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Absorption of Antisera for Studies on Specific Enzyme Turnover

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Antisera were raised to acetyl-CoA carboxylase and 6-phosphogluconate dehydrogenase from mammary glands of lactating rabbits, and cytochrome oxidase from rat liver. The enzymes were all highly purified but gave rise to multispecific antisera when tested against tissue extracts. Absorption procedures were devised to free the antisera of contaminating antibodies. Antisera to acetyl-CoA carboxylase and cytochrome oxidase were absorbed with fractions discarded during enzyme purification. The antiserum to 6-phosphogluconate dehydrogenase was absorbed with a tissue extract from an early stage in mammary-gland differentiation. Monospecific antisera are essential for enzyme turnover studies and therefore antisera should be extensively tested and absorbed before use. A general procedure for the absorption of antisera to purified enzymes has been devised on the basis of accepted principles of antisera absorption.

In recent years, antisera have become increasingly important as tools for biochemical research. This is especially true of studies on the turnover (i.e. synthesis and degradation) of specific enzymes (Emery & Baldwin, 1967; Glass & Doyle, 1972; Cihak *et al.*, 1973; Werner, 1974; Hizi & Yagil, 1974; Peavy & Hansen, 1975; Speake *et al.*, 1975). Such studies depend critically on the purity of the antiserum, and yet often antisera have not been sufficiently characterized before use. It should never be assumed that an antiserum is monospecific simply because the antigen is pure by biochemical criteria (Kabat, 1961; Harboe & Ingild, 1973). For example, in this laboratory, acetyl-CoA carboxylase (EC 6.4.1.2) (Manning *et al.*, 1976) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (Betts & Mayer, 1975) were purified to homogeneity by the criterion of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and yet gave rise to antisera containing contaminating antibodies.

In the present paper procedures are given for the characterization and purification of antisera to acetyl-CoA carboxylase and 6-phosphogluconate dehydrogenase from rabbit mammary gland and to cytochrome *c* oxidase (EC 1.9.3.1) from rat liver. All the procedures adopted are based on the principle of antisera absorption (Kwalpinski, 1972) by fractions that contain contaminating antigens (Philippidis *et al.*, 1972; Harboe & Ingild, 1973; Rietra *et al.*, 1974). The general procedures that have been adopted are applicable to antisera for studies on the turnover of soluble and membrane-bound proteins and indeed for work on any antigen for which monospecific antisera are essential.

Materials and Methods

Animals

The sheep used in these studies were housed at the Joint Animal Breeding Unit, Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. New Zealand White rabbits and Wistar rats were obtained from the same source.

Materials

Triton X-100 was purchased from Lennig Chemicals Ltd., Croydon, London CB9 3NB, U.K. Cytochrome *c* (grade VI) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Agarose and barbitone were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Glycine (A.R.) and barbitone sodium (S.L.R.) were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. All the other materials used have been described previously (Betts & Mayer, 1975; Manning *et al.*, 1976; Walker & Mayer, 1976).

Methods

Preparation of an antiserum to acetyl-CoA carboxylase. Acetyl-CoA carboxylase was purified from mammary gland of lactating rabbits as described previously (Manning *et al.*, 1976). An antiserum was raised in a sheep by injecting 450 μg of purified enzyme together with Freund's complete adjuvant on three occasions. The antigen was injected at five or six sites (subcutaneously and intramuscularly) on each occasion at 14-day intervals. The volume injected at each site was 1 ml (0.5 ml of antigen

