Bender, M. L., *et al.* (1966), *J. Amer. Chem. Soc. 88*, 5890.
Bender, M. L., and Brubacher, L. J. (1966), *J. Amer. Chem. Soc. 88*, 5880.
Brubacher, L. J., and Bender, M. L. (1966), *J. Amer. Chem. Soc. 88*, 5871.
Finkle, B. J., and Smith, E. L. (1958), *J. Biol. Chem. 230*, 669.
Glazer, A. N., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 2948.
Glazer, A. N., and Smith, E. L. (1965), *J. Biol. Chem. 240*, 201.
Heinrikson, R. L. (1970), *Biochem. Biophys. Res. Commun. 41*, 967.
Husain, S. S., and Lowe, G. (1969), *Biochem. J. 114*, 279.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw Hill, pp 78–111.
Kimmel, J. R. (1967), *Methods Enzymol. 11*, 584.
Klein, I. B., and Kirsh, J. F. (1969a), *Biochem. Biophys. Res. Commun. 34*, 575.
Klein, I. B., and Kirsh, J. F. (1969b), *J. Biol. Chem. 244*, 5928.
Light, A., Frater, R., Kimmel, J. R., and Smith, E. L. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1276.
Mitchel, R. E. J., Chaiken, I. M., and Smith, E. L. (1970), *J. Biol. Chem. 245*, 3485.
Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 893.
Rick, W. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic Press, p 807.
Sanner, T., and Pihl, A. (1963), *J. Biol. Chem. 238*, 165.
Shields, G. S., Hill, R. L., and Smith, E. L. (1959), *J. Biol. Chem. 234*, 1747.
Sluyterman, L. A. E. (1967), *Biochim. Biophys. Acta 139*, 430.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Wallenfels, K., and Eisele, B. (1968), *Eur. J. Biochem. 3*, 267.
Whitaker, J. R., and Bender, M. L. (1965), *J. Amer. Chem. Soc. 87*, 2728.
Wolthers, B. G., Drenth, J., Jansonius, J. N., Koekoek, R., and Gwen, H. M. (1970), Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., New York, N. Y., Academic Press, p 272.

Physicochemical Studies of the Relation between Structure and Function in Hemoglobin Hiroshima (HC3β, Histidine → Aspartate)*

Kiyohiro Imai,† Howard B. Hamilton,‡ Takaoki Miyaji,§ and Susumu Shibata ¶

ABSTRACT: Various physicochemical properties of Hb Hiroshima (HC3β, histidine → aspartate) were studied to clarify the mechanism for the altered oxygen equilibrium functions of the hemoglobin and the role of the C-terminal histidine residues of the β chains. The cooperativity in oxygen binding of Hb Hiroshima is markedly diminished by stripping it of 2,3-diphosphoglycerate (DPG). The effect of DPG on the oxygen equilibrium of Hb Hiroshima is, however, apparently normal because its overall oxygen affinity exhibits the same dependence on the concentration of DPG as that of Hb A and the overall free energy of interaction among the binding sites of oxygen of Hb Hiroshima increases on the addition of DPG by an extent similar to that of Hb A. Ultraviolet difference spectra suggest that the conformational changes of the $\alpha_1\beta_2$ contacts that occur during oxygenation are impaired in Hb Hiroshima corresponding to its diminished cooperativity and that the penultimate tyrosine residues of the β chains in Hb Hiroshima undergo environmental changes similar to those which occur in Hb A during oxygenation. The intrinsic microscopic equilibrium constant for the fourth stage of oxygenation, k_4, for Hb Hiroshima is insensitive to the concentration of DPG and nearly equal to that for Hb A, whereas the constant for the first stage of oxygenation, k_1, decreases on the addition of DPG and is always larger than that of Hb A, irrespective of DPG concentration, indicating that deoxy-Hb Hiroshima probably assumes a more unconstrained form than deoxy-Hb A. The reaction rates of 4,4′-dipyridine disulfide with the sulfhydryl groups of oxy- and deoxy-Hb Hiroshima were more rapid than for Hb A, particularly that of the deoxy form. Electron paramagnetic resonance spectra of NO-Hb Hiroshima are identical with those of NO-Hb A.

