine side chain on octanal binding to luciferase: (●) FMN; (○) ω-phosphopentylflavine. Octanal (3–100 μl of a 0.01% solution) was added to 1.0 ml of 0.02 M phosphate–0.2% bovine serum albumin (pH 7.0) containing luciferase. The reaction was initiated by injection of 1.0 ml of 5×10^{-5} M flavine catalytically reduced over platinized asbestos. The reciprocal of the initial light intensity ($1/v$) is plotted *vs.* the reciprocal of the microliters of 0.01% octanal ($1/[S]$) added to the assay medium.

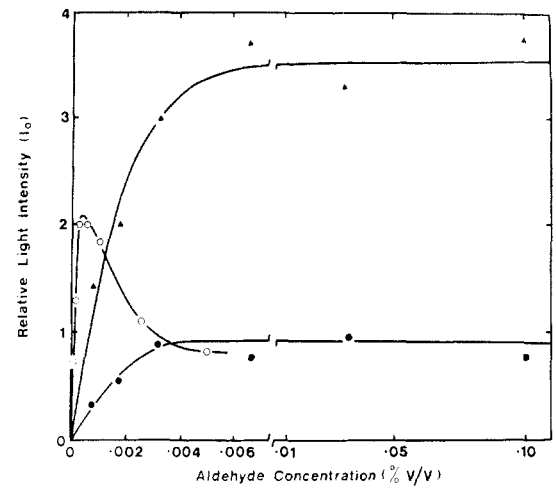

FIGURE 3: The effect of aldehyde concentration on initial light intensity in a modified dithionite assay system containing luciferase and FMN (▲) or ω-carboxyalkylflavine (●). One milliliter of the appropriate concentration of decanal in 0.1% Triton X-100 was injected into 2 ml of 0.01 M phosphate–0.025 M mercapthoethanol (pH 7.0) containing luciferase and 2.5×10^{-5} M flavine reduced with a small amount of $Na_2S_2O_4$ (~0.5 mg). A standard assay for luciferase (○) at different decanal concentrations is included for comparison (see Experimental Section). The decanal concentration is given as the final concentration in the assay medium.

and Molecular Biology, Cornell University, Ithaca, N. Y. The analogs of riboflavine with neutral side chains were converted to FMN derivatives (Flexser and Farkas, 1953) and purified by chromatography on DEAE-cellulose columns. The isolated compounds moved as single spots in butanol–acetic acid–water (2:1:1) on thin-layer chromatograms. The concentrations of side-chain flavine analogs and 3-MeFMN were determined using $\epsilon_{450} = 12.2 \times 10^3$ cm^{-1} M^{-1} for FMN (Whitby, 1953). A value of $\epsilon_{450} = 8.0 \times 10^3$ was used for isoFMN (Föry and Hemmerich, 1967).

Standard Assay. The standard assay for luciferase activity (Hastings *et al.*, 1965) was carried out by injection of 1.0 ml of 5×10^{-5} M flavine (reduced catalytically over platinized asbestos) into 1.0 ml of 0.2% bovine serum albumin–0.02 M phosphate (pH 7.0), containing luciferase (approximately 10^{-8} M in all assays) and aldehyde (10–20 μl of a 0.1% suspension). Light intensity was measured with a photometer similar to that described by Mitchell and Hastings (1971) and recorded graphically on a high-speed recorder (Esterline-Angus, Model S-601-S). The light intensity rapidly rises to a maximum (~1 sec) and then decays exponentially. The maximal light intensity is a measure of enzyme activity in light units (LU) or quanta per second. One light unit (LU) is equivalent to 2.5×10^9 quanta/sec in the present experiments. Decay rates were measured between 15 and 70% of maximal light intensity (I_0).

Results

Preliminary experiments with flavine analogs in the bioluminescent reaction were attempted with a dithionite assay system developed for use in binding studies with FMN (Meighen and Hastings, 1971). In this assay system, one ml of a suspension of aldehyde (0.005–0.01%) is injected rapidly into a solution containing luciferase and FMN reduced with sodium dithionite (~0.5 mg). The assay medium also contains bovine serum albumin at a concentration of 1 mg/ml. However, great difficulty was encountered using this assay system with flavine analogs for two reasons: (a) the albumin preferentially binds the more hydrophobic flavines and (b) the aldehyde concentration was not sufficient to give maximal activity with the analogs. Apparently the binding of aldehyde to luciferase depends on the type of flavine molecule. This latter result is illustrated in Figure 2 which shows the activity of luciferase at different octanal concentrations with two different flavines. Activity measurements were made by injection of reduced flavine into a solution containing the enzyme, bovine serum albumin, and different amounts of aldehyde. The aldehyde concentrations in these assays are not known absolutely since the albumin binds aldehyde and aldehyde solutions are not stable. Since dilutions were made from the same stock solution of aldehyde and the different flavines were assayed consecutively at each aldehyde concentration the relative concentrations of aldehyde in these assays are considered to be accurate. The results show that the dissociation constant for aldehyde depends on the type of flavine substrate used in the bioluminescent reaction. The binding of octanal to luciferase is substantially weaker if ω-phosphopentylflavine is the substrate rather than FMN. These results indicate that the substrates may have a preferred order of addition in the bioluminescent reaction with flavine binding to luciferase prior to aldehyde.

