Production of Antibodies that Bind Biotin and Inhibit Biotin Containing Enzymes†

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ABSTRACT: Methods were developed for the coupling of biotin to bovine serum albumin and bovine γ-globulin using a water-soluble carbodiimide. The use of [^{14}C]biotin as a tracer allowed quantitation of the incorporation of biotin into the conjugates: 2.55 mol of biotin was incorporated per mol of γ-globulin and 7–9 mol of biotin was incorporated per mol of serum albumin in different preparations. These conjugates were highly immunogenic in the rabbit and antibodies reactive with the biotinyl group itself could be detected by their ability to precipitate the heterologous biotinated carrier but not the unmodified heterologous carrier. These antisera rapidly inactivated transcarboxylase and pyruvate carboxylase and this inactivation could be blocked by pretreatment of the antisera with biotin or biocytin. Using enzyme inhibition to detect free antibody, the binding constant for biotin was found to be 5.0×10^{-8} *M* and that for biocytin 3.5×10^{-8} *M*.

As part of a study of the immunological reactions of transcarboxylase (methylmalonyl-CoA:pyruvate carboxyltransferase EC 2.1.3.1.), it was desired to determine if biotin could serve as a hapten and if biotin binding antibodies would inhibit the enzyme. The production of biotin binding antibodies in response to immunization with biotin conjugated to carrier proteins is reported here. These antibodies were found to inhibit biotin containing enzymes and this enzyme inhibition could be used as a convenient measure of free antibody in binding studies of biotin and biocytin to the antisera.

Experimental Section

Materials. Bovine γ-globulin (Cohn Fraction II) and crystalline bovine serum albumin were obtained from Pentex-Miles Laboratories, Kankankee, Ill.; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl was from the Ott Chemical Company, Muskegon, Mich.; [2′-^{14}C]biotin (58 Ci/mol) was from Amersham-Searle; and (+)-biotin was from Calbiochem. Biocytin (N^{ϵ}-biotinyl-L-lysine, L-511, 199-00V02) was the generous gift of Dr. Lewis Mandel, Merck Sharp and Dohme Research Labs, Inc. All other chemicals were of the highest purity commercially available. Radioactivity was determined using a Packard Tri-Carb scintillation spectrometer and dioxane base scintillant.

Preparation of Biotinyl Bovine γ-Globulin. Biotin (50 mg) was dissolved in 10 ml of 0.018 *N* NaOH solution. This solution was brought to pH 5.5 by the addition of 0.14 ml of 0.2 *N* HCl in small increments, yielding a final biotin concentration of 20 m*M;* 6.8×10^6 cpm of [2′-^{14}C]biotin was added to this solution which was then kept at room temperature; 1 ml of the biotin solution was used to dissolve 250 mg (1.3 mmol) of the carbodiimide and this solution was held at room temperature for 6 min by which time the pH had risen to about 7. Then, 0.02 ml of 2 *N* HCl was added and the pH returned to about 5 and the solution was held at room temperature for another 7 min. The biotin–carbodiimide reaction mixture was then added to a solution of 100 mg of γ-globulin in 6 ml of 0.05 *M* Tris-HCl (pH 9.0) with gentle stirring at room temperature, and stirring was continued for 4.5 hr. The reaction mixture was dialyzed at room temperature against four changes of 2 l. each of 0.1 *M* potassium phosphate buffer (pH 6.8) over a period of about 24 hr, during which slight turbidity was noted. About 54,000 cpm was retained on dialysis corresponding to 1.58 μmol of biotin or 2.55 μmol of biotin/μmol of γ-globulin.

