ACTA CHEMICA SCANDINAVICA 9 (1955) 1587—1599

# Kinetics and Equilibria in Flavoprotein Systems

## III. The Effects of Chemical Modifications of the Apoprotein on the Dissociation and Reassociation of the Old Yellow Enzyme

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The dissociation of the old yellow enzyme (O.Y.E.) and its resynthesis from FMN and protein have been measured spectrophotometrically and fluorimetrically on the same solutions. The two methods were found to give equal results.

O.Y.E. and its apoenzyme have been chemically modified by functional group reagents and the effects on the binding of FMN have been studied. Specific acetylation of a small fraction of the 60 primary amino groups strongly decreased the rate of resynthesis of O.Y.E. and increased its rate of dissociation. However, the acetylated apoenzyme was able to bind one mole of FMN when excess FMN was added. The essentiality of amino groups for the tight binding of FMN has been confirmed by the use of other chemical reagents. Formaldehyde inhibition of the apoenzyme was reversible, except when very low concentrations of the apoenzyme were exposed to formaldehyde.

Specific substitution of a small fraction of the 24 tyrosyl groups with iodine strongly decreased the reassociation reaction and increased the dissociation reaction of O.Y.E. However, in the presence of excess FMN, an iodinated apoenzyme was able to bind one mole of FMN.

Iodinated and acetylated apoenzyme appeared homogeneous in the ultracentrifuge with practically the same sedimentation constants as for the unmodified protein.

FAD and a riboflavin diphosphate have been found to combine loosely with the apoprotein.

Atabrin, which has been shown to interfere with the activity of other yellow enzymes also appears to combine loosely with the apoenzyme of O.Y.E., as indicated by a quenching of the fluorescence of atabrin by the apoprotein.

Implications of some of these findings have been discussed.

The observed effects [1] of pH, anions, and temperature on the reaction

$$\text{FMN} + \text{apoprotein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{FMN} \cdot \text{apoprotein (Old Yellow Enzyme)}$$

suggested strongly the dominant role of electrostatic forces in the binding of

FMN to the protein. Furthermore, evidence was presented to show that the doubly anionic phosphate group of FMN in juxtaposition to positively charged amino groups may furnish an important part of the energy of attraction.

However, the kinetic studies did not give information about the attachment of the isoalloxazine nucleus to the protein [2]. In the present work we have attempted to identify combining sites of the protein by subjecting the enzyme and the apoprotein to functional group inhibitors and measuring the effects on the kinetics and equilibria of the FMN-apoprotein-O.Y.E. system.

Also included in this paper are some comparative experiments of the fluorimetric and spectrophotometric technique for measurements of association velocity constants.

A short communication of this study has already been presented [3].

## MATERIALS AND METHODS

Pure preparations of O.Y.E. were necessary for quantitative experiments. Thus, considerable effort was spent in improving the isolation procedure. The partially crystalline preparations of Vestling [4] were still inhomogeneous in electrophoresis. By use of paper electrophoresis we obtained limited amounts of entirely homogeneous material, containing 1 mole of FMN per 72 000 g of protein. The absorption coefficient of O.Y.E. at 465 m$\mu$ was redetermined and found to be $\beta = 2.52 \times 10^7$ cm$^2$/mole, on the basis [5] of $\beta_{\text{FMN, 450 m}\mu} = 2.81 \times 10^7$. The details will be described in a forthcoming paper. For some qualitative experiments somewhat less pure material was used.

Flavin mononucleotide (FMN) was purified from a commercial preparation by paper electrophoresis, as described previously [6]. A solution of FMN kept in the dark at 0° was 50 % destroyed in about two weeks, but if kept in a frozen state, remained stable for months.

Flavin adenine dinucleotide (FAD) was kindly supplied by Drs. O. and E. Walaas in this institute; the sample was obtained from baker's yeast with paper ionophoresis used for the final purification.

Acetylation of the enzyme and apoenzyme was accomplished by the addition of 1 $\mu$l quantities of acetic anhydride to the protein solution buffered with 2.5 M sodium acetate and kept at 0°. Not more than 1—3 $\mu$l acetic anhydride were required for extensive acetylation of 5 ml of a 50 $\mu$M solution of the apoenzyme. The acetylation of amino groups was followed by Van Slyke aminonitrogen analyses [7], which were carried out at room temperature on dialyzed samples containing approximately 15 mg of protein. A fifteen minute reaction time was found sufficient to give maximum values.

