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Kinetics and Equilibria in Flavoprotein Systems

I. A Fluorescence Recorder and its Application to a Study of the Dissociation of the Old Yellow Enzyme and its Resynthesis from Riboflavin phosphate and Protein

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For the first time we are now able to study in detail the kinetics of the reversible reaction between a coenzyme and its apoprotein. We have made use of the disappearance of the fluorescence of flavin mononucleotide when it combines with the specific protein to form the "old yellow enzyme". The fluorescence was recorded with a highly sensitive apparatus that enabled us to record "on" and "off" velocities in the formerly inaccessible concentration region, $10^{-6}-10^{-9}$ *M*. The combination reaction was found to be of the second order, the dissociation reaction of the first order. The influence of pH, salt concentrations and temperature will be reported later.

A few data may be found in the literature on the equilibrium constants of coenzymes with their specific proteins,

$$K = \frac{[\text{Coenzyme}] \times [\text{Protein}]}{[\text{Coenzyme-Protein}]}$$

These values are mainly based upon determinations of overall reaction velocities with excess of substrate, and varied concentrations of coenzymes and proteins. However, kinetic data on the combination and dissociation velocities, according to the equation:

$$\text{Coenzyme} + \text{protein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{coenzyme-protein}$$

have been lacking, but for one exception. By using rapid spectrophotometry refined to the limits of amplification and stability, Theorell and Chance [1] obtained an approximate value for k_1 in the reaction:

$$2\ \text{DPNH} + \text{ADH (alcohol dehydrogenase)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (\text{DPNH})_2\text{—ADH}.$$

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k_1 was computed on the assumption that the two DPNH molecules were bound independently and that the effective molarity of ADH was twice its actual concentration. This assumption may or may not be completely valid. It was not possible to determine k_2 because this would have required dilutions exceeding the practical limits of spectrophotometry. k_2 thus had to be calculated from k_1 and a rather uncertain value of the equilibrium constant K, according to the relationship:

$$K = \frac{k_2(\text{sec}^{-1})}{k_1(M^{-1} \times \text{sec}^{-1})}$$

It should be pointed out that even in the simpler situation when one molecule of coenzyme combines with one molecule of protein, as is the case for the "old yellow enzyme", it can by no means be taken for granted that the combination and dissociation reactions should follow the simple law of mass action, since flavin nucleotides [2] and pyridine nucleotides (see *e. g.* [3]) both are attached to their proteins by more than one linkage.

The velocity constants, k_1 and k_2, are of decisive importance for the overall reaction velocity in the case of "mobile" coenzymes. For instance, in the system DPN + ethanol (both in excess) + ADH, the limiting factor for the reaction velocity is the dissociation velocity (k_2) of $(DPNH)_2$—ADH.

It was desirable to find a more sensitive method than spectrophotometry in order to study the kinetics of the formation and dissociation of coenzyme-enzyme compounds at high dilutions. This would give conveniently low "on" velocities and high enough degrees of dissociation for studying "off" velocities and equilibria.

Fluorescence measurements were found to be around 1 000 times more sensitive than spectrophotometry and in addition to cover a much wider range of concentrations: with our apparatus 10^{-5}—10^{-9} M.

The old yellow enzyme in its oxidized form was found to be a very suitable compound for kinetic studies with the aid of our fluorescence recorder.

In a preliminary communication [4] we have briefly reported some of the results already obtained. We have made use of the fact that the fluorescence of FMN (flavin mononucleotide) is quenched by coupling to the protein [5].

In this paper we shall describe the fluorescence recorder used in these studies, and a means of purifying commercial FMN. The data and calculations of some typical "on reactions" (FMN + apoenzyme → FMN-apoenzyme /O.Y.E./) and "off reactions" (FMN-apoenzyme → FMN + apoenzyme) will be reported.

THE RECORDING FLUORESCENCE METER

Fig. 1 is a block diagram of the different parts of the apparatus.

