



The Journal of Immunology

Immunologic Properties of Glucose Oxidase

This information is current as of May 18, 2010

Ira Green and John K. Inman

J. Immunol. 1970;104;1094-1100

Subscriptions

Information about subscribing to *The Journal of Immunology* is online at <http://www.jimmunol.org/subscriptions/>

Permissions

Submit copyright permission requests at <http://www.aai.org/ji/copyright.html>

Email Alerts

Receive free email alerts when new articles cite this article. Sign up at <http://www.jimmunol.org/subscriptions/etcoshtml>

Downloaded from www.jimmunol.org on May 18, 2010

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994. Copyright ©1970 by The American Association of Immunologists, Inc. All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.



IMMUNOLOGIC PROPERTIES OF GLUCOSE OXIDASE

IRA GREEN AND JOHN K. INMAN

*From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases,
National Institutes of Health, Bethesda, Maryland*

Received for publication November 17, 1969

Glucose oxidase, an enzyme obtained from *Aspergillus niger*, is a potent antigen. Guinea pigs and rabbits immunized with this antigen in complete Freund's adjuvant produced high serum concentrations of precipitating antibody. In addition, heat-inactivated enzyme and apoenzyme can be used to detect cellular immunity in the guinea pigs.

Glucose oxidase retains its enzymatic activity when bound to antibody, thus allowing direct visualization of the latter upon incubation with a suitable mixture of substrate and chromogenic reagent. Plasma cells producing antibody to the enzyme were visualized in smears and in tissue sections by sequentially reacting fixed preparations with glucose oxidase and chromogenic reagent. Anti-DNP antibody-producing cells elicited by immunization of guinea pigs with dinitrophenylated-ovalbumin were also visualized in a similar manner by employing dinitrophenylated glucose oxidase as a test antigen.

The purpose of this report is to call attention to an enzyme glucose oxidase (G.O.) obtained from the fungus *Aspergillus niger* with properties which make it a useful antigen for immunologic investigation (1, 2). Glucose oxidase is a glycoprotein, M.W. 150,000, containing 16% carbohydrate; each molecule of G.O. is associated with two molecules of the cofactor flavin adenine dinucleotide (FAD) (2). The major characteristic which makes glucose oxidase a useful antigen is that its enzyme activity can be readily detected; the reaction catalyzed is the autoxidation of D-glucose to yield gluconic acid and hydrogen peroxide. If a suitable electron transfer system is present, organic dyes can be oxidized to form insoluble colored compounds. Thus, Avrameas (3) has previously reported that G.O. coupled to antibodies or to antigens could be used for the visualization of antibody-forming cells.

In the present investigation glucose oxidase itself was used as the antigen. Anti-G.O.-forming cells can be easily detected in animals immunized with G.O. Furthermore, anti-2,4-dinitrophenyl (DNP) antibody-producing cells from animals immunized with 2,4-dinitrophenylated proteins can be detected using DNP-conjugated G.O. (DNP-G.O.). We have also studied the levels and specificities of antibodies produced in guinea pigs and rabbits to G.O. as well as the cellular immune reactions to the antigen.

MATERIALS AND METHODS

Glucose oxidase (G.O.) type V, 880 units/ml, phenazine methosulfate and MTT tetrazolium 3 (4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide were purchased from Sigma Chemical Co., St. Louis, Mo. 1-Fluoro-2,4-dinitrobenzene (DNFB) was obtained from Eastman Organic Chemicals, Rochester, N. Y. Ovalbumin (OVA) was purchased from General Biochemicals, Chagrin Falls, Ohio, and bovine γ globulin (BGG) from Armour Pharmaceutical Co., Kankakee, Ill. Freund's adjuvant containing 0.5 mg/ml *Mycobacterium butyricum* (CFA) was obtained from Difco Laboratories, Detroit, Mich.

Immunization. Guinea pigs and rabbits were obtained from the Animal Production Section, National Institutes of Health. Hartley guinea pigs were immunized with 100 μ g G.O. in CFA; 0.1 ml of emulsion was injected into each of the four footpads. Rabbits were immunized with 2 mg G.O. in CFA; 0.5 ml of emulsion was injected into each footpad. The animals were bled 2 to 3 weeks after immunization. Other guinea pigs were immunized in the four footpads with 100 μ g DNP₁₈OVA or with 100 μ g BGG in CFA.

