the work of Snyder & Speck (1939) and Snyder, Smith & Stewart (1944) on the benzylation of thiol compounds, acetoacetate and malonate provides useful information. From this and the data in Tables 1 and 4, the comparisons given in Table 5 have been made. Thus it can be seen that chemical affinity for a reactive hydrogen does not appear to run parallel with biological activity.

As has been suggested in the case of contact insecticides (Davies & Sexton, 1948), it is probable that biological activity depends upon having the correct degree of chemical reactivity in the group concerned, balanced with physical properties which ensure maximum ease of penetration to the site of action.

After our work had been completed, a paper by Mitchell, Wirwille & Weil (1949) appeared, recording plant-growth regulatory activity in certain quaternary salts containing benzyl and substituted benzyl radicals. Prill, Barton & Solt (1949) have also observed a reduction of the growth of wheat roots by various surface-active compounds of which the most active were two quaternary ammonium salt derivatives, both of which reduced root growth by 50% at concentrations of 5 p.p.m.

SUMMARY

1. The herbicidal activity of quaternary ammonium salts has been examined by a laboratory seed-germination technique, using rape and oats.

2. In the homologous series of *n*-alkyltrimethyl ammonium salts activity against both species rose as the series was ascended. In the case of rape, activity diminished after C₁₄. In the case of oats the position of the maximum was not determined, but it appeared to be higher.

3. In a series of quaternary salts containing benzyl or substituted benzyl radicals, oat seeds were more susceptible than rape.

4. In contrast to the results in the phenoxyacetic acid series, the herbicidal activity was but little affected by the introduction of substituent groups (e.g. chlorine) into the benzene ring. A sulphonic acid group brought about complete inactivation.

5. The activity was greatly influenced by the nature of the other substituent groups on the nitrogen atom.

6. The results are discussed in relation to the known ability of certain quaternary salts to alkylate thiol groups.

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Fluorescence of Riboflavin and Flavin-adenine Dinucleotide

By G. WEBER (Beit Memorial Fellow)
Biochemical Laboratory, University of Cambridge

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The fluorescence of riboflavin has been extensively studied with a view to the determination of this substance. Relatively little work has been done on the variations of this property in the compounds of riboflavin of biological importance such as flavin-adeninedinucleotide (FAD) and the flavoproteins.

The variations in fluorescence under different conditions, and notably after addition of quenchers, can give some indication of the configuration of a

molecule. The following is a study of the fluorescence of riboflavin and FAD from this point of view.

EXPERIMENTAL

The intensity and the degree of polarization of the fluorescent light and the absorption spectrum were determined with solutions of riboflavin and FAD under various conditions.

For the measurement of the intensity of the fluorescence, the exciting light was filtered through a combination of blue and green filters (Corning glass 5850 and 978), while the fluorescent light reached the photocell through an orange filter (Corning glass 351). The photocell was of the barrier-layer selenium type. The same combination of filters was used in the observations of the polarization of the fluorescence. The exciting light was linearly polarized.

The percentage of polarization of the fluorescent light is given by the identity (Wood, 1934)

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}},$$

where $I_{\parallel}$ is the intensity of the polarized wave with its electric vector parallel to that of the exciting light, while $I_{\perp}$ is the intensity of the polarized wave with its electric vector normal to that of the exciting light. The measurement of the polarization of the fluorescence was made by means of a Savart plate and an Arago compensator. The latter was calibrated by viewing the light reflected from the face of a glass prism, the fraction of polarized light being calculated by the use of the Fresnel equations (Gaviola & Pringsheim, 1926). For the measurements of low degrees of polarization a pile of glass plates was placed between the source and the compensator (Perrin, 1936) so as to increase the amount of polarized light in the latter, and a new calibration curve was made. The general arrangement of the apparatus was similar to that described by Perrin (1929) and will be described in detail in another paper.

