electron flow are functionally separate pathways with differential sensitivity to phloretin.

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# Properties of the Active Sites of Antibodies Specific for Folic Acid*

William A. Rubenstein and J. Russell Little

ABSTRACT: Certain pteridine haptens undergo characteristic shifts in their absorbance spectra when bound by rabbit antifolate antibodies. These shifts are closely reproduced when the same haptens form complexes with L-tryptophan. Folic acid undergoes a similar spectral shift when bound by chicken liver dihydrofolic reductase. Specific inactivation of rabbit antifolate molecules by *N*-bromosuccinimide can be prevented by hapten protection of the antibody sites suggesting, together with the spectral shift of bound ligands that rabbit antibodies specific for folic acid contain tryptophan residues in their active sites. There was no significant increase in affinity or in cross-reactivity between the antifolate antibodies isolated 48 days after immunization and those obtained after a booster injection 7 months after primary immunization.

Evidence presented previously (Little and Eisen, 1967; Little and Donahue, 1968; Little *et al.*, 1969) suggests that one or more tryptophan residues forms a part of the active sites of antibodies to polynitrobenzene determinant groups. This evidence was derived primarily from the characteristic ligand spectral shifts observed on binding and the qualitatively and quantitatively similar spectra produced when the same ligands form complexes with L-tryptophan as the free amino acid. It has also been observed that the polynitrobenzenes have a high affinity for electrons while tryptophan, of all the naturally occurring amino acid residues, has the greatest ability to serve as an electron donor. Therefore, it has been proposed (Little and Eisen, 1967) that charge transfer complex formation may account for the red shifts in the absorbance spectra of DNP and TNP ligands bound in the antibody combining sites. In order to examine this possibility, we have studied here the reactions between folic acid and antifolate antibodies.

Folic acid was chosen as a haptenic determinant because it is a good electron acceptor and readily forms complexes with a variety of electron donors (Fujimori, 1959). The availability of a large number of folate analogs also provided an opportunity to examine the antibody combining sites by comparison of their affinity for a homologous series of ligands. Specifically, the following study was addressed to

* From the Department of Medicine, The Jewish Hospital of St. Louis, and the Departments of Medicine and Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received December 11, 1969.* This work was supported by Research Grant AI 07951 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, by Research Grant T-486 from the American Cancer Society, Inc., and by a Public Health Service research career program award (1-K3-AM-38,620) from the National Institute of Arthritis and Metabolic Diseases.

these questions: Would antibodies formed against an electron-attracting determinant (folate) exhibit hypochromic–bathochromic changes in the absorbance spectra of bound ligands? Would quenching of antibody fluorescence result from ligand binding by antifolate molecules in view of the direct relationship often observed between the electron affinity of a ligand molecule and ability to accept excitation energy transfer? In addition, we have compared the antifolate antibodies isolated at different times with respect to possible increase in affinity and cross-reactivity with time after immunization. Finally, we have made a preliminary examination of the possibility that the active site of a folate binding enzyme (dihydrofolic reductase) might exhibit structural features in common with antifolate antibodies.

## Materials and Methods

*Folate–Protein Conjugates.* The immunizing and precipitating hapten protein conjugates were prepared from folic acid and bovine γ-globulin (fraction II of bovine plasma from Armour and Co., Chicage, Ill.) and crystallized human serum albumin (Pentex Inc., Kankakee, Ill.), respectively. Each preparation was similar to that described by Ricker and Stollar (1967) except that 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Ott Chemical Co., Muskegon, Mich.) was used in the preparation of folate–human serum albumin conjugate[1] (folate–HSA) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Aldrich Chemical Co., Milwaukee, Wis.) was used to prepare the folate–bovine γ-globulin conjugate (folate–BγG). An additional conjugate of folate–methylated bovine serum albumin (folate–mBSA) was prepared with methylated bovine serum albumin (Mandell and Hershey, 1960) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and was used only for determination of its absorbance spectral properties. Minor amounts of insoluble aggregates were discarded from each hapten–protein conjugate preparation and the folate–BγG required 18-hr dialysis at 4° against 0.015 M HCl to achieve complete solubility. Folate–HSA preparations had a tendency to shed free folate groups gradually while in solution at 4° and were therefore applied to a 0.9 × 35 cm Sephadex G-25 (Pharmacia Fine Chemicals, Inc., New York, N. Y.) column before each use and only material appearing with the void volume was used. From dry weight and absorbance measurements at 348 mμ, it was calculated that folate–BγG had 57 moles of folate/160,000 g of BγG; folate–HSA had 20 moles of folate/70,000 g of HSA; and folate mBSA had 37 moles of folate/70,000 g of BSA.

*Folic Acid and Other Pteridines.* Folic acid (pteroylglutamic acid) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and purified on DEAE-cellulose (microgranular DE52 from Whatman, W. and R. Balston Ltd., England) using a linear gradient of $NH_4HCO_3$, 0.1 to 0.4 M. The purified product was freed of salt by lyophilization and stored dry and shielded from light. At pH 7.0, the $\epsilon_{1\,cm}$ for folate was taken to be 8500 at 345 mμ and 31,800 at 280 mμ (Huennekens, 1968). Pteroic acid was a gift from Charles M. Baugh (Krumdieck and Baugh, 1969). Dihydrofolate, tetrahydrofolate, oxidized and reduced TPN, and *p*-aminobenzoylglutamic acid were also obtained from Sigma. Ptn-COOH was the product of the Aldrich Chemical Co. (Milwaukee, Wis.). Aminopterin and amethopterin (methotrexate) were provided by the Lederle Laboratories, American Cyanimide Co. (Pearl River, N.Y.). All pteridine ligands gave a single spot on ascending thin-layer chromatography in 1-butanol–pyridine–water (1:1:1 by volume) on silica gel G (Eastman Chromagram Sheets, Distillation Products Industries, Rochester, N. Y.) except for dihydrofolate and tetrahydrofolate, which were not tested, and aminopterin which was chromatographically heterogeneous. Tritiated folic acid was 43 Ci/mmole as obtained from Amersham Searle (Des Plaines, Ill.).