solution and 0.5ml of adjuvant). At 1 week after the final injection the animal was bled by jugular cannulation and the blood processed to give the immunoglobulin fraction as described by Speake *et al.* (1975). Immunodiffusion studies (Ouchterlony, 1949; Piazzi, 1969) were performed in 1% (w/v) agarose gels containing 50mM-Tris/HCl buffer, pH 7.5, and 150mM-NaCl. Gels (1.5mm thick) were cast on glass plates (10cm × 10cm) that had been precoated with agarose. Diffusion was allowed to proceed for 2 days at room temperature (25°C) in a moist atmosphere before pressing, washing and staining the gels for protein (Weeke, 1973*a*). The immunoelectrophoretic techniques were exactly as described elsewhere using the Svendsen buffer system (Axelsen *et al.*, 1973). Absorption of the antiserum (i.e. removal of contaminating antibodies by precipitation with the corresponding macromolecular antigen) was achieved by incubating a fraction discarded during enzyme purification (see the Results section) with the antiserum for 30min at 37°C and then for 24h at 4°C. A precipitate was removed by centrifugation at 4°C for 30min at $27000g_{av.}$. Immunotitrations were performed in 20mM-sodium phosphate buffer, pH 7.0, containing 150mM-NaCl, 20mM-potassium citrate and 1% (w/v) Triton X-100. Incubations were carried out at 37°C for 30min and then for 24h at 4°C. The enzyme was assayed spectrophotometrically (Manning *et al.*, 1976) in a supernatant prepared by centrifugation of each incubation mixture for 20min at maximum speed in a bench centrifuge. One unit of acetyl-CoA carboxylase catalyses the carboxylation of 1 μmol of acetyl-CoA/min.

Preparation of an antiserum to cytochrome c oxidase. Cytochrome *c* oxidase was purified from rat liver by the method of Walker & Mayer (1976). An antiserum was raised in a sheep by injecting 1mg of the purified enzyme together with Freund's complete adjuvant on six occasions. Antigen was administered at four sites (two subcutaneously and two intramuscularly) on each occasion at 14-day intervals. The volume injected at each site was 1ml (0.5ml of antigen solution and 0.5ml of adjuvant). At 1 week after the final injection the animal was bled and the blood processed as described by Speake *et al.* (1975). Immunodiffusion studies (Ouchterlony, 1949; Piazzi, 1969) were performed in 1% (w/v) agarose gels containing 20mM-sodium phosphate buffer, pH 7.0, 150mM-NaCl and 1% (w/v) Triton X-100. Immunoelectrophoretic methods were as described (Axelsen *et al.*, 1973) except that the gels contained 1% (w/v) Triton X-100. Rat liver homogenates (1:2, w/v) were prepared in 0.25M-sucrose previously adjusted to pH 7.0 with solid $NaHCO_3$. Particle-free supernatant (20mg of protein/ml) was obtained by centrifuging the homogenate at $100000g_{av.}$ for 60min. A Triton X-100 extract (5%, v/v) of the homogenate was obtained by adding the appropriate volume of detergent to the homogenate. The resulting suspension was centrifuged at $100000g_{av.}$ for 60min to give a clear supernatant of solubilized proteins.

Preparation of an antiserum to 6-phosphogluconate dehydrogenase. 6-Phosphogluconate dehydrogenase was purified from the mammary glands of lactating rabbits as described previously (Betts & Mayer, 1975). An antiserum was raised in a sheep by injecting 450 μg of the purified enzyme together with Freund's complete adjuvant on four occasions. Antigen was administered at eight sites (four subcutaneously and four intramuscularly) on each occasion at 14-day intervals. The volume injected at each site was 1ml (0.5ml of antigen solution and 0.5ml of adjuvant). At 1 week after the final injection the animal was bled and the blood processed as described by Speake *et al.* (1975). Immunodiffusion studies (Ouchterlony, 1949; Piazzi, 1969) were performed as described above in 1% (w/v) agarose gels containing 20mM-sodium phosphate buffer, pH 7.0, and 150mM-NaCl. Immunoprecipitates which formed on immunodiffusion were stained for enzyme activity by means of Nitro Blue Tetrazolium as described by Betts & Mayer (1975). Absorption was achieved by incubating the appropriate tissue extract (see the Results section) with the antiserum for 30min at 37°C followed by 24h at 4°C. A precipitate was removed by centrifugation at 4°C for 20min in a bench centrifuge at maximum speed. Immunotitrations of 6-phosphogluconate dehydrogenase activity were performed as described for acetyl-CoA carboxylase except that potassium citrate and Triton X-100 were not included in the reaction mixture. One unit of 6-phosphogluconate dehydrogenase catalyses the reduction of 1 μmol of $NADP^+$/min at 30°C.

