The Influence of Antibody on the Interaction between Nucleotides and Glutamic Dehydrogenase*

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ABSTRACT: Rabbit antisera to chicken or beef liver glutamic dehydrogenase have been observed to influence the interaction between the purified enzymes and various nucleotides. At saturating levels of substrates and without preincubation of antiserum with enzyme the antiserum need not inhibit the initial velocity of the reaction: α-ketoglutarate + ammonia + reduced diphosphopyridine nucleotide → glutamate + diphosphopyridine nucleotide + water. Under conditions where antiserum does not inhibit the above reaction it has been found to prevent, to a significant extent, the inhibitory effects of guanidine triphosphate and excess reduced diphosphopyridine nucleotide as well as the activating effect of adenosine diphosphate. It also effectively reduces both the inhibition by adenosine triphosphate at pH 8 and the stimulation by large amounts of adenosine triphosphate at pH 7. These results have not been obtained with normal rabbit sera. If the antisera are absorbed with enzyme and the enzyme-specific antibodies thereby precipitated removed, the remaining sera no longer exhibit any influence on the action of the nucleotides. Possible mechanisms by which antibody might influence the action of regulators on glutamic dehydrogenases are discussed.

At a recent conference (Cinader, 1963) much evidence was presented to substantiate the conviction that antibody–enzyme systems will provide further insight into reaction mechanisms, molecular structures, and the nature of catalytic sites of enzymes. Antibodies to beef liver glutamic dehydrogenase have been utilized to confirm the existence of a variety of molecular configurations of this enzyme (Talal *et al.*, 1963). A number of reagents that affect enzymatic activity were shown to alter antibody–enzyme interaction as evaluated by agar diffusion and immunoelectrophoresis. The converse can also occur; that is, the combination of enzyme and antibody can alter the influence of many of these same reagents on catalytic activity. It has been shown that antibodies to beef liver glutamic dehydrogenase are capable of markedly inhibiting the enzyme obtained from a variety of sources (Bollet *et al.*, 1962). In this paper, evidence will be presented indicating that under certain circumstances the antibody does not inhibit but, rather, protects the enzyme molecule against both inhibition and activation induced by a variety of nucleotides.

Materials and Methods

Chicken liver glutamic dehydrogenase was purified for the most part according to the method described by Kubo *et al.* (1959). The essential step in this method simply consists in protecting the enzyme against denaturation by heating an homogenate of chicken livers and water at 58° for 5 min in the presence of 5% sodium sulfate. After removal of the precipitate the concentration of sodium sulfate in the supernatant fluid is rasied to 20% at room temperature. After centrifugation the precipitate is dissolved at room temperature in 0.1 M sodium phosphate buffer, pH 6.4, and the procedure repeated with the concentration of protein maintained at about 10 mg/ml. After the second precipitation and at a protein concentration of 10–15 mg/ml and about 10% sodium sulfate, crude crystals formed in approximately 7 days if the solution were left undisturbed in the cold. In our hands recrystallization was most readily accomplished by redissolving the crystals in 0.1 M sodium phosphate, pH 6.4, spinning out the insoluble material and adding small quantities of ammonium sulfate. The crystals at first glance appeared to be hexagonal plates, but closer examination indicated they were actually cuboidal, presenting an hexagonal appearance when viewed on end. The enzyme was found to be homogeneous upon ultracentrifugation, and electrophoretically and immunologically pure.

Beef liver glutamic dehydrogenase was obtained as an ammonium sulfate–free suspension in glycerin from Calbiochem. It was recrystallized from ammonium sulfate and extensively dialyzed against 0.05 M K_3PO_4, pH 7.5, before use.

Preparation of Antisera. Antisera were obtained by the following general procedure. Before injection into rabbits the enzymes were dialyzed extensively against 0.05 M K_3PO_4, pH 7.5, to remove ammonium sulfate. Immunization was initiated by the administration of 5–10 of mg antigen in Freund's Adjuvant *via* the toepads

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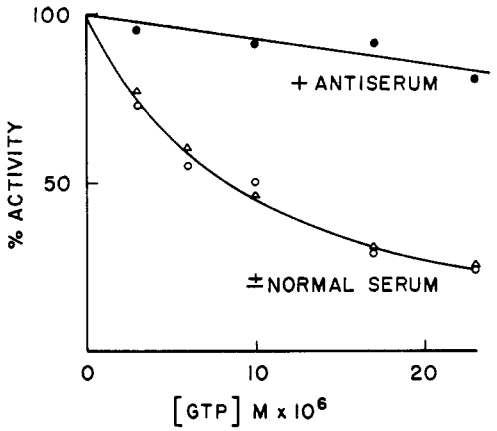

FIGURE 1: Effect of antiserum *vs.* increasing amounts of GTP. The reaction mixture consisted of DPNH (1.5×10^{-4} M), α-ketoglutarate (5×10^{-3} M), NH_4Cl (0.067 M), K_3PO_4 buffer (pH 7.5, 0.1 M), chicken liver glutamic dehydrogenase (1.7 μg), and GTP as indicated. The upper curve contained, in addition, anti-chicken liver glutamic dehydrogenase serum protein (1 mg), whereas the lower curves contained either normal serum protein (1 mg) or no addition. The reaction was started by addition of enzyme.