Hemoglobin Hiroshima is a mutant hemoglobin with greatly altered oxygen equilibrium functions: it has an oxygen affinity 5-fold higher than that of Hb A[1] at pH 6.5, a decreased Bohr effect about half that of Hb A, and reduced heme–heme interaction (Hill's constant, 2.0–2.6, compared to 3.0 for Hb A) (Imai, 1968). The carriers of this hemoglobin have no apparent clinical symptoms save a mild erythrocytosis in com-

* From the Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka, Japan, from the Department of Clinical Laboratories, Atomic Bomb Casualty Commission, Hiroshima, Japan, and from the Third Division of Internal Medicine, Yamaguchi University School of Medicine, Ube, Japan. This paper was presented in part at the 8th International Congress of Biochemistry, Interlaken, Switzerland, Sept 3–9, 1970.

† Present address: the Department of Physicochemical Physiology, Medical School of Osaka University, Osaka, Japan.
‡ To whom correspondence should be sent at the Department of Clinical Laboratories, Atomic Bomb Casualty Commission. The Commission is a cooperative research agency of the U. S. National Academy of Sciences, National Research Council, and the Japanese National Institute of Health of the Ministry of Health and Welfare, with funds provided by the U. S. Atomic Energy Commission, the Japanese Institute of Health, and the U. S. Public Health Service.

§ Third Division of Internal Medicine, Yamaguchi University School of Medicine.
¶ Department of Medicine, Kawasaki Medical College Hospital.

pensation for its less efficient oxygen-transport function (Hamilton *et al.*, 1969).

Although the biochemical lesion in Hb Hiroshima was initially judged to be an amino acid substitution, histidine → aspartate, at the site H21(143)β, recent X-ray crystallographic studies of deoxyhemoglobin Hiroshima at 3.5-Å resolution and confirmatory chemical examination (Perutz *et al.*, 1971) showed that the amino acid substitution in Hb Hiroshima is at HC(146)β, *i.e.*, the C-terminal residue of the β chain.

Previously, on the basis of a tentative atomic model for oxyhemoglobin at 5.5-Å resolution (Perutz, 1965), we suggested that the altered oxygen binding properties of Hb Hiroshima might be explicable in terms of interference with the cross-links between sister β chains, specifically, those involving H21(143)β, where the amino acid substitution was thought to be (Imai, 1968; Hamilton *et al.*, 1969). A more recent atomic model at 2.8-Å resolution (Perutz *et al.*, 1969), however, showed that the bridges between sister β chains are of minor importance and do not involve the H21β residue.

Recent evidence suggests that certain amino acid residues at or near the C terminal of the β chain play important roles in the binding of ligands by normal hemoglobin: HC3 histidine in oxygen-linked protonation (Perutz *et al.*, 1969); HC2 tyrosine in triggering conformational changes (Perutz, 1970); H21 histidine in binding DPG (Bunn and Briehl, 1970; Perutz, 1970). Thus it would appear that further studies of Hb Hiroshima might yield more detailed information on the contribution of the C-terminal of the β chain to these reactions. We report here results of the following studies: the effect of DPG on oxygen equilibrium, uv difference spectra, reactivity of sulfhydryl groups, and epr spectra of NO-Hb derivatives. In the light of these results we discuss the relation between the functional and structural abnormalities of this mutant hemoglobin, and also the role of the C-terminal residues of the β chain in the oxygen-binding functions of hemoglobin.