Results

Absorption of the antiserum to acetyl-CoA carboxylase

Immunodiffusion studies with either conventional Ouchterlony (1949) or Piazzi (1969) techniques seemed to indicate that the antiserum was monospecific since a single line was obtained over a wide range of concentrations of the antiserum and of a particle-free supernatant that contained the enzyme. However, rocket immunoelectrophoresis demonstrated the presence of antibodies responding to two antigens in the particle-free supernatant prepared from an homogenate of mammary glands from lactating rabbits (Fig. 1*a*). Crossed-rocket immunoelectrophoresis confirmed the presence of two antigen–antibody systems and the techniques of tandem-crossed and rocket-line immunoelectrophoresis (Axelsen *et al.*, 1973) were used to demonstrate that the two antigenic components were not related (results not shown). Fractions discarded during the purification of the enzyme were checked by means of rocket immunoelectrophoresis to find

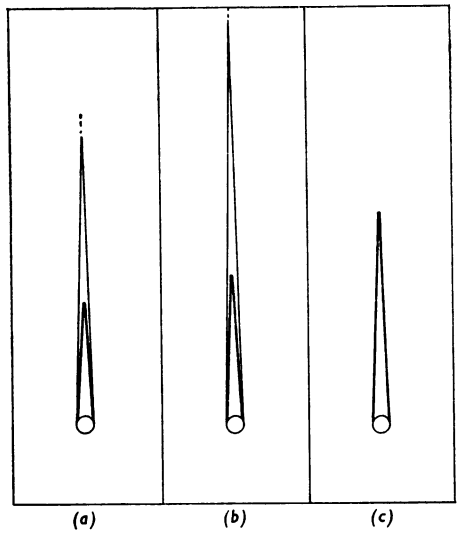

Fig. 1. *Rocket immunoelectrophoretic analysis of the absorption of an antiserum to acetyl-CoA carboxylase from rabbit mammary gland*

Each well contained 10 μl of a particle-free supernatant prepared from mammary glands of 15-day lactating rabbits. Immunoelectrophoresis was carried out in 0.1% (v/v) antiserum in 1% (w/v) agarose gel. The antiserum was unabsorbed (*a*), partially absorbed (0.5 vol. of waste fraction/vol. of antiserum) (*b*) and completely absorbed (10 vol. of waste fraction/vol. of antiserum) (*c*). Immunoelectrophoresis was carried out for 16 h at 10 V/cm at 15°C.

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if any particular fraction was enriched in the contaminant. The supernatant from the calcium phosphate-gel treatment (Manning *et al.*, 1976) contained significant amounts of this contaminating antigen. This supernatant, however, contained only a small amount (approx. 5%) of the total activity of acetyl-CoA carboxylase compared with that which was in the solution that had been originally treated with the calcium phosphate gel. The optimum amount of the supernatant from calcium phosphate treatment required for the absorption of the contaminating antibodies was determined by absorbing several samples of the antiserum with increasing amounts of this fraction. The results of some of these absorption procedures are shown in Fig. 1. The contaminating rocket increases in height after partial absorption (Fig. 1*b*) and totally disappears when absorption is complete (Fig. 1*c*). The rocket immunoprecipitate due to acetyl-CoA carboxylase increases in height, indicating a loss of antibodies to the enzyme. This increase in height corresponds to a 33% decrease in the antibodies to the enzyme and this value was confirmed by immunoinhibition titrations of enzyme activity (results not shown). It should be noted that enzyme activity was completely inhibited by the addition of very small amounts of antiserum (1 μl of absorbed antiserum per 30 munits of enzyme activity). This may be due to depolymerization of the highly polymerized enzyme complex that is required for the activity of acetyl-CoA carboxylase (Lane *et al.*, 1975). In contrast 20 μl of absorbed antiserum was required for maximum precipitation of the amount of enzyme protein corresponding to 30 munits of enzyme activity.

The absorbed antiserum was tested by rocket immunoelectrophoresis (Fig. 1*c*) and by crossed immunoelectrophoresis (results not shown) and was found to be monospecific. At equivalence, 1 ml of the absorbed antiserum precipitates 7.4 mg of protein with a tissue extract that contains 1.5 units of enzyme activity. This corresponds to 290 μg of acetyl-CoA carboxylase in the immunoprecipitate when calculated from the specific activity of the purified enzyme (Manning *et al.*, 1976).