*Immunization.* New Zealand white rabbits (20) were immunized with the folate–BγG conjugate in complete Freund's adjuvant. The immunization schedule began with injection of each animal with 1 mg of folate–BγG distributed equally in the four footpads. Twenty-one days and 31 days later each animal was injected with 5 mg of folate–BγG subcutaneously and at 38 days after primary immunization each animal received an additional 10 mg of the same antigen. Ten days after the latter dose, capillary precipitin tests were positive (48 days after the first injection of antigen) and a serum pool was made from 3 successive day's bleedings of the 13 animals with the highest serum antibody concentration. This pool was used as a source of "early antibody" since capillary precipitin assays had been negative prior to each antigen dose. Six of the 13 surviving rabbits were given a booster injection of 10 mg of folate-BγG in complete Freund's adjuvant 7 months after the first antigen exposure at a time when their sera were either negative or contained no more than 100 μg/ml of precipitating antibody as measured with folate–HSA. The six boosted animals were bled 14 days after boost and their antiserum pool was a source of "late antibody."

*Isolation of Antifolate Antibodies.* The procedures used for the isolation of rabbit antifolate antibodies were analogous to those described for anti-DNP antibodies (Eisen *et al.*, 1967). The following sequential steps were employed. (1) Antibodies were precipitated from serum by the addition of an optimum (equivalence) amount of folate–HSA. Since the immunogen was prepared with BγG as the carrier protein and a morpholinethylcarbodiimide, the precipitating antigen employed HSA as the carrier and a dissimilar carbodiimide as the coupling agent. The specific precipitate formed at 37° was incubated 18–24 hr at 4° and harvested by centrifugation. (2) Hapten elution of the washed specific precipitate was performed with 0.05 M folic acid in 0.1 M $NH_4HCO_3$, pH 8.1, for 1 hr at 37°. (3) The soluble hapten–antibody–antigen mixture was then applied to a DEAE-cellulose–Dowex 1-×8 (200–400 mesh) column in which the upper layer of DEAE-cellulose was ten times the volume of the lower Dowex 1 layer. The column was eluted with 0.1 M $NH_4HCO_3$ at 22°. (4) The antibody recovered from the ion-exchange resin column was dialyzed 18 hr against 0.02 M potassium phosphate–0.15 M NaCl, pH 7.4 (buffered saline), at 4° and then against 0.001 M phosphate, pH 7.6, to remove euglobulins. The final product was soluble in 0.001 M phosphate, was free of detectible absorbance at 350 mμ (at a protein concentration of approximately 1 mg/

[1] Abbreviations used are: HSA, human serum albumin; BγG, bovine γ-globulin; mBSA, methylated bovine serum albumin; Ptn-COOH, pteridine-6-carboxylic acid.

ml), and had an absorbancy ratio 278:251 mμ greater than 2.5. The yield of antibodies isolated relative to the amount precipitated at equivalence varied between 65 and 78%.

*Fluorescence Quenching.* The binding of haptens by purified antifolate antibodies was estimated by the method of fluorescence quenching in an Aminco–Bowman spectrophotofluorometer as described elsewhere (Velick *et al.*, 1960; Little and Eisen, 1966). Samples (1 ml) with antibody at a concentration of 40–50 μg/ml were titrated with increments of a hapten solution at a concentration of 4.0–6.0 mμmoles/ml. Antibody fluorescence was activated with incident light at 280 mμ and emission intensity was measured at 350 mμ. All titrations were performed at 0–4° with buffered saline as solvent unless otherwise indicated.

*Equilibrium Dialysis.* Measurements of antibody affinity by equilibrium dialysis were performed as described elsewhere (Eisen, 1964). Plastic chambers (Gateway Immunosera, Inc., Cahokia, Ill.) with 0.1-ml capacity in "inside" and "outside" compartments were employed (Eisen *et al.*, 1968). Purified antibody concentrations in buffered saline were 40–60 μg/ml in each experiment. The experimental chambers were rotated 5–7 rpm for 24 hr at 4° and then equilibrium across the membranes was verified by counting aliquots of control chambers containing ligand but no antibody. The extent of "bag binding" was less than 4% of the free ligand concentration with tritiated folic acid. At equilibrium, aliquots were sampled from inside and outside compartments for liquid scintillation counting in Bray's solution (1960). Average intrinsic association constants, $K_0$, were calculated as described (Nisonoff and Pressman, 1958; Karush, 1962). Binding constants for a variety of unlabeled ligands were measured by competitive inhibition of the binding of tritiated folic acid. The equilibrium inhibition constant, $K_i$, for the unlabeled ligand was calculated as described by Karush (1956).

*Ligand Spectral Shift.* Absorbance spectra were obtained in a Cary Model 14 recording spectrophotometer equipped with a temperature-controlled cell housing. Difference spectra were performed in tandem rectangular cells (Eisen *et al.*, 1968) (available from Pyrocell Mfg. Co., Westwood, N. J.) and were usually obtained with a slide-wire that gave full-scale pen deflection for an absorbancy of 0.100.

*Purification of Dihydrofolic Reductase.* Dihydrofolic reductase was isolated from chicken liver (100 g of starting material) as described by Mathews and Huennekens (1963), and assayed for enzymatic activity by the rate of decrease in absorbancy at 340 mμ due to the oxidation of TPNH to TPN and reduction of dihydrofolate to tetrahydrofolate (Osborn and Huennekens, 1958). After chromatography on hydroxylapatite, the pooled fractions with maximum enzyme activity were lyophilized, reconstituted in water, and dialyzed at 4° (4 hr) against 4 l. of 0.05 M potassium phosphate, pH 7. This dialyzed preparation had a catalytic rate at 22° of 0.017 μmole/min of dihydrofolate reduced at pH 7.4 and this activity was totally inhibited by $3.08 \times 10^{-6}$ M amethopterin in assays in which the initial concentration of dihydrofolate was $6 \times 10^{-5}$ M. The specific activity of the enzyme preparation was 0.2 μmole of dihydrofolate reduced per min per mg of protein.