The acetylation of phenolic groups was followed by means of Folin's phenol reagent [8], the protein being first denatured with lauryl sulfate as described by Miller [9].

Iodination of the enzyme and apoenzyme was carried out in 0.1 M phosphate pH 7.4 at 0° C. The iodinating agent was 0.1 M $I_2$ dissolved in alcohol, and 1 $\mu$l quantities were added to approximately 50 $\mu$M protein solutions. Tyrosine was estimated by the Millon-Lugg procedure [10], and the diiodotyrosine formed was calculated from the difference in tyrosine content before and after iodination. The formation of diiodotyrosine in the apoenzyme was also demonstrated qualitatively in hydrolysates by paper electrophoresis [11]. Monoiodotyrosine also reacts with the Millon reagent, in contrast to diiodotyrosine.

Azobenzene sulfonic acid reagent was prepared as described by Jorpes [12].

Fluorimetric experiments using the modified apoprotein were conducted to determine the following:

1. The association velocity constant, $k_1$, in the way previously described [6].

2. The total combining capacity of the modified apoprotein for FMN, and the equilibrium constant for the combination. In measurements of the combining capacity, increasing quantities of FMN were added to a given quantity

of apoprotein until no more FMN was coupled. Because most of the chemical modifications of the apoprotein resulted in an increased dissociation of the FMN-apoprotein, a considerable excess of FMN was sometimes necessary to saturate the protein. The experiments were less accurate when the equilibrium constant was high *. The equilibrium constant, $K$, was calculated from the amount of combination when the protein was only partially saturated with FMN.

3. The dissociation velocity constant. This could be determined directly when functional group reagents were added to the O.Y.E. However, when the apoprotein had been treated with such agents, $k_2$ was more readily obtained from the relation $k_2 = k_1 \times K$ **.

## RESULTS

### A comparison between spectrophotometric and fluorimetric measurements of the association velocity constant

Since our previous experiments [1,3,6] had been done with the fluorimetric technique it was of interest to examine whether the disappearance of the fluorescence when FMN combines with the apoprotein took place with the same rate as the shift of the absorption band from 445 to 465 m$\mu$ [13]. Since the greatest difference in the light absorption of FMN and O.Y.E. was found to be around 495 m$\mu$ this wavelength was chosen for the measurements. A highly sensitive recording spectrophotometer of our own construction was used, but the spectrophotometric measurements were nevertheless far less sensitive than the fluorimetric ones. Therefore, concentrations of FMN and apoenzyme which were considerably higher than applied in previous experiments [1,3] had to be used in order to determine the association velocity at the same concentrations of reactants with both methods. In the absence of inhibitors, these high concentrations around 3 $\mu$M gave too rapid reactions to be recorded with our apparatuses; thus, polyvalent anions were added to decrease the velocity. Easily measurable association rates were obtained using phosphate and versene buffers. The results are shown in Table 1. They show that the spectrophotometric and fluorimetric methods gave similar results, the variations being within the experimental errors. We may thus feel confident that the results obtained by the aid of fluorimetry in all probability are identical with those that would have been obtained by rapid spectrophotometry.

### Functional group reagents

Practically all protein reagents react with thiol groups and their presence makes the test for the essentiality of other groups difficult. It was therefore a great advantage for the further investigation of this problem that the pure

* In our first experiments published in a preliminary communication [3], highly acetylated or iodinated apoproteins were studied. In these the dissociation constant was so high that the total combining capacity appeared to be decreased, because high enough concentrations of FMN to saturate the protein were not applied.

** For the meaning of $k_1$, $k_2$ and $K$ in modified protein preparations see the discussion p. 1597.

*Table 1. Spectrophotometric and fluorimetric measurements of the reaction apoenzyme + FMN* $\xrightarrow{k_1}$ *O.Y.E. at pH 7.0, 23° C.*

| Buffer | | $k_1$, $M^{-1} \times sec^{-1}$ | |
|---|---|---|---|
| | | Spectrophotometric | Fluorimetric |
| Phosphate<br>Versene | 0.5 M<br>0.0016 M | $2.5 \times 10^4$ | $2.0 \times 10^4$ |
| Phosphate<br>Versene | 0.03 M<br>0.0016 M | $10.0 \times 10^4$ | $10.9 \times 10^4$ |