Optical parts. Stabilized 220 V A.C. (1) is connected with the mercury lamp (3) over the choke (2). In order to avoid stray light the lamp is entirely enclosed in a water-cooled housing (4) equipped with water inlet and outlet (5). The adjustable condensor lens (6) makes the light parallel and sends it through the filter (7), which has a maximal transmission around 360 mμ, thus letting the strong mercury lines at 366 mμ pass through nearly unabsorbed

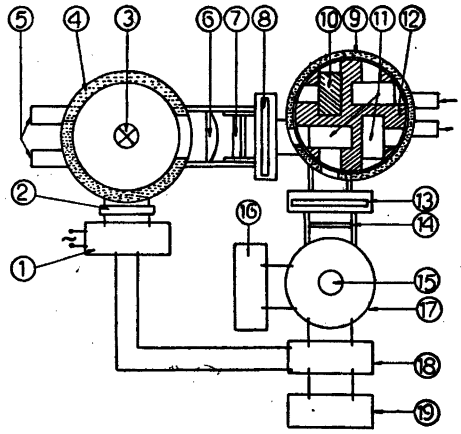

Fig. 1. Fluorescence recording instrument.

1. Stabilizer, Philips, 0—1 000 VA
2. Choke for the lamp
3. Mercury lamp, Philips, H.P. 80 watt (outer bulb removed)
4. Water cooled lamp housing
5. In- and outlets for tap water
6. Condenser lens, f = 65 mm, diameter 40 mm
7. UV filter "Jena UG 2"
8. Entrance slit selector
9. Thermostated water
10. Plexiglas standard
11. Cuvettes, maximum size 20 × 30 mm
12. Cylindrical cuvette holder
13. Exit slit selector
14. Filter for visible light, "Jena GG 5"
15. Photomultiplier RCA 1 P21
16. Battery for 15, 10 × Tudor 90 V
17. Housing for photomultiplier
18. Preamplifier and Power Supply, see Fig. 3
19. Recorder Speedomax, Leeds and Northrup

and minimizing the visible light. The entrance slit selector (8) is a revolving brass disc with eight rectangular openings, 17 mm high, and 1, 2, 3, 4, 5, 6, 7 or 10 mm wide. The entering light intensity used can thus be varied tenfold. The plexiglas standard prism (10), made of colorless, "nonfluorescent" plexiglas and the three cuvettes (11) are located in a revolving aluminium cylinder with windows at right angles for light entrance and exit. The cylinder is surrounded by circulating thermostated water of desired temperature and covered by an aluminium disc with holes for the addition of solutions to the cuvettes. The hole can be shut to exclude external light. Three cuvettes can thus be used intermittently, readings being taken on cuvettes and standard by turning the cylindrical cuvette holder (12). The dark current value is obtained in intermediate positions. The fluorescence light is allowed to fall on an exit slit selector (13), which is similar to (8) and has openings of 17 mm height

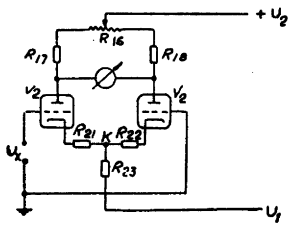

Fig. 2. Simplified diagram showing the general principle of preamplifier.

and 1—6 mm width. The light filter (14) transmits only visible light to the photomultiplier (15) in its housing (17). The distance from the condenser lens to the center of the cuvette is 7 cm, from the cuvette to the photomultiplier 8 cm.

Electrical parts. The preamplifier (18) for the recorder (19) was built especially for the purpose by Mr. K. O. Särnesjö at this institute. A simple diagram is given in Fig. 2. It is "push-pull coupled" and works in the following way. Suppose a positive voltage, U_x, is imposed on the grid of the triode, V_1, the anode current through V_1 and the voltage at point K will increase. This results in an increased negative voltage on the grid of V_2, thereby decreasing its anode current. It can be shown that if the fixed resistance, R_{23}, is sufficiently high the anode current will decrease in V_2 about as much as it will increase in V_1. With this arrangement the voltage variations at point K are kept very small, and negative current feed-back is avoided. The reverse feed-back function is taken care of by resistors R_{21} and R_{22}.

One of the favourable properties of this push-pull coupled amplifier is its high stability. The operating point of the tubes, V_1 and V_2, is determined by the high resistance R_{23} and the fixed grid bias U_1. If U_1 and R_{23} are chosen so that the voltage drop over R_{23} is compensated by U_1, the operating point becomes fairly independent of moderate variations in the power supply or the parameters of V_1 and V_2.

