

## Preparation of Monospecific Antiserum to Lupin Nodule Glutamate Dehydrogenase

Y. T. Tchan,<sup>A</sup> Z. Wyszomirska-Dreher<sup>A</sup> and I. R. Kennedy<sup>B</sup>

<sup>A</sup> Department of Microbiology, University of Sydney, N.S.W. 2006.

<sup>B</sup> Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006.

### Abstract

Immunization of rabbit, using biochemically homogeneous glutamate dehydrogenase, proved to be unsuitable to produce monospecific antiserum. The presence of traces of contaminating immunogen (undetected by physicochemical methods) induced the production of other antibodies. Procedures for rigorously establishing monospecificity of antisera and a technique for preparation of monospecific antiserum, using immunologically impure antigens, are described.

### Introduction

Biochemical studies of symbiotic nitrogen fixation have shown that the initial product of dinitrogen fixation is ammonia (Bergersen 1965; Kennedy 1966). For transformation of the ammonia to amino acids, two pathways have been proposed—either solely via plant glutamine synthetase (Scott *et al.* 1976) or with plant glutamate dehydrogenase as a complement (Kennedy 1979; Kennedy *et al.*, unpublished data). Stone *et al.* (1979) purified glutamate dehydrogenase from lupin nodule to 8000-fold homogeneity and studied its properties and kinetics of the amination and deamination reactions (Stone *et al.* 1980a, 1980b). As the intracellular localization of this enzyme is unable to be defined by normal biochemical techniques (Kennedy *et al.*, unpublished data) immunocytochemical techniques are indicated for such studies.

This paper reports the difficulties involved in the successful preparation of monospecific antiserum to glutamate dehydrogenase (GDH, EC 1.4.1.2) from lupin nodule, together with some properties of the GDH (antigen)–antibody reaction.

### Methods

#### (a) Biochemical Techniques

##### *Preparation of nodule crystal*

Cytosol was prepared by homogenizing lupin nodules in sucrose–phosphate buffer, pH 7 (0.4 M sucrose, 0.05 M potassium phosphate, 5 mM mercaptoethanol; 2% (w/v) soluble polyvinylpyrrolidone; 1 g nodules per millilitre buffer). This yielded a pinkish red supernatant when filtered through cheesecloth and centrifuged at 49 000 *g* for 15 min. Intact *Rhizobium lupini* bacteroids and other nodule particulates were sedimented in the pellet. Such cytosol preparations contained about 0.3–0.6 units ( $\mu\text{mole min}^{-1}$ ) of GDH aminating activity per millilitre, equivalent to 1–2  $\mu\text{g}$  of pure enzyme. Where necessary, extracts were concentrated by freeze-drying or by centrifugation through Amicon membranes (Minicon cones, 25 000 molecular weight cut-off). This cytosol, containing about 10 mg protein per millilitre, was used throughout the experiments described here.

### *Preparation of biochemically homogeneous GDH*

GDH from the plant fraction of lupin nodules was prepared by the methods of Stone *et al.* (1979), including affinity chromatography on NAD-Agarose. The enzyme was homogeneous as judged by the single symmetrical boundary obtained on ultracentrifugation ( $S_{20,w} = 10.4$ ), by four charge isozymes observed on polyacrylamide electrophoresis and by four species of different isoelectric points revealed by electrofocusing. Its molecular weight was determined as 266 000 (Stone *et al.* 1979), apparently a hexameric protein with subunits of equal molecular weight. This enzyme (antigen) was considered to be biochemically homogeneous.

### *Assay of GDH*

Enzyme activity was measured in cuvettes by the rate of decrease of absorbance at 340 nm during the  $\text{NH}_4^+$  + 2-oxoglutarate-dependent oxidation of NADH. The standard assay medium for this reaction contained in a final volume of 1 ml: 0.2 M Tris-HCl buffer (pH 8.2), 0.1 mg NADH, 12.5 mM 2-oxoglutarate, 0.2 M  $\text{NH}_4\text{Cl}$  and enzyme equivalent to 1–2 ng of purified GDH (3–6 milliunits of enzyme activity).

GDH activity was detected in gels following electrophoresis using a reaction mixture containing in 100 ml: 0.1 M Tris-HCl (pH 8.5), 0.1 M L-glutamate, pH 8.5, 150 mg  $\text{NAD}^+$ , 25 mg iodonitro-tetrazolium violet and 2.5 mg phenazine methosulfate. In all cases the GDH activity in the gel was tested by immersing the plate in the above mixture at room temperature in the dark.