The development of an assay system in which there is no bovine serum albumin and which gives optimal activity was thus necessary to study the flavine analogs. It was found that injection of higher concentrations of aldehyde into a medium containing 0.025 M mercaptoethanol, in the absence of the albumin, gave reproducible and maximal activities with the different flavines. With FMN as substrate, this new assay system (modified dithionite) gave a maximal light intensity 1.7 times greater than that obtained in the standard assay. Furthermore, the inhibition of activity at high concentrations of aldehyde that is observed in the standard assay does not

TABLE I: Relative Activities and Decay Rates of Analogs of FMN with Bacterial Luciferase.[a]

	Octanal		Nonanal		Decanal		Dodecanal	
Flavine[b]	% I_0	Rel Decay Rate[c]	% I_0	Rel Decay Rate[c]	% I_0	Rel Decay Rate[c]	% I_0	Rel Decay Rate[c]
ω-Phosphohexylflavine	80	5.7	70	3.0	70	2.8	70	3.4
ω-Phosphopentylflavine	140	4.0	90	2.3	110	3.0	120	3.2
ω-Phosphobutylflavine	80	2.7	60	1.5	100	2.1	150	3.7
ω-Phosphopropylflavine	0.8	3.0	0.7	2.3	0.7	2.6	2	4.3
ω-Carboxypentylflavine	26	2.0	18	0.6	32	0.9	70	2.6
ω-Carboxyethylflavine	0.06		0.1		0.1		0.1	
Riboflavine	0.4	1.7	0.5	1.4	0.3	1.2	0.7	1.8
IsoFMN	0.3	0.7	0.6	0.4	0.4	0.4	0.5	0.6
Control (no flavine)	0.05	1.0	0.05	1.0	0.06	1.0	0.05	1.0

[a] Enzyme assays were initiated at 25° by injection of 1.0 ml of a 0.1% aldehyde suspension in 0.1% Triton X-100 into 2.0 ml of 0.01 M phosphate–0.025 M 2-mercaptoethanol (pH 7.0) containing luciferase and 2.5×10^{-5} M flavine reduced by a small excess of sodium dithionite. All assays with a given aldehyde suspension were conducted over a short period of time (<1 hr) to minimize fluctuations in temperature and aldehyde concentration. The initial light intensities and decay rates were divided by those measured for FMN in control assays with the same aldehyde to give the relative activities (% I_0) and decay rates. The initial light intensities of luciferase and FMN with octanal, nonanal, decanal, or dodecanal were in the ratios of 15:100:100:12, and the decay rates were 3.5, 23, 23, and 3.3 min^{-1}, respectively. The quantum yields (total light emitted) for each flavine relative to FMN can be calculated by dividing the relative light intensities (% I_0) by their relative decay rates. The quantum yield of luciferase with FMN is essentially identical with these aldehydes. [b] Other flavine analogs with very low activity under these assay conditions are given in Table III. The activity of 3-MeFMN was found to be 1% of FMN. However, its decay rates with all aldehydes were identical with those of FMN which indicates the presence of contaminating FMN. [c] The decay rate may reflect decomposition of the intermediate (EFOA) *via* one or more pathways, including dissociation of the aldehyde and decay of the EFO complex. Decay through this latter pathway might occur even under the present conditions where high aldehyde concentrations has converted all EFO to EFOA if the decay rate of EFO is much more rapid than EFOA (Cline and Hastings, 1972).

occur in the modified assay with either FMN or ω-carboxyalkylflavine (Figure 3). Similar results were obtained with other flavines and/or aldehydes. Comparison of the relative aldehyde concentrations between the two assays is not possible since the albumin binds aldehyde in the standard assay, and the mercaptoethanol complexes aldehyde as a thiohemiacetal in the modified dithionite assay.

The following assay system (modified dithionite assay) was adopted for the study of the various flavine analogs, in particular for the measurement of their binding to luciferase. One milliliter of 0.1% aldehyde in 0.1% Triton X-100 is rapidly injected into 2 ml of 0.01 M phosphate–0.025 M mercaptoethanol (pH 7.0) containing luciferase and the flavine reduced with a small amount of $Na_2S_2O_4$ (~0.5 mg). This assay system is identical with the dithionite assay except that mercaptoethanol has replaced the albumin and higher concentrations of aldehyde are injected.

Dependence of the Activity and Decay Rate of Luciferase on Flavine Structure. The relative activities and decay rates of bacterial luciferase with different flavines and aldehydes are given in Table I. The activities with the different flavines are expressed as percentages of the activity obtained with FMN and the corresponding aldehyde. Luciferase has a very low activity with flavines that contain an uncharged side chain as illustrated by riboflavine which has 0.5% the activity of FMN. Similar results were obtained with other flavines containing neutral side chains. In contrast, high activities were observed with flavines possessing negatively charged side chains with the negative charge located >8.4 Å[4] from the ring. The ω-phosphopropylflavine derivative, in which the negative charge is located 7.6 Å from the ring, has an activity of only 1% of FMN, which illustrates the importance of the location of the negative charge of the flavine side chain at greater than a minimal distance from the ring.

The relative activity (I_0) and decay rate for riboflavine with dodecanal are approximately 15% of the previously reported values (Hastings *et al.*, 1969) and may reflect differences in the assay methods. It is interesting to note, however, that essentially identical quantum yields were obtained in both studies.

It may be noted that the secondary hydroxyl groups on the flavine side chain are not necessary for the activity with luciferase. In addition, the negatively charged phosphate group can be substituted by a negatively charged carboxyl group as in ω-carboxypentylflavine. The negative charge is located 8.4 Å from the ring in this derivative and fairly high activity, 25–70% of that of FMN, is observed with the different aldehydes. However, the shorter ω-carboxyethylflavine derivative is essentially inactive, presumably because the negative charge is not located far enough from the ring.