Fig. 3 shows a complete wiring-diagram of the amplifier with photomultiplier, recorder and power supplies. As can be seen, the sensitivity, S, of the apparatus can be regulated at the amplifier within the limits 0.1 V to 100 V per full deflection (10 inches) of the recorder. For checking the performance, a standard cell is provided, and adjustment can be made for full scale deflection of the recorder in the 1 volt range by regulating the variable resistor R_{19}.

The sensitivity of the apparatus for the fluorescence of FMN may be expressed as follows: At sensitivity, $S = 0.1$ V/10 inches, anodic resistance of photomultiplier = 100 kohm, slit openings 6 mm, the recorder makes a deflection of 2 to 2.5 inches for a 10^{-9} M solution of FMN. Under these conditions the recording system is still very stable. If needed, it would be possible to increase the sensitivity still more by increasing the anodic resistance above 100 kohm. However, the limiting factor in the practical use of the apparatus was the fluorescence of the cuvettes and the buffers used. Common glass cuvettes fluoresce too strongly, and thus we had to use cuvettes of "non-fluorescent" glass. The fluorescence of these cuvettes containing distilled water caused deflections around 3 inches at $S = 0.1$ V/10 inches and slit openings 1 and 6 mm. Under these conditions 10^{-8} M FMN adds 3 inches to the

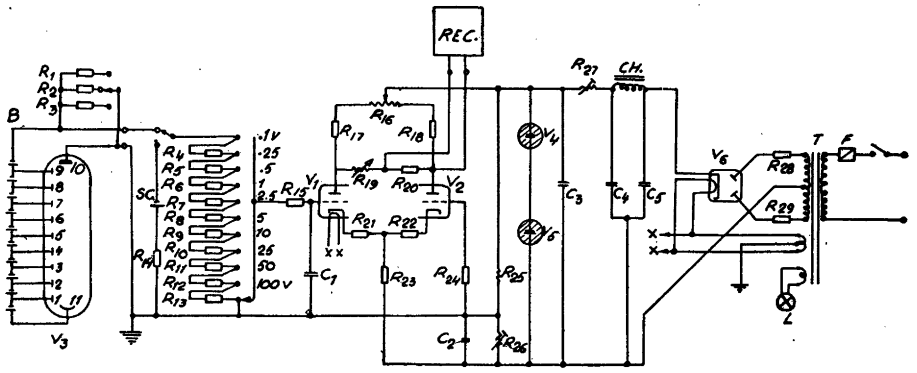

Fig. 3. Wiring-diagram.

R_1 — 20 kohm 1/2 W
R_2 — 50 kohm 1/2 W
R_3 — 100 kohm 1/2 W
R_4 — 600 kohm 1/2 W
R_5 — 200 kohm 1/2 W
R_6 — 100 kohm 1/2 W
R_7 — 60 kohm 1/2 W
R_8 — 20 kohm 1/2 W
R_9 — 10 kohm 1/2 W
R_{10} — 6 kohm 1/2 W
R_{11} — 2 kohm 1/2 W
R_{12} — 1 kohm 1/2 W
R_{13} — 1 kohm 1/2 W
R_{14} — 50 kohm 1/2 W
R_{15} — 620 kohm 1/2 W
R_{16} — 1 kohm (pot.) 1 W
R_{17} — 30 kohm 1 W
R_{18} — 30 kohm 1 W
R_{19} — 20 kohm (pot.) 1 W
R_{20} — 1 kohm 1/2 W
R_{21} — 4 kohm 1/2 W
R_{22} — 4 kohm 1/2 W
R_{23} — 30 kohm 1 W
R_{24} — 1.5 M 1/2 W
R_{25} — 10 kohm 6 W
R_{26} — 3.7 kohm (to be adjusted) 2 W
R_{27} — to be found by testing
R_{28} — 160 ohm 1 W
R_{29} — 160 ohm 1 W

C_1 — 2 000 pF
C_2 — 16 μF 150 V
C_3 — 16 μF 350 V
C_4 — 25 μF 350 V
C_5 — 25 μF 350 V

V_3 — 1P21
V_1, V_2 — E80CC
V_4 — VR150
V_5 — VR105
V_6 — EZ40
L — Lamp, 6,3 V
T — Transformer 2 × 260V, 60 mA
CH — Choke 20 H, 60 mA
F — Fuse 0.5 A
Rec. — Recorder Speedomax 0—10 mV Leeds & Northrup
S. C. — Standard cell for checking recorder sensitivity
B — Battery, 10 × 90 V Tudor

deflection. Of course the salts and buffer substances used had to be pure enough not to cause inconvenient increase of the blank fluorescence. So far it has not been necessary to subject any of the substances employed to further purification. It might be mentioned that our tap water gives a considerable fluorescence.