### *Effect of antibodies on GDH activity*

10–20  $\mu\text{l}$  of enzyme solution (c. 3 milliunits) were added to the standard assay medium in the cuvette at 30°C and increasing amounts of antiserum or normal serum added. Reaction rate was measured until a constant rate of NADH oxidation was reached (1–5 min). The order of addition of substrates and antibodies was found to have no effect on the final degree of inhibition observed.

### *Agarose gel electrophoresis*

Electrophoresis was performed using 1.5% (w/v) Agarose A (Pharmacia, Uppsala, Sweden) on glass plates for 1 h at 200 V (9 V/cm) with no serum in the gel. Protein was fixed in a mixture of picric acid:acetic acid:water (14 g:200 ml:1000 ml) for 10 min. After removal of picric acid by washing with 96% (v/v) ethanol, the plate was pressed, dried and stained for protein as described below. The enzyme test was performed in duplicate immediately after electrophoresis.

### *Protein staining*

Coomassie brilliant blue R-250 was used for staining, by the procedure recommended by Weeke (1975, pp. 26–7).

## *(b) Immunological Techniques*

### *Preparation of K-series antiserum*

The biochemically homogeneous GDH (0.5 mg) was mixed with Freund's complete adjuvant (1:1, v/v), and 2 ml of the suspension was injected into the thigh muscle of a 5-kg rabbit. A subsequent intramuscular injection of 0.2 mg GDH in 2 ml saline without adjuvant was made at intervals of 3 weeks. The animal was bled from the ear vein after 1 more week, by which time a satisfactory titre was obtained. The serum obtained here was designated K-series antiserum. Control serum was taken prior to the first injection.

### *Preparation of immunologically homogeneous GDH*

Line immunoelectrophoresis, using cytosol as antigen and K-series antiserum, was performed as described below. The precipitin complex (GDH–antibody) formed in the contact gel was enzymically active and well separated from other precipitin (see Figs 3a, 3b). To harvest this complex, the gel was extensively washed with 0.85% (w/v) NaCl saline to remove unprecipitated proteins. The precipitin line with a minimal amount of gel was excised from the plate with a razor-blade and used for immunization of the rabbit.

*Preparation of monospecific anti-GDH serum*

Immunologically homogeneous GDH from 12 plates, as prepared by line immunoelectrophoresis, was ground into a suspension in a mortar with a minimum volume of saline. This was mixed with an equal volume of Freund's complete adjuvant and 2 ml of the mixture injected into the thigh muscle of a rabbit. A booster injection was given 3 weeks later. Serum was collected 1 month after the original injection and used for affinity chromatography as described below. The titre of the serum was low, approximately equal to 1/200 of the K-series antiserum. This method of preparation of monospecific antiserum using the precipitin complex was modified from Koch and Nielsen's (1975) technique.

*Affinity chromatography*

Affinity chromatography was used to obtain a larger quantity of immunologically homogeneous GDH. Low-titred monospecific serum (1 ml) was absorbed onto a protein A-Sepharose column [50 by 0.5 cm; 0.75 g Pharmacia (Uppsala, Sweden) product] and washed with 3 ml of phosphate-buffered saline (Goding 1976). Biochemically homogeneous GDH (200  $\mu$ g) was passed through the column bed where the complex IgG-GDH was formed. The column was then washed with 3 ml of phosphate-buffered saline, pH 7.4, to remove unbound protein. The IgG-GDH complex was eluted with 2 ml of 0.58% (v/v) acetic acid in 0.15 M NaCl. The eluate was collected in 0.5 ml of carbonate-bicarbonate buffer (0.2 M, pH 9.3), using a hand refractometer to monitor elution of protein.

*Preparation of high-titred monospecific anti-GDH serum*

1 ml of the IgG-GDH complex eluted from the Sepharose column was mixed with an equal volume of Freund's complete adjuvant for interdermal injection at 20 sites in the back of the rabbit and a booster (100  $\mu$ l of complex) was given at 5 weeks. Serum was collected at weekly intervals from the third week after the primary injection. This serum were designated M-series antiserum (monospecific), and the titre obtained was comparable to the K-series antiserum.

