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Purification of nucleotide-requiring enzymes by immunoaffinity chromatography

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Monospecific (affinity-purified) anti-(yeast glucose-6-phosphate dehydrogenase) IgG inhibits three different NADPH-requiring enzymes, chicken liver dihydrofolate reductase, pigeon liver fatty acid synthetase and chicken liver malic enzyme. The inhibition of all three enzymes was approx. 50% in a 2h incubation with 100 μg of IgG. Similarly, with several different NADH-requiring enzymes, an immunocross-reactivity was observed. Monospecific anti-(rabbit muscle glyceraldehyde-3-phosphate dehydrogenase) IgG inhibited yeast alcohol dehydrogenase and pig heart malate dehydrogenase by 39% and 55% respectively. The cross-reactivity observed was tested by affinity chromatography. Immunoaffinity columns made with each monospecific IgG were able to bind each of the enzymes it immunotitrated. Enzymes were eluted with a nondenaturing solvent with little loss of activity. The immunoaffinity column with monospecific anti-(glucose-6-phosphate dehydrogenase) IgG as the bound ligand was also used to purify partially (over 150-fold) both isocitrate dehydrogenase and dihydrofolate reductase from crude rat liver homogenate.

Affinity chromatography has universal application in the purification of proteins. Column matrices can be made with ligands ranging from organic substances to enzymes to antibodies. Many column purification procedures are quite specific while others are more general. A system which incorporated general ligands in affinity purification of a broad range of enzyme was demonstrated by Mosbach *et al.* (1972). They chose to immobilize AMP and NAD^+ on a Sepharose gel matrix and subsequently showed the binding and elution of proteins requiring that particular nucleotide. This type of affinity technique has been used in the preparation of homogeneous enzymes as demonstrated by Caldés *et al.* (1979) and Mayer & Durrant (1979). Also, Thompson *et al.* (1975) and Wilson (1976) demonstrated the use of dyes coupled to Sepharose in the purification and subsequent structural studies of some nucleotide-requiring enzymes. The general complexation of the dyes with the nucleotide-requiring enzymes is indicative of a conserved relationship between these proteins. Many structural similarities have already been noted between nucleotide-requiring enzymes (Sund, 1969; Rossmann *et al.*, 1974), as well as immunological cross-reactivities (Katiyar & Porter, 1983*a*,*b*; S. R. Stapleton, S. S. Katiyar & J. W. Porter, unpublished results). The cross-reactivities previously observed and additionally defined in the present paper indicated the applicability of a novel immunoaffinity technique in the purification of nucleotide-requiring enzymes. Immunoaffinity chromatography provides a rapid and relatively specific means for purification of proteins (Livingston, 1974). Our results demonstrate antigen–antibody cross-reactivity which can effectively be applied in the purification of nucleotide-requiring enzymes.

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Materials and methods

Materials

The enzymes malate dehydrogenase (pig heart; L-malate:NAD oxidoreductase, EC 1.1.1.37), alcohol dehydrogenase (yeast; alcohol:NAD oxidoreductase, EC 1.1.1.1), glyceraldehyde-3-phosphate dehydrogenase [rabbit muscle; D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12] and glucose-6-phosphate dehydrogenase (yeast; D-glucose 6-phos-

phate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Dihydrofolate reductase (chicken liver; 5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) and malic enzyme [chicken liver; L-malate:$NADP^+$ oxidoreductase (oxaloacetate decarboxylating), EC 1.1.1.40] were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Pigeon liver fatty acid synthetase was purified by a procedure previously reported by this laboratory (Muesing & Porter, 1975).

Glucose 6-phosphate, dihydrofolic acid, oxaloacetate, malate, $NADP^+$, NADPH, NAD^+, and NADH were purchased from Sigma Chemical Co. Complete and incomplete Freund's adjuvant was obtained from Difco Laboratories, Detroit, MI, U.S.A. CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. All other chemicals were of analytical grade.

Immunization protocol and isolation of antibodies

New Zealand white rabbits (3–4 kg) were immunized with purified preparations of glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. Initially, 1 mg of enzyme emulsified with 2 ml of Freund's complete adjuvant was injected subcutaneously at different sites. The animals were boosted with enzyme in Freund's incomplete adjuvant at 2 and 4 weeks after the first injection. At 10 and 20 days after the third injection, blood was withdrawn from an ear vein. The blood was allowed to clot at 37°C for 1 h and then stored at 0°C overnight. After the blood clot was removed, the rabbit serum IgG fraction was isolated by precipitation with 50% $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose (Katiyar *et al.*, 1980). This IgG fraction was further purified by immunoaffinity chromatography.