*N-Bromosuccinimide Oxidation of Antifolate Antibodies.* *N*-Bromosuccinimide (Fisher Scientific Co., St. Louis, Mo.) was recrystallized from glacial acetic acid. Purified antifolate antibodies in 0.05 M acetate buffer, pH 5.0, were titrated at 22° with 10-μl increments of $6 \times 10^{-4}$ M *N*-bromosuccinimide as described by Spande and Witkop (1967). Using antibodies at 287 μg/ml, a series of absorbancy measurements were obtained at 278 mμ representing up to a 40-fold molar excess of NBS to antibody. Following each addition of *N*-bromosuccinimide, the number ($n$) of moles of tryptophan residues oxidized per mole of antibody was calculated from eq 1 (Spande and Witkop, 1967)

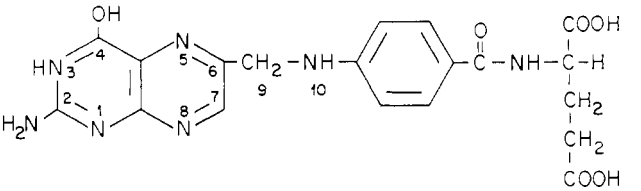
$$n = \frac{1.31 \times A_{278\ m\mu}}{5500 \times \text{antibody molarity}} \quad (1)$$

where $A_{278\ m\mu}$ is the decrease in absorbance, 1.31 is an empirical factor based on oxidation of model tryptophan peptides by *N*-bromosuccinimide (Patchornik *et al.*, 1958), and 5500 is the $\epsilon_{278\ m\mu}$ of tryptophan.

Using these titrations as a guide, hapten protection experiments were performed in 0.05 M acetate buffer, pH 5.0, using $1.4 \times 10^{-6}$ M antifolate antibodies in the presence and absence of $1.4 \times 10^{-4}$ M folate as protecting hapten and employing $3.13 \times 10^{-5}$ M *N*-bromosuccinimide. Folate was added to the same final concentration to the unprotected sample after 2-min exposure to *N*-bromosuccinimide. The reacted antibody populations were then chilled to 0° and dialyzed exhaustively against buffered saline. Each preparation was then applied to a 0.9 × 4 cm Dowex 1-×8 ion-exchange column equilibrated with buffered saline. The eluted protein preparations were free of detectible absorbance at 350 mμ and recovery of protein was 82% of the unprotected and 87% of the hapten-protected sample. Hapten binding measurements on aliquots of each preparation were then performed by fluorescence quenching, equilibrium dialysis, and ligand spectral shift.

## Results

*Antibody Specificity and Purity.* Since the immunogen, folate–BγG, was prepared by reaction with a water-soluble carbodiimide, several potential problems were posed regarding the chemical nature of the haptenic determinant. Despite the unreactive nature of the 2-$NH_2$ and 4-OH groups of the pteridine portion of the molecule we wished to obtain evidence that coupling had been achieved primarily through the glutamate carboxyl groups. This was determined by comparison




FOLIC ACID

of the absorbance spectra of folate–HSA, folate–mBSA, folate–BγG, and folic acid at neutral pH and in alkali. In 0.1 M NaOH folic acid and folate–BγG had 3 $\lambda_{max}$ which were coincident (256, 283, and 365 mμ) and agreed well with published values for folate (Angier *et al.*, 1945). At pH 7.4 in 0.02 M phosphate buffer, folate and folate–BγG had

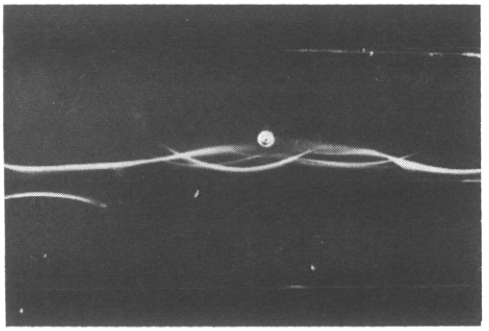


FIGURE 1: Immunoelectrophoresis of rabbit antifolate antibodies isolated from early antiserum pool: upper well, normal rabbit serum; lower well, rabbit antifolate, 1.7 mg/ml, isolated from pooled antisera obtained 48 days after the first injection of antigen. The trough contained a goat antiserum to whole rabbit serum. The analysis was performed in 1.5% Noble agar, 0.04 M barbital buffer (pH 8.2) and the anode was at the right.

$\lambda_{max}$ at 280 and 348 m$\mu$. Folate–HSA and folate–mBSA also had spectra at neutral pH and in alkali which were very similar to free folate. Since folate–mBSA had few, if any, available carboxyl groups, it was likely that conjugation occurred at the primary amino groups of each protein carrier. In agreement with the spectral evidence hapten inhibition of precipitation (Table I) indicated that the pteridine portion of folic acid rather than the glutamate moiety constituted the major determinant of antibody specificity.

Following purification, each antibody preparation was evaluated by quantitative precipitin analysis using folate–HSA as antigen and by immunoelectrophoresis (Figure 1). At a concentration of 1.1–1.3 mg/ml, the purified antibodies were 76–85% precipitable with antigen and each preparation gave a single precipitin arc of $\gamma$G mobility on immunoelectrophoresis developed with a goat antiserum to whole rabbit serum.

*Affinity of Early and Late Antifolate Antibodies.* In Figure 2 are shown the data plotted from equilibrium dialysis experiments of [$^3$H]folic acid binding by early and late purified antibodies. $K_0$ for the early antibody preparation was $4.1 \times 10^6$ M$^{-1}$ and for the late antibody was $3.2 \times 10^6$ M$^{-1}$. This difference was not thought to be significant and the lower value of the late antibodies may relate to a minor contaminant in the latter preparation since the extrapolated value with the abscissa, $r$, was 1.78 while the early antibody preparation gave an extrapolated value of $r = 2$. The heterogeneity index calculated from the slope of the insert Sips plot of Figure 2 was 0.92. When [$^3$H]folate was used as the reference in competition experiments with other ligands performed by the equilibrium dialysis method, these data (Table II) also showed little difference in affinity of the early and late antibodies for a series of structurally related haptens.