*Rocket immunoelectrophoresis*

Weeke's (1975, pp. 37-46) method was used with the following specifications:

- (1) Antigen: cytosol was used at a rate of 20  $\mu$ l per well.
- (2) Antiserum: undiluted and 200-fold diluted K-series antisera were used at a rate of 5.2  $\mu$ l/cm<sup>2</sup> of gel (37  $\mu$ l of serum per millilitre of 1.5% Agarose A).

Immunoelectrophoresis was carried out at 80 V for 16 h followed by a further 3 h at 280-300 V (field strength: 8-10 V/cm).

*Crossed immunoelectrophoresis*

Weeke's (1975, pp. 47-56) method was followed with the following specifications:

- (1) Antigen: 10  $\mu$ l (0.1-0.5 milliunits) of biochemically homogeneous GDH was applied per well.
- (2) Cytosol: 20  $\mu$ l per well.
- (3) Antiserum: see 'Rocket immunoelectrophoresis' above.

The first-dimension electrophoresis on Agarose was run at 280-300 V for 1 h and the second dimension was carried out as in 'Rocket immunoelectrophoresis' above.

*Line immunoelectrophoresis*

The technique of Krøll (1975) was used with the following specifications:

- (1) Gel strip: 4.2 ml of cytosol was mixed at 40°C with 3.5 ml of 3% (w/v) Agarose B (Pharmacia) and strips 0.3 by 0.14 cm and of different lengths were cut and placed on a contact gel 8 mm from the antibody-containing Agarose B.
- (2) Antisera: the undiluted K-series and M-series antisera were used as described above.

Electrophoresis was carried out as for rocket immunoelectrophoresis.

#### *Preparation of absorbed serum*

To remove unwanted antibodies from the K-series antiserum prepared in initial experiments, absorption according to the method of Harboe and Ingild (1975) was performed. Cytosol was incubated with antiserum in a ratio of 10:1 (v/v) at 37°C for 1 h and refrigerated for 48 h. After centrifugation at 10 000 *g* for 20 min, the supernatant was used for immunological testing or absorbed further to achieve a final ratio by volume of the crude antigens (cytosol) to antiserum of 36:1.

#### *Preparation of cytosol free of active GDH antigen*

A constant amount of cytosol was mixed with different quantities of M-series antiserum in a final volume of 1.05 ml with saline. The absorption was carried out according to the method of Harboe and Ingild (1975) and the absorbed cytosol was tested by rocket immunoelectrophoresis and electrophoresis on Agarose.

#### *Fluorescent antibody staining and photomicrography*

Lupin nodules were sectioned manually and deposited on a slide. After a few seconds, the sections were removed and the impression made on the slide was fixed briefly by heat. One drop of undiluted antiserum was added and the slide incubated for 1 h at room temperature in a moist chamber. The preparation was washed several times with physiological saline and stained with rhodamine-conjugated swine antirabbit immunoglobulins (Dako, Copenhagen) for 15 min. It was washed again with physiological saline to remove excess antibodies and mounted in phosphate-glycerol for fluorescence microscopy with a green excitation filter ( $\lambda = 546$  nm) and red barrier filters ( $\lambda = 590$  and 610 nm). For photomicrography, a phase-fluorescence microscope with white light supply to the phase ring was used (Tchan 1957). For one-step antibody labelling of preparations the method of Kawamura (1969), using rhodamine lissapol B200 (I.C.I. Chemicals, Melbourne) and phosphorus pentachloride, was used to conjugate rabbit IgG. Photomicrographs were taken using Polaroid film and an automatic camera.