apoenzyme as well as a preparation denatured by incubation with lauryl sulfate were found not to contain thiol groups. These preparations did not react with *p*-chloromercuribenzoate [14], as measured by the spectral change at 250 mμ, and gave a negative nitroprusside test. Further, typical thiol reagents did not affect the binding of FMN to the protein; $Cu^{++}$, $Fe^{+++}$, $Hg^{++}$, and $Ag^{+}$ in concentrations as high as $10^{-3}$ M and incubation of the apoenzyme with $1.5 \times 10^{-3}$ M *p*-chloromercuribenzoate for 72 hours at 0° C, pH 6.7, caused no inhibition of the "on" reaction.

The apoenzyme was also very resistant to strong oxidizing agents like periodate and hydrogen peroxide. Thus, 100 equivalents of periodate per mole of apoenzyme (protein concentration 15 μM) at pH 5.5 [11] caused no inhibition after two hours, and only after 72 hours was 50 % of the apoenzyme unable to couple with FMN. There was no effect of 0.1 M $H_2O_2$ after 30 minutes incubation at 25° C, pH 6.6.

*Acetic anhydride* is claimed to be the reagent of choice for the specific acetylation of amino groups on proteins [15]. Under the conditions used, the only other types of groups which could possibly react are sulfhydryl and phenolic hydroxyl. In the old yellow enzyme, which does not contain thiol groups, inhibition after acetylation could be due to blocking of essential amino groups or phenolic groups, or both. Fortunately, incubation of acetylated tyrosine at pH 11 for 5 minutes hydrolyzes this compound [15] without affecting acetylated amino groups. Therefore, the inhibition which remains after this alkaline hydrolysis of acetylated O.Y.E. can be attributed to acetylated amino groups alone.

Acetylation with acetic anhydride was found to inhibit the "on" reaction strongly, and none of this inhibition was reversed by incubation at pH 11. This alkalinity was in itself harmless to the protein. The Folin's phenol color was the same in the acetylated sample as in the control, within the experimental error (±5 %). It may therefore be concluded that acetic anhydride is specific for amino groups in the apoenzyme under the conditions used in our experiments. In order to determine quantitatively the importance of amino groups, the apoenzyme was subjected to different degrees of acetylation and $k_1$ determined for the different samples. It is shown in Fig. 1 that for example

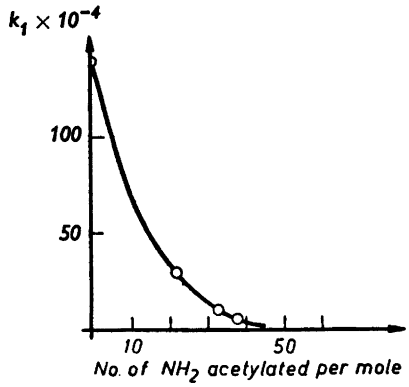



*Fig. 1. The effect of acetylation of apoenzyme on $k_1$, as measured in 0.1 M glycine pH 9.0.* 1 $\mu$l portions of acetic anhydride were added to 3 ml of a 27 $\mu$M solution of the apoenzyme in 2 M NaAc, pH 6.5, 0° C.

the acetylation of 22 of the 60 free amino groups resulted in 80 % decrease of the reaction velocity.

It was reported in the two previous papers in this series [1,6] that the dissociation constant, $K = k_2/k_1$, of the old yellow enzyme in water or non-dissociating electrolytes was less than $10^{-12}$ M between pH 4 and 10. This was mainly due to an extremely low "off" velocity constant ($k_2$) of the enzyme. Appreciable dissociation took place, however, in the presence of anions of strong acids, like chloride and sulfate. This effect was explained as a displacement of the phosphate group of FMN from its salt-like linkage to the amino groups of the protein. In view of these findings, it was interesting that an acetylated sample of the enzyme dissociated into FMN and protein even in the absence of chloride at pH 9.7, whereas no measurable dissociation in the absence, but an increased dissociation in the presence of chloride, took place at pH 5.3. A possible explanation is that, at pH 5.3, the number of positively charged, not acetylated amino groups was sufficient to keep the negatively charged FMN tightly bound, whereas this was not the case at pH 9.7, where the high pH would cause discharge of some amino groups.