Materials and Methods

Preparation of Hemoglobins. Hb Hiroshima and Hb A as control were obtained from the same hemolysate in oxygenated form. The hemoglobins were separated from each other by two methods: column chromatography and starch-block electrophoresis. The hemolysate containing Hb Hiroshima was put on a CM-Sephadex column equilibrated with 0.05 M phosphate buffer (pH 6.0), and Hb F followed by Hb Hiroshima were eluted with 50 mM Na^+; Hb A and Hb A_2 were eluted together with 100 mM Na^+. Hb A was separated from Hb A_2 on a DEAE-Sephadex column equilibrated with 0.05 M Tris-HCl buffer having a pH gradient of 8.6–7.0. Starch-block electrophoresis was employed using Tris–EDTA–borate buffer (pH 8.6). The purities of the separated hemoglobins were tested by agar-gel electrophoresis. No significant contamination could be detected for either Hb A separated by column chromatography, or for Hb Hiroshima. Hb A separated by electrophoresis, however, was contaminated with a small amount of Hb F (less than 3%). Hemoglobins separated by column chromatography were used for oxygen equilibrium and uv difference spectra, and hemoglobins separated by electrophoresis for reactivity of the sulfhydryl groups and epr of nitric oxide derivative experiments. Hemoglobins, stripped of DPG as described previously by Benesch *et al.* (1968), contained no detectable DPG when assayed by the method of Ames and Dubin (1960). Hemoglobin concentrations were determined by spectrophotometry of pyridine hemochromogen derivatives (Paul *et al.*, 1953) and are given on heme basis unless otherwise stated. DPG concentration was determined by titration (Benesch *et al.*, 1969). DPG was obtained as the pentacyclohexylammonium salt tetrahydrate (Calbiochem, Los Angeles) and converted into the free acid by passage through a column of the H form of Dowex 50; Bis-Tris and 4-PDS were used without further treatment (Aldrich Chemical Co., Milwaukee).

Uv Difference Spectra. Deoxyhemoglobin as reference for difference spectra of oxy- *vs.* deoxyhemoglobin in 0.1 M glycine–NaOH buffer was prepared in a 200-ml cylindrical tonometer with a square-fused silica cuvet of 10-mm light path. Deoxyhemoglobin as reference for uv difference spectra of oxy- *vs.* deoxyhemoglobin in the presence and absence of DPG was prepared in Benesch's versatile tonometer (Benesch *et al.*, 1965) which contained stripped hemoglobin solutions without and with DPG in the cuvet and reservoir, respectively. The uv difference spectrum in the absence of DPG was determined using the deoxyhemoglobin solution in the cuvet and the spectrum in the presence of DPG by mixing the solutions in the cuvet and reservoir. In each instance, deoxygenation was performed by repeated alternate evacuations and flushings with pure nitrogen gas (99.999%), accompanied by mild shaking of the tonometer. The concentration of oxyhemoglobin was adjusted to that of reference deoxyhemoglobin by using an isosbestic point of 548 mμ. The difference spectra were recorded by a Hitachi Model 124 spectrophotometer (Hitachi Ltd., Tokyo) with a fixed slit width and servo control of photomultiplier voltage. The band width of the incident light was constantly 1 mμ over the entire range of wavelength used for the determination. Uv absorption spectra between 240 and 350 mμ of oxyhemoglobin and of reoxygenated hemoglobin previously used for the reference coincided with each other within 2%. Methemoglobin content, determined as described by Benesch *et al.* (1965), was less than 8% of total hemoglobin after the determination except in the case of stripped hemoglobin where the content amounted to 15%.

Reactivity of the Sulfhydryl Groups with 4-PDS. The rate of reaction of the reactive sulfhydryl groups of hemoglobin with 4-PDS was determined as described by Ampulski *et al.* (1969) with minor modifications. For the reaction of 4-PDS with deoxyhemoglobin Benesch's versatile tonometer was used. Oxyhemoglobin and 4-PDS solutions put into the cuvet and the reservoir of the tonometer, respectively, were deoxygenated and the reaction was started by mixing them with each other. The change of optical density at 324 mμ over time was recorded by a Hitachi Model 124 spectrophotometer.

