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Immunologic Studies of the Old Yellow Enzyme. The Inhibition of Enzyme Activity by Antibodies

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The effect of precipitating antibodies against the Old Yellow Enzyme on the activity of the enzyme and its apoprotein has been investigated. Immediately after the addition of antiserum to the enzyme a partial inhibition of the activity occurred, evidently due to the reversible formation of enzyme-antibody complexes. The inhibition increased if enzyme and antiserum were incubated for some time prior to the activity determination. The antibodies and reduced triphosphopyridine nucleotide, which is oxidized by the enzyme, did not compete for the same binding sites on the enzyme molecule. No immediate effect of antiserum on the association reaction between apoprotein and coenzyme could be demonstrated. After incubation, the apoprotein was precipitated by the antibodies. The influence of the complexity of the enzyme-inhibitor system on the kinetics of the inhibition is discussed.

In a previous study [1] precipitating antibodies reacting with crystalline Old Yellow Enzyme (OYE) * were obtained after immunization of rabbits with impure enzyme preparations. The experimental results indicated the existence of specific antibodies against the pure OYE in the antisera. A comparison between the immune reactions of the holoenzyme and the apoprotein, using the agar-gel diffusion technique, showed an identical behaviour of the two proteins. It was concluded that the coenzyme group, FMN, was not involved in the antigenically active part of the enzyme molecule, or in its binding sites to antibody. This, however, is no obstacle for the inhibition of enzyme activity by antibody. Preliminary studies showed that inhibition did occur.

The precipitin reactions had thus provided some information concerning the relationship between active groups of the enzyme and its binding sites to antibody, suggesting that these had different localizations on the enzyme molecule. It then appeared of interest to further investigate the mechanism of inhibition of enzyme activity by antibodies.

* In this paper the following abbreviations are used: OYE = Old Yellow Enzyme; FMN = flavin mononucleotide; TPN = triphosphopyridine nucleotide; TPNH = reduced TPN.

MATERIALS AND METHODS

OYE was prepared by the method of Theorell and Åkeson [2], and the *apoprotein* obtained from the enzyme according to Warburg and Christian [3]. Evaluation of enzyme and apoprotein solutions with regard to purity, concentration, and equivalence was carried out as in the previous investigation [1]. OYE and apoprotein preparations used in the activity studies were of purity 0.54—0.70.

Glucose-6-phosphate (di-sodium salt), *glucose-6-phosphate dehydrogenase (Zwischenferment)*, *FMN*, *TPN* and *TPNH* used in the experiments were all commercial preparations (Sigma Chemical Company). The concentrations of the FMN, TPN and TPNH solutions were determined in a Beckman DU spectrophotometer at 445 mμ[2], 260 mμ[4], and 340 mμ[5], respectively. The activity of the Zwischenferment preparation was stated to be approximately 800 Kornberg units [6] per gram under optimal conditions. The reduction of TPN in the presence of Zwischenferment and excess glucose-6-phosphate in 0.13 M sodium phosphate buffer of pH 7.4 at +37°C was studied spectrophotometrically at 340 mμ[7]. At TPN concentrations from 1 to 20 × 10^{-6} M a fluorimetric technique [8] was employed by measuring the fluorescence of the TPNH formed and comparing it to that of a TPNH standard.

Immunization and testing of the antisera with precipitin reactions were performed as described before [1]. Two rabbits were used in these experiments. Each received 51.5 mg of an enzyme preparation of purity 0.54, mixed into an adjuvant, in the first injection, and 25.8 mg of the same preparation as a boosterdose. Control sera were collected from the same animals before immunization. Antisera used in the inhibition studies reacted with the crystalline enzyme, forming one single precipitin line, when tested with the agar-gel diffusion technique according to Ouchterlony [9]. Also, they showed identical reaction with the OYE and an equivalent apoprotein solution.