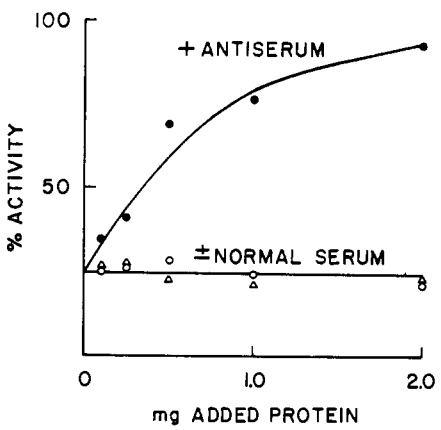

FIGURE 2: GTP inhibition in the presence of increasing concentrations of antiserum. The experiment was performed in K_3PO_4 buffer (0.1 M, pH 7.5). The concentrations of the reactants were as follows: DPNH (1.4×10^{-4} M), α-ketoglutarate (5×10^{-3} M), NH_4Cl (0.1 M), GTP (10^{-5} M), beef liver glutamic dehydrogenase (5 μg), and anti-beef liver glutamic dehydrogenase or normal serum as indicated. The data are expressed as per cent of activity in the absence of GTP or added serum.

and 20–30 mg intramuscularly. The second course of immunization was administered 3 weeks later by intravenous injection of 2 mg of antigen every other day for 1 week. In some instances a second course of intravenous boosting was carried out. Seven days after the last injection 40 ml was bled from the rabbit's ear.

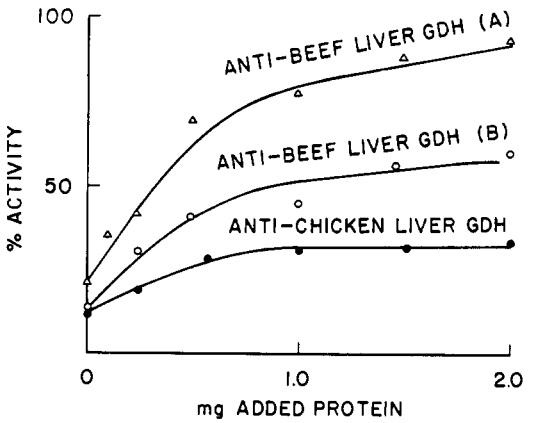

FIGURE 3: Comparison of different antisera *vs.* GTP inhibition of beef liver glutamic dehydrogenase. The experimental conditions were the same as in Figure 2.

Clotted red cells were removed by centrifugation and the sera were stored frozen at −20°. Normal sera were obtained from rabbits that had not previously been immunized. During the period in which these experiments were done several samples of normal serum from different rabbits were used. Both normal and antisera were subjected to heat inactivation at 60° for 20 min before use. Estimation of protein content was made according to the equation $1.55D_{280} - 0.76D_{260}$ based on the data of Warburg and Christian (Layne, 1957).

Complement fixation studies were done by the method of Wasserman and Levine (1961). Nucleotides were obtained from Pabst and Sigma Laboratories and α-ketoglutarate from Calbiochem.

Enzymatic activity was measured by following the decrease in absorbance at 340 mμ on a Zeiss spectrophotometer or Cary automatic recording spectrophotometer at 25°. In the experiments described no attempt was made to allow the antigen–antibody system to preincubate. All reactions were initiated by the addition of the enzyme to the reaction mixture. Thus, the enzyme was confronted by all the reactants simultaneously, including antibody. Under these circumstances inhibition of the initial velocity was rarely observed. (Some of the antisera actually increased the initial velocity.) If the reactants are added to the cuvet in the following order: buffer, DPNH,[1] NH_4Cl, antiserum, enzyme, then incubated at room temperature (27°) for varying periods of time before α-ketoglutarate is added to initiate the reaction, no inhibition is seen up to 2-min incubation. The maximum inhibition observed after 20-min incubation with any of the antisera was 30%.