Preparation of DNP-proteins. G.O. concentration was determined by micro-Kjeldahl nitrogen analysis (4). DNP-G.O. was prepared as follows: A solution of 12 mg G.O. in 2.5 ml of water (as ob-

tained from the supplier) was rendered alkaline by adding 0.5 ml of 5% Na_2CO_3 . To this solution was added 0.1 ml of 1% v/v DNFB in 1,4-dioxane. The mixture was stirred at room temperature for 2 hr and dialyzed against PBS, pH 7.2, overnight. The derivative was found to have an average of 13 groups of DNP/molecule. This was determined by measuring the absorbance of a suitable concentration of the dialysate at 360 and 452 nm (A_{360} and A_{452} , respectively). Since the absorption spectra of flavine adenine dinucleotide cofactor and DNP groups overlap (5, 6), it was necessary to make both measurements and employ the following expression for n , the average number of DNP groups/molecule of enzyme (MW 150,000):

$$n = \frac{A_{360} \cdot E_{452}^{\text{GO}} - A_{452} \cdot E_{360}^{\text{GO}}}{A_{452} \cdot E_{360}^{\text{DNP}} - A_{360} \cdot E_{452}^{\text{DNP}}}$$

where $E_{452}^{\text{GO}} = 22,360$, $E_{360}^{\text{GO}} = 17,880$ (from data of Massey and Curti (6)), $E_{360}^{\text{DNP}} = 17,400$ (5), and $E_{452}^{\text{DNP}} = 3200$ (determined for $\text{DNP}_{66}\text{BGG}$). E 's represent molar extinction coefficients for underivatized G.O. or DNP proteins at the several wavelengths as indicated by superscripts and subscripts.

DNP_{16} ovalbumin was prepared and characterized as previously described (7).

Preparation of apoenzyme. The enzyme was freed of the cofactor, flavine adenine dinucleotide (FAD), by exposing the purified enzyme three times to 1.5 M KBr at pH 9.1 followed by precipitation with ammonium sulfate at pH 1.5 in the cold according to the procedure of Strittmatter (8). Heat-inactivated G.O. was prepared by heating an aqueous solution to 70°C for 30 min. The apoenzyme and the heat-inactivated enzyme, when tested with the substrate, were devoid of enzyme activity.

Precipitin analysis. Measurements of the amount of precipitating antibody were carried out as described by Kabat (4). Antiserum, 0.2 ml, was mixed with 0.2 ml of antigen in various concentrations, and 0.1 ml of sodium phosphate-buffered normal saline or 1×10^{-3} M FAD (sodium salt) was added. The washed precipitates were dissolved in 1.00 ml of 0.02 M aqueous sodium dodecyl sulfate and absorbances were measured at 278 and 452 nm.

In the case of precipitation with the unmodified enzyme (holoenzyme) the contribution of antigen to the absorbance at 278 nm was calculated from the absorbance at 452 nm employing

the ratio of absorbance at 278 nm to 452 nm ($= 10.6$) measured with a solution of the purified enzyme in 0.02 M sodium dodecyl sulfate. The concentrations of antibody were calculated from the corrected 278 nm readings and corresponding specific absorbances for guinea pig and rabbit immunoglobulin of 1.32 and 1.51, respectively, for 1 mg/ml. In the case of precipitations with the apoenzyme, the 278 nm absorbance of the dissolved precipitate was corrected for absorbance due to antigen by subtracting the calculated value derived from the total antigen added and the specific absorbance of 0.576 (278 nm, 1 mg/ml) for the apoenzyme. The assumption was made that all added antigen was in the precipitate at the equivalence zone.

Detection of antibody-forming cells. Lymph node cell smears were prepared as previously described (9). To detect anti-G.O.-producing cells, slides were incubated for 20 min with G.O. (250 $\mu\text{g}/\text{ml}$) in isotonic sodium phosphate-buffered saline, pH 7.2 (PBS). The slides were then washed and G.O. activity subsequently detected as described by Avrameas (3). Slides were incubated in the dark for 30 min with a solution prepared by dissolving 50 mg D-glucose, 10 mg MTT tetrazolium and 2 mg phenazine methosulfate in 20 ml 0.1 M sodium phosphate buffer, pH 7.0. They were then rinsed in PBS and mounted in glycerine-gelatin (10). In animals immunized with DNP-OVA , anti-DNP antibody-producing cells were detected in an analogous fashion except that the lymph node cell smears were first incubated with $\text{DNP}_{16}\text{G.O.}$

Anti-G.O. antibody-forming cells were also detected in lymph node sections prepared by the cold alcohol technique of Sainte-Marie (11). The sections were cut and then treated in the same manner as cell smears.