Oxygen equilibrium curves of hemoglobin in the absence or the presence of DPG were determined by the automatic recording apparatus of Imai *et al.* (1970). Methemoglobin content (Benesch *et al.*, 1965) was 11 and 15% of total hemoglo-

[1] Abbreviations used are: Hb, hemoglobin; DPG, 2,3-diphosphoglycerate; epr, electron paramagnetic resonance; NO-Hb, nitric oxide derivative of hemoglobin; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; 4-PDS, 4,4′-dipyridine disulfide; P_{50}, oxygen pressure at 50% oxygenation of hemoglobin; n, Hill's constant, empirical measure of cooperativity; Y, fractional oxygen saturation; p, oxygen pressure; ΔF_I, free energy of interaction; ΔOD, change in optical density. The location of an amino acid residue, *e.g.*, H21(143)β, is given according to Perutz's system that specifies its position within a segment of the chain and permits comparison of homologous sections of myoglobin, and the α, β, and γ chains (Perutz, 1965). The figure in parentheses is the residue's position numbered from the amino terminal.

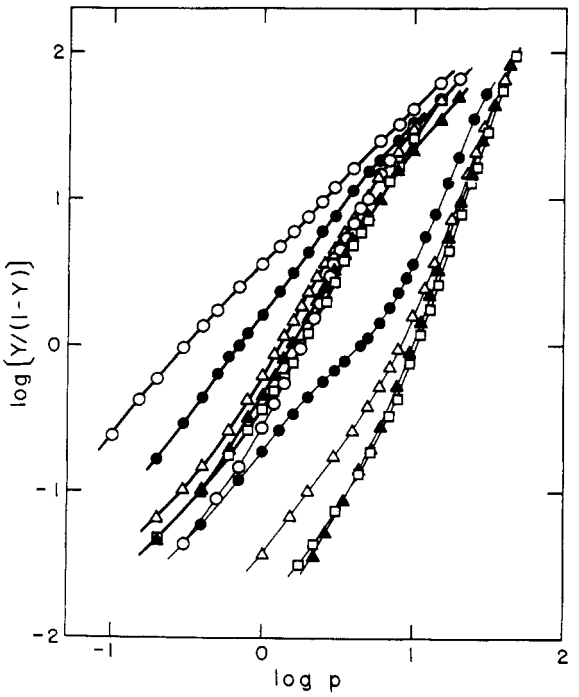

FIGURE 1: Effect of DPG on the oxygen equilibrium curves of Hb Hiroshima and Hb A. p, oxygen pressure (mm of Hg); Y, fractional saturation of hemoglobin with oxygen. (○) DPG-free (stripped); (●) 0.01 mM DPG; (△) 0.05 mM DPG; (▲) 0.15 mM DPG; (□) 0.3 mM DPG. Bold and fine lines are for Hb Hiroshima and Hb A, respectively. Hb concentration, 6×10^{-5} M; in 0.01 M Tris-HCl buffer, pH 7.0; temperature, 20°.

bin, respectively, before and after determination of oxygen equilibrium.

Epr Spectra of NO-Hb. Epr spectra of NO-Hb were measured as described by Shiga *et al.* (1968) using a Varian Model E12 epr spectrometer (Varian Associates, Palo Alto, Calif.).

Results

Effect of DPG on Oxygen Equilibrium Curves. Oxygen equilibrium curves of Hb Hiroshima and Hb A in the absence (*i.e.*, stripped) or in the presence of various amounts of DPG are shown in Figure 1, where they are represented by Hill plots (Wyman, 1964). The plot was biphasic as reported previously (Benesch *et al.*, 1968; Imai *et al.*, 1970) for Hb A in 0.01 mM DPG where the hemoglobin was not saturated with DPG.

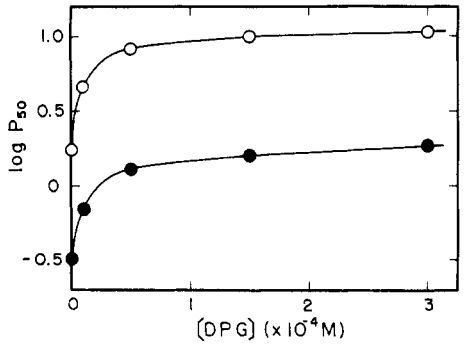

FIGURE 2: The dependence of the oxygen affinities of Hb Hiroshima (●) and Hb A (○) on the concentration of DPG. P_{50} is oxygen pressure (mm of Hg) at 50% saturation. Conditions as for Figure 1.

