J. Pharm. Pharmacol. 1987, 39: 190–195
Received June 30, 1986

The effects of pH, calcium and chloride ions on the binding of tolmetin to human serum albumin: circular dichroic, dialysis and fluorometric measurements

KENJI MATSUYAMA†, ASOK C. SEN AND JOHN H. PERRIN*

College of Pharmacy, University of Florida, Gainesville, Florida 32610, USA

The binding of the non-steroidal anti-inflammatory drug, tolmetin (1-methyl-5-*p*-toluoylpyrrole-2-acetic acid) to human serum albumin (HSA) has been shown by circular dichroism, fluorescence and equilibrium dialysis to be dependent on the N-B conformational change of the albumin. The influence of calcium and chloride ions on the interaction was also investigated using the same techniques. Experiments suggested that calcium ions increased the binding constant of tolmetin to HSA whereas chloride ions decreased it. The displacement study showed that tolmetin caused a significant increase in the affinity of diazepam to HSA whereas it decreased the binding of warfarin to HSA. Tolmetin seems to cause an allosteric change in the diazepam binding site in spite of it sharing a primary site with warfarin.

Albumin is an important carrier for drugs and endogenous substances such as hormones, bilirubin and fatty acids. Interest has been shown in its physiological function and its physicochemical properties (Saroff & Lewis 1963; Leonard et al 1963; Harmsen et al 1971; Nikkel & Foster 1971). Leonard & Foster (1961), using optical rotation studies at 314 nm, first discovered a conformational change in human serum albumin (HSA) in the pH range of 7–9. This conformational change is now commonly referred to as the neutral to base or N–B transition. Histidine, whose imidazole residues have a pK_a of 6·4–7·0, seems to be involved in this transition, and it is hypothesized that 10 imidazonium residues are 'hidden' in the N-form and become available in the B-form (Harmsen et al 1971). At physiological pH, albumin can exist in these two forms. Small inorganic ions such as chloride (Cl^-) and calcium (Ca^{2+}) ions have been shown to affect the N–B transition (Wilting et al 1979; Wilting et al 1980a). Calcium ions are thought to compete with imidazonium residues for carboxylate binding sites (Pederson 1972). These studies also show that Ca^{2+} ions react reversibly with 12 of the 16 imidazole groups on HSA with the calcium binding increasing over the pH range 5–9.

No workers had shown the involvement of the N–B transition in drug-HSA interactions until the first work reported with warfarin (Wilting et al 1979; Wilting et al 1980a), the drug having an increased affinity for HSA in the B-form. Ca^{2+} ions also increase the affinity of the drug for HSA, presumably by altering the conformation, but the chloride ions, at high concentrations, decrease the affinity.

Subsequently, a paper on the diazepam-HSA interaction was published which again showed an increased affinity for the drug when HSA is in the B-form, however, calcium ions decreased the affinity of the drug for HSA (Wilting et al 1980b).

The binding of tolmetin (1-methyl-5-*p*-toluoylpyrrole-2-acetic acid) has been quantitatively investigated by two different groups (Selley et al 1978; Behm et al 1981). However, they did not investigate the effect of the N–B transition on the HSA-tolmetin interaction. In this paper, a study of possible linkage between the N–B transition and the binding is described.

MATERIALS AND METHODS

Human serum albumin, fraction V, Lot No. 35F-9542 was obtained from Sigma Chemical Company, St. Louis, MO. Sodium tolmetin (Lot No. 7501166) and diazepam (Lot No. 380121) were donated by McNeil Laboratory (Ft. Washington, PA) and by Hoffman LaRoche (Nutley, N.J.), respectively. Sodium warfarin (Lot No. 71-167-1) was obtained from Endo Laboratory Inc., Garden City, N.Y.

* Correspondence.
† Present address: Faculty of Pharmaceutical Science, Kyushu University, Fukuoka 812, Japan.

Albumin was deionized by passing through a mixed bed ion exchange column (Dowex 50 W-X8, Na^+ cation exchange and Dowex 1-X8 anion exchange resin, Baker Chemical Co.). The concentration of HSA was determined spectroscopically after the deionization process taking an extinction coefficient of $3{\cdot}73 \times 10^4$ L mol^{-1} cm^{-1} at 278 nm.