Two flavine analogs, modified in the isoalloxazine ring structure, isoFMN and 3-MeFMN, were also studied in the

[4] The distance in Ångstroms refers to the maximal linear distance from the center of the N-10 of the ring to the center of the negatively charged atom, as measured using CPK space-filling models.

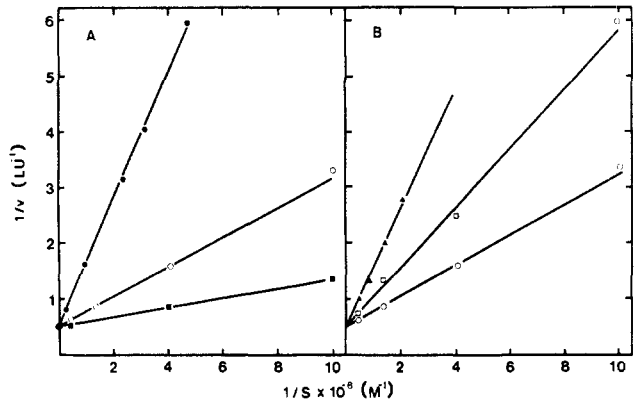

FIGURE 4: (A) Relationship between the reciprocal of the initial light intensity ($1/v$) and the reciprocal of the reduced flavine concentration for different flavines with luciferase (ω-carboxypentylflavine (●), FMN (○), and ω-phosphohexylflavine (■)). The light intensities at saturating flavine concentrations for ω-carboxypentylflavine and ω-phosphohexylflavine have been normalized to that of FMN. (B) Competitive inhibition of FMN (○) by 2.5×10^{-5} M ω-hydroxypentylflavine (□) and 2.5×10^{-5} M ω-hydroxylpropylflavine (▲). The initial light intensities were obtained by the modified dithionite assay described in the text. The flavine concentrations in the above plot were those prior to injection of dodecanal.

TABLE II: Dissociation Constants of the Flavine–Enzyme Complex.[a]

Flavine	Dissociation Constant (M × 10^7)	Method of Anal.
FMN	5	K_d
ω-Phosphohexylflavine	2	K_d
ω-Phosphopentylflavine	2	K_d
ω-Phosphobutylflavine	5	K_d
ω-Phosphopropylflavine	400	K_i
ω-Carboxypentylflavine	25	K_d
ω-Carboxyethylflavine	200	K_i
Riboflavine	300	K_d, K_i
ω-Hydroxyhexylflavine	200	K_i
ω-Hydroxypentylflavine	250	K_i
ω-Hydroxybutylflavine	150	K_i
ω-Hydroxypropylflavine	100	K_i
isoFMN	200	K_i
3-MeFMN	≥1000	K_i

[a] Experimental details are given in Figure 4.

present investigation. Although 3-MeFMN had 1% the activity of FMN with luciferase, its decay rates with all aldehydes were identical to that of FMN indicating that its activity may arise from contaminating FMN. IsoFMN, on the other hand, had different decay rates from FMN.

The relative decay rates of the flavine analogs lacking secondary hydroxyl groups on the side chain were generally more rapid than those with FMN with all aldehydes as shown in Table I. These results indicate that the hydroxyl groups on the flavine side chain stabilize the enzyme–flavine–oxygen–aldehyde complex. However, carboxypentylflavine has a slower decay rate with nonanal and decanal and a faster decay rate with octanal and dodecanal than FMN. Thus, the relative decay rate of the EFOA intermediate is dependent on both the flavine and aldehyde and their interaction in this complex. The slower decay rate of isoFMN with all aldehydes indicates that the removal of the methyl group from the 8 position on the ring and/or substitution onto the 6 position of the ring also stabilizes the EFOA intermediate.

Binding of Reduced Flavine to Luciferase. Since the relative activities given in Table I were obtained at a flavine concentration of 2.5×10^{-5} M, it is possible that the differences in activities simply reflect the relative affinity of luciferase for the different flavines. The dissociation constants of the enzyme–flavine complex (EF) were obtained either directly by activity measurements (K_d) or by competitive inhibition with $FMNH_2$ (K_i) as illustrated in Figure 4. The dissociation constants for the various flavine analogs are summarized in Table II. Relatively low dissociation constants were obtained for complexes of luciferase with flavine analogs possessing a negatively charged side chain located >8.4 Å from the ring. The higher dissociation constant obtained for ω-carboxypentylflavine as compared to that of the phosphoalkylflavine derivatives may simply reflect the slightly shorter side chain rather than the substitution of the carboxyl for a phosphate group. The ω-phosphopropylflavine and ω-carboxyethylflavine analogs, in which the negative charge is ≤7.6 Å from the ring, have substantially higher dissociation constants than the other flavines with negatively charged side chains. The secondary hydroxyl groups on the side chain do not appear to be important for binding as shown by comparison of the dissociation constants of ω-phosphopentylflavine and FMN. Flavines with neutral side chains have dissociation constants between 1×10^{-5} and 4×10^{-5} M which may primarily reflect the intrinsic binding of the flavine ring to luciferase.

The importance of the ring structure for binding is reflected in the dissociation constants obtained for 3-MeFMN and isoFMN, both of which contain negatively charged side chains. The result with 3-MeFMN indicates that the hydrogen on the 3 position of the ring may be absolutely necessary for binding of flavine to luciferase.

Since the activities for luciferase were obtained at a flavine concentration of 2.5×10^{-5} M (Table I) and the dissociation constants obtained for the different flavines are approximately equal to or less than this value, the very low activities given by some of the flavine molecules cannot be primarily attributed to a decreased affinity for bacterial luciferase.