The absorption spectrum of the solutions were measured with a Beckman photoelectric spectrophotometer. The viscosity of the glycerol-water mixtures used in some experiments was obtained with an Ostwald viscometer.

RESULTS

Fluorescence of riboflavin and the action of quenchers

The fluorescence of flavin-adeninedinucleotide (FAD) was found to be much weaker than that of free riboflavin and this was traced, as later shown, to a quenching effect of the adenine part of the molecule. A similar quenching effect is produced by the addition of adenine compounds or of purines to solutions of riboflavin. The quenching effect of purines differs considerably from that of electrolytes like potassium iodide or potassium thiocyanate (Weber, 1948). The fluorescence of riboflavin is quenched by a number of substances: electrolytes, diphenols, purines and pyrimidines. Table 1 shows the relative efficiency of these quenchers. I/I_0 is the ratio of the fluorescence intensities after and before the addition of the quencher respectively. It is also the ratio of the corresponding fluorescence efficiencies, provided no change in the absorption spectrum of the fluorescent solution over the range of absorbed and emitted wavelengths takes place on addition of quencher (Sveshnikoff, 1936). Table 1 also shows that silver ions are the most effective of all quenchers studied. They are followed by the purines, which exhibit small differences among themselves. Breaking of the pyrimidine ring is followed by complete loss of

the quenching ability as shown by allantoin. Potassium iodide, the most effective of the electrolytes is slightly less effective than the diphenols.

Table 1. *Quenching of the fluorescence of riboflavin by different substances*

(I/I_0 is the relative fluorescent efficiency of an aqueous solution containing 10^{-2} M-quencher and 6.9×10^{-6} M-riboflavin. Measurements made at room temperature. pH values varied from 6 to 8.)

Quencher	I/I_0
Adenine	0.50
Adenosine	0.50
Hypoxanthine	0.51
Caffeine	0.53
Muscle adenylic acid	0.63
Thymine	0.71
Cytosine	0.77
Allantoin	0.99
Potassium iodide	0.72
Hydroquinone	0.66
Catechol	0.65
Silver nitrate	0.03
Silver acetate	0.03

Action of potassium iodide. The quenching of the fluorescence of dyes in solution has been explained as due to collisions of the second kind, i.e. collisions

Fig. 1. Quenching of the fluorescence of riboflavin by KI. Riboflavin concentration, 7×10^{-6} M; I_0/I , ratio of the intensities of the fluorescence before and after adding the quencher.

in which the electronic excitation energy is transformed into kinetic energy, as opposed to collisions of the first kind, in which the reverse process takes place. In this type of quenching the efficiency of the quencher at low concentrations is constant and proportional to the lifetime of the excited state of the fluorescence, while at higher concentrations the efficiency of the quencher increases steadily. This is apparent from Fig. 1, which depicts the quenching

of the fluorescence of a solution of riboflavin by potassium iodide at two different temperatures. From the slopes of the linear part of the curves it is possible to calculate the mean life of the excited state using an equation derived by Wawilov (1928). In this way it is found that the lifetime of the excited state of riboflavin is approximately 1×10^{-8} sec. On comparing the two curves shown in Fig. 1, it is clear that the quenching is more effective at the higher temperature owing to the increase in the number of collisions and the decrease in the viscosity of the solvent (Wawilov, 1928).

The quenching by collisions of the second kind results in a shortening of the lifetime of the excited state. In this case, according to Perrin (1929),

$$\frac{I_0}{I} = \frac{\tau_0}{\tau}, \quad (1)$$

where τ_0 and τ are the mean values of the lifetime of the excited state before and after quenching. The ratio τ_0/τ can easily be deduced from measurements of the polarization of the fluorescence (Sveshnikoff, 1936). If p_i is the initial polarization of the fluorescence before quenching, p_o the fundamental polarization* and p the observed polarization after addition of quencher, we have

$$\frac{\tau_0}{\tau} = \frac{1 - \frac{1}{p_i}}{1 - \frac{1}{p}} \cdot \frac{p_o}{p_o} \quad (2)$$

Table 2 gives the values of I_0/I and τ_0/τ , the latter calculated from equation 2, for a series of solutions of

Table 2. *Effect of quenching by potassium iodide on the lifetime of the excited state of the fluorescence of riboflavin*

(Glycerol-water mixture of viscosity 0.040 c.g.s. unit at 17°. Riboflavin concentration 7×10^{-6} M.)