For control studies lymph node cell smears were made from animals immunized with bovine γ globulin.

Effect of anti-glucose oxidase on enzyme activity. Glucose oxidase, 1.5 ml (0.3 mg/ml) in PBS, pH 7.2, was mixed with 0.5 ml of antiserum (containing 1.5 mg of antibody) and 1.0 ml PBS and allowed to stand at room temperature for 1 hr. Controls were prepared with normal serum from the same species in place of antiserum. Samples were assayed for enzyme activity by measuring gluconic acid formation.

One milliliter of enzyme-antiserum mixture was added to a solution of 0.75 g of anhydrous

glucose in 25 ml of 0.035 M sodium acetate, 0.015 M acetic acid, 0.025 M NaCl (pH 5.1) at 35°C. Continuous aeration was provided through a gas dispersion tube. Two drops of capryl alcohol were added to prevent foaming. Fifteen minutes after adding the enzyme mixture the reaction was stopped with 10.0 ml of 0.1 N NaOH. Gluconic acid production was measured by a back titration with 0.05 N HCl (phenolphthalein endpoint) that was compared with a parallel run in which glucose had been omitted.

Delayed sensitivity to G.O. One-tenth milliliter of PBS containing 20 μ g of G.O., heat-inactivated G.O., or G.O. apoenzyme was injected intradermally into guinea pigs. The skin reactions were evaluated 24 hr later.

Lymph node cell stimulation in vitro. Lymph node cells were obtained from guinea pigs 2 to 3 weeks after immunization. The cells and antigen were incubated for 24 hr, 1 μ Ci of ^{3} H thymidine was added, and the cells were incubated for an additional 24 hr. The details of this method as well as the method of harvesting and counting the cells for ^{3} H thymidine incorporation into DNA are given in earlier reports (12, 13).

RESULTS

Antibody levels and specificity. Substantial levels of precipitating antibody to G.O. were observed with all guinea pigs and rabbits which had been immunized with the unmodified (holo-) enzyme in complete Freund's adjuvant. The serum of all animals immunized with G.O. when tested in double diffusion analysis in agar gave dense

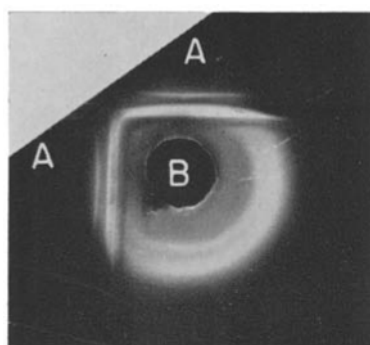


Figure 1. Antibody well (A) contains rabbit antibody 80P, antigen well (B) contains G.O. holoenzyme 250 μ g/ml. The larger dense line near the antigen well stains for enzyme activity. The minor component does not stain for enzyme activity.

TABLE I
Concentration of anti-glucose oxidase antibody in antisera from animals immunized with glucose oxidase holoenzyme

	Serum Antibody Concentration		
	Precipitating antigen		
	Holo-enzyme	Apo ^a enzyme	Holo-enzyme ^b + FAD
	mg/ml		
Guinea pig serum 79-7/7	3.1	0.2	
Guinea pig serum 79-7/10	3.1	0.3	3.8
Rabbit serum 80P	2.1	0.9	2.4
Rabbit serum 80	1.6	4.6	

^a Apoenzyme prepared by acidified ammonium sulfate precipitation which removes FAD co-factor. See *Materials and Methods*.

^b FAD sodium salt added to the precipitin tube in a final concentration of 2×10^{-4} M.

precipitation lines. In addition to the major band, which stained for enzyme activity, there was a minor component which did not stain for enzyme activity (Fig. 1). Representative values of the antibody concentrations of the antisera as measured by quantitative precipitin analysis are shown in Table I. The addition of a 30- to 60-fold molar excess of free FAD over enzyme-bound FAD to each tube in the precipitin assay did not significantly alter the amount of antibody precipitated. The sera of two individual rabbits immunized with the holoenzyme contained different amounts of antibody capable of precipitating with the apoenzyme (Table I).