[1] Abbreviations used in this work: DPNH, reduced diphosphopyridine nucleotide; DPN^+, oxidized diphosphopyridine nucleotide; ADT and ATP, adenosine di- and triphosphate, respectively; GTP, guanidine triphosphate.

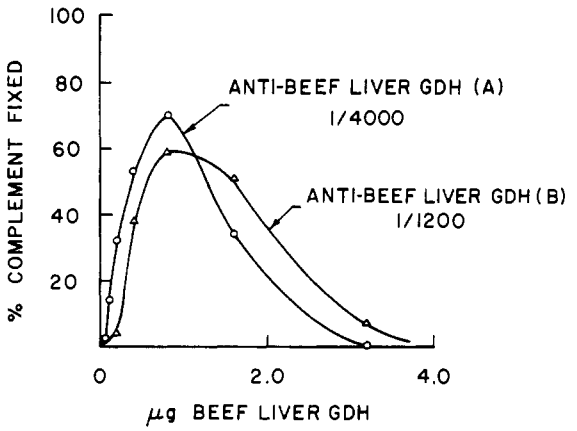

FIGURE 4: Comparison of the complement-fixing titer of the two anti-beef liver glutamic dehydrogenases used in Figure 3.

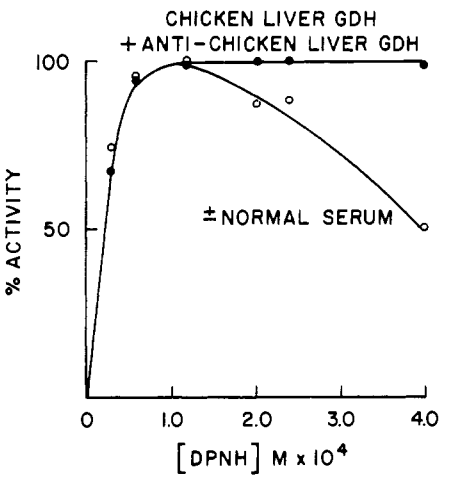

FIGURE 5: Effect of excess DPNH in the presence of antiserum. The experiment was performed in Tris acetate buffer (0.1 M, pH 8) with the following concentrations of reactants: α-ketoglutarate (0.01 M), NH_4Cl (0.1 M), chicken liver glutamic dehydrogenase (0.8 μg), anti-chicken liver glutamic dehydrogenase or normal serum (0.3 mg), and DPNH as indicated.

Results

Effect of Antibody on GTP Inhibition of Glutamic Dehydrogenase. One of the most effective inhibitors of beef liver glutamic dehydrogenase has been shown to be GTP (Frieden, 1962). It was of interest to ascertain whether the presence of antiserum would alter the catalytic activity of the enzyme with and without added GTP. In the absence of GTP the antiserum affects the reaction velocity no more than an equivalent protein concentration of normal serum. However, when GTP is included in the reaction mixture the degree of inhibition observed in the presence of normal serum is not seen in the presence of antiserum. Figure 1 illustrates the protection afforded chicken liver glutamic dehydrogenase by its homologous antiserum against the inhibitory influence of increasing quantities of GTP. In this instance the data are shown as the per cent of activity seen in the absence of GTP.

To corroborate the suggestion that antibody alone was responsible for the observed phenomena the antiserum was absorbed with excess enzyme. The resultant precipitate was removed by centrifugation. It was readily demonstrable after inactivating residual enzyme by heating at 60° for 10 min that the remaining supernatant fluid could no longer prevent GTP inhibition. Several samples of normal sera were tried as well as bovine serum albumin and antisera to other enzymes, but no protective effects against the GTP could be observed. It was also found that a 33% ammonium sulfate precipitate of the antiserum could produce an effect similar to whole antiserum, indicating that the responsible agent is in the γ-globulin fraction of the serum.

An example of increasing protection against GTP inhibition is shown in Figure 2. The concentration of GTP has been held constant while varying amounts of antiserum or normal serum were added. In this particular instance beef liver glutamic dehydrogenase and anti-beef liver glutamic dehydrogenase were used, but essentially the same results are obtained with an homologous system of the chicken liver enzyme and antiserum. A qualitatively similar effect is observed for heterologous systems. The anti-beef liver glutamic dehydrogenase protects the chicken liver enzyme against GTP inhibition whereas equivalent protein concentrations of normal serum do not. The protective capacity of a particular antiserum appears to be related to its ability to fix complement. In Figure 3 three different antisera are compared in terms of their ability to forestall GTP inhibition of beef liver glutamic dehydrogenase. The most effective (A) is three times stronger in its complement fixation titer than the second (B) (see Figure 4), whereas the third and least effective is the heterologous anti-chicken liver glutamic dehydrogenase.