Preparation of Biotinyl Bovine Serum Albumin. The procedure of Abraham and Grover (1971) was modified as follows: 500 mg of biotin and 15×10^6 cpm of [^{14}C]biotin were suspended in 7.5 ml of 50% aqueous pyridine with stirring at room temperature; 2.5 g of carbodiimide was dissolved in 12.5 ml of 50% aqueous pyridine. This mixture was added dropwise to the biotin suspension and stirring was continued at room temperature for 30 min, during which time a clear solution was formed; 250 mg of serum albumin in 6.25 ml of distilled H_2O was added dropwise to the biotin–carbodiimide reaction mixture and stirring was continued at room temperature for 4.5 hr following which the reaction mixture was dialyzed for 24 hr at room temperature against 4 l. of 0.09% NaCl. Calculations of the amount of [^{14}C]biotin retained on dialysis indicated that 7.3 μmol of biotin had been fixed per μmol of serum albumin. Other similar preparations contained up to 10 μmol of biotin/μmol of serum albumin.

Immunization. New Zealand White rabbits were obtained from a local supplier and were maintained on a standard lab chow diet in the animal facilities of Case Western Reserve University School of Medicine. Complete Freund's adjuvant (Bacto No. 0638-59) was obtained from Difco, Inc. Protein solutions were diluted in 0.9% NaCl to a final concentration of about 2 mg/ml. Equal volumes of protein solution and adjuvant were emulsified by repeated pumping through an 18 gauge needle using 10-ml glass syringes.

Initially, 1 ml of the emulsion was injected into each of the hind foot pads of each rabbit. Booster injections of 2 mg of the antigen in 1 ml of 0.9% NaCl were given in the peripheral ear vein at 14-day intervals. Test bleedings from the central artery of the ear were drawn immediately before each immunization. Terminal bleeding by cardiac puncture

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Table I: Precipitin Ring Tests with Anti-biotinyl-γ-globulin.[a]

Protein	Highest Serum Dilution Forming Precipitate
1. Biotinyl-γ-globulin	256
2. γ-Globulin	128
3. Biotinyl serum albumin	8
4. Serum albumin	None

[a] Biotinyl-γ-globulin (2.5 biotins/mol), γ-globulin, biotinyl serum albumin (9.7 biotins/mol), and serum albumin solutions, 1 mg/ml, and serum dilutions were made in 0.9% NaCl; 40 μl of diluted serum was placed in the bottom of a 6 × 50 mm tube and 40 μl of protein solution was carefully layered on top. The mixtures were incubated at room temperature for 30 min and then examined for the presence of precipitate at the interface between the two solutions. Results are expressed as the highest serum dilution still giving a visible precipitate.

was performed 7–9 days after the last immunization. In all cases, blood was allowed to clot at 37° for 1 hr and then stored at 0° overnight. Sera were separated from the clots by centrifugation and stored at −10° in the presence of 0.02% sodium azide. Normal rabbit serum was purchased from Gibco, Inc. and was also stored as above.

Enzymes and Assays. Transcarboxylase (methylmalonyl-CoA:pyruvate carboxytransferase, EC 2.1.3.1.) was purified from extracts of *Propionibacterium shermanii* by the method of Wood et al. (1969) except that gel filtration on Bio-Gel A 1.5m was substituted for chromatography on TEAE-cellulose and Sepharose 2B. The enzyme used in these studies had a specific activity of 38 units/mg and appeared pure by sedimentation velocity profile and polyacrylamide gel electrophoresis. The enzyme was assayed by measuring the rate of formation of oxalacetate from methylmalonyl-CoA and pyruvate using malate dehydrogenase and following the rate of NADH oxidation in a Gilford Model 2000 recording spectrophotometer. A unit is defined as 1 μmol of oxalacetate produced/min.

Purified chicken liver pyruvate carboxylase (pyruvate: CO_2 ligase (ADP) EC 6.4.1.1.) was the gift of Drs. Barry L. Taylor and M. F. Utter of this department. This enzyme was assayed by following the formation of oxalacetate from pyruvate, ATP, and HCO_3^- in the presence of 15 m*M* acetyl-CoA using a linked assay system similar to that used for transcarboxylase (Scrutton et al., 1969).