The value of $k_1$ decreased continuously with increasing degree of acetylation (Fig. 1); $k_2$, on the other hand, had reached a maximum already when 7 of the 60 primary amino groups present in one molecule had been acetylated (See Table 2). Further, acetylation of up to 40 groups seemed not to increase but rather to decrease $k_2$ (however, see the discussion). The total combining capacity for FMN remained the same, 1 mole per mole, even in the most acetylated samples, where $k_1$ had decreased to 5 % of the value for the native apoprotein.

*Formaldehyde* is known to react with a number of groups in proteins and to form different crosslinkages between such groups. However, the reversible reaction that takes place with short exposure time and low concentrations of the reagent has been reported to involve mainly primary amino groups [15]. In

*Table 2. The equilibrium constant (K) and dissociation velocity constant ($k_2$) as a function of the degree of acetylation.*

| No. of acetylated $NH_2$ groups per mole apoprotein | Per cent acetylated | $k_1 \times 10^{-6}$ $sec^{-1}$ $M^{-1}$ | $K \times 10^7$ M | $k_2 \times 10^2$ (from $k_2 = Kk_1$ $sec^{-1}$) |
|---|---|---|---|---|
| 0 | 0 | 1.4 | $<10^{-5}$ | 0.00 |
| 7 | 12 (calc) * | 0.79 | 0.23 | 1.8 |
| 21 ** | 35 » | 0.30 | 0.63 | 1.9 |
| 21 ** | 35 | 0.29 | 0.52 | 1.5 |
| 26 *** | 43 (calc) | 0.19 | 0.74 | 1.4 |
| 26 *** | 43 » | 0.19 | 0.63 | 1.2 |
| 38 | 63 | 0.05 | 1.8 | 0.9 |
| 40 | 67 (calc) | 0.04 | 2.7 | 1.1 |

* The degree of acetylation was calculated from Fig. 1 on the basis of $k_1$ obtained.
** Two different apoprotein preparations.
*** » » » »

the first studies with formaldehyde the old yellow enzyme or the apoenzyme were added at $t = 0$ to test solutions containing buffer, formaldehyde, and FMN (for the apoenzyme). The rate of dissociation or association was measured within the first few seconds. In this way, the "instantaneous" effect of formaldehyde on the dilute (0.1—1.0 $\mu$M) protein could be observed. Above pH 7 both the dissociation ($k_1$) and the association reaction ($k_2$) were affected by very low concentrations of formaldehyde. The effect on the dissociation was observed as an increased $k_2$ in the presence of sodium chloride. This is shown in Fig. 2. The effect of formaldehyde on the association reaction is shown in Fig. 3. It is seen that even 0.02 M formaldehyde had a large effect in the alkaline range, a much smaller one in the acidic range. This is what would be expected, since only uncharged amino groups combine with formaldehyde.

However, the reaction of the dilute apoenzyme with formaldehyde was not readily reversible. For instance, if the apoenzyme was allowed to react with formaldehyde in borate buffer at pH 9.3 for a few seconds, and the pH adjusted to 6.5 by the addition of hydrochloric acid, $k_1$ for this sample was much lower than for a control containing the same amount of formaldehyde, borate and sodium chloride, but not incubated at pH 9.3.

In order to obtain a reversible effect of formaldehyde, a more concentrated solution of apoenzyme (52 $\mu$M) was incubated at 0° C with 0.4 M formaldehyde in 0.1 M phosphate buffer, pH 8.0. The association reaction was tested in 0.1 M borate buffer, pH 9.0 and $k_1$ was found to decrease from $28 \times 10^4$ $M^{-1}$ $sec^{-1}$ to $6 \times 10^4$ $M^{-1}$ $sec^{-1}$ after 5 minutes and to $3 \times 10^4$ $m^{-1}$ $sec^{-1}$ after 20 minutes incubation. When the formaldehyde-treated apoenzyme was dialyzed against 0.03 M phosphate, pH 7.1, over night, $k_1$ was restored to $24 \times 10^4$ $M^{-1}$ $sec^{-1}$. Thus, the reaction with formaldehyde around neutrality is reversible when a fairly high concentration of apoenzyme is used, but not when the concentration of apoenzyme is of the order of magnitude 0.1—1.0 $\mu$M. Also the sensitivity to formaldehyde inhibition was much less for concentrated samples