Activity studies of the OYE. The activity of the OYE was tested by the conventional Warburg manometric technique [10] in an oxidative system with glucose-6-phosphate, Zwischenferment, and TPN [11]. The activity of the test system is inhibited by the presence of phosphate [12,13] and other ions [14]. Nevertheless, in the present investigation a sodium phosphate buffer of pH 7.4 in a final concentration of 0.13 M was used, since it was considered appropriate to obtain a similar ionic atmosphere as in other OYE-antibody experiments. The reaction mixture also contained 0.2 ml 0.04 M KCN, and 0.2 ml 10 % KOH was added in the central well of each Warburg flask. In the different experiments the concentration of OYE or TPN varied. The addition of Zwischenferment was 1 mg, and glucose-6-phosphate was always added in excess. The volume of the reaction mixture was 3.0—4.0 ml.

In the inhibition studies with antiserum, the OYE was usually added after the serum and other reactants to the main compartment of the reaction vessel, and the reaction was started by the addition of Zwischenferment from the sidearm. When the effect of incubation time was studied, the mixing of the reactants followed a different procedure, which is described in the result section.

The experiments were performed at +37.0°C. The filled reaction vessels were equilibrated with oxygen for 15 min, then with stopcorks closed for another 15 min, before the reactions were started. The activity was expressed as μl oxygen consumed during the first 30 min of the reaction, when the oxygen uptake was constant.

Activity studies of the apoprotein. The activity of the apoprotein could be determined by the method used for the OYE, when FMN was also added to the reaction mixture. In order to estimate the influence of antiserum on the association between apoprotein and FMN, it was necessary to consider its effect on the reaction product OYE. The experiments were arranged in the following way (also illustrated in Table 1). OYE was formed in the presence of antiserum by incubating apoprotein and serum and, later, adding FMN equivalent to the apoprotein. The activity of this enzyme was compared to the activity of that formed in the presence of control serum. To eliminate the effect of the antibodies on the formed OYE, antiserum was also added in the second case. Besides the effect of a different incubation time, which could be controlled in separate experiments, a difference in activity could now be attributed to the fact that in the first case antiserum reacted with the apoprotein before the FMN was bound.

The reaction between apoprotein and FMN was also studied fluorimetrically [15] by adding small aliquots of a FMN solution to an apoprotein solution in 0.13 M sodium

Table 1. A scheme of the method for testing the influence of antiserum on the apoprotein, which later reacted with FMN to form OYE, the activity of which was measured.

Incubation, +22°C, 10 min.	Incubation, +22°C, 10 min.	Enzyme activity measured
Apoprotein Antiserum	+ FMN	+ Control serum
Apoprotein Control serum	+ FMN	+ Antiserum

phosphate buffer of pH 7.4 and observing the quenching of the fluorescence of the FMN. In the inhibition studies, antiserum was mixed with the apoprotein a few minutes before the FMN was added. The experiments were performed at +37°C.

RESULTS

The activity of the OYE in the Zwischenferment-OYE system. The oxygen consumption of the test system varied with the concentration of the OYE, when all other reactants were added in excess, *i.e.* further addition of anyone of these did not increase the activity. Using an enzyme solution of purity 0.64, 0.10 mg OYE * caused an uptake of 100 μl oxygen per 30 min. The turnover number corresponding to this value is 155, expressed as moles of oxygen per mole of OYE per minute **.

Keeping the OYE concentration constant, the activity varied with the concentration of TPN. Plotting the oxygen consumption against the negative logarithm of the TPN concentration resulted in a sigmoid curve (Fig. 1). Raising the TPN concentration to values higher than those shown in Fig. 1 did not cause increased activity. The same reaction is drawn as a double reciprocal plot [16] in Fig. 2, bottom line. The Michaelis constant determined from such a plot was 0.90×10^{-5} M. Spectrophotometric and fluorimetric studies of the reduction of TPN by the amount of Zwischenferment, used in each Warburg experiment, showed that the rate of reduction exceeded the rate of oxygen uptake in the Zwischenferment-OYE system at all TPN concentrations tested.

* In the following the amounts of OYE are always expressed as the amount of pure OYE, as calculated from the light absorption at 464 mμ.

** The corresponding value, expressed as moles of oxygen per mole of protein-bound FMN, is 77.5. This figure is higher than the previously reported values [3,11]. This may be explained by the fact that purer reagents, especially Zwischenferment, were used in the present investigation. Similar results were obtained with other OYE preparations.