Absorption of the antiserum to cytochrome c oxidase

Immunodiffusion studies were carried out by the methods of Ouchterlony (1949) and Piazzi (1969). The particle-free supernatant prepared from an homogenate of rat liver contained two antigens which showed no reaction of identity with purified cytochrome oxidase (Fig. 2*a*). Rocket and crossed-rocket immunoelectrophoresis of a Triton X-100 extract (5%, v/v) of an homogenate of rat liver confirmed the presence of three antigen–antibody systems. Rocket-line immunoelectrophoresis was used to demonstrate that the antigens in the particle-free supernatant were not related to cytochrome oxidase (results not shown). The antiserum to cytochrome oxidase was absorbed with a particle-free supernatant prepared from homogenate of rat liver. The proportions required for the absorption were determined by quantitative precipitation with particle-free supernatant and the unabsorbed antiserum; 4 vol. of the particle-free supernatant was required to absorb each volume of antiserum. After centrifugation to remove the immunoprecipitate, the supernatant was taken to 50% saturation with solid $(NH_4)_2SO_4$. The precipitated protein was dialysed against 20 mM-sodium phosphate buffer, pH 7.0, containing 150 mM-NaCl. Immunodiffusion studies with the absorbed antiserum (Fig. 2*b*) demonstrate the complete removal of contaminating antibodies in the antiserum and these results were confirmed by crossed immunoelectrophoresis (results not shown). Rocket immunoelectrophoresis of samples of a Triton X-100 extract (5%, v/v) of homogenate of rat liver against antisera absorbed to different extents has been performed (Fig. 3). The results show that

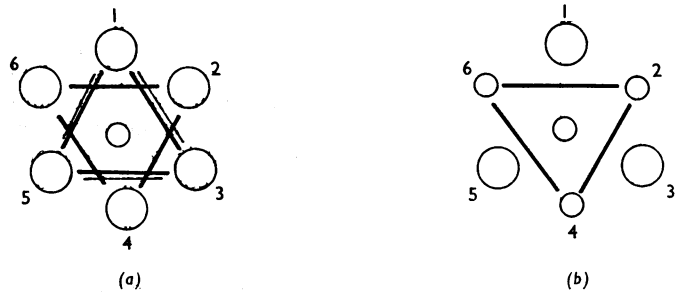

Fig. 2. *Ouchterlony double-diffusion analysis of the absorption of an antiserum to cytochrome oxidase from rat liver*

Wells 1, 3 and 5 contained purified enzyme, and wells 2, 4 and 6 contained samples of a particle-free supernatant prepared from homogenate of rat liver. The central wells contained (*a*) unabsorbed antiserum; (*b*) completely absorbed antiserum. Immunodiffusion was carried out for 2 days at room temperature in a moist atmosphere.

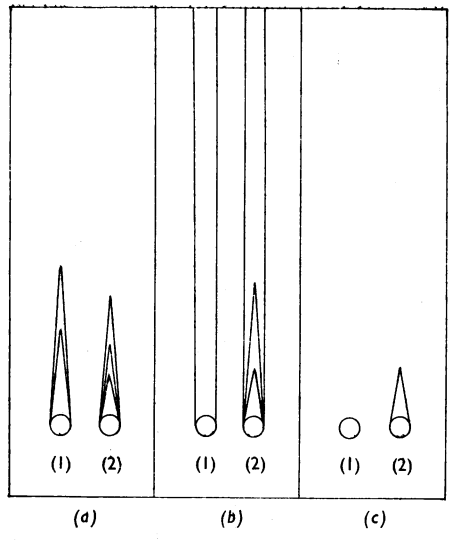

Fig. 3. *Rocket immunoelectrophoretic analysis of the absorption of an antiserum to cytochrome oxidase from rat liver*

The wells contained samples (10μl) of particle-free supernatant prepared from homogenate of rat liver (1) and Triton X-100 extract (5%, v/v) of homogenate of rat liver (2). Immunoelectrophoresis was carried out with 0.25% (v/v) antiserum in 1% (w/v) agarose gel. The antiserum was unabsorbed (*a*), partially absorbed (1 vol. of particle-free supernatant/vol. of antiserum) (*b*) and completely absorbed (4 vol. of particle-free supernatant/vol. of antiserum) (*c*). Immunoelectrophoresis was carried out for 16h at 10V/cm at 15°C.