Phosphate Activation of the Bioluminescent Reaction. The previous results show that a negative charge on the flavine side chains is important for activity (Table I) and binding (Table II). Consequently, the effect of high concentration of phosphate on the bioluminescent reaction was investigated. These results are summarized in Table III. The addition of phosphate stimulated the activity of flavines with uncharged side chains in the bioluminescent reaction. Stimulation was not observed with any flavine which has a negatively charged side chain even for those analogs in which the negative charge is located very close to the ring and have very low activity.

The extent of stimulation is dependent on the length of the flavine side chain. Marked stimulation of the bioluminescent reaction by phosphate is obtained with ω-hydroxyethylflavine, ω-hydroxypropylflavine, ω-hydroxybutylflavine, and riboflavine. Little or no stimulation is observed with ω-hydroxypentylflavine and ω-hydroxyhexylflavine. These results indicate that phosphate added independently of flavines with neutral side chains can perform a function similar to that of the phosphate ester of FMN and its analogs. The lower stimulation of the neutral longer chain derivative by phos-

TABLE III: Effect of Phosphate on the Activity of Luciferase with Flavine Analogs.[a]

Flavine	Octanal		Nonanal		Decanal		Dodecanal	
	Low Phosphate	High[b] Phosphate	Low Phosphate	High[b] Phosphate	Low Phosphate	High[b] Phosphate	Low Phosphate	High[b] Phosphate
Riboflavine	0.3	2	0.3	1.6	0.3	1.3	0.6	3
ω-Hydroxyhexylflavine	0.2	0.3	0.3	0.4	0.5	0.4	0.5	0.5
ω-Hydroxypentylflavine	0.4	0.5	0.5	0.6	0.6	0.7	0.6	1.8
ω-Hydroxybutylflavine	0.5	7	0.4	7	0.8	7	1.3	22
ω-Hydroxypropylflavine	0.5	10	0.9	15	1.2	15	2.8	28
ω-Hydroxyethylflavine	0.05	0.5	0.05	0.5	0.07	0.4	0.1	0.6
Control (no flavine)	0.05	0.03	0.05	0.03	0.06	0.03	0.05	0.04

[a] The activities are given as percentages of the initial light intensities obtained with FMN and the corresponding aldehyde in the modified dithionite assay (low phosphate). No activation was observed with any flavine possessing a negatively charged side chain. The decay of the light intensity of luciferase (k_R) with all the neutral flavines was more rapid than with FMN under the same conditions. The addition of phosphate had no effect on the decay rate with FMN and had either no effect or decreased the decay rate (up to 6-fold) with the neutral flavines. [b] The assay conditions were identical with the modified dithionite assay except 0.50 M phosphate was substituted for 0.01 M phosphate (pH 7.0).

phate may arise from the interference of the side chain with binding of free phosphate to the enzyme. In contrast, ω-hydroxypropylflavine and ω-hydroxybutylflavine in high phosphate give up to 20–30% of the activity of FMN.

Activation of the Bioluminescent Reaction by Salts. The specificity of the ion activation of luciferase with uncharged flavines was investigated and the results are presented in Table IV. Since NaCl and KCl have little effect, the activation by phosphate buffer is clearly due to the phosphate anion. The results also show that other anions can activate the bioluminescent reaction with neutral flavines, and in particular, sulfate is even more effective than phosphate, at pH 7.0. Very little activation is obtained with the monovalent anions, Cl^-, HCO_2^-, and $CH_3CO_2^-$. Divalent anions are thus very effective for activation of the bioluminescent reaction and play an important role in the emission of light by luciferase.

Competitive Inhibition by Phosphate of the Binding of $FMNH_2$ to Luciferase. Phosphate activation of the bioluminescent reaction with flavines having neutral but not charged side chains, indicates that phosphate binds to luciferase at the same site as the phosphate ester of $FMNH_2$. Accordingly, experiments were conducted to determine if phosphate is a competitive inhibitor of $FMNH_2$. It was found, however, that increasing the phosphate concentration initially decreased rather than increased the apparent K_d of $FMNH_2$. This observation is probably an ionic strength dependent change in affinity of luciferase for $FMNH_2$. Only above 0.05 M phosphate did the apparent K_d increase with increasing phosphate concentration (Figure 5), as expected for competitive inhibition. Since for competitive inhibition, the apparent K_d equals $K_d(1 + [I]/K_i)$, the slope of apparent K_d *vs.* phosphate concentration (above 0.05 M) is simply K_d/K_i. A K_d for $FMNH_2$ of 2.2×10^{-7} M at high phosphate concentrations was obtained by extrapolating the linear portion of the curve to zero

TABLE IV: Specificity of Anion Activation of Luciferase with Flavine Analogs.[a]

Salt[b]	ω-Hydroxypropylflavine		ω-Hydroxybutylflavine		Riboflavine	
	% I_0	% Quantum Yield	% I_0	% Quantum Yield	% I_0	% Quantum Yield
None	3	0.5	2	0.3	0.6	0.3
NaCl	5	0.7	7	0.7	0.8	0.3
KCl	5	1.0	7	0.9	0.8	0.3
NaO_2CH	5	0.8	5	0.7	1.3	0.6
NaO_2CCH_3	8	1.7	4	0.8	0.9	0.5
Na_3AsO_4	16	5.0	10	1.9	1.6	1.4
Na_2SO_4	40	7.5	45	7.0	12	5.0
Na–KPO_4[c]	25	8.3	20	4.0	4	2.5

[a] Initial light intensities and quantum yields were obtained by injection of 1.0 ml of 0.1% dodecanal into 2.0 ml of 0.01 M phosphate–0.025 M mercaptoethanol (pH 7.0) containing 2.5×10^{-5} M flavine, $Na_2S_2O_4$ (~0.5 mg), luciferase, and the indicated salt at a concentration of 0.5 M. The light intensities and quantum yields are given as percentages of a control assay with FMN. [b] The stock solutions were adjusted to pH 7.0 with HCl or NaOH. [c] The phosphate buffer was prepared by mixing NaH_2PO_4 and K_2HPO_4 solutions.