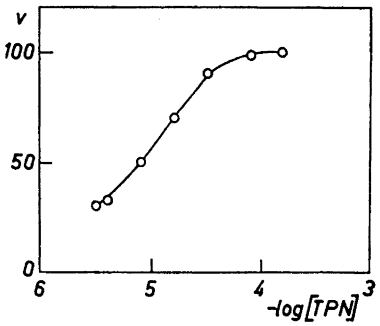

Fig. 1. The activity of 0.10 mg OYE at different concentrations of TPN, expressed as μl O_2 consumed per 30 min (v), and marked along the ordinate. Along the abscissa the negative logarithm of [TPN], expressed in moles per liter, is marked. Each reaction vessel also contained 1.75×10^{-2} M glucose-6-phosphate, KCN, and 0.13 M sodium phosphate buffer, pH 7.4. The reaction was started by adding 1 mg Zwischenferment. The total volume of the reaction mixture was 3.3 ml.

The results of the Warburg experiments were influenced by the ionic conditions. Thus it was found that lowering the phosphate concentration had no effect at high concentrations of TPN, but caused increased enzyme activity when less TPN was used, (*cf.* Refs.[12-13]).

The inhibition of the OYE by antiserum at different concentrations of TPN. The activity of 0.10 mg OYE (same preparation as in the preceding group of experiments) was tested at different TPN concentrations in the presence of antiserum of two different concentrations. The results are summarized in Fig. 2, where they are shown in a double reciprocal plot. The inhibition

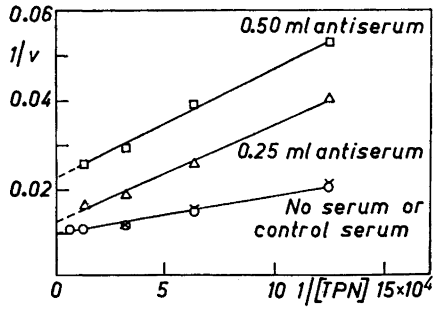

Fig. 2. The inhibition by antiserum of the activity of 0.10 mg OYE at different concentrations of TPN, shown in a Lineweaver-Burk plot. Along the ordinate $1/v$ is marked, along the abscissa 1/TPN. Antiserum and TPN were added before the OYE to the main compartment of the Warburg flask. Otherwise, the experimental conditions were as described under Fig. 1. Bottom curve O — O shows the activity without any serum added, × marks the activity in the presence of 0.50 ml control serum, □ — □ shows the activity in the presence of 0.50 ml antiserum, △ — △ the activity in the presence of 0.25 ml antiserum. Quantitive precipitin reaction between the OYE and antiserum used showed equivalence when 0.17 mg OYE was added to 1.00 ml antiserum.

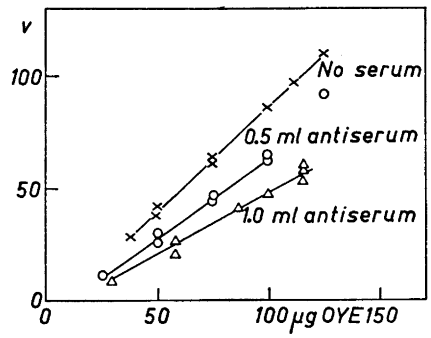

Fig. 3. The inhibition by antiserum at different concentrations of OYE. The activity (v) is marked along the ordinate, the amount of OYE added along the abscissa. Excess TPN was added in each reaction. Other experimental conditions essentially as described under Figs. 1 and 2. The top line × ——— × represents activity studies without the addition of antiserum, the other lines experiments where 0.5 ml (O — O) or 1.0 ml (△ — △) was added in each reaction. In quantitative precipitin reaction between the enzyme and serum used, 0.12 mg OYE was in equilibrium with 1.0 ml antiserum.

caused by the antiserum appeared to be non-competitive [16]. However, prolonging the lines, representing the inhibited and uninhibited reactions, to the left of the ordinate showed that it did not completely follow the kinetics of a non-competitive inhibition, but probably represented some intermediate form.