no change occurs in the height of the rocket immunoprecipitate associated with cytochrome oxidase. The two other rockets increase in height on partial absorption of the antiserum and disappear when absorption is complete. Therefore no loss of antibodies to cytochrome oxidase occurred on absorption and the absorbed antiserum is monospecific. At equivalence, 1 ml of the absorbed antiserum precipitates 2.1 mg of protein with a tissue extract that contains an enzyme activity (0.57 μmol of cytochrome *c* oxidized/min), corresponding to 710 μg of cytochrome oxidase. This value is calculated from the specific activity of the purified enzyme (Walker & Mayer, 1976).

Absorption of the antiserum to 6-phosphogluconate dehydrogenase

Ouchterlony (1949) immunodiffusion and rocket immunoelectrophoresis with the antiserum against a particle-free supernatant from mammary gland of lactating rabbits seemed to indicate that the antiserum was monospecific (results not shown). However, immunodiffusion studies (Fig. 4) by the method of Piazzi (1969) indicated the presence of contaminating antibodies in the antiserum which interact with an antigen in a particle-free supernatant prepared from homogenate of mammary gland of lactating rabbits. The contaminating antigen was also present in a particle-free supernatant prepared from an homogenate of mammary gland of mid-pregnant rabbits. The specific activity of 6-phosphogluconate dehydrogenase in a particle-free supernatant prepared from mammary gland of mid-pregnant rabbits was approximately one-eighth of that measured in a particle-free supernatant from mammary gland of lactating rabbits. The residual activity of 6-phosphogluconate dehydrogenase in immunoprecipitin lines obtained with a particle-free supernatant prepared from mammary gland of mid-pregnant rabbits was therefore negligible compared with that obtained with particle-free supernatant from mammary gland of lactating rabbits.

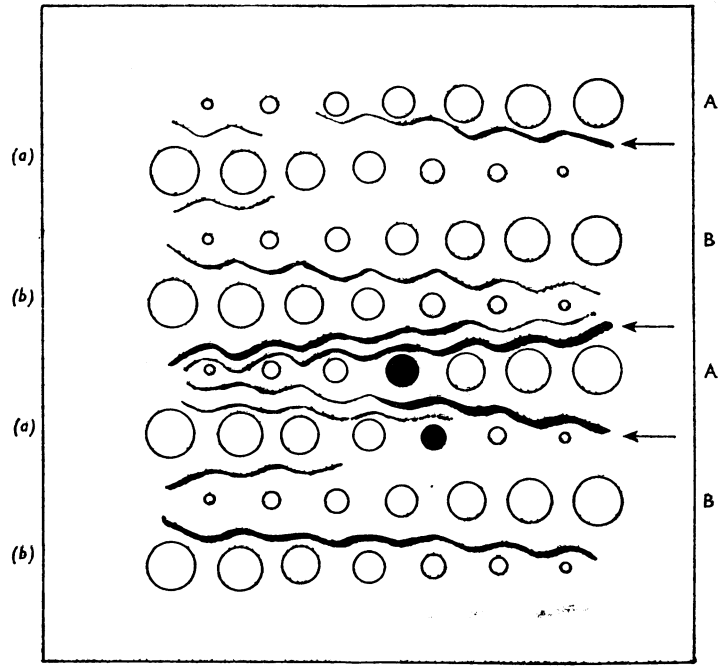

Fig. 4. *Plazzi* (1969) *immunodiffusion analysis of the absorption of an antiserum to 6-phosphogluconate dehydrogenase from mammary gland of lactating rabbits*

Wells contained particle-free supernatant prepared from mammary gland of mid-pregnant (*a*) and 15-day lactating (*b*) rabbits. Opposing wells contained unabsorbed antiserum (A) and completely absorbed antiserum (B). The arrowed immunoprecipitin lines did not stain for 6-phosphogluconate dehydrogenase activity. The filled wells indicate the point at which equivalence was judged to occur for the contaminating antigen–antibody system. Immunodiffusion was carried out for 24 h at room temperature in a moist atmosphere.