KI concn. (M)	I_0/I	p	τ_0/τ
0	1	0.038	1
0.050	1.46	0.053	1.43
0.110	2.18	0.076	2.17
0.191	3.03	0.098	2.95
0.335	5.0	0.135	4.35
0.620	10.0	0.200	7.7

riboflavin in a glycerol-water mixture of viscosity 0.040 c.g.s. units at 17°, containing increasing amounts of potassium iodide. It was necessary to

* The so-called fundamental polarization, p_o , is the polarization attained in a medium where the fluorescent molecules cannot rotate appreciably in the interval between the absorption and the emission of light. In practice p_o differs little from the polarization in a medium of high viscosity, such as glycerol. For riboflavin p_o , as shown later, reaches approximately the theoretical maximum of 0.50.

use glycerol-water mixtures as solvent in these experiments, in order to have an initial polarization which could be measured with some accuracy.

The agreement of the values quoted in Table 2 with Perrin's rule (equation 1) can be considered good except at high values of the ratio I_0/I . For an interpretation of this see Wawilov & Franck (1931) and Weber (1948).

Action of purines. The results obtained for the quenching by purines, stand in contrast to those just discussed for the quenching by potassium iodide. From several facts to be reported presently we have concluded that a non-fluorescent complex is formed between the riboflavin and the purine molecules.

Let $[F_o]$ denote the total concentration of riboflavin, $[C_o]$ that of quencher, and $[FQ]$ the concentration of complex the degree of dissociation of which is denoted by α . Then

$$\begin{aligned} [F] &= \text{Free riboflavin concentration} = \alpha[F_o], \\ [FQ] &= (1 - \alpha)[F_o], \\ [Q] &= \text{Free quencher} = [C_o] - (1 - \alpha)[F_o]. \end{aligned}$$

Since $[F_o]$ is negligibly small compared with $[C_o]$ we can write $[Q] = [C_o]$. Applying the law of mass action, we have

$$K = \frac{[F][Q]}{[FQ]} = \frac{\alpha}{1 - \alpha} [C_o]. \quad (3)$$

If only the free molecules can emit, $I/I_0 = \alpha$, and

$$\frac{[C_o]}{K} = \frac{I_0}{I} - 1. \quad (4)$$

If the concentration of quencher is plotted against $(I_0/I) - 1$ the reciprocal of the slope gives the dissocia-

Fig. 2. Quenching of the fluorescence of riboflavin by caffeine. Riboflavin concentration, 7×10^{-6} M; I_0/I , ratio of the fluorescent intensities before and after addition of quencher.

tion constant of the complex. The measurements show (Fig. 2) that, in contradistinction to the quenching by potassium iodide, K is a true constant

for the whole range of concentrations studied. On the other hand, measurements of K at two temperatures reveal an increase of the dissociation of the non-fluorescent complex when the temperature is raised. Table 3 gives the dissociation constants at

Table 3. *Dissociation constants of purine-riboflavin complexes*

(Solvent 0.02M-phosphate buffer pH 7.5.)

Quencher	Temp. (°)	$K \times 10^3$	S.E.
Adenosine	5	7.4	0.4
Adenosine	17	8.3	0.3
Caffeine	5	9.8	0.5
Caffeine	17	11.0	0.3

two temperatures for the quenching of the fluorescence of riboflavin by adenine and by caffeine at neutral pH. The variation of the quenching with temperature proved to be a perfectly reversible phenomenon. The figures of Table 3 show that the ratio K_{25}/K_{17} is approximately the same for both purines and equal to 1.13. Assuming that the constants are determined with an accuracy of $\pm 5\%$, the van't Hoff isochore gives the heat of dissociation of the complex as -1.6 ± 0.6 kg.cal./mol.