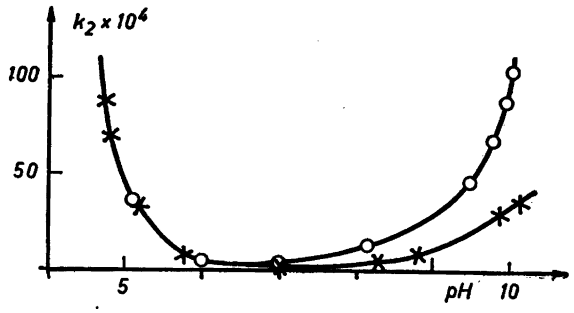



*Fig. 2. The effect of 0.12 M formaldehyde on $k_2$ in 0.4 M sodium chloride, 23° C.*

○○○○ 0.12 M formaldehyde
×××× no formaldehyde

50 μl O.Y.E., 64 μM, was added to 3 ml of a solution containing sodium chloride, 0.1 M sodium acetate + acetic acid (in the acidic range) or sodium hydroxide (in the alkaline range), and formaldehyde. $k_2$ was taken as the tangent at $t = 0$. pH was determined at the end of the experiment.

of the protein. For instance, a 52 μM solution of apoenzyme was not at all affected by 0.02 M formaldehyde even after 20 minutes incubation in borate buffer at pH 9, whereas the dilute samples of apoenzyme were affected strongly and instantaneously by 0.02 M formaldehyde (See Fig. 3).

*Iodine*, in all but one of the many proteins studied [15], reacts preferentially either in oxidation of thiol groups or substitution of tyrosine. The one exception is lysozyme where the imidazole groups were more readily iodinated than

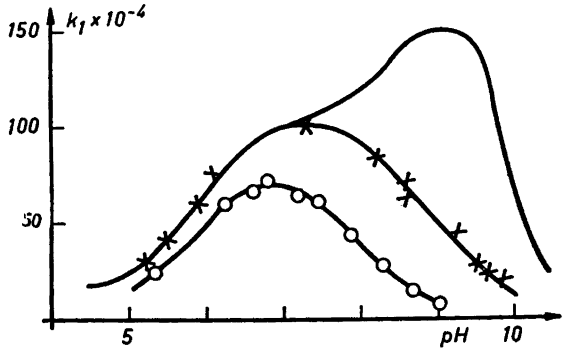


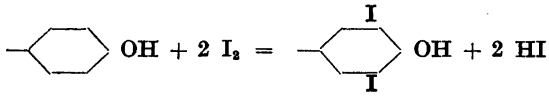

*Fig. 3. The effect of formaldehyde on $k_1$ at 23° C.*

– – – – without formaldehyde
×××× 0.02 M »
○○○○ 0.12 M »

15 μl apoenzyme, 42 × $10^{-6}$ M, was added to 3 ml of a solution of 0.15 μM FMN, 0.1 M sodium acetate, and formaldehyde. $k_1$ was measured within a few seconds. In the alkaline range the protein acted as buffer. pH was determined at the end of the experiment.

the tyrosyl groups [11]. Since the old yellow enzyme does not contain thiol groups, the iodine could be anticipated to enter into the 3- and 5-positions of tyrosine. Monoiodotyrosine has been proved to be a stable intermediate in the iodination of proteins, but in the cases investigated, relative small amounts of this compound were formed.

The iodination reaction proceeds according to the formula




Two moles of $I_2$ are thus required to form one molecule of diiodotyrosine.

An iodinated sample of O.Y.E. dissociated more rapidly in the presence of sodium chloride than did the control. The effect of iodination of the apoenzyme on $k_1$ and on $k_2$ (calculated from $k_2 = K\ k_1$) is shown in Fig. 4. Determination of the tyrosine content of the most iodinated sample, 13 moles of $I_2$ used per mole apoenzyme, showed that over 90 % of the iodine added had been used for the substitution reaction of tyrosine to form 6 moles of diiodotyrosine. The actual formation of diiodotyrosine was demonstrated qualitatively by paper chromatography of a hydrolysate [11]. The one mole of iodine per mole of enzyme which was not used for the formation of diiodotyrosine may have formed monoiodotyrosine or have caused some oxidation. In view of the great stability of the apoenzyme to other oxidizing agents, it is unlikely that a minor oxidation would cause inhibition. The iodine not present as diiodotyrosine would be sufficient only for the monosubstitution of one imidazole group; such a substitution appears unlikely. Even the most iodinated sample of the apoenzyme with 6 moles of diiodotyrosine per mole protein was able to quench the fluorescence of one mole of FMN per mole protein.