Of course, the apparatus can be adjusted so as to use the fluorescence level of the blank (cuvette + buffer) as base line instead of the dark current level. However, in this way it is difficult to test for changes in the standard deflec-

tion. The plexiglas standard gave a deflection of about 5 inches at $S = 0.25$ V/10 inches and slit openings 1 and 6 mm. Variations of the deflections were corrected for by measurements of the fluorescence of the standard. For each experiment the deflection of a known solution of FMN was also determined. The relationship between the deflections caused by the plexiglas standard and fresh solutions of FMN has remained the same as long as the apparatus has been in use (six months). This strongly suggests that the fluorescence of the plexiglas standard has kept constant.

MATERIALS AND METHODS

The preparation of the old yellow enzyme (O. Y. E.)

"Gelbes Rohferment" was prepared from brewer's bottom yeast, kindly supplied by Hamburgerbryggeriet, Stockholm. We used the procedure of Warburg and Christian [6] but omitted their methanol and chloroform treatments which involve rather heavy losses. A product which was about 25 % pure was obtained from "Rohferment" by adsorption on $Al(OH)_3$(Cγ) and elution [7] followed by precipitation between 50 and 60 % saturation of ammonium sulphate, dialysis, and repeated adsorption on Cγ and elution. A further purification of the 25 % pure product could be accomplished by electrophoresis in the Tiselius apparatus, using phosphate buffer of pH 7. The best fraction thus obtained contained 5.3 γ FMN per mg of protein and was 83 % pure as calculated from the data obtained for the crystalline enzyme [8]. The electrophoretic pattern showed some colorless components to be present in amounts roughly corresponding to the degree of purity found by the above analysis of the FMN content. Apoenzyme was prepared from this preparation and from a preparation which was only 25 % pure, and the rates of combination with FMN were compared under different conditions. The rates were found to be the same with both preparations. Furthermore, the addition of serum albumin to an apoenzyme did not affect the reaction velocity. Accordingly, we felt confident in using such 25 % pure preparations for our kinetic measurements after having exhausted our supply of the 83 % pure product.

We had available some "Gelbes Rohferment" which was kindly given to H. T. by Otto Warburg as early as 1935. It was of interest to compare O.Y.E. prepared from this product with O.Y.E. prepared from our yeast. It was found that the aluminum-hydroxide and ammonium sulphate purification procedures, when applied to either of the "Rohferments", gave products with very similar degrees of purity and electrophoretic patterns. The two yellow proteins from Berlin and Stockholm had the same sedimentation constants ($S_{20°} = 5.7-5.8$ in the analytical ultracentrifuge, Spinco) in excellent agreement with the sedimentation constant, $S_{20°} = 5.76$, reported in 1936 [9]. Furthermore, the kinetic data obtained in dissociation and recombination experiments with the FMN protein systems from the two preparations gave indistinguishable results. It may be concluded that the O.Y.E. in the form of dry "Rohferment" keeps for at least 19 years.

Apoenzyme was prepared according to the method of Warburg and Christian [10]. We have observed that the apoenzyme is less stable in glass-distilled water than in a dilute (~ 0.01 *M*) sodium-phosphate or ammonium sulphate solution.

Flavin mononucleotide

We have used FMN purified from a commercial product, kindly supplied by Sigma Chemical Company. When this product was subjected to paper electrophoresis in 0.05 *M* ammonium acetate at pH 7 (5 mA, 100 Volts per 22 cm paper), about 10 % of the flavin material did not move in the electric field (called component 1), 80 % (component 2) moved with the same rate, as FMN prepared from O.Y.E., 2.6 cm/hour. 10 % moved faster than FMN, 3.0 cm/hour (component 3). Component 2 contained as much phosphorus as FMN, 1 P per 1 riboflavin, whereas component 3 contained about twice as much. Components 2 and 3 had the same absorption spectrum as riboflavin. Component

2 combined with apoenzyme at the same rate as did FMN, whereas component 3 behaved quite differently. The "synthetic" compound of component 2 and the apoenzyme had the same dissociation velocity constant in 0.01 *M* acetate buffer, pH 3.72, as had O.Y.E.