Antibody-forming cells. Lymph node cell smears prepared from guinea pigs immunized with G.O. 2 to 3 weeks after immunization and stained for anti-glucose oxidase-producing cells revealed many plasma cells with specific red-violet staining of their cytoplasm; examples of such cells are shown in Figures 2 and 3. The other cells in the smear were completely unstained. Smears in which the G.O. incubation step was omitted but which were stained for glucose oxidase activity were entirely negative. Lymph node cell smears obtained from animals immunized with bovine γ globulin and treated in the same way as the specific smears did not show any stained cells.

Lymph node cell smears prepared from animals immunized with DNP-OVA and stained for anti-DNP-producing cells demonstrated many stained

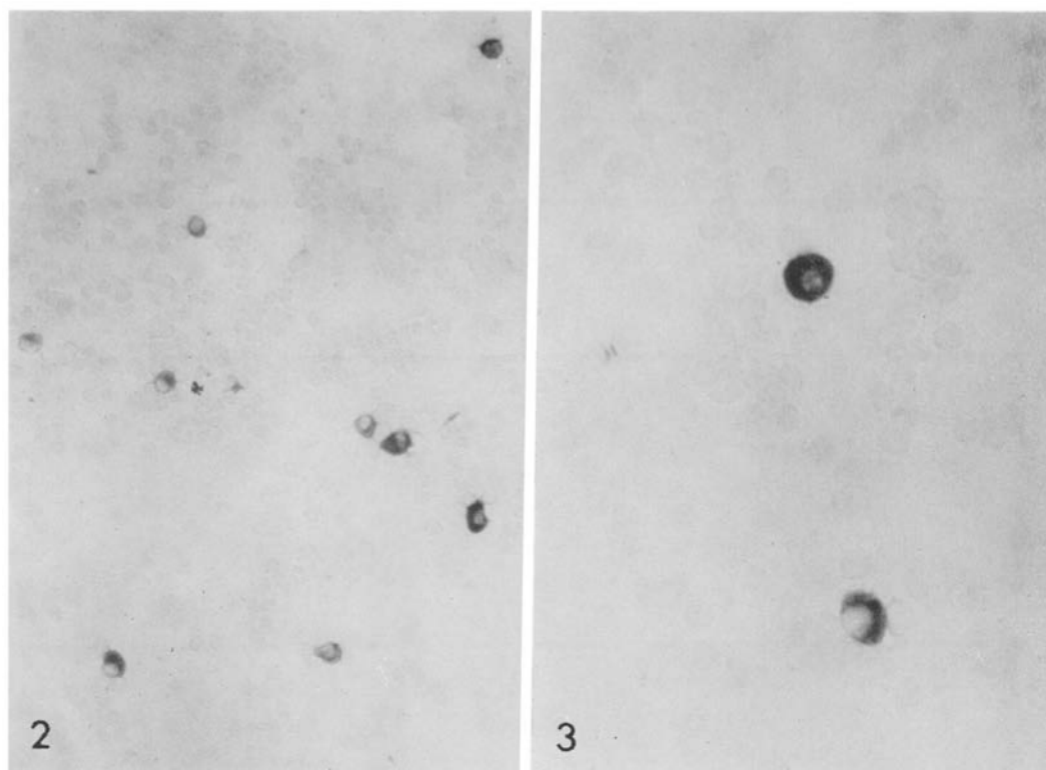


Figure 2 (left). Lymph node smear from a guinea pig immunized with G.O. Nine stained plasma cells are seen. The other cells are completely unstained ($\times 250$).

Figure 3 (right). Lymph node smear from a guinea pig immunized with G.O. Two stained plasma cells are seen. The other cells are unstained ($\times 400$).

plasma cells (Fig. 4). In these slides, however, there was some staining of the lymphocyte cell membrane, and, in addition, there was intense staining of the cytoplasm of the occasional polymorphonuclear leukocytes that were present. These could easily be distinguished from plasma cells (Fig. 5). This non-specific staining of the lymphocytes and polymorphonuclear leukocytes was probably due to increased tendency of some cell components to bind the more negatively charged DNP-G.O. Again in smears in which incubation with DNP-G.O. was omitted, or in those obtained from animals immunized with bovine γ globulin and treated in the usual way, no stained plasma cells were observed; in the latter case an occasional stained polymorphonuclear leukocyte was observed.