### The sedimentation of acetylated and iodinated apoenzyme in the ultracentrifuge

An acetylated sample of the apoenzyme with $k_1 = 43 \times 10^4\ M^{-1}sec^{-1}$ as compared to $140 \times 10^4\ M^{-1}sec^{-1}$ for the control, appeared homogeneous in the ultracentrifuge with $S_{20} = 5.42$ as compared to $S_{20} = 5.36$ for the unmodified protein.

An iodinated sample containing about 8 moles of $I_2$ per mole protein with $k_1 = 19 \times 10^6$ likewise appeared homogeneous in the ultracentrifuge with $S_{20} = 5.61$.

### The reaction of flavin adenine dinucleotide and riboflavin diphosphate with the apoenzyme

Warburg and Christian [16] have shown that the apoenzyme of O.Y.E. is nearly as enzymatically active with flavinadenine dinucleotide (FAD) as with FMN. It was therefore of interest to investigate whether FAD could form a nonfluorescent compound with the apoprotein. This was found to be the case, but the dissociation constant, $K$, of this compound around neutrality was

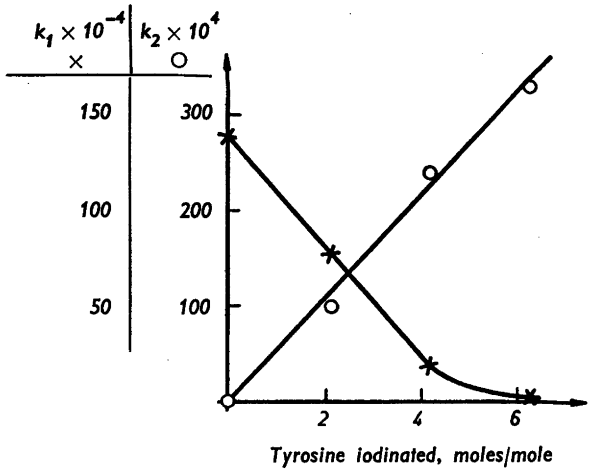



*Fig. 4. The effect of iodination of apoenzyme on $k_1$ and $k_2$, as determined from rate of reassociation and equilibrium constant in 0.1 M glycine pH 9.0.*

3 × 1.85 μl of $I_2$ in alcohol, 0.083 M, were added to 0.8 ml apoenzyme, 40.8 μM, which contained 23.8 moles tyrosine per mole apoenzyme. After iodination the tyrosine content was decreased to 17.5 moles tyrosine per mole apoenzyme. The apoenzyme was tested kinetically after each addition of 1.85 μl $I_2$.

some 200 000 times larger than for O.Y.E. The relatively high activity of FAD + apoprotein in Warburg's test system could have been due to the use of a large excess of FAD. With a large excess of riboflavin, the test system has been found to have a moderate activity [2]. The dissociation constant of riboflavin with the apoenzyme is ten times greater than for FAD + apoprotein. In Table 2, $K$, $k_2$, and $k_1$ for FMN, FAD, or riboflavin + apoprotein have been presented. It is seen that $k_1$, representing the number of successful collisions, is not grossly different for the three substances, but that the stability of the complexes formed, as expressed in $k_2$, is much greater for FMN-apoenzyme than for the two others. $k_1$ for FAD + apoenzyme has, like the FMN and riboflavin systems, a pH maximum around 9.

Some preliminary experiments with a riboflavin fraction from the paper electrophoresis of FMN, probably a riboflavin diphosphate, indicated that this compound formed a complex with the apoenzyme, with $K$, $k_2$, and $k_1$ of the same order of magnitude as for FAD and riboflavin.

## Some preliminary observations

*Phenylisocyanate*, which primarily reacts with amino groups in proteins not containing thiol groups [15], was also found to decrease the "on" reaction. Incubation took place at pH 7.0, 0° C, with equal amounts (by weight) of reagent and protein. After 15 minutes with shaking, $k_1$ was decreased several fold. Unfortunately turbidity appeared gradually.