Hence the antiserum to 6-phosphogluconate dehydrogenase was absorbed with particle-free supernatant from mammary gland of mid-pregnant rabbits. The proportions required for absorption of the antiserum were estimated from the form (sharpness and intensity) of the immunoprecipitate on immunodiffusion (Fig. 4; Williams, 1971). Equivalence for the contaminating antigen–antibody system was judged to occur when 2 vol. of antiserum and 1 vol. of particle-free supernatant from mammary gland of pregnant rabbits were opposed as indicated in Fig. 4. The particle-free supernatant was freeze-dried and the appropriate volume of antiserum added. The precipitate was removed by centrifugation as described in the Materials and Methods section. The absorbed antiserum gave a single immunoprecipitin line on immunodiffusion against particle-free supernatants from homogenates of mammary glands from both pregnant and lactating rabbits (Fig. 4). Immunotitrations of the partially purified enzyme from mammary gland of lactating rabbits with absorbed and unabsorbed antisera demonstrated no loss of antibodies to 6-phosphogluconate dehydrogenase. At equivalence 1 ml of the absorbed antiserum precipitates 1.74 mg of protein with a tissue extract that contains 22.6 units of enzyme activity. This corresponds to 1.03 mg of 6-phosphogluconate dehydrogenase in the immunoprecipitate when calculated from the specific activity of the purified enzyme (Betts & Mayer, 1975).

Discussion

The purity of an antiserum is of critical importance for measurement of rates of enzyme synthesis and degradation by quantitative immunoprecipitation of specific enzymes from radioactively labelled tissue extracts. This is particularly true for studies on antigens during development and differentiation where the antigen of interest may be initially present

in much smaller concentrations than contaminants which will be precipitated by the multispecific antiserum. The results presented in this paper illustrate the elements of a general procedure for the absorption of antisera to purified enzymes. This procedure involves the assessment of antiserum specificity; the identification of the antigen–antibody system of interest; the selection of an absorption procedure to produce a monospecific antiserum and finally the reassessment of antiserum specificity.

As stated above, biochemical criteria of antigen purity are by no means sufficient to ensure the formation of monospecific antisera. Acetyl-CoA carboxylase and 6-phosphogluconate dehydrogenase were pure by biochemical criteria and yet both gave rise to multispecific antisera. Other antigens must therefore have been present. Cytochrome oxidase is thought to consist of six or seven subunits depending on the species from which it is purified (Werner, 1974; Ross *et al.*, 1974; Briggs *et al.*, 1975; Bucher & Pennial, 1975; Poyton & Schatz, 1975; Phan & Mahler, 1976*a*). Although it is possible to purify an active form of the complex with as few as four subunits (Phan & Mahler, 1976*b*), it seems likely that all six or seven subunits are required for the fully functional enzyme complex. Evidence for the presence of 'NADH-linked reductase' activity in several highly purified preparations of cytochrome oxidase from ox heart has been reported using both immunochemical techniques (Pennial *et al.*, 1974) and activity measurements (Bucher & Pennial, 1975). Absorption is thus likely to be a necessary treatment for any antiserum raised to cytochrome oxidase.

The results in the present paper demonstrate that once an antiserum has been raised it should be extensively assessed for specificity by a variety of immunodiffusion (Ouchterlony, 1949; Piazzi, 1969) and immunoelectrophoretic techniques (Axelsen *et al.*, 1973) over a wide range of concentrations of antiserum and antigen. It must be stressed that the use of only one technique may be insufficient to determine multispecificity. If the antiserum is multispecific these techniques should also be used to identify the antigen–antibody system of interest (*a*) with specific stains for enzyme activity (Uriel, 1971; Brogsen & Bøg Hansen, 1975) or (*b*) by a reaction of identity with pure enzyme, by using rocket-line, crossed-line or tandem-crossed immunoelectrophoresis. Clearly stains for enzyme activity may fail to work if the enzyme is initially present in an inactive form or if it is totally inactivated in the immunoprecipitate. It should be remembered that multiple bands can occasionally be artifacts due to refilling wells (Kabat, 1971), the presence of subunit and holoenzyme in the same extract (Poyton & Schatz, 1975), the presence of different amounts of lipid bound to the antigen (Hackenbrock & Hammon, 1975) or the presence of immunologically related antigens in the same extract (Weeke, 1973*b*; Beeley, 1976). Further, the presence of different antigenic determinants on the same antigen molecule may result in multiple bands on immunodiffusion (Jennings & Kaplan, 1960; Clausen, 1969) and immunoelectrophoresis (Weeke, 1973*b*). Reactions of identity on immunodiffusion or immunoelectrophoresis should enable the above possibilities to be tested.