Circular dichroism (CD) measurements were made using a JASCO Model J-500 spectropolarimeter in cells of pathlength 5 or 10 mm. The dynode voltage was kept below 600 V and measurement was made at room temperature (22 °C). Observed ellipticities (θ_{obs}) are the actual CD of the tolmetin-HSA complex while the induced ellipticity is the observed ellipticity of the complex minus the ellipticity of albumin alone at the corresponding wavelength. The optical anisotropy or dissymmetry factor ($\Delta E_\lambda/E_\lambda$) was calculated from the expression:

$$\frac{\Delta E_\lambda}{E_\lambda} = \frac{[\theta]_\lambda}{3300\, E_\lambda}$$

where E = molar extinction coefficient of the drug, λ = wavelength of circular dichroism maxima and $[\theta]$ = molar ellipticity, which is further defined by the expression:

$$[\theta]_\lambda = \frac{100\,\theta_{obs}}{l.C}$$

where θ_{obs} = observed ellipticity n degrees, l = pathlength in cm, C = molar concentration, $[\theta]_\lambda$ is expressed in deg cm^2/decimole. Fluorescence was measured at room temperature (22 °C) using a Perkin-Elmer MPF-44A fluorescence spectrophotometer. With an excitation wavelength of 278 nm and a slit width of 4 to 6 mm, the emission was scanned from 290–400 nm, the maxima occurring near 330 nm. The fluorometric titrations were carried out as follows: 3·5 mL of the protein solution of an appropriate concentration in a 10 mm pathlength cell were titrated by successive addition of 2·0 μL volumes of tolmetin solution such that the final drug concentration in the cell was around $1{\cdot}8 \times 10^{-5}$ M. Equilibrium dialysis experiments were performed with a Dianorm Equilibrium Dialyzer (Diachema A. G. Ruschlikon, Switzerland) using cells of 10 mL total volume. Hydrated cellulose membrane (Diachema, type 10·15, molecular weight cut off of 5000) were washed in deionized water and dried with tissue. Adsorption of tolmetin onto membranes was negligible and the volumes of the solutions on either side of the membrane stayed constant during the dialysis procedure. After the 6 h dialysis at 37 °C, free concentrations of tolmetin, diazepam and warfarin were determined by injection onto a Zorbax ODS (4·6 mm × 15 cm) column in a Varian 5000 liquid chromatograph, detection being effected by a Spectroflow 757 (Kratos, Ramsey, N.Y.) UV detector at 238 nm. A mobile phase of 40% acetonitrile in deionized water (pH 5·2 with 0·05 M of phosphate buffer) was used for tolmetin, diazepam and warfarin.

The phosphate buffers were prepared by adding 0·1 M sodium dihydrogen phosphate to 0·1 M disodium hydrogen phosphate until the desired pH was obtained. The 0·1 M borate buffer was prepared by taking 250 mL 0·2 M boric acid and adding 0·2 M sodium hydroxide until the desired pH was obtained. The solution was diluted to 500 mL with deionized water and the final pH adjustment made. The data from circular dichroism and dialysis were analysed using the Scatchard relationship $r/D_f = Kn - Kr$ where r = no. of moles of drug bound per mole of protein, D_f = molar concentration of free drug, K (M^{-1}) is the association constant and n is the number of ligand binding sites per molecule of the protein.

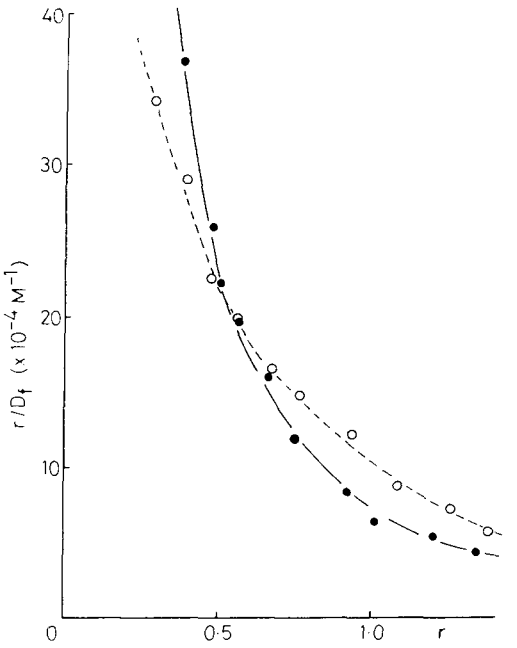

FIG. 1. Scatchard plots of the dialysis data at pH 7·4 in a 0·1 M phosphate buffer: dashed line represents the data at 22 °C, solid line at 37 °C. [HSA] = $2{\cdot}5 \times 10^{-4}$ M.