Effect of purine quenching on the lifetime of the excited state. When a solution of riboflavin is quenched by caffeine or adenosine, in contradistinction to potassium iodide, no increase in the polarization of the fluorescence took place, up to the highest ratio of I_0/I experimentally attained (about 20). Quenching by these substances has therefore no effect on the lifetime of the excited state of the fluorescence. In the event of complex formation (Weber, 1948)

$$\frac{\tau_0}{\tau} = \frac{I_0}{I} \alpha$$

$$\text{and} \quad \frac{\Sigma}{\tau_0} = \frac{\left(\frac{I_0 \tau}{I \tau_0} - 1 \right)}{\left(\frac{\tau_0}{\tau} - 1 \right)}, \quad (5)$$

where Σ is the mean life of the complex. Experimentally, as we have just mentioned, $\tau_0/\tau = 1$ for all accessible concentrations of quencher, and therefore $I/I_0 = \alpha$ which justifies the introduction of this expression in deriving equation 4. Equation 5 allows a calculation of Σ/τ_0 to be made from the experimentally determined values of I/I_0 and τ/τ_0 . In the quenching of the fluorescence of riboflavin by purines it is only possible to get a minimum value for Σ/τ_0 as the change in the lifetime of the excited state on quenching falls within the experimental error of its determination, and it may be concluded that

$$\frac{\Sigma}{\tau_0} > 100 \quad \text{or} \quad \Sigma > 10^{-6} \text{ sec.}$$

Modification of the absorption spectrum of riboflavin on addition of purines. Fig. 3 shows the absorption spectra of (1) a riboflavin solution (dark circles), (2) the same containing 0.050M-adenosine (open circles), and (3) the same containing 0.052M-caffeine (crosses). In the last two solutions the value of I_0/I was very approximately the same, namely 4.5. The modifications of the absorption spectrum consist of a decrease in the height of the two absorption maxima at 375 and 454 m μ . and in a displacement of the red edge of the absorption band towards longer wavelengths. The absorption spectrum of riboflavin in the presence of purines strikingly resembles the spectrum of FAD described by Warburg & Christian (1938).

Fig. 3. Absorption spectrum of riboflavin solutions in the presence of purines. Riboflavin, ●—●; riboflavin in 0.05M-adenosine, ○—○; riboflavin in 0.052M-caffeine, ×—×. Riboflavin concentration 1.07×10^{-5} M.

Effect of pH on purine quenching. The quenching efficiency of caffeine on riboflavin is not affected by a change in pH of the solution. Adenine or adenosine, on the other hand, lose much of their quenching ability in acid media. If the pH of a solution of riboflavin containing a fixed amount of adenosine (Fig. 4) is plotted against the intensity of the fluorescence, a maximum of the latter is attained at pH 2.6–2.8. This maximum is due to a combination of two simultaneous effects, the decrease in the quenching efficiency of adenine and the direct quenching of the fluorescence by the hydrogen ions. The last effect is discussed below.

Quenching of the fluorescence of riboflavin by acids and alkalis. The fluorescence of riboflavin vanishes in strongly acid or alkaline solutions, a fact that has been explained by the assumption that the fluorescent form is the dipolar ion while the cationic and anionic forms are completely non-fluorescent (Kuhn & Moruzzi, 1934). These authors calculated the dissociation constants of the two forms on the assumption that the degree of dissociation, $\alpha = I/I_0$, in every case. As we have seen, this is a fair approximation only if the lifetime of the excited state of the fluorescence is not appreciably changed on quenching.