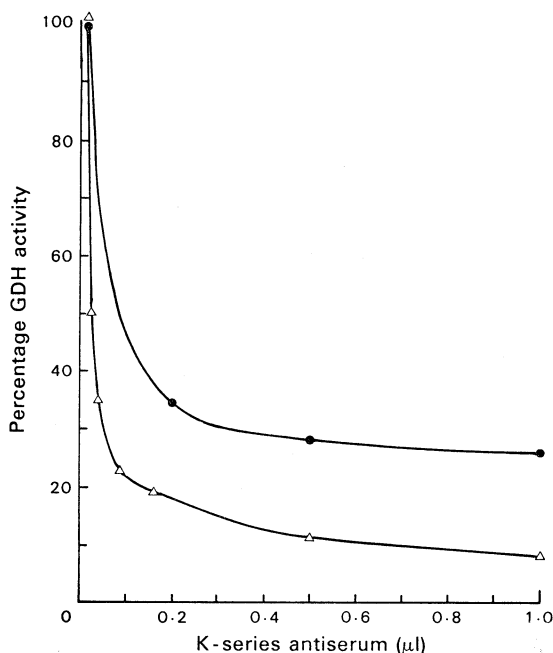
## **Results**

### *K-series Antiserum*

Spectrophotometric tests demonstrated the occurrence of a specific antibody reaction with GDH, since the enzyme in crude nodule extracts was found to be inhibited by the serum to a maximum of 75% (Fig. 1). Control serum from the same rabbit was ineffective. Initial tests of K-series antiserum (see Fig. 2*a*) against biochemically homogeneous GDH by crossed immunoelectrophoresis revealed a single precipitin line (A1) in the contact gel zone after protein staining. This line also gave a positive test for GDH enzyme activity (Fig. 2*a*). When antiserum was diluted 200-fold with saline, one double peak (A1) occurred within the antibody zone, coinciding with the enzyme activity (Fig. 2*b*). The antigen responsible for the formation of this precipitate was designated as antigen 1. As can be seen in Figs 2*a* and 2*b* sufficient enzymic activity remained to provide a positive GDH reaction in the presence of antibodies, despite the enzyme inhibition. These results suggested monospecificity of the K-series antiserum.

Antiserum diluted 200-fold gave a single enzymically active precipitin line in the serum zone when tested by crossed immunoelectrophoresis with cytosol similar to that used to prepare the purified GDH. However, with undiluted sera, where antigen 1 precipitated as a line (A1) in the contact gel, three additional peaks, antigens 2, 3 and 4 (A2, A3, A4), appeared in the serum zone. None of these additional precipitin peaks gave a positive GDH reaction (Figs 3*a*, 3*b*). It was known that purified GDH used for immunization consisted of four isoenzymes (Stone *et al.* 1979) and it seemed reasonable to suspect that these three peaks (antigens 2, 3 and 4) could also be GDH

with their biochemical activity completely inhibited by specific antibody as in the cases of tyrosinase and tryptophanase (Cinader 1966). Therefore their enzymic activity needed to be tested in the absence of their respective antibodies and antigen 1.



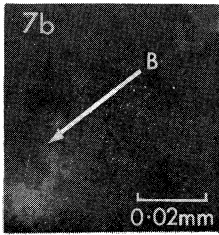
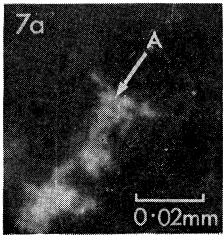
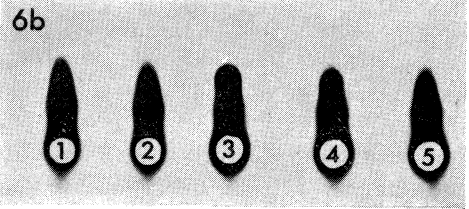
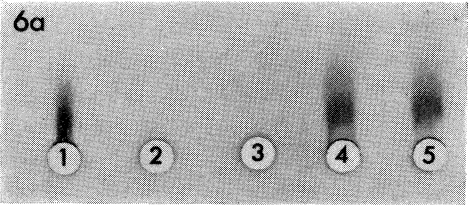
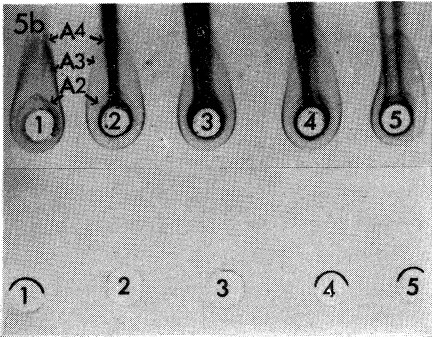
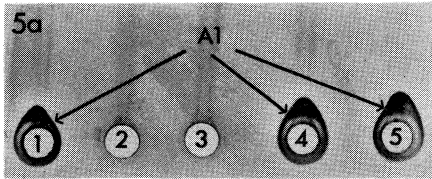
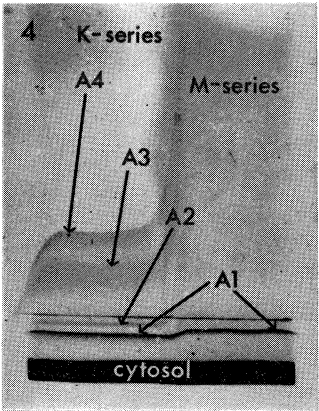
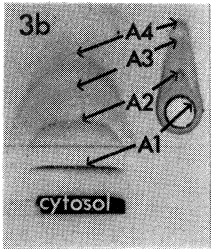
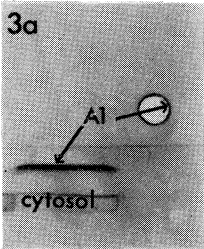
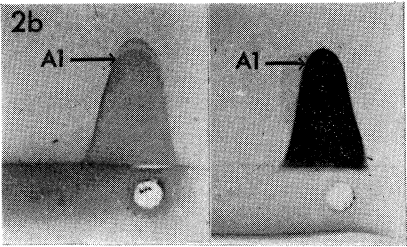
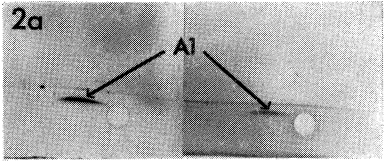
**Fig. 1.** Inhibition of GDH activity by antibody.  $\Delta$  Purified GDH.  $\bullet$  Cytosol. 1  $\mu$ l of antiserum  $\equiv$  1–3 milliunits of enzyme activity in 1.0 ml reaction mixture.