Results

Antibody Response to Biotinylated Proteins. Initially, antibody titer was followed by measuring the highest serum dilution (in 0.9% NaCl) which gave a ring of precipitation at the interface when antigen (in this case biotinyl-γ-globulin) at a concentration of 1 mg of protein/ml was layered over the serum dilution in 6 × 50 mm tubes. Immediate and heavy precipitates were formed with the undiluted sera drawn 14 days after initial immunization with biotinyl-γ-globulin, but no precipitates were observed with nonimmune sera. The precipitation titer was 1:32, 14 days after the second immunization, and the same serum dilutions gave positive precipitation rings with both the antigen and unmodified γ-globulin, indicating that most of the response was directed against the carrier. The serum drawn at 10 days after the fourth immunization gave the results shown in Table I: the titer had continued to increase and the antigen gave a higher titer than the γ-globulin alone. Furthermore, the serum precipitated biotinyl serum albumin but not unmodified serum albumin itself, indicating that antibodies reacting with the biotinyl group had been formed. As will be shown below, these sera rapidly inactivated transcarboxylase and this enzyme inactivation was then used to follow the response to biotinyl serum albumin or biotinyl-γ-globulin.

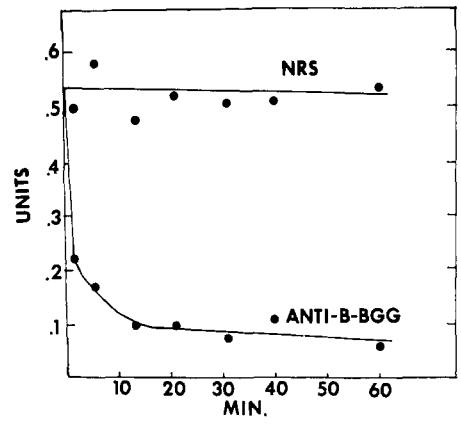

FIGURE 1: Time course of inhibition of transcarboxylase by anti-biotinyl-γ-globulin; 0.5 ml of undiluted serum, anti-biotinyl-γ-globulin (BGG) or normal rabbit serum (NRS), was added to 0.5 ml of transcarboxylase (16 μg) in 0.2 *M* potassium phosphate buffer (pH 6.8) and incubated at room temperature; 10-μl aliquots were assayed at the indicated times. Blanks without methylmalonyl-CoA were run at each time point and those values were subtracted to correct for NADH oxidase activity of the sera. Units are expressed on the basis of the total reaction mixture.

Inhibition of Transcarboxylase and Pyruvate Carboxylase by Anti-biotinyl-γ-globulin and Anti-biotinyl Serum Albumin. The presence of antibody against the biotinyl group suggested that the antiserum might inhibit biotin enzymes and the results obtained with transcarboxylase are shown in Figure 1. Over 50% of the enzyme activity was lost within 1.5 min of mixing the antiserum and the enzyme and approximately 85% inhibition was observed within 15 min. This corresponds to the inhibition of about 1 unit of enzyme/ml of serum. A parallel experiment with chicken liver pyruvate carboxylase gave similar results but that enzyme was less stable than transcarboxylase in the control incubation. The rapidity of the effect of antiserum on transcarboxylase and the ease with which the enzyme is assayed led to the use of enzyme-inhibition following a 30-min incubation as an assay for the production of biotin-binding antibodies. With biotinyl serum albumin, which was more highly substituted, as the antigen, the sera drawn 14 days after the first immunization already had the capacity to inhibit 0.52 unit of enzyme/ml of serum and that drawn 14 days after the second immunization inhibited 0.86 unit/ml of serum. Immunization with biotinyl serum albumin was continued and after the eighth injection, the inhibition capacity had risen to 55 units of transcarboxylase/ml of antiserum. Two of three rabbits gave similar responses whereas the third produced antiserum with the capacity to inhibit only 8.7 units/ml.

A typical inhibition study using serum obtained by cardiac puncture 9 days after the 16th immunization of the rabbit immunized with biotinyl serum albumin and producing serum with the highest inhibiting capacity is shown in Figure 2. The result obtained in this study was 70 units inhibited/ml of antiserum. This serum was used in the remainder of the studies shown below.

Measurement of Antibody–Biotin Binding Constants by Inhibition of Enzyme Activity. The inhibition of enzyme

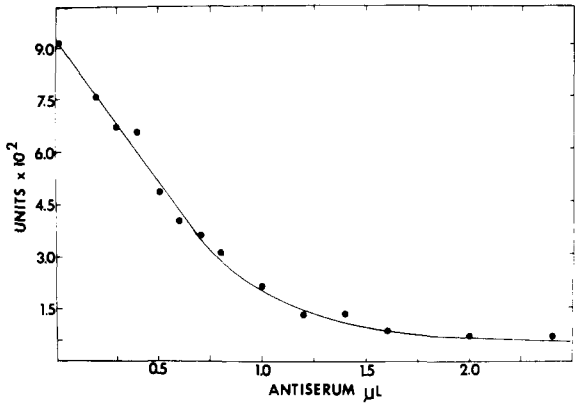

FIGURE 2: Inhibition of transcarboxylase by anti-biotinyl serum albumin; 2.8 μg of transcarboxylase in 0.15 ml of 0.3 M potassium phosphate buffer (pH 6.8) and 0.10 ml of a variable combination of normal rabbit serum and antiserum were incubated for 30 min at room temperature. Aliquots of 0.02 ml were assayed for transcarboxylase activity with appropriate corrections for NADH oxidase activity in the serum. Units are expressed on the basis of the total reaction mixture.

activity by antibody was linear with increasing antibody concentration over a considerable range (Figure 2). It therefore appeared likely that enzyme inhibition could be used as a measure of the concentration of free antibody present in antibody–biotin mixtures, allowing the measurements of the binding constant of antibody for biotin. For this method to be feasible, there must be a direct correspondence between the number of free antibody molecules and the number of enzyme molecules inhibited. Thus, it is necessary that the amount of free antibody is negligible in the presence of excess enzyme and that the antibody–biotin complex should be sufficiently stable so that biotin is not displaced from antibody when the enzyme is added for the measurement.

To determine whether or not there is a significant amount of free antibody remaining in the presence of excess transcarboxylase the following experiment was done. Incubations containing transcarboxylase and antiserum or nonimmune serum were set up and held at room temperature for 30 min and then a portion of each mixture was assayed for enzyme activity. The amount of antiserum had been chosen such that the enzyme activity in the tube with antiserum was about 30% of that in the control tube. Additional transcarboxylase was then added to aliquots of each mixture and the enzyme activity was determined again. If there was free antibody remaining in the mixture with antiserum and enzyme, the increase in enzyme activity would be less than that observed in the mixture containing control serum and enzyme. The increases in both tubes were the same within the limits of experimental error and did not change over a 30-min period. These results indicate that there was little or no free antibody available in the presence of the original excess of enzyme.

Evidence that the antibody–biotin complex does not dissociate when transcarboxylase is added to assay the amount of free antibody present was obtained by comparing the time course of enzyme inhibition by antibody–biotin mixtures and by antibody alone. Various amounts of biotin were incubated with the antiserum for 30 min at room temperature, then the transcarboxylase was added and incubation was continued. Aliquots were assayed at various time intervals and, again, it was found that the transcarboxylase activity in all cases decreased rapidly during the first 5–10 min and then levelled off. Only a slight further decrease in enzyme activity occurred after 30 min at room temperature but this decrease was also found in control tubes containing antiserum but no biotin and in tubes containing nonimmune serum. This decrease probably results from denaturation of a small amount of enzyme during the 30-min incubation period. If there was significant dissociation of the antibody–biotin complex it would be expected that dissociated antibody would combine with the excess transcarboxylase and the enzyme activity would continue to decrease with time in the mixtures containing biotin, but this was not observed.

An example of the binding studies with biotin is shown in Figure 3A. Again, amounts of antiserum and transcarboxylase were chosen so that the maximum inhibition of enzyme was about 65%. Various amounts of biotin were incubated with the antiserum for 30 min at room temperature, then the transcarboxylase was added to this mixture and the enzyme activity was determined after another incubation. The enzyme activity increased with increasing biotin indicating that the antiserum was being neutralized by the biotin although complete restoration of enzyme activity was not achieved except at much higher concentrations of biotin. The data were replotted on a double reciprocal plot as shown in Figure 4A, allowing the calculation of an apparent binding constant of 5.0×10^{-8} M for biotin to antibody (Dixon and Webb, 1964).

Since biotin is covalently attached to the ϵ-NH_2 group of certain lysine residues in the enzyme (Wood et al., 1963) it is possible that the antibody binding site also recognizes the lysyl side chain. To study this question, similar experiments were run using biocytin (N^{ϵ}-biotinyl-L-lysine) to neutralize the antiserum. As can be seen in Figure 3B, the results were quite similar and when these data were replotted on a double reciprocal plot, as shown in Figure 4B, the apparent binding constant was calculated to be 3.5×10^{-8} M.

Discussion

Although there are many known examples of antibodies against enzymes (reviewed by Arnon, 1973) there are relatively few examples in which the antibodies bind to a single, identifiable site on the enzyme molecule. In this case, the coupling of biotin to bovine serum albumin or bovine γ-globulin allowed the production of antibodies that react with biotin. Since the biotinyl group is covalently attached to the enzymes (Wood et al., 1963; Moss and Lane, 1971) these antibodies can be used to inhibit the biotin enzymes as illustrated in this study. The ease with which the enzyme assays are carried out, the rapidity of the reaction of antibody with enzyme, and the small amounts of enzyme and antiserum required make the use of enzyme inactivation a very convenient tool in the study of the antibodies that react with biotin.

Although the immune response to the conjugates was quite strong and immediate in both cases, the presence of transcarboxylase-inhibiting antibodies was not observed until after the fourth immunization with the lightly substituted biotinyl-γ-globulin. With the more highly biotinated serum albumin derivative, however, antibodies with the capacity to inhibit transcarboxylase appeared after only one immunization and the amount of this antibody and/or its affinity continued to increase with continued immunization as demonstrated by an increasing capacity of the serum to inhibit the enzyme. Examination of the weight ratios of biotin in the conjugates: $(2.5 \times 244 \times 100/155{,}600) = 0.3\%$ for the biotinyl γ-globulin, and $(7.3 \times 244 \times 100/69{,}800)$

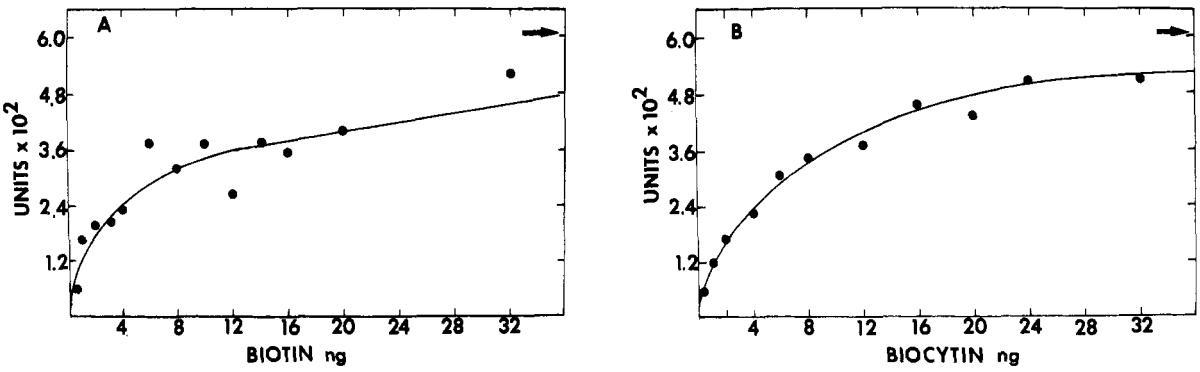

FIGURE 3: Neutralization of antiserum by preincubation with biotin (A) or biocytin (B). In A, 0.32 ml of 0.3 *M* potassium phosphate buffer (pH 7.0) containing varying amounts of biotin were incubated for 30 min at room temperature with 0.08 ml of a 50-fold dilution of antiserum in normal rabbit serum; 0.10 ml of transcarboxylase (5.6 μg) in 0.25 *M* potassium phosphate buffer (pH 6.5) was added and incubation was continued for 30 min at room temperature; 0.02-ml aliquots were assayed for transcarboxylase activity and the sum of controls with no biotin added and with no methylmalonyl-CoA in the assay was subtracted. The experiment in B was run in the same way but all volumes were reduced to half and biocytin was substituted for the biotin. To facilitate comparison, values in A are expressed on the basis of the 0.25-ml reaction mixture. Values of control incubations with normal rabbit serum in place of antiserum are indicated by arrows.

= 2.5% for the biotinyl serum albumin, indicates that the biotinyl group itself must be a highly antigenic hapten.

It is of interest that although the antisera do precipitate biotin attached to a carrier other than that used in the antigen, neither pyruvate carboxylase nor transcarboxylase was precipitated during any of these studies. In addition, no indication of precipitation with the enzymes was obtained in the "ring tests" or on double immunodiffusion (Ouchterlony) plates, even when very concentrated enzyme solutions (26 mg of protein/ml = 48 μg of biotin/ml, equivalent to 0.96 μg of biotin/well) were allowed to react with antiserum for over 72 hr at 0°. Under similar conditions, strong precipitin lines are observed in less than 24 hr when the biotin serum albumin conjugate is used at 25 μg of biotin/ml (0.25 μg of biotin/well). Since the antibodies involved in these reactions are probably divalent IgG molecules, this is rather surprising because the chicken liver pyruvate carboxylase contains four biotins and transcarboxylase (in the 18S form) six biotins. This lack of precipitation could be due to the inability of the IgG molecule to span the distance that might be needed to bridge the gap between biotins on different enzyme molecules. Alternatively, each IgG molecule might be saturated by two adjacent biotins from the same enzyme molecule. The latter possibility seems especially pertinent in the case of transcarboxylase since the biotins in that enzyme are believed to be on exposed, extended portions of flexible peptide chains (Green et al., 1972) and few steric problems would be predicted.

The use of the enzyme inhibition assay system as a convenient measure of the amount of free antibody present also facilitated the study of the binding of biotin and biocytin to the antiserum. These competitive binding studies are based on the assumption that there is a negligible concentration of free antibody in the presence of excess transcarboxylase. This is supported by the findings that with the proportion of antibody and enzyme used, the decrease in enzyme activity is linear with increasing antibody concentration; and that the antibody–enzyme mixture is incapable of inhibiting additional aliquots of enzyme.

It has also been assumed that little or no reequilibration of the antibody–biotin complex occurs upon the addition of transcarboxylase. The basis of the test of this assumption is the kinetics of other antibody–hapten reactions (Hammes, 1968; Sachs et al., 1972) in which it has been found that the rate of reaction for the binding of antigen to antibody approaches the maximum limits set by diffusion, whereas the rate of dissociation of the antigen–antibody complex is very much slower, with a half-time of several minutes. If the preformed antibody–biotin complex released biotin at a significant rate during the incubation in the presence of transcarboxylase, one would expect a continuing drop in the enzyme activity during the course of the second incubation. As discussed in the Experimental Section, however, the time course of the inhibition of transcarboxylase by antibody–biotin complex was indistinguishable from the course of enzyme inhibition by antibody alone. It is, of course, possible that as the antibody–biotin complex dissociates, the antibody rebinds biotin much more rapidly than it binds

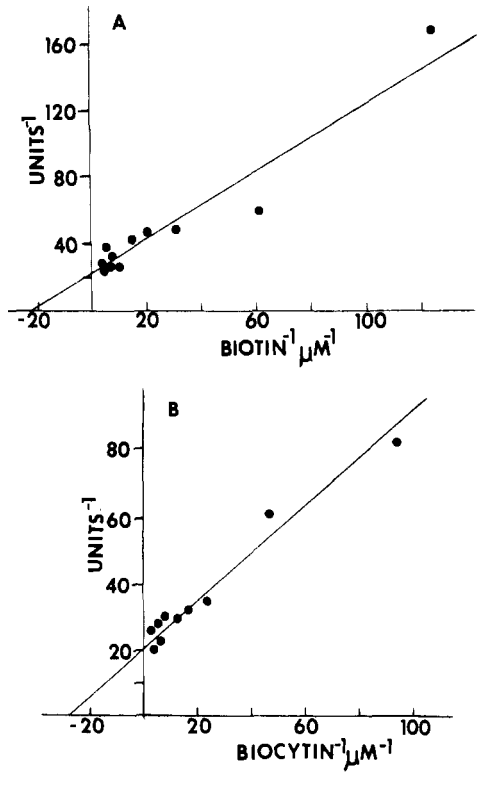

FIGURE 4: Double reciprocal plot of neutralization data from Figure 3 for biotin (A) and biocytin (B). These lines were drawn with the aid of a Hewlett-Packard 9100 calculator using a least-squares linear regression program. The correlation coefficients were 0.96 for biotin (A) and 0.98 for biocytin (B).

transcarboxylase because of the much faster diffusion of the smaller biotin molecule. It thus seems quite likely that the decrease in enzyme activity corresponds very closely to the amount of free antibody remaining after the initial incubation of enzyme with biotin or biocytin.

It is conceivable that the results obtained in these studies are due to the action of free biotin or biocytin as a carboxyl carrier in place of enzyme bound biotin which is occupied by antibody, thereby allowing the enzyme-antibody complex to carry out the enzymatic reactions. This is very unlikely because recent studies have demonstrated that neither free biotin nor biocytin is capable of functioning as a carboxyl carrier in the partial reactions of transcarboxylation which are catalyzed by the enzyme subunits (Chuang et al., 1975).

The results obtained resemble classical enzyme–substrate interactions and thus the data were replotted on double reciprocal plots (Dixon and Webb, 1964). The apparent binding constants calculated from these plots are quite similar for biotin and biocytin and indicate that the majority of the domains in the antibody binding site probably interact with the biotin ring system and/or the valeric acid side chain rather than a larger determinant which would include the lysyl residue. These plots also allow the calculation of maximal velocity of the enzymatic reaction in the presence of antiserum and hapten and the values are 0.049 unit and 0.046 unit for biocytin and biotin, respectively. These calculated values are somewhat lower than the expected values of 0.062 unit which correspond to the activity of controls incubated with nonimmune serum. This discrepancy may be due to the presence of another population of antibodies with a lower affinity for the haptens since complete neutralization of enzyme inhibiting antibody is obtained only at biotin concentrations 10–20 times higher than those used here. Nevertheless, this method of analysis seems quite useful and valid over most of the range of antibody binding and enzyme activity and suggests that a restricted population of antibodies may be responsible for these results.

These antisera could be quite useful in studies of the nature of antibody-enzyme reactions since the same antibodies should react with the biotinyl group of any one of a number of biotin containing enzymes regardless of their source or the reactions they carry out. The binding constant of 10^{-8} M also suggests that these antibodies should be suitable for coupling to an insoluble matrix (Wofsy and Burr, 1969) and subsequent use in the purification of biotin enzymes by affinity chromatography.

This general approach, in which antibodies against enzymes are prepared by immunization of animals with conjugates in which the coenzyme is used as a hapten, might also be applicable to other enzymes with covalently attached prosthetic groups.

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