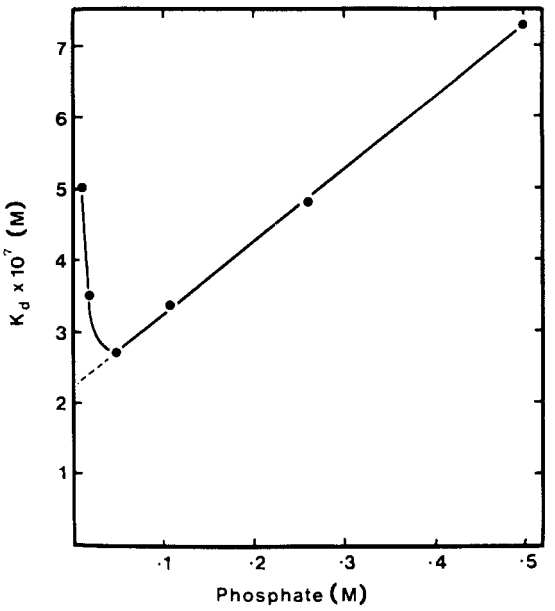

FIGURE 5: The dependence of the apparent K_d of $FMNH_2$ on the concentration of phosphate in the assay medium. The dissociation constants were measured as described previously by injection of dodecanal into 2.0 ml of phosphate buffer (pH 7.0) containing 0.025 M mercaptoethanol, luciferase, flavine, and a small excess of $Na_2S_2O_4$. The phosphate concentration is that prior to injection of aldehyde.

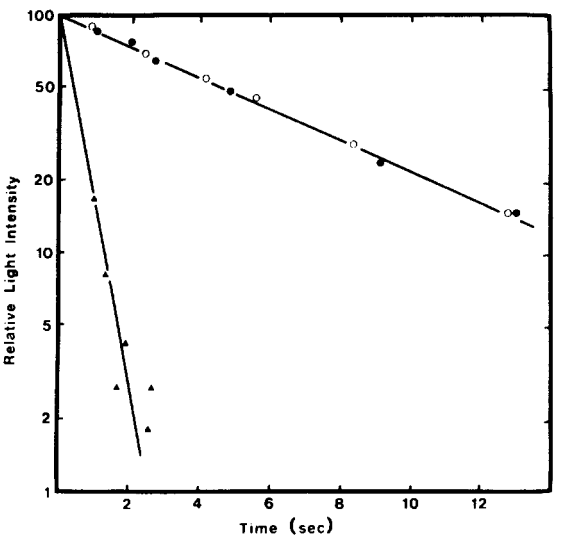

FIGURE 6: The dependence of the decay rate (k_2) of the enzyme–flavine–oxygen complex (EFO) on the flavine side chain (ω-phosphobutylflavine in 0.01 M phosphate (O) and 0.50 M phosphate (●); ω-hydroxybutylflavine in 0.50 M phosphate (▲)). The reaction was initiated by injection of 1.0 ml of 5×10^{-5} M catalytically reduced flavine into 1.0 ml of 0.02 or 1.0 M phosphate (pH 7.0) containing luciferase and 0.2% bovine serum albumin. At subsequent times, 1.0 ml of 0.1% dodecanal was injected which resulted in the light intensity rising to a maximum and then decaying exponentially. These maximal light intensities are plotted on a log scale as a function of the time elapsed between injection of the reduced flavin and the aldehyde, and are given as percentages of the light intensity obtained if aldehyde is added prior to reduced flavine.

phosphate concentration. The K_i for phosphate was calculated to be about 0.22 M.

Binding Constant of Phosphate Determined by Activation. Experiments were conducted to measure the extent of activation of the bioluminescent reaction with ω-hydroxypropylflavine and ω-hydroxybutylflavine at different concentrations of phosphate in the modified dithionite assay. From double-reciprocal plots of the initial light intensity in the bioluminescent reaction with ω-hydroxypropylflavine at varying phosphate concentrations, a dissociation constant (K_d) of 0.2 M was obtained for phosphate. A similar experiment with ω-hydroxybutylflavine gave a K_d for phosphate of about 0.15 M. These results are in good agreement with the value (0.22 M) obtained for the dissociation constant of phosphate by competitive inhibition of $FMNH_2$ and indicate that the added phosphate binds at the same site on the enzyme as the phosphate of FMN.

Determination of the Binding of Uncharged Flavines by Activity Measurements. Experiments were conducted on ω-hydroxypropylflavine and ω-hydroxybutylflavine in high concentrations of phosphate buffer to elucidate if their binding constants were affected by phosphate. The dissociation constants of ω-hydroxypropylflavine and ω-hydroxybutylflavine measured in the modified dithionite assay with 0.5 M phosphate (pH 7) gave values of K_d of 1.1×10^{-5} and 1.6×10^{-5} M, respectively. A comparison of the K_d values with the previous dissociation constants measured by competitive inhibition of $FMNH_2$ in low phosphate (Table II) shows the phosphate has little if any effect on the binding of these flavines to luciferase. These results clearly show that the addition of phosphate to the assay medium does not activate the bioluminescent reaction with uncharged flavines by increasing their affinity for luciferase.