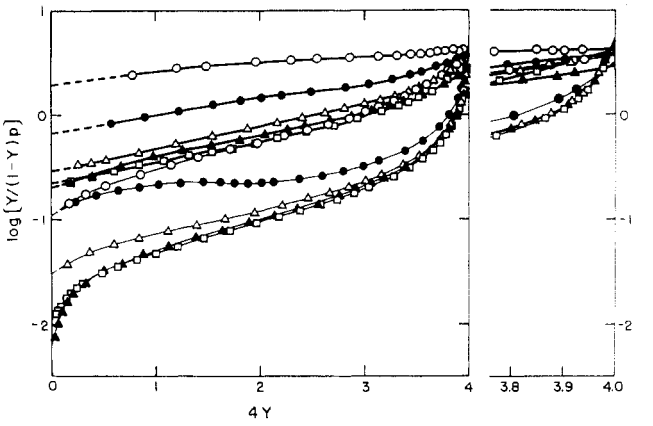

FIGURE 3: Scatchard plots of the data shown in Figure 1. Symbols are as for Figure 1. In the portion on the right hand the scale of abscissa is expanded.

The logarithm of oxygen pressure at 50% oxygen saturation of hemoglobin, log P_{50}, which is a simple measure of overall oxygen affinity, was obtained from the data shown in Figure 1 and plotted against DPG concentration (see Figure 2). The effect of DPG on the oxygen affinity of Hb Hiroshima was similar to that of Hb A: the ratio of P_{50} in the presence of 0.3 mM DPG to P_{50} in the absence of DPG was 5.94 for Hb Hiroshima and 5.91 for Hb A. Contrary to the normal dependence of oxygen affinity of Hb Hiroshima on DPG, the cooperativity in oxygen binding of Hb Hiroshima showed a different dependence on DPG from that of Hb A: the cooperativity of Hb Hiroshima was diminished markedly by the stripping of DPG while that of Hb A was diminished only slightly. Hill's constant, n, which is regarded as an empirical measure of cooperativity, in Hb Hiroshima was decreased from 1.9 in 0.3 mM DPG to 1.1 by stripping, yielding an almost hyperbolic oxygen equilibrium curve, while that of Hb A decreased slightly from 2.8 to 2.4 without altering appreciably the shape of the curve. These results are summarized in Table I. The values of n, 1.9 and 2.8, are somewhat smaller than those which were previously obtained for Hb Hiroshima and Hb A in 0.1 M phosphate buffer (pH 7.0), *i.e.*, 2.2 and 3.0, respectively (Imai, 1968). This may be due to the larger amount of methemoglobin in the samples used for the present studies (15%) than in those of the previous studies (3%). The upper ends of all the Hill plots in Figure 1 apparently converge toward a common asymptote while the lower ends diverge from one another. Scatchard plots (Edsall *et al.*, 1954) of the data demonstrate this feature more clearly (see Figure 3). The extrapolation of the Scatchard plots to $4Y \rightarrow 4 (p \rightarrow \infty)$ yielded intercepts around 0.6 on the right coordinate irrespective of the kind of hemoglobin and concentration of DPG. This shows that Hb Hiroshima and A have similar intrinsic microscopic equilibrium constants for the fourth stage of oxygenation, k_4, in Adair's equation for successive oxygenation (Adair, 1925), and that k_4 is almost independent of DPG concentration (see Table I). On the other hand, the intercepts on the left coordinate, which corresponded to the equilibrium constant for the first stage, k_1, differed from each other depending on the kind of hemoglobin and the concentration of DPG. The values of k_1 for both Hb Hiroshima and Hb A increased by about 10-fold by stripping of 0.3 mM DPG, but those for Hb Hiroshima were always larger than those for Hb A irrespective of the concentration of DPG (see Table I). From the values of k_1 and k_4, a free energy of interaction, ΔF_1, which

TABLE I: Effect of DPG on Oxygen Equilibrium.