Table 4. *Effect of the hydrogen-ion concentration on the intensity and polarization of the fluorescence of riboflavin*

(Solvent: glycerol-water mixture of viscosity 0.031 c.g.s. unit at 17°; E is the extinction value at 450 m μ . The last column of the Table is the degree of dissociation of the anionic form of riboflavin calculated on the assumption $\alpha = E/E_0$, E_0 being the value of the extinction in the absence of acid.)

HCl concn. (M)	I_0/I	τ_0/τ	$E_{450 \text{ m}\mu}$	$\alpha = E/E_0$
0	1	1	0.080	1
0.62	3.31	3.34	—	—
1.25	5.44	5.72	0.075	0.94
2.50	10.7	9.7	0.063	0.79
3.75	19.2	14.5	0.058	0.72
5.00	38.5	18.7	—	—
6.25	50	20	0.050	0.62

The quenching of the fluorescence of riboflavin by hydrochloric acid showed a shortening of the lifetime of the excited state much the same as that found in the quenching by potassium iodide. The results are given in Table 4. It is apparent that equation 4 cannot be applied to the quenching of riboflavin by

This means that at this particular hydrogen-ion concentration, less than one-tenth of the excited molecules emit radiation, the other nine-tenths passing into the non-fluorescent form before emission can take place. The shortening of the mean life is thus explained. The molecules which emit are those which have such a short lifetime of the excited state that they manage to fluoresce before passing into the non-fluorescent species. In this case, therefore, there is little doubt that the value of Michaelis *et al.* (1936) is correct.

Table 5. *Effect of potassium hydroxide on the intensity and polarization of the fluorescence of riboflavin*

(Solvent: glycerol-water mixture of viscosity 0.0658 c.g.s. unit at 16°. The solutions were kept in the dark up to the time of the observations, to avoid the well known photochemical splitting of lumiflavin. The calculated polarizations were obtained by introducing the experimental value of I_0/I into equation 5, assuming $\Sigma/\tau_0 = 5$, solving for τ_0/τ and using this last value in equation 2 to obtain $1/p$.)

I_0/I	Polarization	
	Calculated	Observed
1	—	0.067
2.18	0.076	0.070
5.08	0.091	0.082
10.4	0.112	0.110
16.4	0.122	0.122
23.8	0.151	0.151

Fig. 4. Effect of pH on the fluorescent efficiency of FAD and of riboflavin in the presence of quenchers. $\times-\times$, riboflavin; $\blacksquare-\blacksquare$, FAD; $\circ-\circ$, riboflavin in 0.05M-adenosine; $\bullet-\bullet$, riboflavin in 0.05M-caffeine. Concentration of riboflavin of the solutions 5×10^{-6} M-concentration of FAD is the same. (Determined by the absorption at 454 m μ .)

hydrogen ions. It is not surprising, therefore, that Michaelis, Schubert & Smythe (1936), who used a spectrophotometric method, obtained for the pK of the anionic form of riboflavin a value of -0.2 , while Kuhn & Moruzzi (1934), using the fluorimetric method, obtained pK = 2. Table 4 shows that, when five-eighths of the original riboflavin dipolar ions are present in the solution, the fluorescence efficiency has been reduced to one-twentieth of the original.

With potassium hydroxide it is found that the lifetime of the excited state changes slowly on quenching. From equation 5 it is found that the mean life of the cationic form is approximately 5×10^{-8} sec. Data for a solution of riboflavin quenched by potassium hydroxide are given in Table 5. Applying equation 5, half dissociation would occur, not at $I/I_0 = 0.50$, but at $I/I_0 = 0.42$; so that the error in the pK value of the cationic form obtained by Kuhn & Moruzzi (1934) is not large.