Control serum was practically without effect. In some experiments a slight inactivation was observed, perhaps due to an alteration of the ionic milieu. As can be seen in Figs. 2 and 5, the effect of control serum was by no means comparable to that of antiserum.

The inhibition of varying concentrations of OYE by antiserum. In these experiments different dilutions of an OYE preparation of purity 0.54 were used. TPN was added in excess. The results are shown in Fig. 3, where the top line shows the reaction without antiserum. The addition of 1 ml of control serum in each experiment gave essentially identical values. The prolongation of the line, representing the activity of the OYE without inhibitor, does not pass through the origin of the coordinate system. This is probably due to the fact that oxygen was released during the experiments, perhaps in connection with the tipping procedure. It might also be that a constant amount of enzyme was blocked by some impurity in the reaction mixture. There was a rectilinear relationship between the amount OYE added and the oxygen consumption. In the presence of antiserum there was a rather constant relative inhibition by the same amount of serum at the different OYE concentrations, at least at the low concentrations tested.

The inhibition by different concentrations of antiserum. The reactants were the same as in the preceding group of experiments, but the amount of OYE was 0.10 mg in each reaction, and the amount of antiserum added varied. Fig. 4 shows the relative activity of the OYE at the different serum concentrations. At the low concentrations the decrease in activity appeared to be pro-

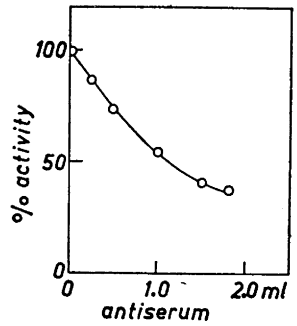

Fig. 4. The activity of 0.10 mg OYE at different concentrations of antiserum. Other experimental conditions were as described under Fig. 3, and the enzyme and antiserum were the same as those, used in the experiments illustrated in that figure. The relative activity is marked along the ordinate, where 100 % represents the activity in the absence of antiserum. (Addition of 1.0 ml control serum gave the same activity.) Along the ordinate the amount of antiserum, used in each experiment, is indicated.

portional to the amount serum added, but further increase in serum concentration was not as effective.

The effect of the incubation time on the inhibition of the OYE by antiserum. In all experiments described OYE was added to the main compartment of the Warburg flask, which contained glucose-6-phosphate, TPN, and antiserum, and Zwischenferment was added 30 min later from the sidearm. A series of experiments were performed in an identical way, except that the antiserum

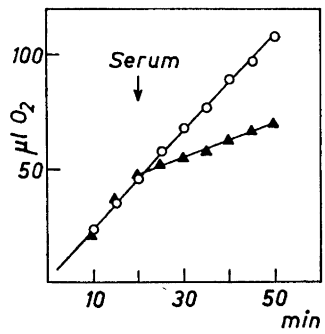

Fig. 5. The influence of antiserum on the "active" enzyme. 0.10 mg OYE was used in each reaction, the TPN concentration was 1.62×10^{-5} M. The reaction was started by the addition of Zwischenferment from one side-arm of the Warburg flask, the experimental conditions being the same as described under Fig. 1. After 20 minutes 0.50 ml serum was added from the other side-arm. Along the ordinate are marked consumed µl of O_2, along the abscissa the time in minutes from the start of the reaction. In the flask where antiserum was added (△ — △) there was a clear effect upon the reaction velocity. The addition of control serum (○ — ○) had some effect, although much less pronounced. Enzyme and antiserum were the same as those used in experiments illustrated in Fig. 2.

was not added at the beginning but kept in a second sidearm of the Warburg flask. After the reaction had been started, and the oxygen uptake studied for 20 min, the serum was added (Fig. 5). The inhibition caused by a given amount of antiserum under these conditions was of the same magnitude as when the antiserum was added at the same time as the TPN.

The inhibition was also studied when the OYE was added to the main compartment of the reaction vessel, containing glucose-6-phosphate, Zwischenferment, and antiserum, and the TPN was added 30 min later from the sidearm. This procedure did not cause any difference in the degree of inhibition except at low TPN concentrations, where a slight increase was noticed.