Immunoaffinity chromatography

An individual affinity column was prepared with either glucose-6-phosphate dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase as the bound ligand. The procedure for coupling the ligand to CNBr-activated Sepharose was as described by Pharmacia. The ligand in a Na_2CO_3 buffer and the gel matrix were rotated end-over-end for 2 h at room temperature. The remaining active groups were blocked with a glycine solution and the excess protein was washed on a sintered glass funnel. Unbound protein in the filtrate was measured by the Bradford (1976) method and the amount of enzyme bound was calculated indirectly. The gel was packed into a column (1 cm × 1.5 cm) and washed extensively before passage of the rabbit serum IgG.

DEAE-cellulose purified rabbit serum IgG produced against either glucose-6-phosphate dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase was loaded onto the affinity column with its respective enzyme attached. The immunoglobulin solution was allowed to adsorb onto the column for at least 2 h and then passed over the column several times before washing and elution began. The flow rate of the column did not exceed 10 ml/h. The affinity gel was washed with 0.9% NaCl containing 1 mM-EDTA, and then eluted with 4.5 M-$MgCl_2$ which had been neutralized to an apparent pH of 6.4 with 1 M-Tris/HCl. The IgG fractions were pooled, dialysed against 10 mM-potassium phosphate, pH 7.5, and concentrated by lyophilization. This monospecific antibody was tested for its ability to immunotitrate the antigen against which it was raised. Monospecific antibody (50 μg) which was able to immunotitrate its specific antigen by at least 70% in 2 h was then stored frozen at −20°C at a final concentration of 5 mg/ml until used for immunotitrations or preparations of immunoaffinity columns.

Monospecific antibody against glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase was bound onto individual affinity columns by the method described above. Columns were made with final ligand: gel matrix concentrations of 4.5 mg/ml and 3.8 mg/ml for monospecific anti-(glucose-6-phosphate dehydrogenase) IgG and monospecific anti-(glyceraldehyde-3-phosphate) IgG, respectively. Enzymes tested for their binding ability to the column were slowly loaded onto the affinity column which had been equilibrated with the assay buffer of choice for that enzyme. The column was washed extensively with buffer containing 0.9% KCl and the bound enzyme was then eluted with 4.0 M-KCl. The fractions containing active protein were pooled and the percent recovery was calculated.

Enzyme assays

All enzyme activities were measured spectrophotometrically at 340 nm by following the rate of oxidation or reduction of the appropriate nucleotide in the presence of substrate. The assay mixture for dihydrofolate reductase was as described by Poe *et al.* (1972) and contained 0.1 mM-NADPH, 1 mM-dihydrofolate, 10 mM-β-mercaptoethanol, 25 mM-potassium phosphate, pH 7.0, and 1 μg of dihydrofolate reductase. Fatty acid synthetase (1 μg) was assayed by the method of Katiyar *et al.* (1975) and each 1.0 ml mixture contained 0.04 mM-acetyl-CoA, 0.1 mM-malonyl-CoA, 0.1 mM-NADPH, 0.2 M-potassium phosphate and 1 mM-EDTA, pH 7.0. Malic enzyme was assayed in the presence of 0.2 mM-$NADP^+$, 4 mM-$MnCl_2$, 70 mM-Tris/HCl, pH 7.0 and 0.5 mM-malic acid

(Hsu & Lardy, 1967). The assay conditions for alcohol dehydrogenase were as described by Vallee & Hoch (1955), and each mixture contained 10mM-NAD^+, 400mM-ethanol, 50mM-potassium phosphate, pH7.0, and 0.01 μg of alcohol dehydrogenase. Malate dehydrogenase was assayed by the method of Thorne & Kaplan (1963) and each 1.0ml mixture consisted of 0.2mM-NADH, 0.5mM-oxaloacetate, 90mM-potassium phosphate, pH7.5, and 0.01 μg of malate dehydrogenase.