	Hb Hiroshima		Hb A	
DPG (mM)	0 (stripped)	0.3	0 (stripped)	0.3
P_{50}[a] (mm of Hg)	0.31	1.84	1.81	10.7
P_{50}^{DPG}/P_{50}^{st}[b]	5.94		5.91	
n[c]	1.1	1.9	2.4	2.8
k_1[d] (mm Hg^{-1})	2.0	0.22	0.11	0.011
k_4[e] (mm Hg^{-1})	4.3	4.3	3.7	4.6
ΔF_I[f] (cal/mole per site)	450	1710	2040	3580
$\Delta(\Delta F_I)$[g] (cal/mole per site)	1260		1540	

[a] Oxygen pressure at 50% saturation. [b] P_{50}^{DPG} and P_{50}^{st} in the presence of 0.3 mM DGP and in its absence (stripped). [c] The Hill constant. [d,e] Intrinsic microscopic equilibrium constants for the first and fourth stages of oxygenation, in mm Hg^{-1}. [f,g] Overall free energy of interaction, and the increment of free energy due to addition of DPG, in calories per mole per site.

is involved in the cooperative interaction among hemes as defined by Wyman (1964), was estimated as previously described (Tyuma *et al.*, 1971). The ΔF_I was increased by the addition of DPG both for Hb Hiroshima and Hb A, but the value for Hb Hiroshima was smaller than that for Hb A whether in the presence or absence of DPG, indicating a parallel dependence of ΔF_I to Hill's constant, n (see Table I). The increment of ΔF_I due to the addition of 0.3 mM DPG was 1260 and 1540 cal per mole per site for Hb Hiroshima and Hb A, respectively, but the discrepancy between these values is probably not significant and may be ascribable to experimental error. The effects of DPG on oxygen equilibrium of Hb A described above coincide well with those reported previously by Tyuma *et al.* (1971).

Uv Difference Spectra. Figure 4 shows the uv difference spectra of carbonmonoxyhemoglobin Hiroshima and A, pH 10.7 *vs.* 8.4. Both the hemoglobins had almost identical ΔOD at 245 mμ. Figure 5 shows the uv difference spectra of oxy- *vs.* deoxyhemoglobin. The ΔOD at 242 mμ for Hb A was very close to zero at pH 8.4 but increased toward positive at pH 10.3 probably because of environmental changes around the HC2β tyrosine residues following their ionization. The ΔOD at 242 mμ of Hb Hiroshima exhibited a dependence on pH similar to that of Hb A.

Slight but significant discrepancies were, however, found between the difference spectra of oxy- *vs.* deoxyhemoglobin Hiroshima and A in the range of 280 to 295 mμ. Narrow banded spectra with two maxima at 283 and 291 mμ and one minimum at 288 mμ, superposed on a broad band with a peak at 275 mμ, appeared in the difference spectra of Hb Hiroshima and A, but the difference between the maximum at 291 mμ and the minimum for the former was somewhat diminished compared to that for the latter, both at pH 8.4 and 10.3 (see Figure 5).

The narrow banded spectra of stripped Hb Hiroshima was considerably diminished corresponding to its greatly impaired cooperativity (n = 1.1) (Figure 6A), but returned to approximately the same magnitude as that in 0.1 M glycine–NaOH buffer (compare Figure 5) on the addition of 0.3 mM DPG, when cooperativity was intermediate (n = 1.9). On the other hand, the narrow banded spectra of Hb A was not affected significantly by the addition of DPG and it exhibited only a small change of n (see Figure 6B and Table I).

Reactivity of the Sulfhydryl Groups to 4-PDS. Figure 7 shows the reaction rates of the sulfhydryl groups in oxy- and deoxyhemoglobins with 4-PDS. The number of reactive sulfhydryl groups in oxyhemoglobin per tetramer was 2.14, 2.21, and 2.37 for Hb Hiroshima, Hb A separated from Hb Hiroshima, and normal adult Hb A, respectively. The reactions of the deoxyhemoglobins could not be followed to the end because the rates were too low but the number of reactive sulfhydryl groups were assumed to be the same as those of oxyhemoglobin. The rate plot was linear for Hb A but only approximately so for Hb Hiroshima indicating a pseudo-first-order reaction. Apparent first-order rate constants were obtained from the initial slope of each plot and are summarized in Table II. Oxy-Hb Hiroshima reacted somewhat more

TABLE II: Apparent First-Order Rate Constants for the Reaction of 4-PDS with Sulfhydryl Groups of Hb Hiroshima and Hb A.[a]

	Oxy Form	Deoxy Form
Hb Hiroshima	0.480[b]	0.250
Hb A[c]	0.190	0.0326
Hb A[d]	0.214	0.0292

[a] Conditions as for Figure 7. [b] In min^{-1}. [c] Hb A separated from sample containing Hb Hiroshima. [d] Hb A from a normal adult.