From these experiments it might appear that the inhibition was practically the same whether the antiserum was allowed to come in contact with the enzyme before, at the same time as, or after the TPN. However, when OYE and antiserum were first incubated for 30 min at + 37°C, then added to the Warburg flask, and the TPN was introduced 30 min later from the sidearm, the inhibiting capacity of the serum increased markedly. The antibodies could thus react with the OYE for 60 min before the appearance of the reduced TPN. Under the experimental conditions described in Fig. 4, 1.0 ml antiserum inhibited the activity of 0.10 mg OYE 45 %. Using the same reactants, but following the preincubation procedure described above, the inhibition was 71 %. This increase could not be due to the mere fact that OYE and antiserum had reacted for 60 min, because in the first case the inhibition was still approximately 45 % when OYE and serum had been mixed for a considerably longer time.

The effect of antiserum on the association reaction between apoprotein and FMN. No influence of the antiserum was observed when this reaction was studied by the manometric technique. These experiments were performed with concentrations of serum which had an immediate effect on an OYE solution, equivalent to the apoprotein used. Incubation with antiserum for a longer period of time, and removal of formed precipitate before the addition of FMN, decreased the activity of the apoprotein solution. When antiserum was added in excess, incubation performed overnight and the precipitate removed, no enzymatic activity remained.

The study of the reaction between apoprotein and FMN, using the fluorimetric technique, encountered certain difficulties, because the presence of serum caused a large background fluorescence, thus making the readings less accurate. Therefore, the experiments were carried out in excess antigen zone, but with serum concentrations that inhibited OYE solutions equivalent to the apoprotein used. The ability of the apoprotein to bind FMN was not impaired by the presence of antiserum. A typical experiment is shown in Table 2. Preincubation of apoprotein and antiserum before the addition of FMN resulted in the appearance of a precipitate, which made the fluorimetric technique unsuitable.

DISCUSSION

The special character of the enzyme-inhibitor system must be considered in interpreting kinetic data of the inhibition of enzyme activity by antibodies. Even if the antibodies possess a very specific affinity to the enzyme molecules,

Table 2. Results of a typical experiment for studying the influence of antiserum on the association reaction between apoprotein and FMN by the fluorimetric technique. The molarity of the FMN bound, and the association reaction velocity constant k_1 were determined as described in Ref.[15]. From the amount FMN bound in the sample of apoprotein, which did not contain serum, it was calculated that 0.19 mg apoprotein was added in each test. 0.17 mg of an equivalent OYE solution was needed to reach the equivalence zone in quantitative precipitin reaction with 1.0 ml of the antiserum used.

Reaction mixture to which FMN was added.	FMN coupled, M.	Association velocity constant, k_1, $M^{-1} \times sec^{-1}$.
3.0 ml apoprotein 0.5 ml buffer	1.058×10^{-6}	16.8×10^4
3.0 ml apoprotein 0.5 ml control serum	1.160×10^{-6}	17.6×10^4
3.0 ml apoprotein 0.5 ml antiserum	1.137×10^{-6}	15.9×10^4

there is no simple relationship in their reaction with the antigen. During the immunization antibodies of different reactivity may develop [17]. The inhibition may depend upon the number of antibody molecules bound to each enzyme molecule [18]. The enzyme-antibody interaction changes considerably with the time, and thus it is of importance at which stage in the reaction the enzyme is brought to act, that is to combine with the substrate [19,20]. The inhibition may be influenced by the fact that the inhibitor molecules are of equal magnitude as, or often larger than the enzyme [21]. Such characteristics emphasize the difficulties in arriving at definite conclusions from inhibition studies of enzyme-antibody reactions.

The OYE preparations used in the activity studies were not pure, and the antisera were obtained after immunization with impure OYE. Still it is reasonable to believe that the enzyme reacted with specific antibodies in the sera. Evidence in favour of this has been presented in a previous paper [1], and the antisera used in the activity studies reacted in an identical way when tested in precipitin reactions. The action of antiserum on Zwischenferment, which is also a yeast enzyme, was tested by measuring spectrophotometrically the ability of this enzyme to reduce TPN, and no effect was found.