These experiments give additional evidence to prove that the main component (~ 80 %) of the commercial product is identical with natural FMN. Component 3 may be a riboflavin diphosphate.

The ammonium salt of FMN was not very stable, even when light was kept out carefully. After some weeks of occasional use, a large percentage of FMN was inactive. The deterioration was observed as an increased proportion of fluorescence that did not disappear upon the addition of apoenzyme ("rest fluorescence").

The part of FMN which had deteriorated did not move in our paper electrophoresis experiments. The unchanged FMN and the immobile spot could be washed out separately and the amount of yellow material determined spectrophotometrically at 445 mμ. The deteriorated FMN could thus be determined in two ways: by the rest fluorescence observed in the presence of excess apoenzyme (the usual method), and by paper electrophoresis of the deteriorated solution. The two methods gave similar results. Thus, a solution which was calculated to contain 51 % deteriorated FMN from titration with excess of apoenzyme, was found to contain 57 % deteriorated FMN by paper electrophoresis. The small disagreement could be due to the fact that the electrophoresis experiment was carried out four days after the titration experiment. The fluorescence and the optical density at 445 mμ of a 50 % deteriorated FMN solution was not less than that of a fresh solution. We have not yet investigated the product formed when FMN deteriorates. Obviously the phosphoric acid group is split off, with or without the ribityl group. Usually we did not use solutions of FMN which contained more than 15 % non-coupling flavin material. The light absorption coefficient, β, was taken as 2.7×10^7 cm^2/mole[11].

In order to calculate the concentration of FMN, it was necessary to subtract the fraction of inactivated flavin material from the value found by spectrophotometry.

FMN was kept cold and in the dark as much as possible. During the experiments, the exposure to ultraviolet light was kept to a minimum by the use of small entrance slits. With the most frequently used slits, 1×17 mm, only 3 % of the total volume of the solution was exposed. Only in experiments with very low concentrations of FMN ($c < 10^{-8}$ *M*) was it necessary to use wider entrance slits. In experiments of longer duration than one minute, the solution was kept in the dark most of the time, readings being taken at suitable intervals.

The fluorescence of FMN was found to be proportional to its concentration in all solutions tested. This, of course, holds true only for solutions in which light absorption does not interfere. The quenching of the fluorescence of FMN by different anions has been described previously[4]. The quenching factor, QF, is defined as

$$QF = \frac{\text{The fluorescence in water}}{\text{The fluorescence in the solution.}}$$

The presence of O.Y.E. did not affect the fluorescence of FMN. Before any coupling had taken place at $t = 0$, the fluorescence of FMN was the same in the presence and absence of apoenzyme. It may be concluded that fluorescence measurements can be used to determine the concentration of FMN present in all mixtures of O.Y.E., apoenzyme and FMN.

EXPERIMENTS

Experiment 24 a. Determination of apoprotein concentration by titration with FMN, and calculation of the velocity constant of the "on reaction"

Conditions: Temp. 23°5. Sensitivity, *S*, = 0.5 V per 10 inches deflection was used in the FMN-additions 1—6, 1.0 V/10″ in addition 7. (A photomultiplier 4—5 times less sensitive than the one described was used in this experiment). Slits: entrance 1 mm, exit 6 mm. Cuvettes: 10 × 10 mm (plexiglas). Speed of recording paper, 3 inches per minute.