The fluorescence quenching curves shown in Figure 3 indicate a surprising efficiency of quenching at relatively low ligand concentrations despite the modest affinities of each antibody preparation. It was not possible to analyze these data in the usual fashion, assuming linear proportionality between antibody fluorescence quenched and sites occupied. Even when it was assumed that antibody site saturation would result in 100% quenching, the percentage quenching observed was more than could be accounted for by the total

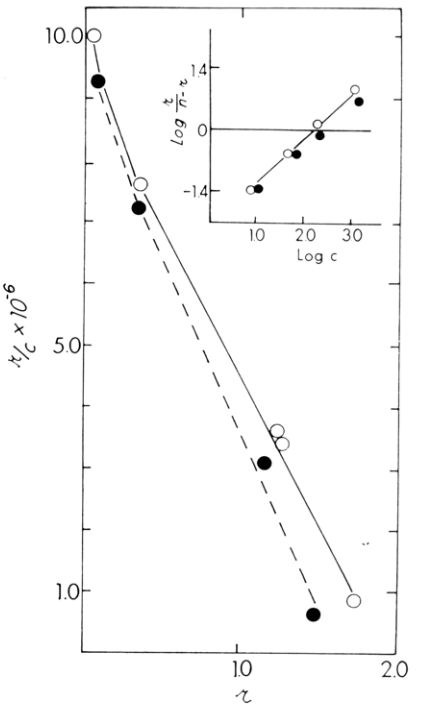



FIGURE 2: Scatchard and Sips (inset) plots of equilibrium dialysis data obtained from the binding of [$^3$H]folate by isolated early and late rabbit antifolate antibodies. Binding experiments were performed in plastic chambers at 4° with 50-$\mu$l volumes on each side of the dialysis membrane. In the notation on the coordinates, $r$ is moles of hapten bound per mole of antibody, $n$ is the limiting value of $r$ (*i.e.*, 2), and $c$ is the molar free hapten concentration. The units for values of $r/c$ are therefore liters per mole (M$^{-1}$) and the abscissa of the Sips plot is expressed as log $c$ in $\mu\mu$moles/ml ($10^{-9}$ M). Early antibodies (open circles) or late antibodies (filled circles) in buffered saline (0.15 M NaCl–0.02 M phosphate, pH 7.4) were found to have very similar average association constants ($3–4 \times 10^6$ M$^{-1}$) and heterogeneity indices ($a = 0.92$). An $r$ value of 2 at saturation was assumed for calculation of $K_0$ for each antibody preparation.

TABLE I: Hapten Inhibition of Precipitation of Antifolate Antibodies.[a]

| Inhibiting Hapten (0.01 M) | Antibody Pptd (mg) | Inhibition (%) |
|---|---|---|
| None | 0.473 | 0 |
| *p*-Aminobenzoylglutamic acid | 0.426 | 10 |
| Pteridine-6-carboxylic acid | 0.270 | 43 |
| Folic acid | 0 | 100 |

[a] Each assay was performed with 1.0 ml of pooled rabbit antifolate early antiserum, 0.1 ml of inhibiting hapten (or buffered saline as a control) previously adjusted to neutral pH with NaOH, and 60 $\mu$g of folate–HSA (the equivalence amount of antigen for this antiserum). After 1 hr at 37° and 18 hr at 0°, the precipitates were collected and washed at 0° with iced buffered saline. The precipitates were then dissolved in 0.1% sodium dodecyl sulfate in 0.02 M sodium phosphate, pH 7.0. Absorbance measurements at 348 and 278 m$\mu$ were used to calculate the amount of antibody in each assay.

TABLE II: Affinity of Early and Late Antibodies for Various Ligands as Determined by Equilibrium Dialysis Competitive Binding.[a]

| Competing Ligand | Total Concentration (M) of Competing Ligand | $K_i \times 10^{-5}$ ($M^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Early Antifolate | | | Late Antifolate | | |
| | | Expt 1 | Expt 2 | Av | Expt 1 | Expt 2 | Av |
| [$^3$H]Folic acid | $2.9 \times 10^{-7}$ | 64 | 60 | 62 | | 39 | 39 |
| Pteroic acid | $8.6 \times 10^{-7}$ | 4.5 | 4.5 | 4.5 | 11 | 13 | 12 |
| Pteridine-6-carboxylic acid | $1.1 \times 10^{-5}$ | 2.0 | 2.0 | 2.0 | 2.6 | 2.8 | 2.7 |
| Methotrexate | $1.2 \times 10^{-4}$ | 1.3 | 1.3 | 1.3 | 2.3 | 2.1 | 2.2 |
| *p*-Aminobenzoylglutamic acid | $7.7 \times 10$ | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |

[a] Equilibrium dialysis was performed as described in Materials and Methods with [$^3$H]folic acid. The unlabeled competing hapten was added to same side of the dialysis chamber containing antibody. For each competing ligand employed, early and late antifolate binding studies were performed in the same experiment, *i.e.*, using the same stock solutions of ligands and the same incubation times (24 hr) and temperatures (0°). $K_i$ values are presented for each experimental chamber and the average was calculated. Equilibrium control chambers containing [$^3$H]folic acid, but no antibody, were included in each experiment. The concentration of each preparation of antibodies was 50 $\mu$g/ml and the initial concentration of [$^3$H]folate was $1.16 \times 10^{-6}$ M.

ligand added for most of the titration points on each curve. Nevertheless, the relative magnitude of quenching for each ligand corresponded to the relative magnitude of their $K_0$ values determined by dialysis equilibrium, and there was little difference in the quenching produced by various ligands with early and late antibodies. *p*-Aminobenzoylglutamate produced no quenching of either antibody preparation.