Lymph node sections from animals immunized with G.O. and stained for the presence of anti-G.O.-forming cells contained many stained plasma cells at the periphery of lymphoid folli-

cles and in the medullary areas of the lymph node (Fig. 6); in addition, specific staining for enzyme activity was also seen in the subcapsular and other interstitial areas as well as in the interior of blood vessels. This staining is due to the binding of G.O. by the antibodies present in the plasma or lymph contained in these areas.

Enzyme inhibition by antibody. G.O. was mixed at neutral pH with either guinea pig or rabbit antiserum to G.O. containing enough antibody to precipitate most of the enzyme. When subsequently assayed for enzyme activity at pH 5.1 (see *Materials and Methods*) no diminution of activity was found as compared with normal serum controls. Duplicate trials were performed on each serum. It was concluded that no significant fraction of the antibody formed in either species was able to modify the enzyme activity (by steric or conformational effects).

Cellular hypersensitivity. Normal guinea pigs injected with 20 μ g of G.O. demonstrated red

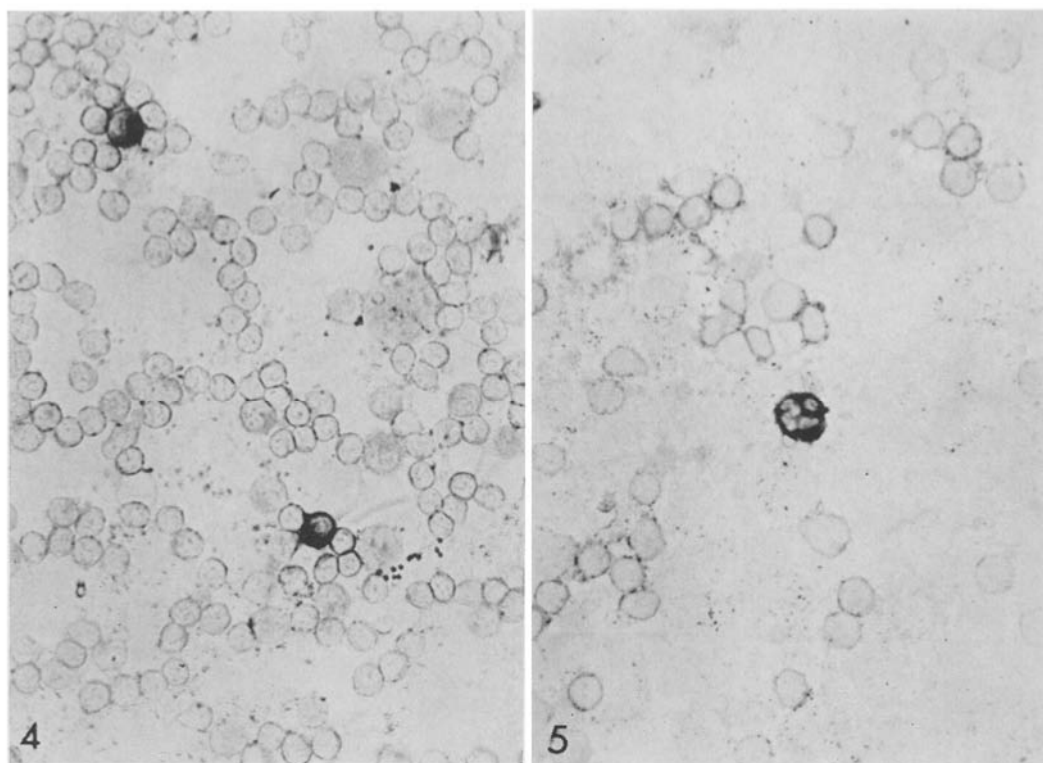


Figure 4 (left). Lymph node smear from a guinea pig immunized with DNP-OVA. Two stained plasma cells are seen. The cell membranes of small lymphocytes are also stained ($\times 400$).

Figure 5 (right). Another portion of the lymph node smear seen in Figure 4. A polymorphonuclear leukocyte shows staining of the cytoplasm.

indurated reactions with an average diameter of 15 x 15 mm. These nonspecific inflammatory reactions were probably the result of the enzyme-mediated release of H_2O_2 at the injection site. However, when the same test doses of G.O. apoenzyme or of heat inactivated G.O. were injected into normal animals only small reactions less than 5 mm in diameter were observed. In guinea pigs immunized with G.O. large, specific, delayed reactions to G.O. apoenzyme and heat-denatured glucose oxidase were observed. Control animals immunized with protamine (a material recently demonstrated to be an antigen in some Hartley guinea pigs (14)) showed no significant reactions to these materials (Table II).