RESULTS AND DISCUSSION

Scatchard plots of the dialysis data from deionized HSA (the molar ratio of fatty acid to HSA; $r_f = 0{\cdot}8$) at pH 7·4, 22 °C and 37 °C are shown in Fig. 1. The data were analysed assuming two independent classes of binding sites using the computer programs described by Perrin et al (1974). The best fit was obtained with the binding data $n_1 = 1$, $K_1 = 3{\cdot}84 \times 10^5$ M^{-1} and $n_2 = 2$, $K_2 = 1{\cdot}24 \times 10^4$ M^{-1} at 22 °C with the constraint $n_1 = 1$ and $n_2 = 2$. K_1 is approximately fifty percent higher at 37 °C with a slight decrease in K_2. From dialysis experiments, Selley et al (1978), have reported tolmetin to have the values of $n_1 = 1$, $K_1 = 3{\cdot}6 \times 10^5$ and $n_2 = 4$, $K_2 = 1{\cdot}2 \times 10^4$ M^{-1} at 23 °C. The present data are in reasonable agreement with these values. Tolmetin, when bound to HSA, gives rise to an extrinsic Cotton effect with a positive maximum near 340 nm and a negative maximum near 290 nm. The isobestic point at 319 nm changes slightly when the HSA becomes saturated.

The changes in observed ellipticities at various drug to protein ratios (D/P) (pH 7·4, 0·1 M phosphate buffer) are shown in Fig. 2. The CD intensity increases with D/P for D/P < 2 and is constant for D/P > 2. Following the method of Rosen (1970), a tangent to the plot of induced ellipticity against (D/P) was drawn, this gives an estimation of free and bound fraction of the drug. A Scatchard plot (Fig. 3) again indicates more than one binding site for tolmetin to HSA. The best fit was obtained with the binding data $n_1 = 0{\cdot}43$, $K_1 = 1{\cdot}4 \times 10^6$ and $n_2 = 0{\cdot}93$, $K_2 = 2{\cdot}6 \times 10^4$ M^{-1} using no constraints. The values are several times higher than the association constant obtained from dialysis data. However, in the dialysis method protein concentrations higher by one order of magnitude were used. This concentration effect is frequently noticed (Bowmer & Lindup 1978a, b) and is probably attributable to the increased protein-protein interaction at higher concentration which leads to a decrease in the availability of the binding sites.

The binding of tolmetin to HSA causes the native fluorescence of HSA to be quenched. The amount of quenching which occurs is directly reflected in the amount of tolmetin bound allowing a quantitation of tolmetin-HSA interaction. The major contribution to the native fluorescence of albumin arises from its lone tryptophan residue. Quenching is thought to occur when there is a close association of the tryptophan group of HSA and the drug which allows a radiationless energy transfer from the tryptophan to the drug to dissipate the tryptophan's absorbed energy. Thus the quenching of the native fluorescence of HSA by the binding of tolmetin suggests that tolmetin is bound at the tryptophan site (site I) of albumin. Relative fluorescence was plotted as a function of drug concentration at fixed protein concentration ($1{\cdot}55 \times 10^{-5}$ M) as shown in Fig. 4. Correction was made for absorbance of the drug (Attallah & Lata 1968; Velick et al 1960) and the

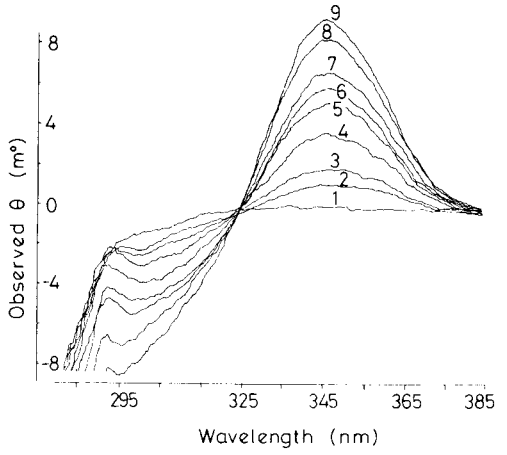

FIG. 2. Observed ellipticities of tolmetin–HSA complexes at various drug to protein ratios (D/P) at pH 7·4 in 0·5 cm cells [HSA] = $6{\cdot}120 \times 10^{-5}$ M. Drug to protein ratios are: 1 0, 2 0·098, 3 0·195, 4 0·39, 5 0·585, 6 0·78, 7 0·98, 8 1·3, 9 1·63.