*Ligand Spectral Shifts in Complexes with Antifolate Antibodies and with Tryptophan.* Figure 4 shows the absorbance spectrum of the long-wavelength band of folic acid and the marked change that resulted from the antibody–hapten reaction. The absorbance spectrum of bound folate is shifted hypochromically and bathochromically. When the same preparation of free and partially bound folate was examined by difference spectroscopy in tandem cells, the solid line difference spectrum of Figure 5 was obtained. In the region of the spectrum 350–600 m$\mu$, only a single $\lambda_{max}$ was observed at 394 m$\mu$. Similar difference spectra were obtained with

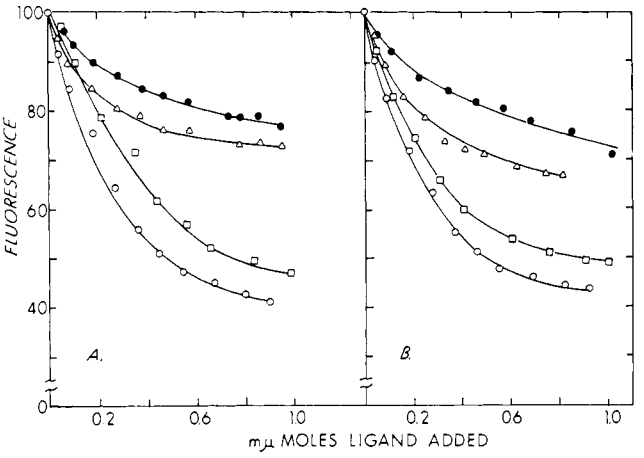



FIGURE 3: Fluorescence quenching of antifolate antibodies isolated from antiserum pools obtained 48 days (panel A) or 7.5 months (panel B) after initial immunization of a single group of rabbits. The ligands employed were folic acid (open circles), pteroic acid (open squares), pteridine-6-carboxylic acid (open triangles), and methotrexate (closed circles). Each titration was obtained with 1-ml aliquots of antibody at 40–50 $\mu$g/ml in buffered saline at 0–2°. Fluorescence values have been corrected for solvent fluorescence and for volume change due to ligand addition. Each curve is the average obtained from duplicate titrations. When dihydrofolic acid was used as a ligand in similar titrations, the quenching curves obtained were nearly superimposable with those shown above for methotrexate.

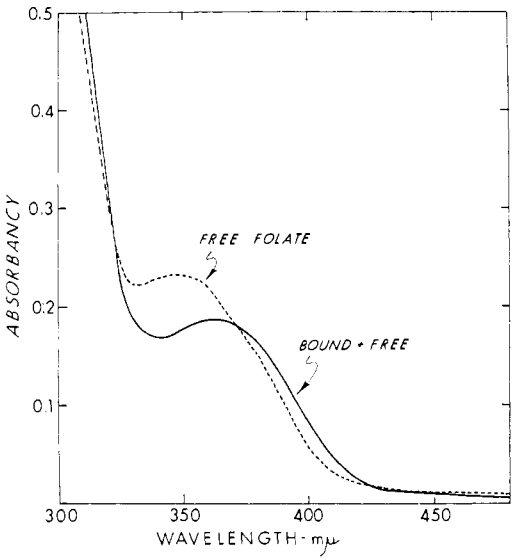



FIGURE 4: Absorbance spectra of free and antibody-bound folic acid The dotted curve was obtained with $2.72 \times 10^{-5}$ M folic acid at 4°, in buffered saline in a Cary Model 14 spectrophotometer equipped with a temperature-controlled cell housing. The solid curve was obtained with the same concentration of folate mixed with $1.38 \times 10^{-5}$ M antifolate antibodies isolated from the early antiserum pool. The $\lambda_{max}$ of free folate was 350 m$\mu$ and the $\lambda_{max}$ of the solid curve (bound + free) was 362–363 m$\mu$.

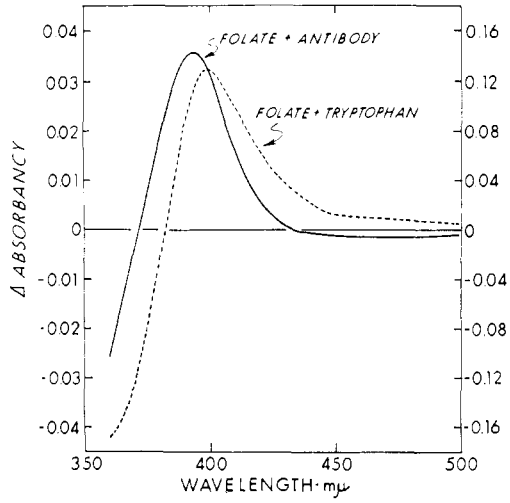



FIGURE 5: Difference spectra between free folate and folate complexes made at pH 7.0 with antibodies or with L-tryptophan. The solid curve (left ordinate) difference spectrum was obtained with the same concentrations of folic acid and antibody stated in the legend of Figure 4. The experimental cell contained the mixture of antibodies and folate while the reference tandem cell contained the same two reagents at the same concentrations in separate chambers. The dotted curve (right ordinate) difference spectrum was obtained with $4.5 \times 10^{-2}$ M L-tryptophan plus $7.4 \times 10^{-5}$ M folic acid in the experimental cell and equal concentrations of each of these reagents in separate chambers of the tandem reference cell. The solvent was buffered saline and the temperature was 10°. Similar difference spectra obtained with nonspecific rabbit γG-immunoglobulins and folic acid gave no significant spectral shift.