Immunotitrations of enzymatic activities

Immunotitrations were performed by a procedure previously reported by this laboratory (Katiyar *et al.*, 1980; Lornitzo *et al.*, 1981*a*,*b*). The direct immunotitration method in which a constant amount of enzyme is incubated with various amounts of monospecific antibody was employed. Fatty acid synthetase, dihydrofolate reductase and malic enzyme were each incubated at 30°C for 2h with increasing amounts (0–200 μg) of monospecific antibody against glucose-6-phosphate dehydrogenase. Additionally, the incubation mixture for fatty acid synthetase and dihydrofolate reductase contained 0.1M-potassium phosphate, pH7.5, and bovine serum albumin at a final concentration of 1mg/ml. The incubation mixture for malic enzyme also contained the bovine serum albumin but the buffer was 0.1M-Tris/HCl, pH7.0. After the 2h incubation, the remaining enzyme activities were measured. Immunotitrations of malate dehydrogenase and alcohol dehydrogenase were carried out with monospecific antibody against glyceraldehyde-3-phosphate dehydrogenase. The immunotitration mixtures contained bovine serum albumin (1mg/ml), potassium phosphate, pH7.5, enzyme (0.01 μg) and various amounts of monospecific anti-(glyceraldehyde-3-phosphate dehydrogenase) IgG (0–200 μg), and were incubated at 30°C for 2h. The remaining enzyme activity was measured following the 2h incubation.

Purification of rat liver dihydrofolate reductase and isocitrate dehydrogenase

Rat livers were homogenized thoroughly in a blender using a 1:1 (w/v) ratio of liver to buffer (50mM-potassium phosphate, pH7.0, with 0.5mM-EDTA). The resulting homogenate was treated with solid $(NH_4)_2SO_4$ to yield 20% saturation. After centrifugation at 20000*g* for 30min the supernatant was removed and dialysed against 0.1M-potassium phosphate, pH7.0. This crude homogenate was loaded onto the monospecific anti-(yeast glucose-6-phosphate dehydrogenase) IgG affinity column previously equilibrated with phosphate buffer. The column was washed extensively with 0.1M-potassium phosphate, pH7.0, containing 0.9% KCl and eluted with 4.0M-KCl. The eluate was assayed for both dihydrofolate reductase (Poe *et al.*, 1972) and isocitrate dehydrogenase (Londesborough & Dalziel, 1968). These enzymes were chosen since both are fairly abundant enzymes in rat liver. The active protein fractions were combined, dialysed against 10mM-potassium phosphate, pH7.0, and concentrated by lyophilization. The analysis of this protein purification was carried out using sodium dodecyl sulphate/13% polyacrylamide-gel electrophoresis according to Laemmli (1970), and the gels were silver stained according to the method of Oakley *et al.* (1980).

Results and discussion

The existence of cross-reactive sites on protein molecules has recently been demonstrated with the use of polyclonal antisera. This laboratory (Katiyar & Porter, 1983*a*,*b*) has reported that conventional rabbit antiserum raised against an enzyme containing a nucleotide binding site (e.g. NADPH) cross-reacts with another apparently unrelated enzyme that also possesses a structural domain for the binding of that nucleotide. Additionally, this type of cross-reactivity using polyclonal antisera has been observed with enzymes that require either NADH or ATP for activity (S. S. Katiyar & J. W. Porter, unpublished work). S. R. Stapleton, S. S. Katiyar & J. W. Porter (unpublished work) have shown inhibition of NADPH-requiring enzymes with the polyclonal antisera generated against a NADH-requiring enzyme and the converse was also found to be true. The observed cross-reactivity suggested a common structural domain in nucleotide-requiring enzymes. Since the only common feature between the enzymes glucose-6-phosphate dehydrogenase, dihydrofolate reductase, malic enzyme and fatty acid synthetase is their requirement for a $NADP^+$/NADPH nucleotide, and if a portion of the polyclonal antisera was directed towards this common structural area between these nucleotide binding proteins, then one should be able to generate affinity-purified antibody which is still inhibitory to all. Fig. 1 illustrates the inhibition of dihydrofolate reductase, fatty acid synthetase and malic enzyme by affinity-purified (monospecific) anti-(yeast glucose-6-phosphate dehydrogenase) IgG. Monospecific anti-(yeast glucose-6-phosphate dehydrogenase) IgG was prepared by purifying the polyclonal IgG fraction over a yeast glucose-6-phosphate dehydrogenase affinity column as described in the Materials and methods section. In a 2h incubation, both dihydrofolate reductase and fatty acid synthetase were inhibited by over 50% with 100 μg of the monospecific IgG, whereas with malic enzyme immunoinactivation