Quenching of the fluorescence of riboflavin by silver ions. When silver nitrate or silver acetate is added to a solution of riboflavin in distilled water the solution

immediately turns red and the tint becomes deeper as time passes showing that a slow reaction is taking place. At the same time the fluorescence drops accordingly. On heating the solution the colour changes, approaching that of a solution of free riboflavin, and the fluorescence reappears. On cooling, the solution again becomes red and less fluorescent, and this cycle of events can be repeated at will. Acidification destroys the colour and on neutralization with ammonia the red colour returns and persists at alkaline pH. Addition of a silver precipitant (chloride or phosphate ion) to the cold solution results in a brown precipitate, but addition of the same precipitant to the boiling solution produces only the usual precipitate of silver chloride or phosphate leaving as supernatant a solution of riboflavin with its full fluorescence.

Clearly we have here the formation of a complex which dissociates on boiling and reassociates on cooling. The formation of the complex takes place slowly and the same is true of its dissociation. The determination of the dissociation constant by the fluorimetric method is made difficult by the change in the absorption spectrum on addition of silver. The polarization of the fluorescence of riboflavin showed no increase on quenching by silver ions.

Fluorescence of flavin-adeninedinucleotide (FAD)

FAD was prepared by the method of Warburg & Christian (1938). The preparations were taken to the stage of extraction from the cresol solution. The absorption spectrum in the visible and near ultra-violet ($\lambda > 300 \text{ m}\mu$) corresponded exactly to that of isoalloxazine. At least 95% of the absorption at $450 \text{ m}\mu$ disappeared on addition of dithionite, and the amount of FAD determined in this way agreed well with the value obtained by the D-amino-acid oxidase method (Warburg & Christian, 1938). Paper chromatograms (Crammer, 1948) showed that FAD was the only flavin present. The fluorescence which varied slightly in the different preparations, due to impurities able to absorb the exciting light, was approximately 0.2 of the fluorescence of riboflavin solutions of the same molar concentration.

Effect of pH on the fluorescence efficiency of FAD. When the fluorescence intensity of the solutions is plotted against the pH a striking difference is observed between riboflavin and FAD. While the fluorescence of the former falls slowly between pH 6 and 4 and then rapidly, the solution of FAD at pH 4.5 already shows an increase in the fluorescence efficiency and at pH 3 this increase is strong enough to be detectable in daylight by the naked eye. The curve of Fig. 4 shows a well defined maximum at pH 2.7–2.8. Comparison with the pH intensity curve of a solution of riboflavin of the same molar concentration indicates that below pH 1.5 the fluorescence of both substances is the same. This

effect is not due to a destruction of the dinucleotide, which is not to be expected at this acidity (Warburg & Christian, 1938). If, after standing at pH 2.8 for 24 hr., the solution is brought back to pH 5 the fluorescence drops exactly to the value corresponding to the latter pH, showing the complete reversibility of the phenomenon.

Effect of pH on the absorption spectrum. Comparison of the absorption spectrum of solutions of FAD at pH 7 and 2.7 (phosphate and acetate buffers respectively) showed in the latter a noticeable increase in the absorption maximum at $450 \text{ m}\mu$, but no change either in the $375 \text{ m}\mu$ maximum or in the position of the red edge of the absorption band.

Mean life of the excited state of the fluorescence of FAD. The purpose of the following experiment was to ascertain whether the diminished fluorescence of FAD, as compared with that of riboflavin, was accompanied by a corresponding shortening of the lifetime of the excited state. The latter was measured by the method of Perrin (1926) which is based on the polarization of the fluorescence in media of different viscosities. According to Perrin (1926)

$$\frac{1}{p} = \frac{1}{p_0} + \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{RT}{\eta V} \tau_0,$$

where p is the polarization of the fluorescence of a substance of mean life in the excited state τ_0 , and

Fig. 5. Polarization of the fluorescence of riboflavin (RF) and FAD in glycerol water mixtures of increasing viscosity. $1/p$, reciprocal of the polarization; $1/\eta$, reciprocal of the coefficient of viscosity in c.g.s. units. Concentration of the fluorescent substances, approximately $2 \times 10^{-6} \text{ M}$.

molar volume V , in a medium of viscosity η , kept at the absolute temperature T ; R is the gas constant and p_0 the limiting, or fundamental, polarization. If

$1/p$ is plotted against $1/\eta$ a straight line is obtained which, extrapolated to $1/\eta = 0$, cuts the ordinate at $1/p = 1/p_0$.