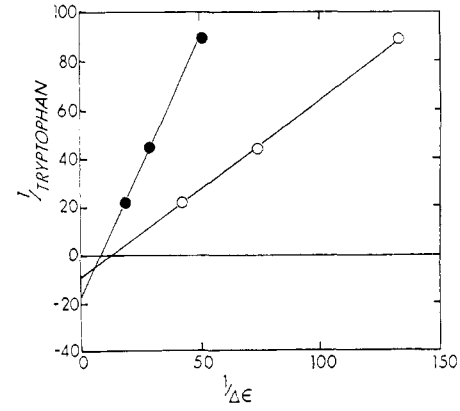



FIGURE 6: Determination of molar extinction coefficients and equilibrium constants for the L-tryptophan–folic acid complex. Δε refers to the difference between the reference cell with folate and L-tryptophan in separate chambers of the tandem cell and the experimental cell in which the same reagents were mixed. Difference spectra were obtained in 0.02 M phosphate buffer either at pH 7.4 where $\lambda_{max}$ of Δε was at 400 mμ (open circles) or at pH 6.3 where $\lambda_{max}$ of Δε was at 394 mμ (filled circles). The folic acid concentrations were $7.4 \times 10^{-5}$ M for the mixtures made at pH 7.4 and $9.3 \times 10^{-6}$ M for the mixtures at pH 6.3. L-Tryptophan concentrations varied between 0.05 and 0.006 M. The abscissa intercept $(1/[L_t]\Delta\epsilon)$ was 8.0 at pH 7.4 and 12.5 at pH 6.3 corresponding to $\Delta\epsilon_M$ values of 1080 and 1360, respectively. Spectra were obtained at 10°.

preparations of late and early antifolate antibodies and nonspecific rabbit γG-immunoglobulin showed no spectral shift with folic acid.

When folate–tryptophan mixtures were examined by difference spectroscopy, a marked dependency of extinction and $\lambda_{max}$ on pH was observed. The dotted difference spectrum showed in Figure 5 which has a $\lambda_{max}$ at 398 mμ was obtained with $4.5 \times 10^{-2}$ M L-tryptophan and $7.4 \times 10^{-5}$ M folic acid at pH 7.0 in phosphate buffer. The $\lambda_{max}$ shifted to 394 mμ and had greater magnitude when the same mixture was examined at pH 6.3. Using an analysis proposed by Isenberg and Szent-Györgyi (1958), the extinction coefficient (at $\lambda_{max}$) and association constant were calculated for the L-tryptophan–folic acid complex at pH 7.4 and 6.3. This was performed by obtaining difference spectra between a reference solution of folate and L-tryptophan in separate chambers of a tandem quartz cell and the same concentrations of folate and L-tryptophan mixed in one side of an experimental tandem cell. On the assumptions that: (1) the complexes form reversibly and consist of one molecule of L-tryptophan plus one molecule of folate, and (2) the absorbancy of the complex obeys Beer's law, it follows that

$$A = \Delta\epsilon[LT]l \tag{2}$$

and

$$K = \frac{[LT]}{[T][L]} \tag{3}$$

where $A$ is the net absorbance (from the difference spectrum, at the $\lambda_{max}$); $\Delta\epsilon$ is the change in the folate molar extinction coefficient when in the complex form; [LT], [L], and [T] are the molar concentrations of the complex, unbound folate, and unbound L-tryptophan, respectively; $K$ is the association constant for the formation of the complex and $l$ is the light path (1.0 cm). With L-tryptophan in large excess, the free and total L-tryptophan concentrations are essentially equivalent; and if $[L_t]$ is the total folate concentration

$$\frac{1}{[T]} = K[L_t]\Delta\epsilon\frac{1}{A} - K \tag{4}$$

a plot of $1/[T]$ *vs.* $1/A$ is linear; the abscissa intercept is $1/[L_t]\Delta\epsilon$, while the ordinate intercept is $-K$. Figure 6 shows that $K$ (at 10°) for the folate–L-tryptophan complex was 9 $M^{-1}$ at pH 7.4 and 18 $M^{-1}$ at pH 6.3. The molar extinction coefficient at 394 mμ at pH 6.3 was 1360 and at pH 7.4 where the $\lambda_{max}$ was 400 mμ the $\epsilon_M$ was 1080. These values agree within experimental error with those reported by Fujimori (1959). At a particular pH, in 0.02 M phosphate, variation of the NaCl concentration between 0 and 1.0 M had no effect on the $\epsilon_M$ or $\lambda_{max}$ of the folate–L-tryptophan complex.

Using Figure 5 to obtain the extinction difference at 394 mμ for the folate–antibody complex and the experimentally determined $K_0$ for the early antifolate antibodies (Figure 2), the calculated $\Delta\epsilon_M$ for this antibody site–ligand interaction was 1430. This agrees well with the value (1360) for the L-tryptophan–folate complex at pH 6.3.

Similar experiments were conducted with methotrexate (amethopterin), which also displayed a dependency of $\lambda_{max}$ and $\Delta\epsilon_M$ on the pH of L-tryptophan–methotrexate mixtures. Figure 7 shows difference spectra obtained from the antibody–methotrexate interaction and the methotrexate–L-tryptophan

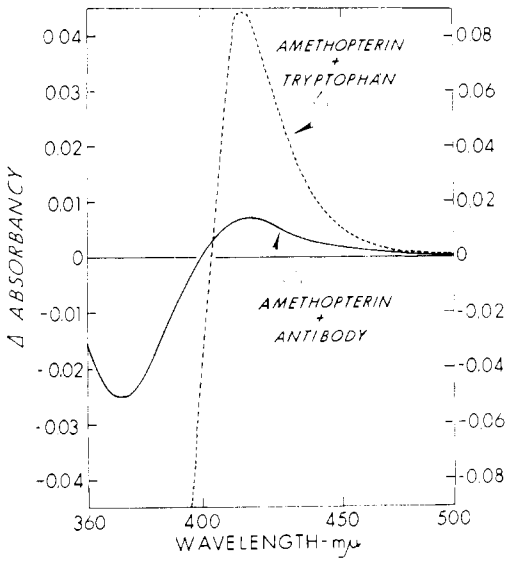



FIGURE 7: Difference spectra between free amethopterin (methotrexate) and amethopterin complexes made at pH 7.4 with antifolate antibodies or with L-tryptophan. The solid curve (left ordinate) difference spectrum was obtained with a mixture of $7.8 \times 10^{-6}$ M antifolate antibodies (from early antiserum) and $3.17 \times 10^{-5}$ M amethopterin in the experimental cell and the same concentrations of each reagent in the separate compartments of the reference tandem cell. The dotted curve (right ordinate) difference spectrum was obtained with a mixture of 0.045 M L-tryptophan and $3.35 \times 10^{-5}$ M amethopterin in the experimental cell and the same concentrations of each reagent in the separate compartments of the reference tandem cell. Both spectra were obtained at 10° in buffered saline as solvent. $\lambda_{max}$ was 417 and 415 mμ for the solid and dotted curves, respectively.