When absorption was used to remove antibodies to antigens 2–4 from the K-series antiserum, and the antiserum then tested by rocket and line immunoelectrophoresis, it appeared to be monospecific. However, detectable amounts of antibodies to at least one of antigens 2–4 were found in the antiserum when tested against nodule cytosol, using increased amounts of antiserum in the Agarose gel. It was concluded that absorption could not provide a means of obtaining a satisfactory monospecific antiserum to antigen 1 for immunocytochemistry.

Consequently, a monospecific serum prepared according to the modified method of Koch and Nielson (1975) was obtained (see Methods). It produced a single GDH-active precipitin peak on immunoelectrophoresis, but with an unsatisfactory titre (about 200-fold lower than K-series antiserum). To obtain an increased titre, the immunoglobulin fraction from this weak serum was bound to protein A–Sephadex in a column and used for affinity chromatography of biochemically pure GDH. This larger quantity of immunologically purified GDH was then used to prepare new antiserum. The resultant antiserum was monospecific for GDH when tested with cytosol under a range of conditions, and possessed a satisfactory titre. A comparison with the K-series antisera by line immunoelectrophoresis is shown in Fig. 4.

#### *Identification of Antigens 2, 3 and 4*

The monospecific (M-series) antiserum described above was then used to remove antigen 1 from the cytosol to allow the testing of the activity of antigens 2, 3 and 4. The following results were obtained:



- (1) Cytosol after absorption by the M-series antiserum was tested by rocket immunoelectrophoresis and biochemically assayed. No GDH activity was detected (Fig. 5a, wells 2 and 3).
- (2) This absorbed cytosol was tested against K-series antiserum. Antigens 2, 3 and 4 (A2, A3 and A4) were clearly visible. The constant height of these peaks testified that their concentration was not affected by the presence of monospecific M-series antiserum (Fig. 5b).
- (3) The treated cytosol was tested by electrophoresis on Agarose gel and biochemically assayed. No GDH activity was detected in wells 2 and 3 (Fig. 6a), although the quantity of protein of all samples revealed by the stain was very similar (Fig. 6b). This was confirmed by negative results obtained after spectrophotometric assay of the treated cytosol. Together with results from (2), antigens 2, 3 and 4 thus failed to demonstrate enzymic activity with their corresponding antibodies absent, proving their non-identity with GDH in crude cytosol.

**Fig. 2.** Crossed immunoelectrophoresis of GDH with K-series antiserum in gel and GDH in well. (a) Undiluted antiserum [0.13 ml per 3.5 ml of 1.5% (w/v) Agarose A] ( $5.2 \mu\text{l}/\text{cm}^2$ );  $10 \mu\text{l}$  (2.5 milliunits) GDH. Only one line in the contact gel (A1) was detected by enzyme (right) and protein (left) stains. (b) Antiserum diluted 200-fold with saline;  $20 \mu\text{l}$  (5 milliunits) GDH. One double-peaked precipitation line (A1) was formed in the antibody-containing gel, for both protein (left) and enzyme (right) stains (the latter not visible).