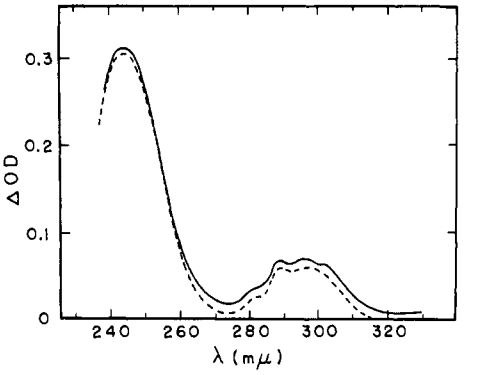

FIGURE 4: Ultraviolet difference spectra of CO-Hb Hiroshima (solid line) and CO-Hb A (dashed line), pH 10.7 *vs.* 8.4. Hb concentration, 3×10^{-5} M; in 0.1 M glycine–NaOH buffer.

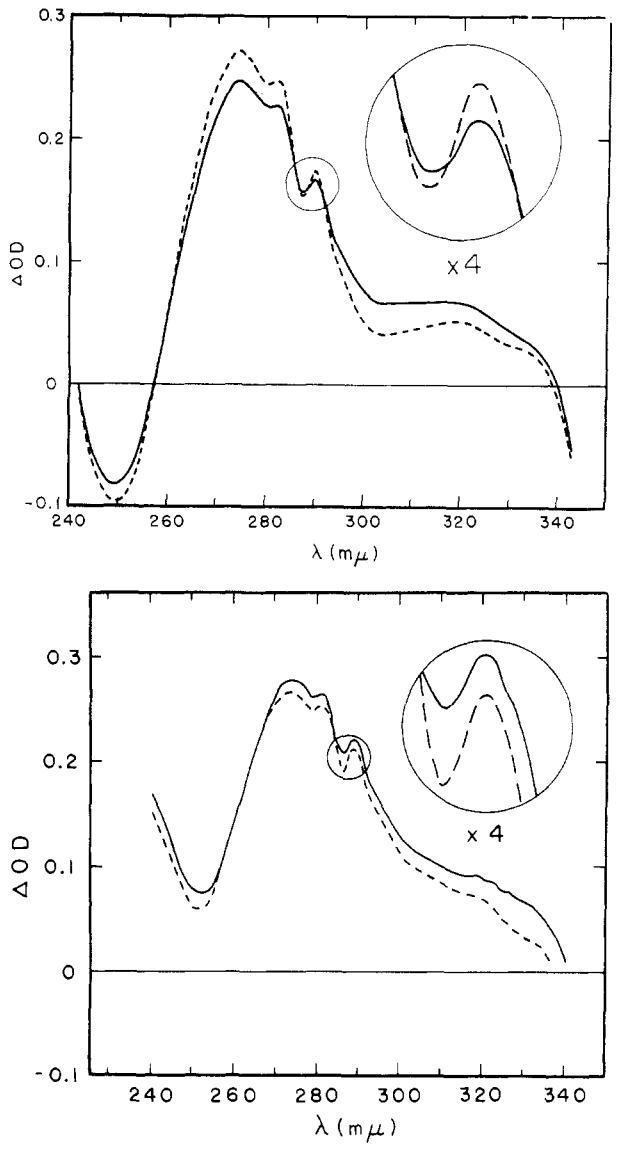

FIGURE 5: Ultraviolet difference spectra of oxyhemoglobin *vs.* deoxyhemoglobin at pH 8.4 (A, top), and 10.3 (B, bottom). —, Hb Hiroshima; ----, Hb A. Hb concentration, 6×10^{-5} M; in 0.1 M glycine–NaOH buffer. Narrow banded spectra around 291 mμ in the circle are enlarged 4-fold.