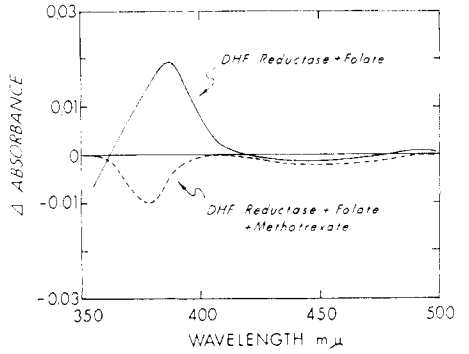



FIGURE 8: Difference spectra between free folate and folate complexes with chicken liver dihydrofolic reductase. The solid curve with $\lambda_{max}$ at 387 mμ was obtained with a preparation of partially purified dihydrofolic reductase and $1.12 \times 10^{-4}$ M folate. See Materials and Methods for enzyme purification and catalytic characteristics. The dotted curve was obtained with the same reference and experimental samples as the solid curve spectrum but after the addition of methotrexate (amethopterin) to each cell at a final concentration of $3.2 \times 10^{-5}$ M and with a total added volume that changed the enzyme concentration less than 5%. Addition of methotrexate resulted in disappearance of the $\lambda_{max}$ and appearance of a $\lambda_{min}$ at 370 mμ. Each spectrum was obtained in 0.05 M phosphate buffer, pH 7.4, at 4° and each was observed to be stable over a 15-min interval of observation. Difference spectra obtained with folate and other chicken liver proteins lacking dihydrofolic reductase activity produced no spectral shifts when examined under the same conditions.

complex. At pH 7.4, the $\lambda_{max}$ values from the difference spectra were 417 mμ for antibody binding and 415 mμ for the complex with L-tryptophan.

*Ligand Spectral Shift with Dihydrofolic Reductase Enzyme.* Figure 8 shows a typical example of difference spectra obtained with partially purified chicken liver dihydrofolic reductase and folic acid. Two separately isolated preparations were examined and control difference spectra were performed with chicken liver proteins devoid of dihydrofolic reductase activity eluted from the hydroxylapatite column used in the final stage of enzyme purification. Fractions without enzymatic activity also failed to produce difference spectra with folic acid. Also shown in Figure 8 is the disappearance of the $\lambda_{max}$ at 387 mμ when methotrexate was added to the folate–enzyme complex. The negative deflection produced by methotrexate had a $\lambda_{min}$ at 370 mμ and probably represented a hypochromic effect on the methotrexate spectrum (370 mμ is the center of a methotrexate absorbance band). Since these experiments were performed at 4° without added TPNH, catalysis was precluded and the observed spectra were stable under these conditions.

*N-Bromosuccinimide Oxidation of Antifolate Antibodies.* When aliquots of early antifolate antibodies were examined by fluorescence quenching, it appeared that folate binding at pH 7.4 and at pH 5.0 were indistinguishable. When aliquots of the same antibodies were titrated with *N*-bromosuccinimide at pH 5.0 in 0.05 M acetate buffer, there was a consistently smaller decline in absorbance after each addition of *N*-bromosuccinimide when antibody sites were protected with excess folate. Control experiments with folate alone at pH 5.0 showed no effect on the absorbance of folate at 280 mμ. At a mole ratio of 22.3 (*N*-bromosuccinimide:antibody) 2.2 tryptophan residues/mole of antibody were oxidized in the presence of protective hapten and 4.3 residues tryptophan/mole of antibody were oxidized in the samples containing antibody but no hapten. This indicates that two tryptophan residues per molecule of antibody were protected from *N*-bromosuccinimide oxidation by folate in excess. Using the *N*-bromosuccinimide titrations as a guide, experiments were performed with hapten-protected and -unprotected antifolate molecules exposed to a 20-fold molar excess of *N*-bromosuccinimide at 22° in 0.05 M acetate buffer, pH 5.0. Each preparation (hapten protected and unprotected) was then freed of hapten and evaluated for its ligand binding properties. Figure 9 shows the difference in fluorescence quenching between the protected sample, which retained almost all of its binding activity with folate, and the unprotected sample which was inactive. The unprotected sample was also inactive when tested for its ability to bind [$^3$H]-folate in equilibrium dialysis or to cause a shift in the absorbance spectrum of folic acid.

## Discussion

The immunogenic conjugate of folic acid we have prepared is similar to those reported previously from two other laboratories (Ricker and Stollar, 1967; Jaton and Ungar-Waron, 1967). Our interest in the antibodies evoked by these conjugates derived from earlier observations of Fujimori (1959) that folate and certain other pteridines made spectrally distinctive complexes with tryptophan and other

indoles. Since folate can act as an electron-withdrawing group (Perault and Pullman, 1960), we speculated that antifolate antibodies might contain one or more tryptophan (the best electron donor among natural amino acids) residues in their active sites. The presence of a tryptophan residue in the active sites of rabbit antifolate antibodies is supported by two kinds of experimental evidence. First, the antifolate antibodies were shown to lose their ligand binding properties after exposure to *N*-bromosuccinimide and this loss was largely prevented if hapten protected the antibody sites during *N*-bromosuccinimide oxidation. In addition, these antibodies had two sites per molecule at which folate was bound and two fewer tryptophan residues were oxidized by *N*-bromosuccinimide when the antibody active sites were occupied by folate than when the sites were vacant. Second, there was a marked spectral change that occurred with ligand binding in the long-wavelength visible absorbance band of folate and we have confirmed Fujimori's observations (1959) that a similar spectral change occurs when folate–tryptophan complexes are formed.