In Fig. 5 the polarization of the fluorescence of solutions of riboflavin (RF) and FAD in glycerol-water mixtures of different viscosities is shown, $1/p$ being plotted against $1/\eta$. Both substances yield $1/p_0 \simeq 2$. Both give straight lines at high viscosities, and at low viscosities both deviate from the simple Perrin law. This occurs with practically all the fluorescent substances of low molecular weight studied (Perrin, 1926; Wawilov, 1936). Over the linear portion of the graph where the Perrin (1926) equation is followed, it is easy to see that the ratio of the slopes of the riboflavin and FAD curves is

$$\frac{S_{\text{RF}}}{S_{\text{FAD}}} = \frac{V_{\text{FAD}} \tau_{\text{RF}}}{V_{\text{RF}} \tau_{\text{FAD}}} = \frac{11}{8}.$$

This ratio approximates to that of the molecular volumes, and consequently

$$\tau_{\text{RF}} \simeq \tau_{\text{FAD}}.$$

If we take for τ_{RF} the value of 1×10^{-8} sec., obtained by the method of Wawilov (1928), we get for the molecular volume of riboflavin in solution $V_{\text{RF}} = 550$ ml. which seems a reasonable value, to judge by the constitution, and the molecular volume of substances of similar configuration (Marinesco, 1927 *a, b*).

Effect of pH on the polarization of the fluorescence of FAD. Changing the pH from 6 to 2.7 did not change the polarization of the fluorescence of FAD in a glycerol-water mixture, although the brightness of the fluorescence increased by about 300 %.

DISCUSSION

Examination of the mode of action of the purine quenchers leaves no doubt that the formation of a complex between flavin and purine takes place, and that the complex has a heat of dissociation of 1–2 kg.cal./mol., a mean life no shorter than 10^{-6} sec., and an absorption spectrum differing appreciably from that of free flavin. The differences between caffeine on one side, and adenosine or adenine on the other in the effect of pH on the quenching, can be attributed to the presence of the amino group in the last two named substances. According to Levine & Simms (1926), the pK of this group is about 3.7, corresponding to the region of rapid increase in the fluorescence efficiency of FAD shown in Fig. 4.

The quenching effect of purines on the fluorescence of certain hydrocarbons has been shown by Weil-Malherbe (1946), who was able to isolate some of the more stable of these complexes. The evidence presented in this paper is based on the thermal coefficient of the quenching, the effect on the life of

the excited state and the changes in the absorption spectrum. The effect on the mean life of the excited state is decisive in differentiating the quenching by complex formation from that due to collisions of the second kind, although there is a smooth transition between these two types (Weber, 1948). The reaction of riboflavin with silver ions represents a limiting case between the formation of a non-fluorescent complex and a non-fluorescent stable substance.

Quenching by hydrogen chloride, on the other hand, shows that when a fluorescent form in solution is in equilibrium with its non-fluorescent conjugate, the apparent fluorescent intensity bears no direct relation to the amount of fluorescent form present. Thus the value obtained by Kuhn & Moruzzi (1934) for the pK of the anionic form of riboflavin can be shown to be considerably in error. Whenever the simple equation 4 is applied to an equilibrium of this type, it is necessary to ascertain previously that the lifetime of the excited state is not changed by addition of quencher. The fluorescence of FAD and its variation with pH are readily correlated with similar changes taking place in riboflavin solutions in the presence of adenine or adenosine. There is no doubt of the existence of an internal complex in FAD, between the alloxazine and the purine parts of the molecule. The presence of some residual fluorescence in FAD may seem puzzling. The simplest explanation is that the non-fluorescent form is in equilibrium with a fluorescent one, and that this equilibrium is displaced by a change of pH. Since the lifetime of the excited state is the same in riboflavin and FAD, it must be concluded that the non-fluorescent internal complex has a long mean life, as found for the adenosine-riboflavin complex. The argument in favour of this internal complex is confirmed by the resemblance between the absorption spectrum of FAD and that of riboflavin in the presence of purines.