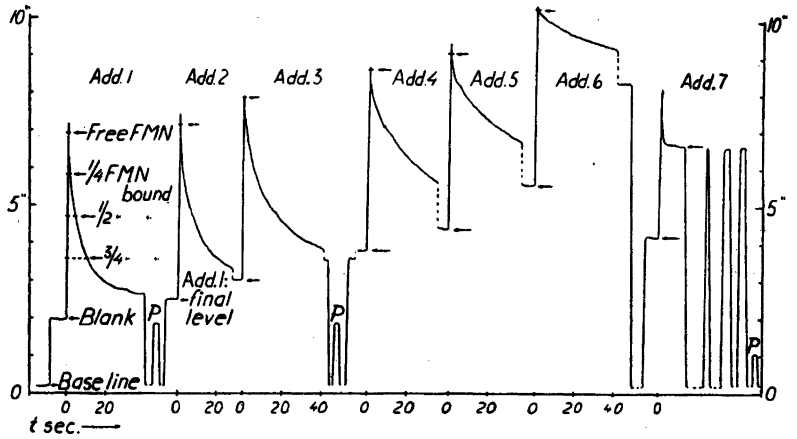

Fig. 4. Titration of apoenzyme with successive additions of FMN (expt. 24).

The levels of "free FMN" (see arrows) were calculated from the deflections caused by the same concentration of FMN without apoenzyme.

The final levels were checked repeatedly after each addition in order to ascertain that equilibrium was reached (indicated only after addition 7). The curves are direct copies of the original records.

P: deflection for plexiglas standard.

Buffer: 3.3 ml Na-phosphate, $\Gamma/2 = 0.1$, pH = 7.05. Apoenzyme: 0.2 ml. FMN solution: 5.55×10^{-4} *M* determined spectrophotometrically. Each addition = 1.96 μl.

The first deflection recorded in Fig. 4 is the blank caused by the fluorescence of the cuvette, buffer and apoprotein. The additions of FMN, from a stirring rod of glass, causes a sudden increase in fluorescence, corresponding to a deflection of about 5 inches. The period of the recorder, 1 sec. per 5 inches, has to be considered in rapid reaction cycles. Immediately after mixing the fluorescence begins to drop, due to the coupling of FMN to the protein. The fluorescence approaches asymptotically a level corresponding to the disappearance of 89 % of the initial fluorescence.

On the assumption that one molecule of FMN couples with one molecule of apoprotein [11] we can calculate the molarity of the apoprotein solution by adding the amounts of FMN coupled in the six first additions. On the seventh addition of FMN no decrease in fluorescence was observed. The results are summarized in Table 1.

Calculation of the "on reaction" velocity constant, k_1.

From the simple relationship

$$1 \text{ FMN} + 1 \text{ apoprotein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 1 \text{ O.Y.E.}$$

one would expect the "on reaction" to be of the second order. However, deviations could occur since the combination of FMN with the apoprotein involves the formation of (at least) two bonds, two unknown groups in the protein being attached to the phosphoric acid residue and the imino group (9) of the FMN[2]. It was therefore of considerable interest to calculate k_1 from different points on the experimental curves to test if it came out as a true constant. Since we knew that the rate of the off reaction was very low compared to the on reaction under the conditions used, we felt justified in neglecting

Table 1. "Titration" of apoprotein with FMN.

FMN Addition No.	FMN coupled, M	Per cent total rest fluorescence
1	0.296×10^{-6}	11
2	0.293 »	11
3	0.290 »	11
4	0.289 »	11
5	0.268 »	15
6	0.135 »	—
7	0	—

Total FMN coupled: 1.573×10^{-6} M.
The concentration of the undiluted apoprotein was
$= \frac{3.5}{0.2} \times 1.573 \times 10^{-6} = 27.5 \times 10^{-6}$ M.

the off reaction in calculating the rate constant, at least for the first three additions. We therefore used the simplified formula:

$$-\frac{dc}{dt} = k_1 \times [\underset{a}{\text{Protein}}] \times [\underset{b}{\text{FMN}}],$$

in its integrated form:

$$k_1 = \frac{2.3}{(a-b)t} \times \log \frac{b(a-x)}{a(b-x)}$$

The results are given in Table 2.

As may be seen from the table, the values for k_1 are nearly constant, the variations being within the experimental error. One may conclude that FMN and protein combine in such a way that the formation of one of the linkages greatly favours the formation of the other, so that the combination nevertheless follows the course of a second order reaction. The average value of k_1 from 1—3 is $= 10.6 \times 10^4$ $M^{-1} \times \text{sec}^{-1}$.

Table 2. Calculation of k_1 from different points on recombination curves (Fig. 4).