The near identity of extinction coefficients of the folate–antibody and folate–tryptophan complexes provides additional confidence that the origin of the ligand spectral shift was reaction with a tryptophan residue in the protein's active sites. The similar methotrexate–tryptophan and methotrexate–antibody difference spectral maxima (respectively, at 415 and 417 mμ) also support this argument. Our results are also consistent with Fujimori's (1959) failure to detect a significant spectral change in complexes of tryptophan and dihydrofolic acid and we also observed no shifts in the ultraviolet absorbance spectrum of dihydrofolate on binding to rabbit antifolate antibodies (unpublished) though the interpretation of these spectra was complicated by the extensive overlap of antibody and ligand absorbance bands.

It lends some support to these data that a folate binding enzyme, chicken liver dihydrofolic reductase, also has a tryptophan residue in its active site (Freisheim and Huennekens, 1969), undergoes extensive fluorescence quenching with ligand binding (Huennekens *et al.*, 1967), and was found to exhibit a ligand spectral shift that was similar to, but not identical with, rabbit antifolate antibodies.

The antibodies described in the present study displayed hapten binding reactions of special interest. First, the hapten inhibition (Table I) fluorescence quenching (Figure 2) and equilibrium dialysis (Table II) data indicated that the pteridine portion of the folate molecule formed the critical portion of the antigenic determinant. This was indicated by the relatively small contribution of the *p*-aminobenzoylglutamate group to the total binding energy of the ligand–antibody reaction and also by the sharp discrimination between the binding of 2-amino-4-hydroxypteridines (folate and pteroate) and the 2,4-diaminopteridines (methotrexate and unpublished data with aminopterin). A second feature of interest was the failure to observe an increasing affinity with time and after a booster injection of a folate–protein conjugate. Other antibody responses that have shown little or no variation in affinity with time were reported by Richards *et al.* (1969), Wu and Rockey (1969), and by Paul *et al.* (1969). As a probable consequence of the absence of the time-dependent affinity change, antifolate molecules from early and late sera had a relatively restricted heterogeneity of association constants (Figure 1, $a = 0.92$). It also seems probable that

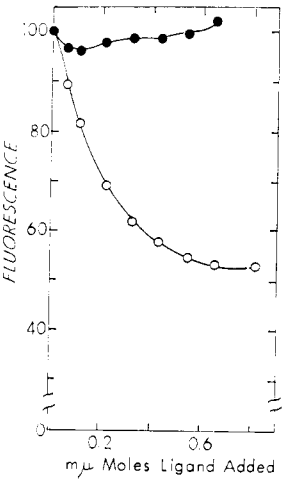



FIGURE 9: Fluorescence quenching of antifolate antibodies after oxidation with *N*-bromosuccinimide in the presence and in the absence of folate. The conditions for *N*-bromosuccinimide oxidation were described in Materials and Methods. Following removal of unreacted *N*-bromosuccinimide and folate, each aliquot of antibody was dialyzed against buffered saline and fluorescence quenching titrations were performed at 2° with folic acid in 0.02 M phosphate, pH 7.4, as described in the legend of Figure 3. The curve provided by the open circles was obtained with antibody sample protected with excess folate during the *N*-bromosuccinimide oxidation. The filled circle data points were obtained with the aliquot of antibody exposed to *N*-bromosuccinimide and then adjusted to the same final folate concentration as in the hapten-protected sample.

the failure to observe increased cross-reactivity in the hapten binding reactions of antifolate molecules produced during the booster response was related to the absence of affinity change. Earlier studies with rabbit antibodies specific for the 2,4-DNP and 2,4,6-TNP groups (Little and Eisen, 1969) and for the 2,6-DNP group (Little *et al.*, 1969) showed a consistent correlation between increasing average affinity and increasing cross-reactivity with time after immunization. The present study provides further evidence for a direct relationship between antibody affinity and cross-reactivity since the "late" antifolate antibodies did not display greater cross-reactions than the antibodies obtained 6 months earlier, nor did they show increased hapten binding constants.

Although our two populations of antifolate antibodies were very similar in affinity and cross-reactivity, our "early" antiserum was obtained 48 days after a multiple dosage schedule of antigen injection, and changes may have occurred during this interval in which we were unable to detect precipitating antibodies. Further studies are in progress to clarify this point.

Although it was of some interest to demonstrate the quenching of antibody fluorescence that occurred with ligand binding, it remains unclear why these data do not permit calculation of average binding constants. Possibly there was considerable variation among antifolate molecules in their ability to be quenched. Some antibody molecules, for example, may have had a higher tryptophan content than most and if these molecules were also more fluorescent and had slightly higher binding constants than the average, the quenching curve for the entire population would show "excessive quenching" of the early titration points. This kind of molecular heterogeneity has been observed in certain

anti-DNP antibody populations reported by McGuigan and Eisen (1968). Other explanations are also possible, *e.g.*, conformational change of the antibody molecules upon binding, the presence of an antibody-bound fluorescent impurity not removed by our isolation procedure or cooperative effects of binding.

A surprising feature of the difference spectrum between folate alone and the folate–tryptophan complex was the pH dependence of $\lambda_{max}$ and its extinction. An ionizable group on the folic acid molecule probably accounts for this change since it was observed that the long-wavelength band of folate had a $\lambda_{max}$ at 348–350 m$\mu$ at pH 7.4 and was at 345 m$\mu$ at pH 6.3 in 0.02 M phosphate buffers. It is possible that the similarity between the difference spectra and molar extinction coefficients of the antibody–folate complex at pH 7.4 and the tryptophan–folate complex at pH 6.3 indicates a change induced by antibody binding on the p$K_a$ of some folate substituent, similar to that observed with a DNP ligand by Metzger *et al.* (1963). However, this may also reflect an environmental effect of the active site on the folate–tryptophyl residue reaction. Either of these possibilities would also explain the different $\lambda_{max}$ observed in difference spectra produced by the folate–antibody and the folate–enzyme complexes. One would anticipate similarly, not identity, in the binding sites of two proteins with such different functions.

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