When FAD or riboflavin phosphate combines with an enzyme protein, in some cases (notatin) complete disappearance of the fluorescence has been noted (Keilin & Hartree, 1946), while in other cases (diaphorase) the fluorescence remains unchanged (Straub, 1939). Proteins, as such, display no conspicuous quenching properties, so that the disappearance of the fluorescence must be sought in the place and mode of attachment of the prosthetic group. Aromatic groups in the protein (tyrosine) are the more likely to act as quenchers. However another explanation is possible when FAD is the prosthetic group; union with the protein might simply prevent the complex from reverting to the fluorescent form in notatin, while for diaphorase the ease of this transition would not be affected.

Adenine and adenosine, and purines in general, are able to deactivate with quenching many fluorescent molecules. It is not perhaps unreasonable to

assume that other forms of electronic energy, not connected with the radiative transition but with other degrees of freedom, might easily be absorbed by purines. This would lead to the existence, in certain degrees of freedom of the purine molecule, of an energy content in excess of that which could be accounted for by a random distribution and the principle of the equipartition of energy. The presence of adenine in several prosthetic groups and coenzymes, where the purine seems to play no part in the catalysed reaction, is an indication that some general property of this type of compound, such as the one suggested, may be involved.

SUMMARY

1. A study of the fluorescence of riboflavin and of flavin-adeninedinucleotide, and the effect of different quenchers on the former has been made.

2. The quenching of the fluorescence of riboflavin by purines is due to the formation of a complex

having a heat of dissociation of 1.6 ± 0.6 kg.cal./mol. and a lifetime longer than 10^{-6} sec.

3. The small fluorescent efficiency of flavin-adeninedinucleotide in comparison with that of riboflavin is due to the formation of an internal complex between the *isoalloxazine* and the adenine of the molecule. The complex is non-fluorescent and exists in equilibrium with its fluorescent form, the equilibrium being easily displaced by changes in pH.

4. A study of the quenching of the fluorescence of riboflavin by acids and alkalis, shows that the fluorimetric method cannot be used for the determination of the dissociation constant of the anionic form of riboflavin, but its use is justified in the case of the cationic form.

5. A complex of riboflavin with silver ions is described.

6. The variations in the fluorescence of flavo-proteins is discussed, and a relation between the quenching properties of adenine and its presence in prosthetic groups and in coenzymes is suggested.

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The Cozymase of Mammalian Brain

BY M. GORE, F. IBBOTT AND H. McILWAIN

Biochemical Laboratories, Institute of Psychiatry (British Post-graduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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The cozymase of brain has been studied because of the central importance of this compound in carbohydrate metabolism, and also because in brain as a whole cozymase exists together with very active systems which degrade it. Inhibition of the degradation appears to be accompanied by disturbance of the balance of carbohydrate metabolism (McIlwain & Grinyer, 1950). The present paper describes studies of the normal cozymase content of mammalian brain, of the stability of cozymase in the tissue during various manipulations, and of its possible origin.

EXPERIMENTAL

Determination of cozymase

Apozymase system

Preparation of apozymase from yeast, and its use for determining cozymase, were based on the descriptions of Myrbäck (1933), Euler (1936), Axelrod & Elvehjem (1939) and McIlwain & Hughes (1948).

Apozymase. Fresh bottom yeast obtained from Barclay Perkins and Co. Ltd., to whom we are indebted, was sucked as dry as possible on a Büchner filter, pressed through a wire sieve (0.1 in. mesh) and dried overnight by a fan. The