If an antiserum proves to be multispecific then either an absorption procedure must be devised or a monospecific antiserum can be raised by injection of the appropriately identified immunoprecipitation line obtained on immunodiffusion (Dingle *et al.*, 1971; Dean, 1974) or immunoelectrophoresis (Koch & Nielsen, 1975). Immunoadsorbant techniques (Campbell *et al.*, 1964; Robinson *et al.*, 1971; Chauvet & Acher, 1972) may be used if sufficiently large quantities of antigen free from the contaminants can be obtained. A great advantage of this method is that the resulting antiserum only contains immunoglobulin molecules that specifically respond to the antigen.

However, when only small amounts of antigen are available alternative procedures are required. In some cases contaminated antigens give rise to antisera where the proportion of antibodies raised to a contaminating antigen may be much less than that expected from the proportion of contaminating antigen in the injected antigen mixture. Here the multispecific antiserum can be absorbed with the injected antigen mixture (Westin, 1975).

The general approach taken in these studies has been to absorb the antiserum with a fraction that contains as much contaminating antigen and as little specific antigen as possible. Examination of the fractions discarded during the purification of acetyl-CoA carboxylase indicated that the supernatant fraction from treatment of the enzyme with calcium phosphate gel (Manning *et al.*, 1976) was selectively enriched in the contaminant. This fraction was therefore used to absorb the antiserum. Clearly the use of a waste fraction from a purification (Harboe & Ingild, 1973) offers the advantage of minimizing the loss of specific antibodies in the absorbed antiserum. Initially this same approach was taken for absorption of the antiserum to cytochrome oxidase since the fraction obtained by washing the DEAE-cellulose column (Walker & Mayer, 1976) was found to be selectively enriched in the contaminating antigens. Fortuitiously, however, it was found that these contaminants were localized in the particle-free supernatant prepared from homogenates of rat liver, a fraction totally free of cytochrome oxidase. The particle-free supernatant was therefore used to absorb the antiserum to cytochrome oxidase without loss of antibodies to the enzyme.

Preliminary observations showed that low activities of 6-phosphogluconate dehydrogenase were

present in a particle-free supernatant prepared from the mammary gland of mid-pregnant rabbits, and that the enzyme activity increased substantially during mammary gland development (S. A. Betts & R. J. Mayer, unpublished observations). Immunodiffusion analysis showed that this low enzyme activity was due to a small amount of the enzyme in extracts of the mammary gland of mid-pregnant rabbits, but that the contaminating antigen was present in relatively large amounts at all stages of mammary gland development. Particle-free supernatant from mammary gland of mid-pregnant rabbits was therefore the obvious fraction to choose for the absorption. Clearly, a similar absorption procedure may be devised for any antiserum prepared for an enzyme that undergoes a similar developmental change (Philippidis *et al.*, 1972). In every case once the absorption has been performed the antiserum should be tested for monospecificity by a variety of immunodiffusion and immunoelectrophoretic techniques. Selective purification of the immunoglobulins (Levy & Sober, 1960; Fahey, 1971; Harboe & Ingild, 1973) could prove advantageous since the absorption procedures result in the introduction of adventitious protein into the antisera.

The monospecific antisera, produced by the methods indicated above, are confidently being used to measure the turnover of acetyl-CoA carboxylase and 6-phosphogluconate dehydrogenase during cytodifferentiation of mammary explants in organ culture. The monospecific antiserum to cytochrome oxidase is being used to study the turnover of cytochrome oxidase in rat liver.

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