Effect of Antibody on Inhibition Produced by Excess DPNH. In addition to GTP other modifiers of glutamic dehydrogenase activity were examined. Apparently, as shown in Figure 5, the presence of antiserum can minimize the decrease in activity associated with excess DPNH. In some experiments the antiserum inhibited up to 10% at low DPNH concentrations but still prevented an appreciable loss in activity at concentrations of DPNH up to 5×10^{-4} M. A similar experiment with the beef liver enzyme and its homologous antiserum yielded virtually identical results.

Effect of Antibody on Activation Induced by ADP. The activation that is known to be induced by ADP can also be prevented, to a considerable extent, when antiserum is present in the reaction mixture. In Figure 6 it is shown that a twofold increase in antiserum at a relatively high concentration of ADP will further reduce the activation, whereas doubling the concentration of normal serum is without effect.

Action of Antibody on Effects Induced by ATP. An

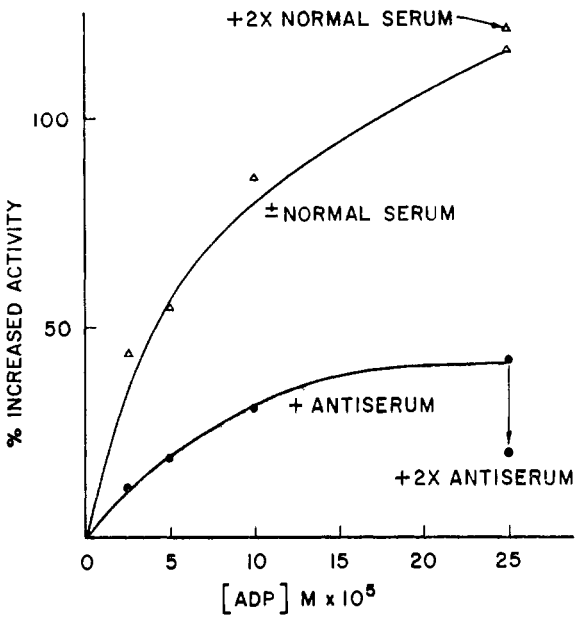

FIGURE 6: ADP stimulation in the presence of antiserum or normal serum. The reaction mixture contained: DPNH (1.4×10^{-4} M), α-ketoglutarate (0.01 M), NH_4Cl (0.1 M), chicken liver glutamic dehydrogenase (0.5 μg), Tris acetate buffer (0.1 M, pH 8), and anti-chicken liver glutamic dehydrogenase or normal serum (0.3 mg). ADP was added as indicated. The data are represented as the percentage increase in initial velocity above that obtained in the absence of ADP.

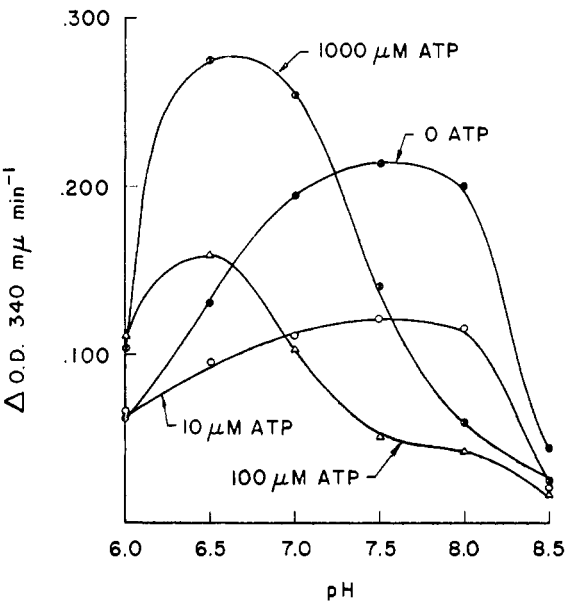

FIGURE 7: Influence of ATP on the pH profile of chicken liver glutamic dehydrogenase. The experiment was performed in Tris acetate buffer (0.1 M) at the indicated pH values. The concentrations of the reactants were as follows: DPNH (1.4×10^{-4} M), α-ketoglutarate (0.01 M), NH_4Cl (0.05 M), chicken liver glutamic dehydrogenase (1 μg), and ATP as indicated.

examination of the effect of ATP on the activity of chicken liver glutamic dehydrogenase indicated that small quantities of the nucleotide were inhibitory, as has been shown for beef liver glutamic dehydrogenase (Frieden, 1963) and observed for the chicken liver enzyme (Frieden, 1962). However, relatively large concentrations of ATP (600–1500 μM) could be shown to activate the enzyme from chicken liver at pH 6.5 and 7.0 (Figure 7) by the comparison of the pH profiles of the enzyme at three different ATP concentrations to the normal pH profile seen in the absence of ATP.