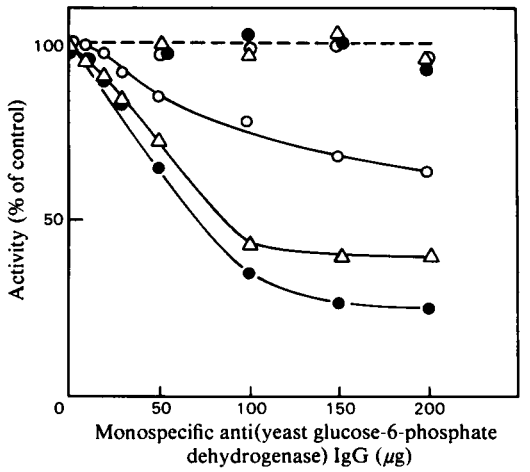

Fig. 1. *Immunoinhibition of dihydrofolate reductase (chicken liver), fatty acid synthetase (pigeon liver) and malic enzyme (pigeon liver) by monospecific anti-(yeast glucose-6-phosphate dehydrogenase) IgG*

Fatty acid synthetase (1 μg) (●), dihydrofolate reductase (1 μg) (△) and malic enzyme (1 μg) (○) were each incubated at 30°C with increasing amounts (0–200 μg) of either monospecific anti-(yeast glucose-6-phosphate dehydrogenase) IgG (—) or preimmune serum IgG (---) for 2h in an incubation mixture also containing bovine serum albumin (1 mg/ml) and assay buffer. After the fixed time interval, residual enzyme activity was measured.

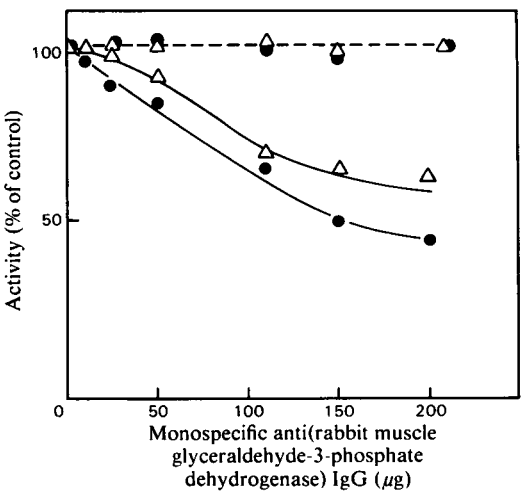

Fig. 2. *Immunotitration of alcohol dehydrogenase (yeast) and malate dehydrogenase (pig heart) by monospecific anti-(rabbit muscle glyceraldehyde-3-phosphate dehydrogenase) IgG*

Alcohol dehydrogenase (0.01 μg) (△) and malate dehydrogenase (0.01 μg) (●) were each incubated at 30°C with increasing amounts (0–200 μg) of either monospecific anti-(rabbit muscle glyceraldehyde-3-phosphate dehydrogenase IgG) (—) or preimmune serum IgG (---) for 2h in the presence of 0.1 M-potassium phosphate, pH 7.5 and bovine serum albumin (1 mg/ml). After the 2h incubation, the enzyme activity remaining was measured.

only approached 40% with 200 μg of antibody. Under the same conditions, incubation of the enzymes with preimmune serum IgG did not result in any loss of enzyme activity.

The monospecific yeast anti-(glucose-6-phosphate dehydrogenase) IgG is still polyclonal in nature but the population of antibody that is non-specific should be at a minimum due to the affinity purification. The only common feature between glucose-6-phosphate dehydrogenase, dihydrofolate reductase, fatty acid synthetase and malic enzyme is a nucleotide-binding region, and hence this must be the site of the inhibitory action of the monospecific antibody. This suggests immunological similarities in the nucleotide-binding regions. This general conclusion is further substantiated by the inhibition of malate dehydrogenase and alcohol dehydrogenase by monospecific anti-(glyceraldehyde-3-phosphate dehydrogenase) IgG and is illustrated in Fig. 2. The preparation of monospecific anti-(rabbit muscle glyceraldehyde-3-phosphate dehydrogenase) IgG was analogous to that for monospecific anti-(yeast glucose-6-phosphate dehydrogenase) IgG and is outlined in the Materials and methods section. Malate dehydrogenase and alcohol dehydrogenase were immunotitrated 55% and 39%, respectively, with 200 μg of monospecific anti-(rabbit muscle glyceraldehyde-3-phosphate dehydrogenase) IgG in a 2h incubation. The inhibition reported is striking considering the unrelatedness of these enzymes and again relates a possible immunological similarity in enzymes with an NADH binding site. The variances observed in the immunotitration by the monospecific antibody may be related to the degree of homology present in the antigenic determinant of each of the enzymes. Again, as illustrated, incubation of the enzymes with preimmune serum IgG did not yield any significant immunoinhibition of enzyme activity.