Addition No.	Fraction FMN bound	Time (sec)	k_1 $M^{-1} \times \text{sec}^{-1}$	k_1 average $M^{-1} \times \text{sec}^{-1}$
1	1/4	1.7	11.1×10^4	10.2×10^4
	1/2	4.7	9.9 »	
	3/4	10.0	9.7 »	
2	1/4	2.0	11.7 »	10.8 »
	1/2	5.5	10.6 »	
	3/4	12.3	10.0 »	
3	1/4	2.4	12.8 »	10.8 »
	1/2	7.3	10.6 »	
	3/4	18.2	9.0 »	

Experiment 24 b

The same apoenzyme was titrated under the same conditions with a solution of FMN which had been prepared some weaks earlier and occasionally exposed to light and to room temperature. It was made up to have the same optical density as the FMN used in expt. 24a and was found to have the same fluorescence. In the presence of excess apoenzyme it had 51 % rest fluorescence. Titration of the apoenzyme with this FMN gave the same value as in 24a, and the values for the velocity constant for the two first additions were 9.8 and 9.2 $\times$ 10^4. It may be concluded that the presence of the deterioration product of FMN does not disturb the on reaction. Nevertheless, the product decreases the accuracy of the experiment by increasing the fluorescence of the blank.

Experiment 51. Determination of dissociation velocity and equilibrium constant

Conditions: Temperature 23.5°. Sensitivity: $S = 0.25$ V/10 inches for expts. (a), (b) and (c); $S = 0.5$ for expt. (d). Slits: entrance 1 mm, exit 6 mm. Cuvettes: 10 $\times$ 10 mm (plexiglas). Speed of recording paper: 3 inches per minute. Buffer: 0.25 *M* with respect to Na-phosphate and 0.4 *M* with respect to NaCl. pH = 5.75. Total volume: 3.5 ml. O.Y.E. solution: 2.38 $\times$ 10^{-5} *M* as determined spectrophotometrically.

Table 3. Dissociation of O.Y.E. in different concentrations (Expt. 51 and Fig. 5). k_2 obtained from initial velocity at $t = 0$. "E" = O.Y.E.

Exp. 51	Initial Molarity of O.Y.E	Initial Velocity $-dc/dt$ $M \times sec^{-1}$	k_2 sec^{-1}	Per cent dissociation at equilibrium	Equilibrium constant, K M	$k_1 = \frac{k_2}{K}$ $M^{-1} \times sec^{-1}$
a	0.102 $\times$ 10^{-6}	12.3 $\times$ 10^{-10}	12.2 $\times$ 10^{-4}	23.5	0.75 $\times$ 10^{-6}	18 $\times$ 10^4
b	0.34 »	42.5 »	12.5 »	13.6	0.70 »	17 »
c	0.95 »	14.3 »	15.1 »	8.4	0.72 »	21 »
d	6.8 »	93 »	13.7 »	2.6	0.67 »	20 »
		Average:	13.5 $\times$ 10^{-4}		Average:	19 $\times$ 10^4

As seen from Table 3, four experiments, a, b, c and d were made with concentrations from 0.102 $\times$ 10^{-6} to 6.8 $\times$ 10^{-6} *M*.

From the equation, FMN-Protein $\xrightarrow{k_2}$ FMN + Protein, one would expect the off reaction to be of the first order, the reaction velocity being proportional to the enzyme concentration

$$-\frac{dc}{dt} = k_2 \times [\text{O.Y.E.}]$$

In one set of calculations we determined the initial velocities, $-dc/dt$ at $t = 0$, from tangents of the dissociation curves drawn at $t = 0$ (see Fig. 5 for expt. c), and obtained the equilibrium constants from the degrees of dissociation after equilibrium had been reached. The on reaction constant, k_1, was then calculated from the formula $K = \frac{k_2}{k_1}$. The results are summarized in Table 3.