As shown in Table I, low concentrations of ATP at pH 8 can, under the given experimental conditions, decrease the activity 60% in the absence of added serum. The inclusion of normal serum does not significantly alter the inhibition, whereas an equal protein concentration of specific antiserum reduces the inhibition by half. When comparatively high concentrations of ATP are used at pH 7, antiserum will effectively reduce the stimulation observed in the presence or absence of normal serum.

TABLE I: Effect of ATP in the Presence and Absence of Normal Serum or Antiserum on the Activity of Chicken Liver Glutamic Dehydrogenase.[a]

	ΔOD at 340 mμ min^{-1}			
[ATP] (mM)	No Additions	+ Normal Serum	+ Antiserum	pH
0	0.193	0.200	0.192	
				8.0
0.1	0.076	0.088	0.144	
0	0.107	0.115	0.108	
1.7	0.142	0.145	0.120	7.0
3.4	0.142	0.140	0.111	

[a] The experiments were performed in Tris acetate buffer (0.1 M), pH 8 and 7. The reaction mixture contained: DPNH (1.2×10^{-4} M), α-ketoglutarate (0.01 M), NH_4Cl (0.1 M), anti-chicken liver glutamic dehydrogenase or normal serum (0.3 mg), and the indicated amount of ATP. The reactions were started by the addition of 1 μg of chicken liver glutamic dehydrogenase.

Discussion

Most of the antisera used in the above experiments will, when incubated with enzyme in sufficient quantity and varying periods of time, lead to a significant reduction in activity. This time-dependent phenomenon can be avoided by the selection of appropriate amounts of antisera and by the exposure of the enzyme to antibody for a minimal period during which the initial velocity

is obtained. It has not been possible to obtain clear results of the influence of antisera on glutamate oxidation inasmuch as higher concentrations of enzyme are required; consequently, turbidity caused by antigen–antibody complex formation obscures the change in absorption. The same difficulty is encountered when one attempts to evaluate the influence of antisera on glutamic dehydrogenase with pyruvate or α-ketobutyrate as substrates. It seems clear that the interaction of antigen and antibody when following the reductive amination of α-ketoglutarate is rapid enough to measure the kinetic effects observed in this study. Moreover, if there were an increase in turbidity detectable by the instruments used, it would tend, for the most part, to minimize the differences that have been seen.

The data indicate that one attribute of glutamic dehydrogenase, its susceptibility to modification by nucleotides, can be affected by antibody preferentially. This fact suggests that the "active or catalytic site" of the enzyme may be less accessible to the antibody than the site or sites responsible for the binding of nucleotides, thus allowing the "catalytic site" to continue to function. Since the site on the enzyme for binding excess DPNH has been shown to be different from the site for binding ATP, GTP, and ADP (Frieden, 1963), it follows that there must be antibodies present in the antiserum directed specifically toward at least three sites.

The suggestion has been made that the nucleotides produce their effects by alterations of the conformation of the enzyme molecule (Yielding *et al.*, 1963). A hypothetical model has been proposed involving equilibria among three forms of the enzyme: a polymer or aggregate form capable of a reversible dissociation to a monomeric form upon dilution, this monomer, in turn, in equilibrium with a configurationally altered monomer. It has been further proposed that the relative concentration of each of these forms is affected by reagents such as the nucleotides used in this study. The second equilibrium is purported to be influenced in one direction by GTP favoring a form with diminished activity toward α-ketoglutarate and in the opposite direction by ADP favoring a form with enhanced activity toward this substrate. Inasmuch as the presence of antibody prevents the influence of ADP and GTP, it cannot be producing the observed effect by modifying this equilibrium.

An alternative possibility is suggested by the work of Gerhart (1964) on aspartate transcarbamylase. It is conceivable that glutamic dehydrogenase has a regulatory subunit intimately associated with it, and this subunit may serve as a very effective antigenic determinant. Nucleotides may evoke their particular response by altering the relationship between this subunit and the other units of the enzyme. The initial function of the antibody would then be to prevent any change in this relationship.

The antibody can protect the enzyme and yet, when preincubated with the enzyme, cause inhibition. Parenthetically, there is a precedent for an antiserum exhibiting paradoxical effects on an enzyme system. Pollock (1964) has demonstrated an initial stimulation by antiserum of penicillinase activity on benzylpenicillin followed by inhibition as the ratio of antiserum to enzyme is increased.

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