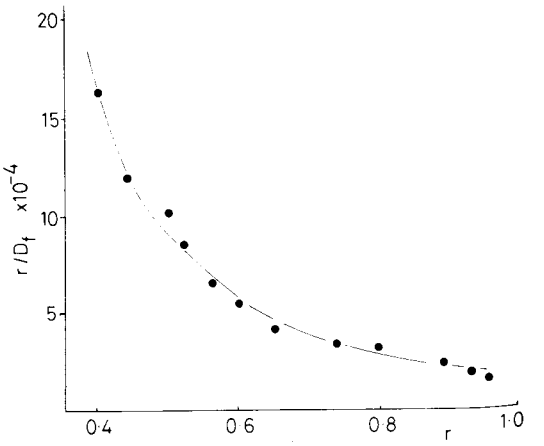

FIG. 3. Scatchard plot of the ellipticities data at 345 nm at pH 7·4 in a 0·1 M phosphate buffer at 22 °C [HSA] = $6{\cdot}12 \times 10^{-5}$ M.

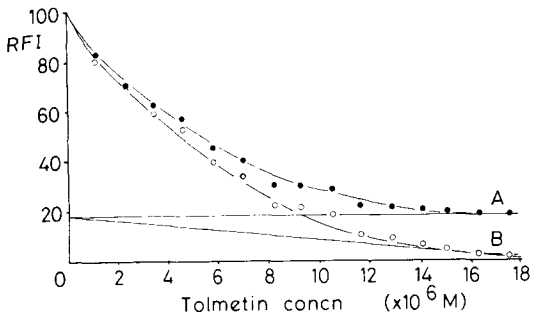

FIG. 4. Quenching of native fluorescence of HSA by tolmetin at pH 7·4 in 0·1 M phosphate buffer [HSA] = 1·55 × 10^{-5} M A (excitation) = 278 nm, B (emission) = 315 nm. Both excitation and emission slits used were of 6 nm.

association constant determined at pH 7·4 from the following equation (Attallah & Lata 1968):

$$K_f = \frac{Q_f}{(1-Q_f)(D_t - nQ_f)C}$$

where C = protein concentration, Q_f = quenching fraction, which is defined as the ratio of the quenching at a point on the curve at or near the stoichiometric point to the maximum quenching, D_t = the total concentration of tolmetin added, n = the drug to protein ratio at a certain point. Following the equation, K_f was found to be 7·7 × 10^5 M^{-1}.

The effect of pH on the ellipticity of tolmetin-HSA complexes was examined. As shown in Fig. 5, the changes in ellipticity as a function of pH cannot be due to the changes in the states of ionization of the drug because its pK_a has been reported to be 3·5 and the drug is fully ionized over the pH region of these investigations. The size of the signal increased from pH 5·5 to pH 7·0 but θ_{obs} decreases as the pH is raised from 7·0 to 9·0.

However, both the dialysis and the fluorescence methods suggest that the pH dependence of tolmetin-HSA complexes parallel the N–B transition over the pH range 6·0 to 9·0.

Judging from the data obtained from dialysis or fluorescence methods, the decreased ellipticity at higher pH values is not due to a decreased affinity of the drug for HSA in the B-form, but may be due to changes in the geometry of the binding site following the N–B transition as was observed in the case of benoxaprofen (Fleitman & Perrin 1982).