*Diazonium compounds* are known to react preferentially with imidazole and phenolic groups of proteins [15] and this reagent should therefore inhibit

*Table 3. The reaction of the old yellow apoenzyme with FMN, FAD or riboflavin in 0.1 M glycine, pH 9.0, 23° C.*

| | $K$ M | $k_1$ $M^{-1}$ $sec^{1-}$ | $k_2$ $sec^{1-}$ |
|---|---|---|---|
| FMN | $<10^{-12}$ | $1.4 \times 10^{6}$ | $<10^{-6}$ |
| FAD | $2 \times 10^{-7}$ | $0.2 \times 10^{6}$ | 0.04 |
| Riboflavin | $2 \times 10^{-6}$ | $0.1 \times 10^{6}$ | 0.2 |

the coupling of FMN to apoenzyme if tyrosyl groups were essential. When apoenzyme (28 $\mu$M) was allowed to react for 15 min at 0° C, pH 8.8, with azobenzene sulfonic acid (96 $\mu$M), $k_1$ for the treated sample was only 5 % of that of the control. In control experiments it was found that sulfanilic acid and nitrite added separately had no effect on $k_1$. The combining capacity was not determined in these experiments.

An acetylated sample of O.Y.E., when tested manometrically with TPN, Zwischenferment, glucose-6-phosphate, and oxygen at pH 7.4, was found to have lost an appreciable part of its activity. This was probably due to a partial dissociation of the acetylated enzyme under these conditions, as indicated by other experiments.

The enzymatic activity of O.Y.E. in the TPN-Zwischenferment system was decreased by iodination of the enzyme, probably again because of increased dissociation.

Walaas and Walaas [17] have demonstrated inhibition of the enzymatic activity of D-amino acid oxidase by structural analogues of the FAD molecule, and it was suggested that it could be due to inhibition of the coupling of FAD to the protein moiety. These investigators found that adenylic acid and ATP exerted 50 % inhibition in as low concentration as $5 \times 10^{-4}$ M, whereas adenosine, adenine, and hypoxanthine were less inhibitory. In the case of the resynthesis of O.Y.E. from apoprotein and FMN we found that adenosine-3-phosphate, adenosine-5-phosphate, adenosine, adenine and hypoxanthine exerted about the same inhibition, a decrease of $k_1$ by 75 % in the concentration $4 \times 10^{-3}$ M. Like the inhibition by polyvalent anions, the adenine inhibition was counteracted by chloride ions.

Atabrin, which has been shown to be a competitive inhibition of FMN in cytochrome reductase [18] and also to interfere with the activity of the FAD linked D-amino acid oxidase [19], was so strongly fluorescent that its possible inhibiting effect on the recombination reaction of FMN and apoenzyme could not be tested with our method. However, the fluorescence of atabrin was found to be somewhat quenched by the apoenzyme, and this quenching was a time reaction which could be recorded in our apparatus, in contrast to the instantaneous quenching caused by some ions. This shows that some reaction occurs between atabrin and the apoenzyme, which may resemble the binding of flavin coenzymes.

## DISCUSSION

The partial modification of some groups in a protein, for example by acetylation or iodination, must necessarily lead to inhomogeneous preparations. Thus, if we acetylate 7 of 60 primary amino groups, 7 is an average value, and some molecules are more and some less acetylated. Furthermore, molecules with the same number of acetylated groups may be different. In our case, the FMN-binding groups may be acetylated in some molecules, in others not.

All this means that we cannot possibly expect the kinetics of the combination of FMN with such preparations to follow the second order for $k_1$ or the first order for $k_2$[1]. Because we determined the association velocity constant as the tangent of the curve at $t \approx 0$, our values of $k_1$ are maximum values, and the significance of changes in $k_1$ is all the more emphasized.