Stability of the Enzyme–Flavine–Oxygen Complex. The decay of the intermediate EFO was investigated to elucidate whether a negative charge on the flavine affected its stability. The stability of EFO is measured by the rate constant k_2. In this experiment, the amount of enzyme–flavine–oxygen complex present after initiation of the reaction was measured by the injection of aldehyde. The maximal light intensity obtained after this latter injection was taken as a measure of the amount of EFO complex present at the time of injection. Figure 6 shows a plot of these light intensities as a function of the time elapsed between the initial flavine injection and the subsequent aldehyde injection for the negatively charged ω-phosphobutylflavine and the neutral ω-hydroxybutylflavine. The linear semilogarithmic plots show that the EFO intermediate decays exponentially. The points extrapolate to a value at zero time that is identical to that obtained for the light intensity if aldehyde is added prior to flavine.

The decay rates for the EFO complexes of luciferase with a variety of different flavines are summarized in Table V. The decay rates of the EFO complex containing a flavine with a negatively charged side chain are 8–12 min^{-1}, whereas with flavines with neutral side chains, the decay rate is approximately 90 min^{-1}. These latter measurements were made in 0.50 M phosphate buffer. The experiment with ω-phosphobutylflavine shows that the phosphate concentration has no effect on its decay rate. Thus, it appears that the presence of a negative charge on the flavine side chain greatly stabilizes a complex of flavine and oxygen.

Phosphate Stimulation of Luciferase Activity after Formation of the EFOA Complex. The effect of phosphate on the rate-limiting step in the bioluminescent reaction scheme is demonstrated in Figure 7. In this experiment, the reaction is initiated by injection of reduced flavine into an assay medium containing aldehyde and luciferase. After the initiation of the reaction

TABLE V: Effect of Flavine Side Chain on Stability of the EFO Complex.[a]

Flavine	Phosphate	Decay Rate (min^{-1})
FMN	−	8
ω-Phosphopentylflavine	−	8
ω-Phosphobutylflavine	±	9
ω-Carboxypentylflavine	−	12
ω-Hydroxybutylflavine	+	90
ω-Hydroxypropylflavine	+	70

[a] The decay rate of enzyme–flavine–oxygen complex (EFO) was obtained by injection of 1.0 ml of reduced flavine into 1.0 ml of 0.02 M (−) or 1.0 M (+) phosphate (pH 7.0) containing bovine serum albumin and luciferase followed by injection of 1.0 ml of 0.1% dodecanal at subsequent times as described in Figure 6.

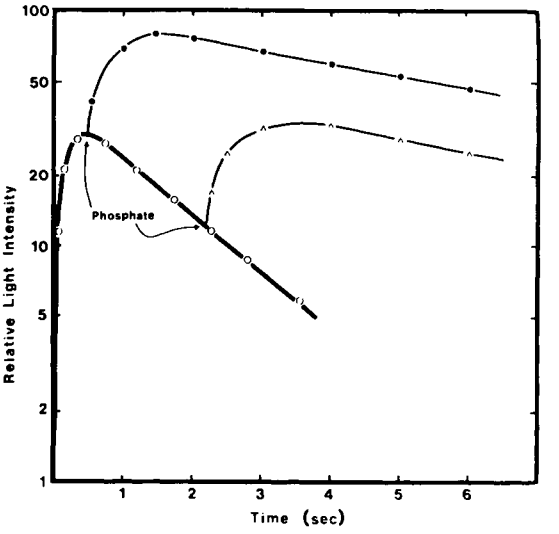

FIGURE 7: Phosphate stimulation of the rate of conversion of the EFOA complex to light. The reaction was initiated by injection of 1.0 ml of 5×10^{-5} M catalytically reduced ω-hydroxybutylflavine into 1.0 ml of 0.02 M phosphate–0.2% bovine serum albumin (pH 7.0) containing luciferase and 50 μl of 0.1% dodecanal. At the times shown above, 0.5 sec (●) and 2.2 sec (△), 1.0 ml of 1.0 M phosphate (pH 7.0) was injected. A control curve in which no phosphate was injected is also shown (○).

by formation of EFO and its conversion to EFOA, a concentrated phosphate solution was injected into the assay medium and, as can be observed in Figure 7 the activity is greatly stimulated. Thus phosphate increases the rate of conversion of the EFOA complex to light. Furthermore, it may be noted that the decay rate in the absence of phosphate for this EFOA complex is about 35 min^{-1}. This was determined both from the data in which phosphate is not injected and by plotting the maximal light intensities after injection of phosphate *vs.* the time elapsed between this injection and the initial injection of reduced flavine. A comparison of this value with the decay rate obtained in the presence of phosphate, about 10 min^{-1}, shows that although the phosphate increases the rate of light formation (light intensity) it actually slows down the turnover rate of the EPOA intermediate.

Discussion

Light emission in the bioluminescent reaction catalyzed by bacterial luciferase is believed to involve an excited state of flavine (Mitchell and Hastings, 1969; Eley *et al.*, 1970). Although the mechanism of the reaction involving reduced flavine, oxygen, and aldehyde is unknown, theories concerning the role of the flavine have centered on the 7,8-dimethylisoalloxazine ring, in particular the 4α and N-5 positions (Eberhard and Hastings, 1972). A possible role of the side chain of FMN in the reaction has not been investigated.