Experiments were also carried out to determine whether or not these antibodies inhibited other enzymes which possess the dinucleotide fold but do not bind NAD^+. Phosphoglycerate kinase was not inhibited by either antibody (results not shown). Some glycolytic enzymes, such as phosphoglyceromutase or triose phosphate isomerase, that retain features of the dinucleotide fold were not tested,

but in light of the lack of inhibition on phosphoglycerate it could probably be assumed that the results would be similar. Several other kinases (creatine kinase, myokinase and hexokinase) were tested to check on potential cross-reactivity of these antibodies with ATP/ADP-requiring enzymes. No immunoinhibition was observed. Some cross-reactivity between antibody generated against NADPH-requiring enzymes with NADH-requiring enzyme has been observed (S. R. Stapleton, S. S. Katiyar & J. W. Porter, unpublished work). The converse of this is also true.

Based on the observed cross-reactivities we decided to utilize this information to develop an immunoaffinity method for purification of nucleotide-requiring enzymes. Two affinity columns were prepared, one utilizing the monospecific anti-(glucose-6-phosphate dehydrogenase) IgG as the coupled ligand and the other with the coupled ligand as monospecific anti-(glyceraldehyde-3-phosphate dehydrogenase) IgG. The columns and conditions for binding and elution are as described in the Materials and methods section. The results of the interaction of the proteins with the affinity columns are recorded in Table 1. The enzymes that were immunotitrated by monospecific anti-(yeast glucose-6-phosphate dehydrogenase) IgG all bound to the affinity column made with this antibody. Using relatively mild conditions, each of these enzymes was rapidly bound and eluted with loss of little of the original enzyme activity. The bound material was removed by using a non-denaturing substance, 4.0M-KCl in appropriate buffer. Other solutions either at various pH values or containing different concentrations of nucleotides were also employed for eluting, but best results were found with 4.0M-KCl. Generally in the past when immunoaffinity columns were used for protein purification, the elution conditions were harsh and sometimes denaturing because of the strong antigen binding. Pigeon liver fatty acid synthetase exhibited the best recovery from the column with loss of 25% of the original activity. An affinity column with monospecific anti-(rabbit muscle glyceraldehyde-3-phosphate dehydrogenase) IgG was also made. Similarly, the enzymes which showed immunoinactivation with this antibody were able to bind to and elute from the column under relatively mild conditions. These results are also recorded in Table 1.

The application of immunoaffinity columns that can selectively aid in the purification of nucleotide-requiring enzymes is obvious. To demonstrate the usefulness of this type of immunoaffinity column, we were able to purify by more than 150-fold both dihydrofolate reductase and isocitrate dehydrogenase from a crude rat liver homogenate with only a single pass of the crude mixture over the anti-(glucose-6-phosphate dehydrogenase) IgG affinity column. Both dihydrofolate reductase and isocitrate dehydrogenase are easily measurable and relatively abundant enzymes in liver preparations, so they are ideal enzymes to use in testing the application of the immunoaffinity column. Enzyme activities and protein concentrations were measured before and after elution from the column and specific activities were determined. Dihydrofolate reductase and isocitrate dehydrogenase were purified 190- and 170-fold, respectively (Table 2). The crude homogenate and the eluted protein were analysed by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as shown in Fig. 3. Lane 1 contains authentic dihydrofolate reductase and isocitrate dehydrogenase; lane 2 is the eluted column material. Lane 3 is crude liver homogenate. Illustrated is the ability of the immunoaffinity column to substantially 'clean up' the crude liver homogenate while partially purifying both dihydrofolate reductase and isocitrate dehydrogenase. Conditions of elution of protein from the column may be varied to elute selectively different NADPH-requiring enzymes. Protein bands in lane 2 comigrate with authentic dihydrofolate reductase and isocitrate dehydrogenase in lane 1, thus pictorially showing the partial purification of these two nucleotide-requiring enzymes.