Fig. 6 shows the effect of calcium and chloride ions on the binding constant for tolmetin to HSA. Chloride ions (0·1 M) decrease the affinity of the drug for HSA in a manner similar to the case of warfarin (Wilting et al 1980a). The large concentration of chloride ions probably compete for the primary

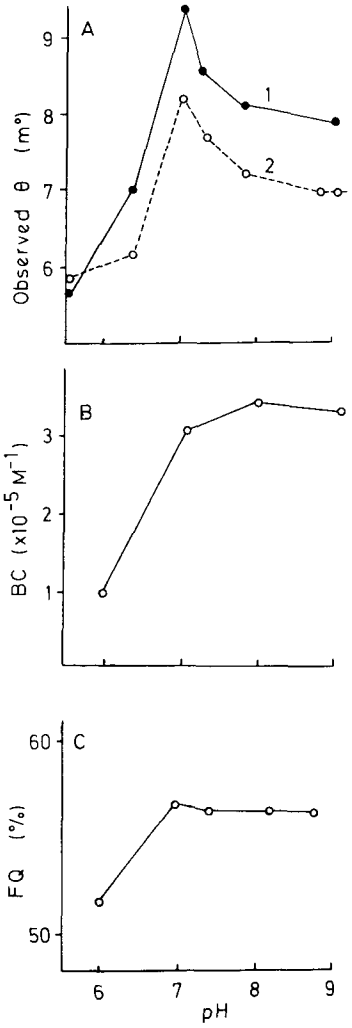

FIG. 5. Effect of pH on tolmetin binding to HSA. A. Observed ellipticities of tolmetin–HSA complex at 342 nm, as a function of pH for fixed drug to protein ratio (D/P) of 0·577. [HSA] = 5·56 × 10^{-5} M, (below pH 8, 0·1 M phosphate buffer, above pH 8, 0·1 M borate buffer was used). The solid line represents data with 0·1 M calcium ions in 0·1 M Tris-HCl buffer, dotted line (2) represents the data without calcium ions under the condition described above. B. Binding constant (calculate from dialysis data) as a function of pH at 25 °C. D/P ratio = 0·5, [HSA] = 2·5 × 10^{-4} M. For pH 6–8, 0·1 M phosphate buffer and for pH 9, 0·1 M borate buffer used. C. pH dependence of quenching of protein fluorescence by tolmetin using 0·1 M phosphate buffer (for pH 8) and 0·1 M borate buffer (for pH 8). [HSA] = 6·7 × 10^{-5} M, D/P ratio = 0·36.

binding site of tolmetin in the same way as they did for the warfarin primary binding site (Wilting et al 1980a). Calcium ions gives rise to an increase in the ellipticity of the complexes (Fig. 5a) and substantially increase the affinity of the drug for HSA as observed by dialysis experiments (Fig. 6).

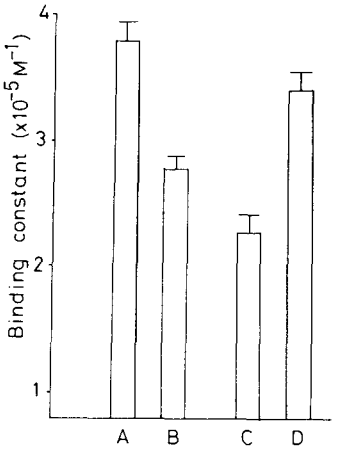

FIG. 6. Effect of calcium and chloride ions on tolmetin binding to HSA at 37 °C. A. 0·1 M of phosphate buffer. B. 0·1 M phosphate buffer with 0·1 M sodium chloride. C. 0·1 M Tris-HCl buffer. D. 0·1 M Tris-HCl buffer with 10^{-2} M calcium chloride.

Table 1. Effect of drugs on tolmetin binding to HSA ($2·5 \times 10^{-4}$ M) in 0·1 M phosphate buffer at 37 °C.

Competitive drug	Concn $\times 10^4$ M	Free concn of tolmetin $\times 10^5$ M	Binding constant $\times 10^{-5}$ M^{-1}
Tolmetin (alone)	1·25	0·248 ± 0·008	3·72
Warfarin	1·25	0·294 ± 0·015**	3·09**
Diazepam	1·25	0·218 ± 0·016*	4·28*

* $P > 0·05$ significant difference between tolmetin alone and tolmetin with diazepam by two tailed t-test.
** $P > 0·01$ significant difference between tolmetin alone and tolmetin with warfarin by two tailed t-test.

Table 2. Effect of tolmetin on warfarin or diazepam binding to HSA ($2·5 \times 10^{-4}$ M) in 0·1 M phosphate buffer at 37 °C.