The effects of charge, length, and secondary hydroxyl groups of the flavine side chain on the bioluminescent reaction were examined. Our studies show that a flavine side chain of minimal length with a terminal negative charge is necessary for tight binding and good activity of the flavine with luciferase. The negative charge on the flavine side chain can be satisfied by either phosphate or carboxyl groups. It was also found that the negative charge must be located at least 8.4 Å from the N-10 position of the flavine ring.[4]

Secondary hydroxyl groups on the flavine side chains had little effect on activity (I_0) and binding to luciferase (Table I and II) or on the stability of the EFO intermediate (Table V) as shown by the results with ω-phosphopentylflavine and FMN. However, the more rapid decay of the EFOA intermediate of ω-phosphopentylflavine (Table I) and its decreased affinity for octanal (Figure 2) as compared with the EFOA intermediate of FMN, indicates that the secondary hydroxyl groups of flavine affect the binding of aldehyde to luciferase. The stabilization of the EFOA intermediate may be due to a reduction in the decay rate of the intermediate *via* the dark pathway in the rate-limiting step (k_n) without an effect on its decay by the light pathway (k_m).

The role of the negative charge on the side chain was also demonstrated by the stimulation by phosphate of the bioluminescence of the neutral flavine analogs. Maximal activity for the neutral flavines in 0.5 M phosphate was obtained for flavines with side-chain lengths of 5.8 Å (ω-hydroxypropylflavine) and 7.0 Å (ω-hydroxybutylflavine). It should be noted that ω-hydroxypropylflavine in 0.5 M phosphate had 15 times the activity (I_0) of ω-phosphopropylflavine. This result suggests that the existence of a specific phosphate binding site that cannot be reached by 3′-phosphate of this analog (7.6 Å), but is accessible to the independently added inorganic phosphate. The lower activity and degree of stimulation of the longer chain neutral flavines may arise by interference with the binding of inorganic phosphate by the longer chains.

The effect of phosphate on binding and activity could arise indirectly by inducing a conformational change in luciferase or directly by interaction with the flavine ring. The possibility of side-chain interaction with the flavine ring has been proposed by Edmondson and Tollin (1971b) for the Shethna flavoprotein. These authors suggest that the side-chain phosphate interacts with N-5 of the flavine ring and stabilizes the semiquinone. In addition, interaction with N-3 of the flavine ring has been shown to be important with certain flavoproteins, but not others (Tsibris *et al.*, 1966). For example, 3-MeFMN binds quite strongly to the Shethna protein (Edmondson and Tollin, 1971b) but is a very poor coenzyme with glycolate oxidase (Tsibris *et al.*, 1966). Accordingly, we have tried to correlate our results with the ability of the negative

TABLE VI: Interaction of Side-Chain Negative Charge with the Flavine Ring.

Flavine	Side-Chain Length (Å)[a]	Potential for Interaction with Ring Positions[b] N-5 (H)	N-3 (H)	Rel Act.[c]
ω-Phosphohexylflavine	11.2	+-	++	70
ω-Phosphopentylflavine	10.0	-+	++	120
FMN	10.0	++	++	100
ω-Phosphobutylflavine	8.8	+-	+	150
ω-Carboxypentylflavine	8.4	++	+	70
ω-Phosphopropylflavine	7.6	+-	-	2
ω-Carboxyethylflavine	4.8	+	-	<0.05

[a] See footnote 4. [b] Qualitative assessment of the ability of terminal negatively charged atom to come within Van der Waal's radii of N-3 or N-5 hydrogen atoms of reduced flavines, using CPK space-filling models ((−) no contact, (+) contact just possible, and (++) contact). [c] Activity (% I_0) with dodecanal.

charge of various flavines to interact with hydrogen atoms on these two positions of the hydroquinone.

Table VI gives the side-chain length of the various flavines and their potential for interaction with the N-5 and N-3 hydrogen atoms of the reduced flavine ring. All flavines have the ability for the negatively charged side chain to contact the N-5 hydrogen whereas only flavines with side chains of length greater than 8 Å can come within Van der Waal's radii of the N-3 hydrogen. The relative activity of these flavines corresponds directly to their potential for interaction at the N-3 position rather than the N-5 position. If the negatively charged side chain was folding over the ring structure to interact at the N-5 position then all the flavines should be active in the bioluminescent reaction. Although the results do not allow exclusion of interaction of the negative charge with other positions of the flavine ring, the N-3 hydrogen would seem a strong possibility in view of its role in the keto–enol tautomerism with the C-4 carbonyl group. It is interesting to note that very little activity could be detected (<1%) with 3-MeFMN and this activity may be due to contaminating FMN. Furthermore, the dissociation constant for 3-MeFMN was greater than 1×10^{-4} M whereas FMN has at least 2×10^3 greater affinity. Even the neutral flavines bind with greater affinity than the negatively charged 3-MeFMN.

These results suggest the importance of the ability of the negative charge of the flavine side chain to reach as far as N-3 of the hydroquinone for activity in the bioluminescent reaction. However, because the negative charge on the side chain can be supplied by either phosphate or carboxyl groups on the flavine, or by other anions with neutral flavines, the interaction with the flavine ring may be of an unspecific nature. This is in contrast with the properties of the Shethna protein where phosphate interaction occurs with the N-5 position of the ring, and carboxyl groups cannot substitute (Edmondson and Tollin, 1971a,b). Thus with luciferase the maintenance of a high negative charge density near the flavine ring, rather than, for example, a specific interaction in the keto–enol tautomerism of positions 3 and 4, may be the role played by the phosphate of FMN.