In the system for testing the activity of the OYE this enzyme acts as an oxidant to the TPNH formed, when glucose-6-phosphate is oxidized in the presence of Zwischenferment and TPN. Observations have been made of the formation of a complex between the OYE and the reduced pyridine nucleotide [22,23], and it appeared likely that such a complex was formed in the present experiments. Confirmatory evidence seemed to be obtained, when the activity of the OYE was tested with different concentrations of TPN in the reaction mixture. Plotting the activity against the negative logarithm of the TPN

concentration resulted in a sigmoid curve (Fig. 1), similar to the dissociation curve typical of enzyme-substrate interactions [24]. This curve evidently described the activity of the OYE, since the activity of the Zwischenferment alone exceeded that of the Zwischenferment-OYE system. Also, further additions of TPN did not cause increased oxygen uptake when all reactants, except OYE, were in excess.

The inhibitory effect of antiserum on the OYE was immediate. From the experiments, illustrated in Fig. 3, where a constant amount of antiserum caused the same relative inhibition at different concentrations of OYE in the test system, it appeared that the enzyme-antibody association was reversible under the existing experimental conditions [25]. Also, the curvilinear relationship obtained, when the relative activity of a constant amount of OYE at different concentrations of antiserum was plotted against the amount antiserum added (Fig. 4), indicated a dissociation of the antigen-antibody complexes. When the OYE and antiserum were allowed to react for a long enough time before the enzymatic activity was tested, the inhibitory capacity of the antiserum increased. Since this occurred, when no changes were made in the experimental conditions except the preincubation of enzyme and serum, the effect must depend upon a firmer association between OYE and antibodies. Allowing the OYE to react with the TPNH seemed to protect the enzyme from the action of the antibodies. This observation is similar to the reduction of the inhibition, which has been found to occur in other enzyme-antibody systems, when the enzyme is incubated with the antiserum in the presence of substrate or coenzyme (see Ref.[21]), and which has been ascribed to the fact that the aggregation of enzyme-antibody complexes is prevented [19].

The studies of the inhibition with different concentrations of TPN in the reaction did not quite clarify, whether the antibodies and the pyridine nucleotide competed for the same binding sites. Since the enzyme-antibody reaction was reversible under the conditions of these experiments, and the K_M value in the OYE—TPNH system indicated a high affinity between enzyme and TPNH [24], the activity of the enzyme would have been completely dependent on the TPN concentration if the binding sites were identical. Therefore it seems more reasonable that the inhibition was non-competitive. That it nevertheless showed some trends of competitiveness could be due to the fact that the large inhibitor molecules caused a steric hindrance for the TPNH to reach its binding sites [21].

It appeared that concentrations of antiserum, having an immediately noticeable effect on the activity of the OYE, did not cause a corresponding inhibition of the ability of equivalent apoprotein solutions to bind FMN. In these experiments apoprotein and antiserum were mixed for a time, long enough to allow interaction, before the FMN was added. The affinity of the apoprotein to FMN is very high [15], and thus it is understandable that the FMN might have displaced the antibodies from the apoprotein, before the activity was tested in the Warburg experiments. However, the use of the fluorimetric technique, allowing direct registration of the association reaction and determination of the velocity constant, did not reveal any influence of the antiserum on the ability of the apoprotein to bind FMN, although this could only be tested at low serum concentrations. Such an effect has been demonstrated

after chemical modification of the apoprotein [26]. On the other hand, the apoprotein was precipitated by the antiserum. These results suggest that the antibodies were bound to other sites on the apoprotein molecule than the FMN, and thus they support the findings of previous precipitin reactions.

Besides the blocking of catalytic sites on the OYE, other factors might have contributed to explain the observed inhibition. The binding of large antibody molecules to the OYE must undoubtedly have altered the physical properties of the enzyme. It may also be that the association caused intramolecular changes in the enzyme, impairing its ability to function as an electron transmittor.

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