**Fig. 3.** Line and rocket immunoelectrophoresis of GDH in cytosol with undiluted K-series antiserum in gel (concn as in Fig. 2a), 0.09 ml cytosol ( $\approx 180$  milliunits GDH) in strip ( $4.0$  by  $0.3$  by  $0.14$  cm) and  $20 \mu\text{l}$  cytosol ( $\approx 40$  milliunits GDH) in well. (a) Biochemical assay showed one active line (A1) in the contact gel (antigen 1—see text) and some activity within the well. (b) Protein stain indicated the presence of one deeper-stained line (A1) corresponding to antigen 1 (GDH-active) and three other peaks, A2, A3, A4 (antigens 2, 3 and 4). The rocket immunoelectrophoresis showed three peaks, A2, A3, A4 (antigens 2, 3 and 4), and antigen 1 (A1) was precipitated within the well.

**Fig. 4.** Comparison of undiluted K-series (left) and M-series (right) antisera in line immunoelectrophoresis of GDH. Content of antisera in gels and of GDH in cytosol in strip and dimensions of strip as in Fig. 3. Note the presence of antigen 1 (A1) only in the gel with M-series antiserum. The gel with K-series antiserum contained antigens 1, 2, 3 and 4 (A1, A2, A3, A4). The height of the antigen 1 peak in both serum-containing gels was similar.

**Fig. 5.** Rocket immunoelectrophoresis assay of enzymic activity of M-series antiserum-treated cytosol. (a) K-series antiserum diluted 1 in 200 in gel (cf. Fig. 2b),  $20 \mu\text{l}$  cytosol in each well. Cytosol was absorbed by M-series antiserum (see text). The supernatant after centrifugation was used for the assays. Ratio of cytosol to antiserum in wells 1, 2 and 3 was 100:1, 100:10 and 100:50 respectively. In well 4 (control 1), ratio of cytosol to physiological saline was 100:50. Well 5 (control 2) contained untreated cytosol. The absence of enzymic activity and the lack of formation of a peak (see wells 2 and 3) indicated the removal of antigen 1 by the M-series antiserum. (b) As for (a) but with undiluted K-series antiserum in gel. The protein stain (upper) showed that all samples possessed antigens 2, 3 and 4. The enzyme assay (lower) demonstrated the GDH activity as a dark crescent in wells 1, 4 and 5. (Compare these results with Fig. 6 where antibody was omitted from the gel.)

**Fig. 6.** Agarose gel electrophoresis of M-series antiserum-treated cytosol. Antibody omitted from gel. Ratios of cytosol to antiserum in wells as in Fig. 5a. (a) Enzymic assay. Note the absence of biochemical reaction with adequate absorption from the cytosol (wells 2 and 3). (b) Protein stain. Samples applied to all wells showed a similar protein-staining reaction.

**Fig. 7.** Crushed lupin nodule stained with rhodamine-conjugated antisera. (a) M-series antiserum: note sharp image of fluorescent material (A); (b) K-series antiserum: note diffuse fluorescent material (B). The background nodule materials were non-fluorescent.

### *Cross-reactivity of GDH Isozymes*

The antigen used for antibody production was composed of four isozymes. It was important to ascertain if all isozymes would also react with the antibodies. With different batches of nodules, up to seven significant active isozymes have been obtained using polyacrylamide gel electrophoresis for separation. All these isozymes were found to be cross-reactive with the antisera when tested in experiments involving initial separation on polyacrylamide gel and rocket immunoelectrophoresis of individual bands.

### **Discussion**

The data obtained demonstrated that biochemically homogeneous GDH was still contaminated by traces of other immunogens. If used as an antigen for immunization, the antiserum obtained was not monospecific. If used for immunocytochemical studies with material containing sufficient quantity of the contaminating antigens, error in location of GDH could result. A demonstration of this was obtained by double staining of a lupin nodule smear. Monospecific antiserum produced sharp fluorescent structures (probably membrane associated) when counter-stained with rhodamine-labelled swine antirabbit immunoglobulins. Diffuse fluorescence resulted from use of K-series antiserum (Fig. 7).