k_1 was also calculated by using a method that is independent of the initial velocity at $t = 0$, but dependent on the equilibrium constant, K. The complete formula for a dissociation reaction with opposing "on" reaction was applied to different points on the curves obtained in expt. c, (see Fig. 5):

$$-dc_1/dt = k_2c_1 - k_1 \times c_2 \times c_3$$

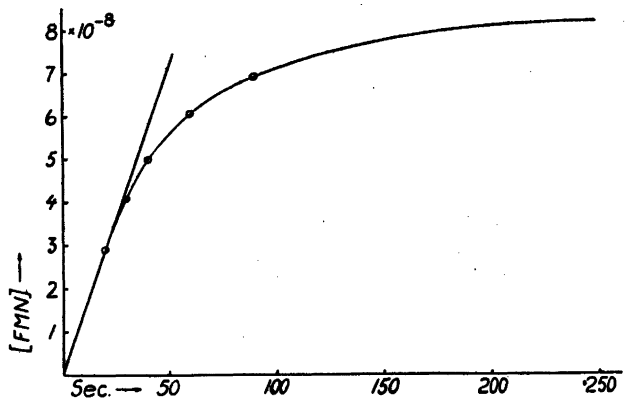

Fig. 5. Dissociation of O.Y.E., expt. 51 c. Ø Points used for the calculations of k_1 according to integrated formula.

where c_1 = [O.Y.E.], c_2 = [FMN], c_3 = [apoenzyme]. If the concentrations at $t = 0$ are $c°_1$, $c°_2$, $c°_3$, the integrated form of this equation is:

$$k_1 t = \frac{1}{2 \times a} \times \ln \frac{c_1 - \frac{2c°_1 + c°_2 + c°_3 + K}{2} - a}{c_1 - \frac{2c°_1 + c°_2 + c°_3 + K}{2} + a} + \text{constant}$$

$$\text{where } a = \sqrt{\frac{(2c°_1 + c°_2 + c°_3 + K)^2}{4} - (c°_1 + c°_2)(c°_1 + c°_3)}$$

We found for expt. c: $k_1 = 22.7 \times 10^4\ M^{-1} \times \text{sec}^{-1}$ after 20 seconds; 23.6×10^4 after 30 seconds; 21.8×10^4 after 40 seconds; 19.9×10^4 after 60 seconds and 17.8×10^4 after 90 seconds. Average: $21.2 \times 10^4\ M^{-1} \times \text{sec}^{-1}$. This value agrees with $k_1 = 21 \times 10^4$ found for expt. c by taking k_2 from the tangent at $t = 0$ and using the equation $K = \frac{k_2}{k_1}$, see Table 3.

In order to determine k_1 by an independent and direct method, we carried out an "on reaction" with FMN + protein in the same buffer and at the same temperature. The value obtained was $k_1 = 17 \times 10^4\ M^{-1} \times \text{sec}^{-1}$, in as good agreement as could be expected with the average value 19×10^4 from expt. 51.

DISCUSSION

The results reported above prove the perfect reversibility of the reaction between FMN and the protein of the O.Y.E. and, furthermore, that the combination reaction is of second order, whereas the dissociation reaction is of the first order under the conditions used in these experiments. Since it must be regarded as established that at least two groups in the FMN — the phosphoric acid residue and the imino group (9) — are bound to two sites in the protein, our results mean that the formation of one linkage so greatly favors the formation of the other one, that no half-coupled intermediates exist in detectable concentrations.

It should be pointed out that the leucoform of FMN or the $FMNH_2$-protein shows no fluorescence and therefore cannot be studied in this way. However, from data on the kinetic and equilibrium constants of the oxidized complex, compared with redox potential measurements to be made we hope to obtain the equilibrium constants of the reduced complex. The velocity constants, k_1 and k_2, are not directly obtainable from such measurements, but on the assumption that k_1 is the same for the oxidized and reduced compounds — as was the case with the DPN- and DPNH—ADH compounds — k_2 can be calculated. We may never be able to determine the dissociation velocity constant of $FMNH_2$-apoprotein (of O.Y.E.) directly, since the redox potential of O.Y.E., as compared with that of riboflavin, is very high. Thus the dissociation velocity of the reduced complex can be expected to be too low to be measured.

One may hope that accurate measurements of the kinetic data on O.Y.E. and other flavoproteins may give some clue to the chemical nature of the FMN-combining groups in the protein.

It may finally be mentioned that we have found fluorescence measurements to be very useful in following the reduction or oxidation velocities in pyridine nucleotide-enzyme systems, since DPNH, but not DPN is fluorescent. In the cases tested so far this fluorescence does not disappear when DPNH is combined with proteins.

Forthcoming papers will demonstrate the profound influence not only of pH and temperature, but also of anions like phosphate and chloride on the kinetics and equilibria of both flavin- and pyridine enzymes.

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