Competitor	Concn $\times 10^4$ M	Concn $\times 10^5$ M	Binding constant $\times 10^{-5}$ M^{-1}
		Free concn of warfarin	
Tolmetin	0	0·638 ± 0·044	1·28
Tolmetin	1·25	0·958 ± 0·024**	0·77**
		Free concn of diazepam	
Tolmetin	0	0·734 ± 0·022	1·08
Tolmetin	1·25	0·480 ± 0·020**	1·78**

Initial concentration of warfarin and diazepam were $1·25 \times 10^{-4}$ M, respectively.
** $P > 0·01$ significant difference between without and with tolmetin by two tailed t-test.

As described in the introduction, calcium ions were reported to decrease the affinity for diazepam to HSA, but for warfarin the presence of calcium increased the affinity. The difference in behaviour of the complexes of warfarin and diazepam with HSA in the presence of calcium ions may be a useful method for distinguishing between the warfarin site and diazepam site. To confirm the tolmetin binding site, mutual displacement experiments were performed using warfarin and diazepam as competitors (Tables 1, 2). In the case of the combination of warfarin and tolmetin, the free concentration of one drug was significantly increased in the presence of the other drug. On the other hand, it was observed that tolmetin increased the affinity of diazepam for HSA and diazepam also caused an increase in the affinity of tolmetin for HSA.

Juni et al (1983) reported that the affinity of indoprofen for albumin rose in the presence of diazepam in spite of its binding site being the diazepam site, and that the thermodynamic parameters for the interaction of indoprofen with HSA indicated that at low concentrations indoprofen interacts with HSA endothermically while at higher concentrations it reacts exothermically. Similarly, as shown in Fig. 1, tolmetin reacts with HSA endothermically at low concentration whereas it interacts with HSA exothermically at high concentration. The endothermic interaction of drug with HSA seems to cause allosteric changes near the diazepam binding site, resulting in an increase of the binding of diazepam to HSA, in the presence of tolmetin.

The evidence presented herein gives more evidence of the N–B transition in albumin and shows the role of this conformational change in the protein binding of the anti-inflammatory drug tolmetin. The physiological meaning of this role in terms of a transport function for the albumin molecule has yet to be elucidated.

REFERENCES

Attallah, N. A., Lata, G. F. (1968) Biochim. Biophys. Acta 168: 321–333

Behm, H. L., Flynn, G. L., Wagner, J. G. (1981) Biopharm. Drug Dispos. 2: 235–244

Bowmer, C. J., Lindup, W. E. (1978a) Biochem. Pharmacol. 27: 937–942

Bowmer, C. J., Lindup, W. E. (1978b) J. Pharm. Sci. 67: 1193–1195

Fleitman, J., Perrin, J. H. (1982) Int. J. Pharm. 11: 227–236

Harmsen, B. J., DeBruin, S. H., Janssen, L. H. M., Rodrigues, J. F., Van, G. A. J. (1971) Biochemistry 10: 3217–3221

Juni, K., Nieves, E. A., Perrin, J. H. (1983) Biopharm. Drug Dispos. 4: 1–7

Leonard, J. W., Foster, J. F. (1961) J. Biol. Chem. 236: 2662–2669

Leonard, J. W., Vijai, K. K., Foster, J. F. (1963) Ibid. 238: 1984–1988

Nikkel, H. J., Foster, J. F. (1971) Biochemistry 10: 4479–4486

Pederson, K. O. (1972) Scand. J. Clin. Lab. Invest. 30: 89

Perrin, J. H., Vallner, J. J., Wold. S. (1974) Biochem. Biophys. Acta 371: 482–490

Rosen, A. (1970) Biochem. Pharmacol. 19: 2075–2081

Saroff, H. A., Lewis, M. S. (1963) J. Phys. Chem. 67: 1211–1216

Selley, M. L., Madsen, B. W., Thomas, J. (1978) Clin. Pharmacol. Ther. 24: 694–705

Wilting, J., Weideman, M. M., Roomer, A. C., Perrin J. H. (1979) Biochim. Biophys. Acta 579: 469–473

Wilting, J., Van der Giesen, W. F., Janssen, H. H. M., Weideman, M. M., Otagiri, M., Perrin, J. H. (1980a) J. Biol. Chem. 255: 3032–3037

Wilting, H., T'Hart, B. J., De Gier, J. J. (1980b) Biochim. Biophys. Acta 626: 291–298

Velick, S. F., Parker, C. W., Eisen, H. N. (1960) Proc. Natl. Acad. Sci. 46: 1470–1482