Table 1. *Binding and elution of active proteins from an immunoaffinity column*

The ligands were bound to CNBr-activated Sepharose as described in the Materials and methods section. The enzymes were loaded on in appropriate buffer and eluted with 4.0M-KCl.

Monospecific column	Enzymes	Activity recovered after binding to and elution from column (%)
Anti-(yeast glucose-6-phosphate dehydrogenase) IgG	Dihydrofolate reductase	67
	Fatty acid synthetase	75
	Malic enzyme	51
Anti-(rabbit muscle glyceraldehyde-3-phosphate dehydrogenase)	Alcohol dehydrogenase	61
	Malate dehydrogenase	74

Table 2. *Partial purification of rat liver dihydrofolate reductase and isocitrate deydrogenase by immunoaffinity purification*
Experimental conditions as described in the Materials and methods section.

Fraction	Specific activity (nmol/min per mg of protein)		Purification (-fold)	
	Dihydrofolate reductase	Isocitrate dehydrogenase	Dihydrofolate reductase	Isocitrate dehydrogenase
Crude	3.0	1.5	1	1
$(NH_4)_2SO_4$ (0–20% supernatant)	8.5	4.0	2.8	2.7
Immunoaffinity column	570	255	190	170

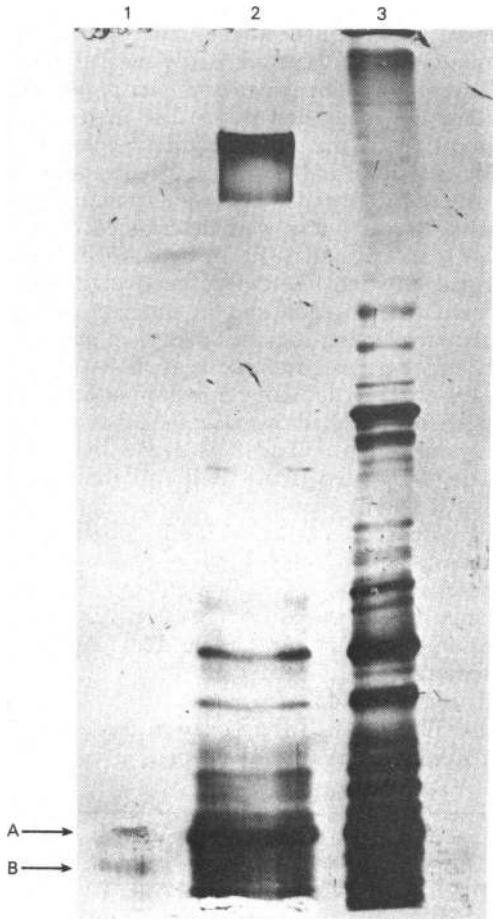

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the partial purification of rat liver isocitrate dehydrogenase and dihydrofolate reductase*
The lanes contain the following: 1, authentic dihydrofolate reductase (B) and isocitrate dehydrogenase (A); 2, eluate from immunoaffinity column with coupled monospecific glucose-6-phosphate dehydrogenase (column conditions were as described in the Materials and methods section); 3, crude rat liver homogenate.

The use of immunoaffinity chromatography for protein purification is not a new one (Cuatrecasas & Anfinsen, 1971). The technique has proved valuable for rapid and specific purification of proteins. Pegg *et al.* (1984) have recently used an immunoaffinity column in the purification of mammalian ornithine decarboxylase. The idea of applying immunochromatography to the purification of a group of enzymes has evolved because of the antigen–antibody cross-reactivity we have observed. This type of purification of nucleotide-requiring enzymes may be compared to ligand chromatography. Blue Dextran and Cibacron Blue coupled to Sepharose are used in the purification of proteins containing the dinucleotide fold (Thompson *et al.*, 1975; Wilson, 1976). The use of immunoaffinity chromatography in this case may, however, exhibit more selectivity (purification of only cross-reacting proteins). Conditions may be varied to yield potential selectivity in protein elution. This technique may then prove useful in abbreviating established protein purification procedures, minimizing loss of enzyme activity due to instability and improving yields.

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