Massey *et al.* (1969) has recently suggested that a negatively charged residue near the flavine ring would be expected to decrease the rate of oxidation of flavine presumably by stabilizing intermediate species of leucoflavine and oxygen. In a similar fashion the role of the negative charge of flavine in the bioluminescent reaction may be simply to stabilize the intermediates EFO, EFOA, and EFOA′ from decay through non-light-emitting pathways. The results in Table V show that the EFO intermediates with neutral flavines decay faster than with the corresponding phosphorylated species. In addition the aldehyde adduct of this intermediate, EFOA, with neutral flavines was stabilized by anions (Figure 7). The higher light yield of the charged flavines as well as the stimulation of activity of neutral flavines with phosphate may be due to stabilization of EFOA′ from decay *via* the dark pathway, k_p, after the rate-limiting step in the reaction.

Eberhard and Hastings (1972) have proposed the EFO and EFOA intermediates in the luciferase-catalyzed reaction to be the 4α-hydroperoxide of the flavine hydroquinone and its aldehyde adduct, respectively. Massey *et al.* (1969) suggested that negative charges near the flavine ring would stabilize such intermediates. Our results show a that negative charge stabilizes the intermediates in the bioluminescent reactions; whether such stabilization involves keto–enol tautomerism or some less specific effect of the negative charge could not be ascertained. However, it is clear that a role for the phosphate of FMN, other than by interaction at N-5, must be included in the overall mechanism of the reaction catalyzed by luciferase.

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Analysis of Oxygen Equilibrium of Hemoglobin and Control Mechanism of Organic Phosphates†

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ABSTRACT: Oxygen equilibrium curves of hemoglobins precisely determined by the automatic recording apparatus have been analyzed according to Adair's (Adair, G. S. (1925), *J. Biol. Chem. 63*, 529) stepwise oxygenation theory, and four successive association constants (k values) for the binding of oxygen have been estimated by the least-squares method. 2,3-Diphosphoglycerate markedly reduces k_1, k_2, and k_3 for human adult hemoglobin without affecting k_4. In contrast, inositol hexaphosphate enormously reduces all four k values. The glycerate also shows a similar but weaker effect on human fetal hemoglobin. The effect of 0.1 M NaCl on the oxygen equilibrium parameters is qualitatively similar to that of the glycerate, except that the neutral salt increases k_4 slightly. Calculation of the hemoglobin fraction of various intermediate oxygenation stages reveals that the fraction of $Hb(O_2)_3$ is negligibly small in the presence of the organic phosphates and/or NaCl except at very high oxygen saturation. Analysis of k values in the presence of 0.1 M NaCl and various concentrations of 2,3-diphosphoglycerate indicates that the phosphates combine not only with the fully deoxygenated hemoglobin but also with the intermediates, $Hb(O_2)_1$ and $Hb(O_2)_2$, with considerable affinity. The binding constant of the phosphate for the fully deoxygenated human adult hemoglobin at pH 7.4 and 25° has been estimated as 3.1×10^4 M^{-1}, which agrees well with that obtained by the direct binding experiment. The overall free energy of interaction among oxygen combining sites is increased by 2 mM 2,3-diphosphoglycerate in the absence of added NaCl by about 5100 and 2700 cal/mol in the adult and fetal hemoglobins, respectively, which corresponds to the formation of four and two, respectively, additional salt bridges between the glycerate and the deoxyhemoglobins. These results are consistent with the model proposed by Perutz [Perutz, M. F. (1970), *Nature (London) 228*, 726] for the cooperative oxygen binding of hemoglobin.

The discovery of the tremendous effect of organic phosphates, especially 2,3-diphosphoglycerate (P_2glycerate)[1] and inositol hexaphosphate (IHP), in lowering the oxygen affinity of human adult hemoglobin (Hb A) (Benesch and Benesch, 1967; Chanutin and Curnish, 1967; Benesch *et al.*, 1968) has drawn much attention to the mechanism and physiological role of this heterotropic interaction. It has been shown that P_2glycerate binds with an overwhelming preference for deoxy Hb A, not for the fully oxygenated hemoglobin, and in a mole:mole ratio in a specified experimental condition (Benesch *et al.*, 1968, 1971). This will undoubtedly reduce the affinity of Hb A to the first oxygen molecule and increase the overall energy of interaction among oxygen binding sites. However, the effect of the phosphates on the oxygen affinity of Hb A in an intermediate stage of oxygenation as well as the overall free energy of interaction in the presence of the phosphates are still unknown. These questions are of critical importance to the understanding of the mechanism of action of the organic phosphates.

The present paper is an approach to these problems from an equilibrium point of view based on the successive oxygenation theory (Adair, 1925). Four successive association constants of hemoglobin for the binding of oxygen estimated by the least-squares method on accurately determined oxygen equilibrium curves in the absence and presence of the organic phosphates will be given together with standard errors involved in the estimation. The binding constant of P_2glycerate for hemoglobin in various oxygenation stages calculated from the four association constants will also be shown. Preliminary accounts of this study, where the association constants were estimated by a trial-and-error curve-fitting procedure on the Hill and Scatchard plots, have been published elsewhere (Tyuma *et al.*, 1971a,b).

Materials and Methods

Materials. Hb A prepared from the blood of normal human adults (nonsmokers) and human fetal hemoglobin (Hb F) isolated from cord blood by the method of Zade-Oppen (1963) were freed from phosphates according to Benesch

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[1] Abbreviations used are: P_2glycerate, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Hb A, human adult hemoglobin; Hb F, human fetal hemoglobin; bisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane.