Antibodies Reactive with Specific Folic Acid Determinants*

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ABSTRACT: Folate and *p*-aminobenzoyl-L-glutamate were coupled to methylated bovine serum albumin and bovine serum albumin, respectively. These compounds were used as antigens to elicit rabbit antibodies reactive with folate determinants. These sera were tested by quantitative complement (C′) fixation and precipitation techniques, respectively. The two sera were found to contain antibodies specific for different ends of the folate molecule.

The characteristics of antifolate and of anti-*p*-aminobenzoyl-L-glutamate combining sites were determined by inhibition of C′ fixation and precipitation with a number of folate analogs and related compounds.

It has recently been shown that antibodies may be produced to pyridoxal, pyridoxal 5′-phosphate, and pyridoxamine 5′-phosphate (Ungar-Waron and Sela, 1966; Cordoba *et al.*, 1966). These antibodies were induced by immunization with antigens prepared by covalently linking the haptenic coenzymes to large carrier molecules (BSA[1] or a synthetic branched polypeptide). It was suggested that antibodies to coenzymes may be useful in studies of enzyme–coenzyme interaction. This article reports the characterization of antibodies reactive with folic acid determinants. Two types of antisera were induced by different hapten–protein conjugates. Each serum reacted with a different terminal region of the folate molecule. One antiserum was directed against the pteridine portion of the coenzyme, and the second against the *p*-aminobenzoyl-L-glutamate portion.

Materials and Methods

Preparation of Antigens. Folate-MBSA was prepared by adding 25.0 mg of a water-soluble carbodiimide reagent (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate, Aldrich Chemical Co.) and then 20.0 mg of folate to 50.0 mg of MBSA (Mandell and Hershey, 1960) which was dissolved in 5.0 ml of water. A yellow precipitate formed. This mixture was then dialyzed extensively against distilled water and then "isotris" buffer (0.01 M Tris-0.14 M NaCl, pH 7.4). Folate-HSA was prepared as above, but remained soluble.

BSA-PaBz-L-Glu was prepared by diazotization of 40.0 mg of PaBz-L-Glu (Calbiochem) dissolved in 6.0 ml of 1 N HCl. A 10% solution of $NaNO_2$ was added to slight excess, as determined by KI–starch paper. The pH was then adjusted to 4.5 with 1 N NaOH. To this 250 mg of crystallized BSA (Armour fraction V) was added. The pH was then adjusted to 9.0 and the mixture was allowed to stand at 4° overnight. The conjugated protein was then precipitated with acetone, redissolved in 8.0 ml of isotris buffer, and then dialyzed against isotris buffer for 72 hr with frequent changes of the bath.

EA-PaBz-L-Glu was prepared by the same diazotization and conjugation procedure. Egg albumin (five times crystallized) was obtained from the Nutritional Biochemical Co. Following dialysis, to remove traces of residual uncoupled hapten, the solution was applied to a 1 × 20 cm Bio-Rad P-10 gel column, and was eluted with isotris buffer, with which the Bio-Rad resin was equilibrated. Fractions of 3.0 ml were collected and those with a constant ratio of light absorbance at 340:260 mμ were pooled.

Immunization Procedures. Two New Zealand white rabbits (Ra-64-13 and Ra-65-50) were immunized according to similar schedules. Ra-64-13 received BSA-PaBz-L-Glu, and Ra-65-50 received folate-MBSA. Each rabbit received 25 mg of antigen in complete Freund's adjuvant subcutaneously and intramuscularly on days 1 and 6, and three intravenous injections of 20 mg of antigen at 3-day intervals over the following 10 days. The first bleeding was performed on day 24. Following four additional 20-mg intravenous injections over the next 10 days, the second bleeding was performed on day 41. A final injection of antigen was given 1 month later, and serum was obtained after 7 days.

Serological Reactions. Two-dimensional immunodiffusion (Ouchterlony, 1949) was carried out in 0.8% agarose with 0.01 M EDTA and 1:10,000 merthiolate on glass microscope slides. Complement (C′) fixation

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[1] Abbreviations used: BSA, bovine serum albumin, folate-MBSA, folate-methylated BSA; folate-HSA, folate human serum albumin; BSA-PaBz-L-Glu, *p*-aminobenzoyl-L-glutamate BSA; EA-PaBz-L-Glu, egg albumin-PaBz-L-Glu; GABA, pteroyl-γ-aminobutyrate.

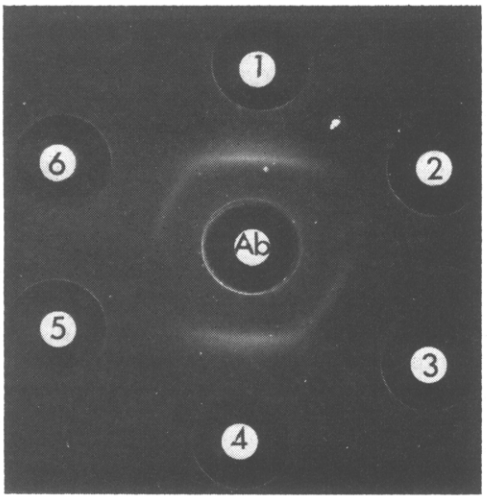


FIGURE 1: Two-dimensional immunodiffusion of serum Ra-65-50 (antifolate-MBSA)-(Ab) with folate-HSA (wells 1 and 4), folate-BSA (wells 3 and 6), HSA (well 2), and BSA (well 5). Each antigen well received approximately 0.01 ml of a solution containing 500 μg/ml.

and C′ fixation inhibition were performed as described by Wasserman and Levine (1961) in a total volume of 7.0 ml and a buffer consisting of 0.14 M NaCl-0.01 M Tris at pH 7.4 with optimal Mg^{2+} and Ca^{2+} added.

Precipitation tests were performed with 0.2 ml of serum (anti-BSA-PaBz-L-Glu) and varying amounts of antigen (EA-PaBz-L-Glu) incubated in a constant volume (1.0 ml) at 37° for 1 hr and then at 4° overnight. The tubes were centrifuged and the supernatant was removed. The precipitate was then washed three times in isotris buffer, dissolved in 2.0 ml of 0.1 N HCl, and the absorbance was read at 280 mμ.

Hapten inhibition of precipitation was performed

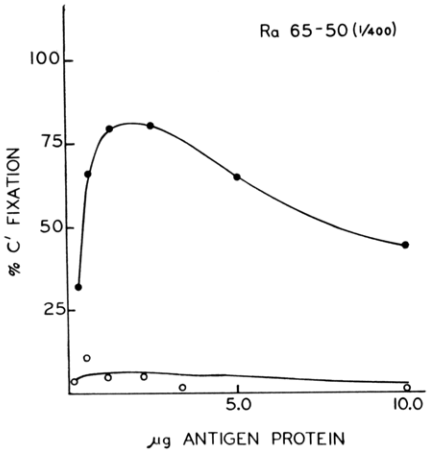
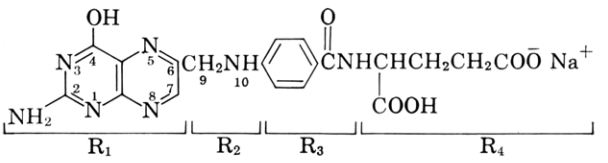
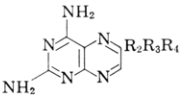
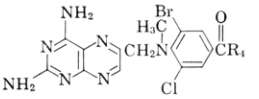
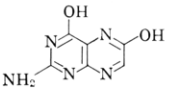


FIGURE 2: C′ fixation of serum Ra-65-50 (antifolate-MBSA) and folate-HSA (●) and HSA (○).

TABLE I: Compounds Used in Inhibition Experiments.



folate

Compound	Structure
Folate	$R_1R_2R_3R_4$
Homofolate	$R_1(CH_2CH_2NH)R_3R_4$
Pteroylglycine	$R_1R_2R_3NHCH_2$ | COOH
Pteroyl-L-alanine	$R_1R_2R_3NHCHCH_2$ | COOH
Pteroyl-GABA	$R_1R_2R_3NHCH_2CH_2$- CH_2COOH
Pteroyl-L-aminoadipate	$R_1R_2R_3NHCHCH_2$- | COOH CH_2CH_2COOH
Pteroyl-D-glutamate	$R_1R_2R_3NHCHCH_2$- CH_2COOH
Pteroyl-L-asparate	$R_1R_2R_3NHCHCH_2$- | COOH COOH
Pteroic acid	$R_1R_2R_3OH$
Aminopterin	
Bromochloromethotrexate	
Xanthopterin	
PaBz-L-glutamate	$NH_2R_3R_4$
L-Glutamate	HR_4
L-Aspartate	NH_2CHCH_2COOH | COOH
L-Glycine	NH_2CH_2 | COOH
L-Alanine	NH_2CHCH_2 | COOH

with constant amounts of serum (anti-BSA-PaBz-L-Glu) and antigen (EA-PaBz-L-Glu) and varying amounts

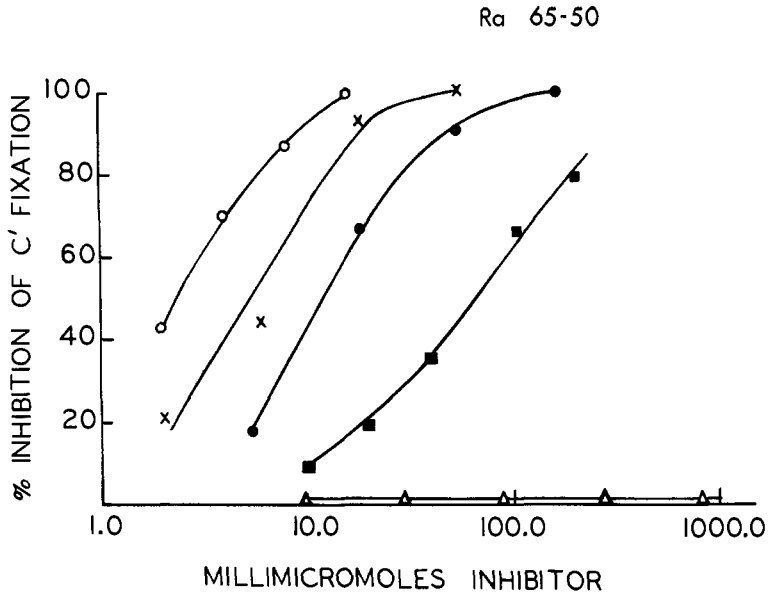


FIGURE 3: C′ fixation inhibition of serum Ra-65-50 (antifolate-MBSA) and folate-HSA by folate (○) pteroic acid (×), aminopterin (●), xanthopterin (■), and PaBz-L-glutamate (△).

of inhibitor in a constant volume. The techniques of the incubation and analysis were the same as above. The compounds tested in inhibition are listed in Table I. L-Alanine, L-glutamate, glycine, and L-aspartate were obtained from Calbiochem Corp.

Inhibitors used in hapten inhibition were dissolved in Tris buffer and the pH was adjusted to 8.4. For use in C′ fixation inhibition the inhibitors were then diluted in the C′ fixation buffer described above. All inhibitors were examined by paper chromatography to assess their purities which were found to be greater than 85%. In calculation of the data, the concentrations of the inhibitors were adjusted according to their purities.

Results

Antibodies to Folate-MBSA. Antifolate-MBSA was studied by precipitation in gel and by quantitative micro-C′ fixation and C′ fixation inhibition. The immunodiffusion pattern is shown in Figure 1, in which strong reactions with folate-HSA and folate-BSA are visible, as well as a faint reaction with native BSA. Because of the latter reaction, BSA was left out of the buffer used in C′ fixation. No line was visible with free HSA.

The C′ fixation reaction of this serum with folate-HSA is shown in Figure 2. Again, no C′ fixation was observed with native HSA.

Five inhibitors were used to study the antibody specificity. The results are shown in Figure 3. Folate achieved 50% inhibition at 2.5 mμmoles and 100% inhibition at 10.5 mμmoles/reaction mixture. Pteroic acid inhibited 50% at a level of 5 mμmoles and 100% at 15 mμmoles. Aminopterin inhibited 50% at 10.2 mμmoles and 100% at 117 mμmoles. Xanthopterin, consisting of only the pteridine portion of folate, was considerably less effective as an inhibitor. PaBz-L-Glu, the opposite end of the folate molecule, caused no inhibition at levels as high as 800 mμmoles.

Antibodies to BSA-PaBz-L-Glu. Anti-BSA-PaBz-L-Glu was precipitated equally well by EA-PaBz-L-Glu and BSA-PaBz-L-Glu (Figure 4). No precipitation occurred when either free BSA or EA were used as antigens. Folate specifically inhibited the precipitation of anti-BSA-PaBz-L-Glu with either BSA-PaBz-L-Glu or EA-PaBz-L-Glu. Folate did not inhibit precipitation or C′ fixation in nonrelated immune systems (BSA–anti-BSA or ribonuclease–antiribonuclease).

Fourteen inhibitors were used to study the specificity of this antiserum. Eleven of these were folate analogs; the majority of this group had variations in the amino acid (glutamate) portion of the folate molecule.

The results of inhibition tests are shown in Figure 5

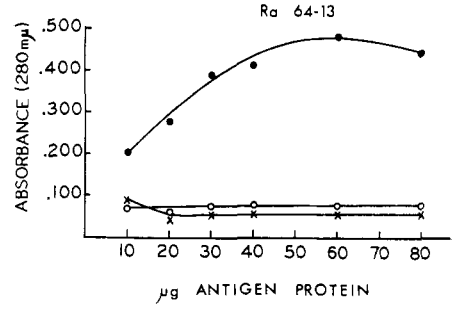


FIGURE 4: Precipitation curves of serum Ra-64-13 (anti-BSA-PaBz-L-glutamate) and EA-PaBz-L-glutamate (●), BSA (○), and EA (×).

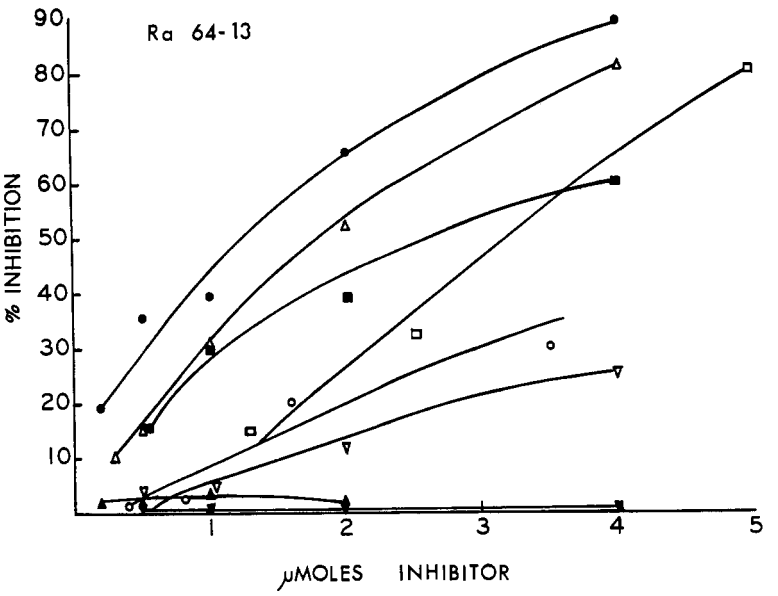


FIGURE 5: Hapten precipitation inhibition of serum Ra-64-13 (anti-BSA-PaBz-L-glutamate) and EA-PaBz-L-glutamate by pteroyl-GABA (▲), PaBz-L-glutamate (□), bromochloromethotrexate (▽), pteroyl-D-glutamate (○), folate (●), homofolate (△), pteroyl-L-aspartate (■), and glycine, L-aspartate, L-alanine, L-glutamate, pteroyl-L-glycine, pteroyl-L-alanine, and pteroylamino-L-adipate (▼).

Suitable controls as well as a complete folate inhibition curve were performed with each series of inhibition reactions.

Folate, causing 95% inhibition at 4 μmoles/ml, was the best inhibitor. Pteroyl-L-aspartate and homofolate achieved 60 and 85% inhibition, respectively, at maximum concentration used. In terms of the amount of inhibitor required for 50% inhibition, folic acid was 1.5 times as effective as homofolate and three times as effective as pteroyl-L-aspartate. PaBz-L-Glu achieved 65% inhibition at 4 μmoles/ml and 100% at 20 μmoles/ml. Bromochloromethotrexate and pteroyl-D-glutamate caused 20 and 30% inhibition, respectively, at 4 μmoles/ml.

Four amino acids (glycine, aspartate, alanine, and glutamate) were tested as inhibitors (Figure 5). The first three showed no activity, in spite of the use of massive quantities of these compounds. Glutamate achieved a maximum level of 20% inhibition when 200 μmoles/ml was used. The remaining four inhibitors, (pteroyl-L-glycine, pteroyl-γ-aminobutyric acid, pteroyl-L-alanine, and pteroyl-α-amino-L-adipate) were virtually ineffective.

Discussion

This article describes the use of two hapten–protein conjugates to induce antibodies to different folate determinants. The antisera induced were specific for the haptens used. One antiserum was induced with conjugated folate-MBSA and C′ fixation was observed with this antiserum and folate-HSA. No C′ fixation occurred when HSA alone was substituted for HSA-folate. As well, the C′ fixation of this serum with HSA-folate was inhibited by hapten (folate), which did not inhibit a nonrelated immune system.

The second antiserum was induced with conjugated BSA-PaBz-L-Glu. The antisera precipitated with EA-PaBz-L-Glu as well as with the immunizing antigen; there was no precipitation with EA alone. Precipitation was inhibited by haptens, containing the PaBz-L-Glu structure, which did not inhibit a nonrelated immune system.

The antisera induced were also specific for either end of the folate molecule. In the conjugation of folate to MBSA, the pteridine portion of the molecule was expected to be free at the surface. This was considered so since all the COOH groups of the BSA were blocked by methyl groups and, therefore, the carbodiimide reagent was likely to link the terminal COOH group of the glutamate of folic acid to the free amino groups of the carrier protein. Folic and pteroic acids were found to be effective inhibitors of this system, whereas PaBz-L-Glu caused no inhibition. Furthermore, it was found that aminopterin, with changes in the pteridine portion of the molecule, caused a greater loss of inhibition than did the removal of the whole amino acid portion (pteroic acid).

There was no pteridine in the immunizing agent BSA-PaBz-L-Glu. However, since PaBz-L-Glu comprises one end of folate, the whole folate molecule was an effective inhibitor. Furthermore, it was determined that changes in the amino acid portion of the hapten inhibitors used caused a marked change in the effectiveness of the inhibitors. Changes in the pteroic acid portion of the inhibitors, such as in homofolate,

caused little change in the effectiveness of the inhibitors.

Anti-PaBz-L-Glu was studied extensively with regard to its specificity for the amino acid (glutamate) portion of folic acid. It was determined that certain changes in the aliphatic portion of the inhibitors rendered them ineffective. Not unexpectedly, the antibody was found to be specific in terms of length and charged groups of the amino acid portion of the antigen.

An important haptenic combining site appeared to be the α-COOH group, as the absence of this group rendered the inhibitor totally ineffective (pteroyl-GABA). Furthermore, the configuration of the α-COOH group was found to be important. A change to the D configuration rendered the inhibitor no better than 20% as effective as the L isomer. The length of the carbon backbone of the amino acid was found to be important, most dramatically when the length was too great. This was illustrated by the fact that pteroyl-α-amino-L-adipic acid caused no inhibition. The only difference between this compound and folate was the presence of an extra methyl group in the longitudinal axis of the amino acid. Since this analog still retained a terminal COOH group, it appeared that the effect of the increased length of adipate was to prevent the coincidence of the α-COOH group with its binding site. This was supported by the fact that shortening the molecule, as in pteroylaspartate, did not prevent the fitting of the α-COOH group and, therefore, the reduction in inhibitory effect was not nearly as marked. However, the presence of a terminal COOH group, on a chain that did allow binding of the α-COOH group, was still required for activity, since pteroylalanine and pteroylglycine were ineffective. It was of interest that the antibodies directed against the pteridine end of the folate were much more easily inhibited in C′ fixation than those directed against the aliphatic terminal; the latter had to be studied by inhibition of precipitation, as they were not inhibited by amounts of free coenzyme that were not anticomplementary.

Sera such as these may be useful as immunochemical assays for the coenzyme. With inhibition of C′ fixation, the sensitivity with the antifolate-MBSA serum was in the order of 1–4 mμmoles (0.4–1.5 μg) of folate/7 ml of reaction mixture. While assays could be performed on a smaller scale, to measure perhaps 0.05 μg, this system is less sensitive than microbiological assays, which can measure 0.001 μg of coenzyme. The usefulness of the immunoassay would, therefore, apply where extreme sensitivity is not required or where one is studying coenzyme analogs which cannot be used by microorganisms in the growth assay. These sera may also be useful in studying conformational changes in coenzyme structure, as may occur in oxidation and reduction, and in examining enzyme–coenzyme interaction.

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