The experiments with the amino group modifying reagents support our earlier conclusion [1] that amino groups in juxtaposition to the phosphate group may serve as binding sites for FMN in the old yellow enzyme. Further, these groups appear to be more reactive than other amino groups. As can be seen from Fig. 1 the acetylation of half of the amino groups (30 of 60) decreases $k_1$ from $1.4 \times 10^6$ to (the maximum value) $0.14 \times 10^6$ which is 10 % of the original values. If the acetylation occurred at random, we would have expected 50 % decrease of $k_1$ if one $-NH_3^+$ and 75 % decrease if two $-NH_3^+$ groups were bound to FMN. This calculation is based on the assumption that the acetylation of non-binding $-NH_3^+$ groups does not affect $k_1$. This assumption may be valid, since the increased negative net charge of the molecule caused by acetylation must be largely neutralized by $Na^+$ ions from the solution of ionic strength 0.1. The total number of collisions between FMN- and acetylprotein molecules can therefore be expected to be roughly the same as for FMN + native apoprotein. The strong decrease of $k_1$, with small degree of acetylation, was not unexpected, since the combining site is likely to be located at the surface of the protein. Because riboflavin combines with the apoprotein, even a total acetylation of all amino groups should not abolish the combining capacity of 1 mole FMN per mole of apoprotein, unless the acetyl groups caused steric hindrance. It was actually observed that acetylation of 40 amino groups did not decrease the FMN combining capacity. This finding demonstrates the auxiliary character of the $PO_3^{-+}(-NH_3)_2$ bonds; they serve to stabilize the complex, but are not essential for the protein-isoalloxazine linkages, which quench the fluorescence, shift the absorption band from 445 to 465 m$\mu$, and activate the coenzyme part of the complex.

As mentioned before, the dissociation constant, $K$, was calculated from one value of the degree of unsaturation of the modified protein in the presence of a small excess of FMN. Since a modified protein is non-homogeneous, the $K$ determined in this way must be a very crude average of the $K$'s for the different protein fractions and depending upon the concentrations of FMN and protein used. The same must apply for the value of $k_2$ that was calculated from $k_2 = k_1 K$, where $k_1$ is a maximum value. The data for $K$ and $k_2$ given in Table 2 and for $k_2$ in Fig. 4 should therefore be regarded as very rough approximations. The sudden increase of $k_2$ after acetylation of 7 amino groups per

molecule must undoubtedly be correct (see Table 2), but the decrease in $k_2$ with increasing acetylation is probably insignificant (because of overestimated $k_1$).

The 3,5-iodination of tyrosyl groups caused a drastic decrease in $k_1$. Thus, iodination of 6 of the 24 tyrosine groups decreased $k_1$ from $1.4 \times 10^6$ to $0.06 \times 10^6$, and a measurable dissociation of the complex appeared. Since the phosphoric acid residue is bound by amino groups it seems very likely that tyrosine-OH groups serve as binding groups for the riboflavin part of FMN and that these tyrosine groups like the binding amino groups are preferentially exposed to chemical reagents. However, the iodination, as far as it could be followed experimentally, did not interfere with the combining capacity for FMN, but as in the case of acetylation, iodination increased the dissociation of the complex strongly. The 3,5-iodination leads to an increase of the acid dissociation constant of the tyrosine hydroxyl group [20], but not to a blocking of the hydroxyl group. It is therefore understandable that the iodination could lead to changes in $k_1$ and $k_2$, but not in the total FMN-combining capacity.

It is not yet possible to decide whether the tyrosine-riboflavin linkage is auxiliary, like the $-PO_3^{-+}(NH_3)_2$ bond, or whether it has essential character. There is thus still no direct experimental evidence to support Weber's [21] suggestion of tyrosine-OH quenching the fluorescence in the old yellow enzyme. But if this happens to be the case, the same linkage is likely to be responsible for the spectrophotometric band shift and the activation of the coenzyme.

The coupling between FMN and the apoprotein of the old yellow enzyme, which results in quenching of the fluorescence of FMN and displacement of its absorption band, appears to be a highly specific one in respect to both interacting molecules. From our data it is evident that such compounds as FAD, riboflavin, and our incompletely defined riboflavin diphosphate, which all contain the isoalloxazine nucleus, become more loosely bound to the apoprotein than FMN. This specific binding of one molecule of FMN to one molecule apoprotein is likely to result from complimentariness in structure of the combining region on the protein molecule [22]. This complimentariness may include both similarity in surface configuration and the juxtaposition of the special combining groups.

Thanks are due to Miss Ann Louise Swanson for skilful technical assistance.

The authors are indebted to *Statens Medicinska Forskningsråd, Knut och Alice Wallenbergs Stiftelse* and *The Rockefeller Foundation* for financial support.

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Received September 10, 1955.