The results of lymph node cell stimulation *in vitro* with G.O. are shown in Table III. Marked inhibition of cell stimulation was observed when G.O. holoenzyme was used *in vitro*. This is probably due to the toxic effect of enzymatically released H_2O_2 in these cell cultures. However,

when heat-inactivated G.O. was used *in vitro*, specific stimulation of DNA synthesis by the cells of specifically immunized animals was observed, but not by the cells of an animal immunized with DNFB.

DISCUSSION

Enzymes have been employed as antigens for a variety of immunologic studies (15). Recently, Avrameas (3) has demonstrated the feasibility of using enzyme-coupled antigens and/or antibodies for visualizing antibody-forming cells. He found that enzyme marker techniques can be as sensitive as other more commonly used methods which employ radioisotope or fluorescent labels. Enzyme markers can be directly visualized after treatment with the substrate and suitable chromogenic reagents.

In the present study we employed the enzyme as immunizing antigen, detecting antigen, and marker. Thereby we obviated the need for cou-

pling together separate enzyme and antigen molecules. Glucose oxidase (G.O.), which is available commercially in relatively pure form, is an ideal marker for the detection of antibody-producing cells since no intrinsic G.O. activity is present in cells found in mammalian lymphoid tissue. Staining of non-antibody-producing cells may be observed with several other enzymes (3). We found, for example, that erythrocytes, which stained by virtue of their intrinsic peroxidase activity, presented a confusing background in the

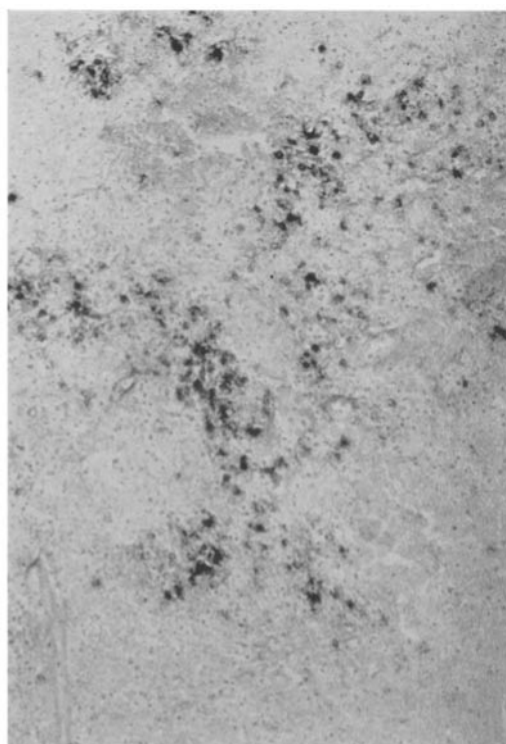


Figure 6. Portion of a section of a lymph node from a guinea pig immunized with G.O. Stained plasma cells can be seen at the periphery of a lymphoid follicle ($\times 250$).

screening of spleen cell smears for antiperoxidase antibody-forming cells (I. Green and J. K. Inman, unpublished).

Guinea pigs and rabbits immunized with G.O. consistently made high levels of antibody as determined by precipitin analysis. Antibody might also be readily measured by the Farr technique (16) because G.O. is soluble in 50% saturated ammonium sulfate.

The fact that G.O. antibody complexes possess enzyme activity in fixed cell preparations and precipitin lines in agar gel is evidence that the bound antibody causes little or no steric hindrance of the active site. This conclusion is upheld by direct assay of enzyme activity following incubation with antibody. Also the FAD cofactor in the active site does not appear capable of combining with antibody as shown by lack of inhibition of antibody precipitation by free FAD.

The enzyme is not inhibited by the dinitrophenylation of 13 of its 30 lysine residues. Thus, DNP-G.O. can be used as a marker antigen for the direct visualization of anti-DNP-producing cells. Anti-hapten antibody-producing cells have been previously demonstrated by hemolysis-in-gel techniques using hapten-coupled erythrocytes (17) or by fluorescence microscopy techniques (9). The ability to visualize anti-hapten antibody-producing cells using hapten-coupled G.O. provides an additional and convenient method of detection. Furthermore, since G.O. is obtained from fungi, it is not likely to cross-react with mammalian proteins commonly used as carriers for haptens.