Affinity chromatography and the modified method of Koch and Nielson (1975) are very suitable for production of monospecific sera. The technique is simple and readily adaptable for general use where absolute homogeneity of immunogens is difficult to achieve. *A priori*, one cannot exclude the possibility of total inhibition of enzymic activity on antibody binding. A negative biochemical test of precipitin complex is not adequate to rule out an identity with a particular enzyme. The enzymic nature of antigens 2, 3 and 4 therefore had to be verified in the absence of antigen 1 and their corresponding antibodies. These conditions were realized using cytosol absorbed with M-series monospecific antiserum, allowing rejection of the hypothesis that antigens 2–4 were GDH since no enzymic activity could be detected in the absence of corresponding antibodies. The antigens are not likely to be inactive or denatured isozymes of GDH, because Talal and co-workers (1964) pointed out that denatured GDH reacted with anti-GDH serum.

### **Acknowledgments**

This work was supported by the University of Sydney and the Australian Research Grants Committee. We are grateful to S. R. Stone and L. Copeland for the initial sample of purified GDH.

### **References**

- Bergersen, F. J. (1965). Ammonia—an early stable product of nitrogen fixation by soybean root nodules. *Aust. J. Biol. Sci.* **18**, 1–9.
- Cinader, B. (1966). Antibodies to enzyme. Discussion of the mechanisms of inhibition and activation. *Proc. 2nd Mtg Fed. Eur. Biochem. Soc.*, Vienna. pp. 85–131.
- Goding, J. W. (1976). Conjugation of antibodies with fluorochromes: modifications to the standard methods. *J. Immunol. Methods* **13**, 215–26.
- Harboe, N., and Ingild, A. (1975). Immunization, isolation of immunoglobulins, estimation of antibody titre. In 'A Manual of Quantitative Immunoelectrophoresis. Methods and Applications'. 4th Edn. (Eds N. H. Axelsen, J. Krøll and B. Weeke.) pp. 161–4. (Universitets Forlaget: Oslo.)



- Kawamura, A. (1969). 'Fluorescent Antibody Techniques and their Applications.' p. 47. (University of Tokyo: Tokyo.)
- Kennedy, I. R. (1966). Primary products of symbiotic nitrogen fixation. II. Pulse-labelling of *Serradella* nodules with  $^{15}\text{N}_2$ . *Biochim. Biophys. Acta* **130**, 295–303.
- Kennedy, I. R. (1979). Assimilating fixed nitrogen. *Proc. Aust. Biochem. Soc.* **12**, Q15.
- Koch, C., and Nielson, H. E. (1975). Fractionation of PHA by crossed immunoelectrophoresis and specific immunosorption. *Scand. J. Immunol.* **4** (Suppl. 2), 121–4.
- Krøll, J. (1975). Line immunoelectrophoresis. In 'A Manual of Quantitative Immunoelectrophoresis: Methods and Applications'. 4th Edn. (Eds N. H. Axelsen, J. Krøll and B. Weeke.) pp. 62–7. (Universitets Forlaget: Oslo.)
- Scott, D. B., Farnden, K. J. F., and Robertson, J. G. (1976). Ammonia assimilation in lupin nodules. *Nature (London)* **263**, 703–5.
- Stone, S. R., Copeland, L., and Kennedy, I. R. (1979). Glutamate dehydrogenase of lupin nodules: purification and properties. *Phytochemistry* **18**, 1273–8.
- Stone, S. R., Copeland, L., and Heyde, E. (1980a). Glutamate dehydrogenase of lupin nodules: kinetics of the deamination reaction. *Arch. Biochem. Biophys.* **199**, 550–9.
- Stone, S. R., Heyde, E., and Copeland, L. (1980b). Glutamate dehydrogenase of lupin nodules: kinetics of the amination reaction. *Arch. Biochem. Biophys.* **199**, 560–71.
- Talal, N., Tomkins, G. M., Mushinski, J. F., and Yielding, K. L. (1964). Immunochemical evidence for multiple molecular forms of crystalline glutamic dehydrogenase. *J. Mol. Biol.* **8**, 46–53.
- Tchan, Y. T. (1957). Phase-fluorescence microscopy. *Nature (London)* **179**, 1243–4.
- Weeke, B. (1975). General remarks on principle, equipment, reagents and procedures. Rocket immunoelectrophoresis. Crossed immunoelectrophoresis. In 'A Manual of Quantitative Immunoelectrophoresis: Methods and Applications'. 4th Edn. (Eds N. H. Axelsen, J. Krøll and B. Weeke.) pp. 26–7, 37–46, 47–56, respectively. (Universitets Forlaget: Oslo.)