Cellular immunity, delayed hypersensitivity and lymphoid cell stimulation *in vitro* can also be studied with this antigen if the enzyme activity is destroyed before its use. Otherwise toxic amounts of hydrogen peroxide are generated by the action of the intact enzyme on glucose *in vivo* or *in vitro*. Cellular immune reactions to heat-inactivated G.O. and G.O. apoenzyme have been

TABLE II

Delayed sensitivity reactions to heat-inactivated glucose oxidase and apoenzyme in five guinea pigs

Immunizing Antigen	Test Antigen 20 μ g/Site	Average Reaction Diameters/mm				
Glucose oxidase	G.O. apoenzyme	14.5	18	17	11	10
	G.O. heat-inactivated	15	14	16	16	12
Protamine	G.O. apoenzyme	5	3	3	2	4
	G.O. heat-inactivated	5	3	2	3	4

TABLE III
In vitro stimulation of ^3H thymidine incorporation by lymph node cells from guinea pigs immunized with glucose oxidase

Animal No.	Immunizing Antigen	Test Antigens			
		Glucose Oxidase		Glucose oxidase heat-inactivated	
		1 μ g	10 μ g	1 μ g	10 μ g
1	Glucose oxidase 100 μ g	0.06 ^a	0.14		
2	Glucose oxidase 100 μ g			1.1	5.7
3	Glucose oxidase 100 μ g				2.8
4	Glucose oxidase 100 μ g				3.2
5	DNFB				0.72

^a Thus number is the ratio counts in acid precipitable radioactivity of cultures with antigen/counts in acid precipitable radioactivity without antigen.

observed. Whether these reactions would be identical with reactions to unaltered G.O. must remain unanswered since the latter cannot be used directly for such studies.

Individual antisera to the holoenzyme differ in their capacity to precipitate the apoenzyme, indicating that a conformational change takes place after removal of the FAD cofactor by the procedure employed. This conclusion is supported by observations with other FAD-containing enzymes, which show that reversible conformational changes as monitored by spectral changes occur upon addition and removal of the FAD cofactor (6).

REFERENCES

1. Pazur, J. H. and Kleppe, K., *Biochemistry*, **3**: 578, 1964.
2. Pazur, J. H., Kleppe, K. and Cepure, A., *Arch. Biochem.*, **111**: 351, 1965.
3. Avrameas, S., *Immunochemistry*, **6**: 43, 1969.
4. Kabat, E. A., *Kabat and Mayer's Experimental Immunochemistry*, 2nd Ed., Charles C Thomas, Springfield, Ill., 1961.
5. Carsten, M. E. and Eisen, H. N., *J. Amer. Chem. Soc.*, **75**: 4451, 1953.
6. Massey, V. and Curti, B., *J. Biol. Chem.*, **241**: 3417, 1966.
7. Levine, B. B. and Benacerraf, B., *J. Exp. Med.*, **120**: 955, 1964.
8. Strittmatter, P., *J. Biol. Chem.*, **236**: 2329, 1961.
9. Green, I., Vassalli, P., Nussenzweig, V. and Benacerraf, B., *J. Exp. Med.*, **125**: 511, 1967.
10. Pearse, A. G. E., *Histochemistry*, p. 578, Little Brown & Co., Boston, 1968.
11. Sainte-Marie, G., *J. Histochem. Cytochem.*, **10**: 250, 1962.
12. Paul, W. E., Siskind, G. W. and Benacerraf, B., *J. Exp. Med.*, **127**: 25, 1968.
13. Foerster, J., Lamelin, J-P., Green, I. and Benacerraf, B., *J. Exp. Med.*, **129**: 295, 1969.
14. Green, I., Paul, W. E. and Benacerraf, B., *Proc. Nat. Acad. Sci.*, **64**: 1095, 1969.
15. *Antibody to Enzymes—A Three-Component System*, Edited by B. Cinader, *Annals N.Y. Acad. Sci.*, **103**: 493, 1963.
16. Farr, R. S., *J. Infect. Dis.*, **103**: 239, 1958.
17. Merchant, B. and Hrabá, T., *Science*, **152**: 1378, 1966.