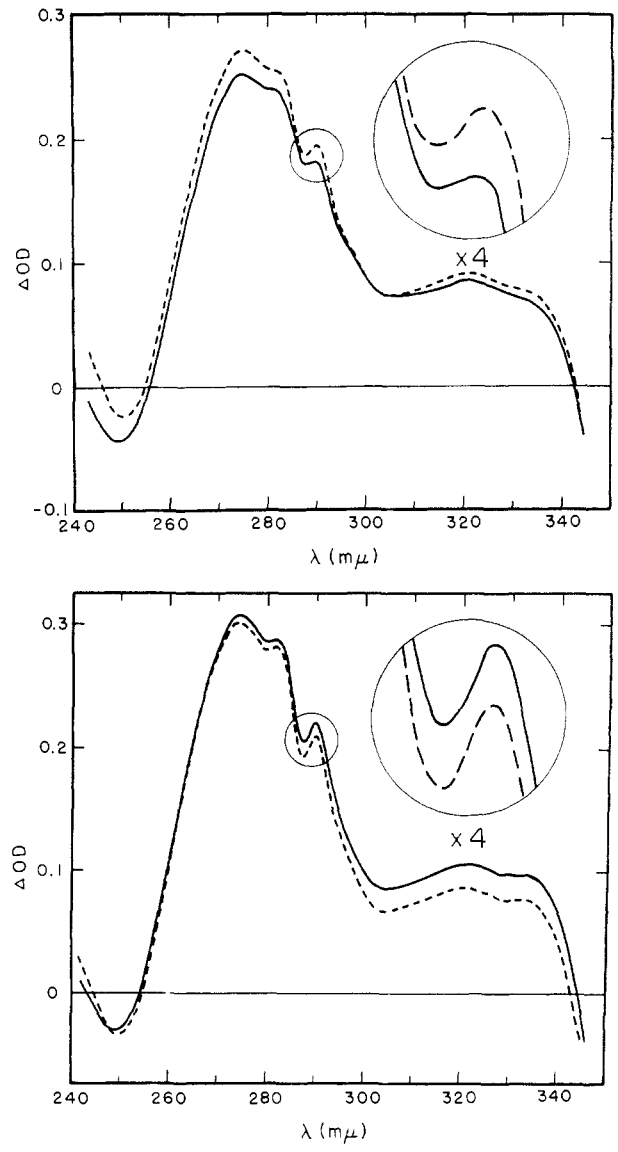

FIGURE 6: Ultraviolet difference spectra of oxyhemoglobin *vs.* deoxyhemoglobin in the absence of DPG (——) and in the presence of 0.3 mM DPG (----). (A, top) Hb Hiroshima; (B, bottom) Hb A. Hb concentration, 6×10^{-5} M; in 0.01 M Bis-Tris buffer, pH 7.0. Narrow banded spectra around 291 mμ in the circle are enlarged 4-fold.

rapidly (2.5-fold) than Hb A but the deoxy form reacted much more rapidly (8-fold).

Epr Spectra of Nitric Oxide Derivatives. Nitric oxide bound to ferrous hemoglobin, unlike oxygen or carbon monoxide, exhibits an epr spectrum which originates from its odd electron and is a reflection of the ambient structure of globin around the hemes to some extent (Gordy and Rexroad, 1961; Shiga *et al.*, 1969). To investigate whether the structure around the hemes of Hb Hiroshima is affected by the amino acid substitution, we compared the epr spectra of NO Hb Hiroshima and Hb A. The spectra obtained at room temperature are shown in Figure 8; there were no significant differences at this temperature, or at +5, −20, −50, −70, −100, and −160° between the spectra of Hb Hiroshima and Hb A.

Discussion

The overall oxygen affinity of Hb Hiroshima shows almost the same dependence on the concentration of DPG as does Hb A. Moreover, the free energy of interaction, ΔF_1, of the mutant hemoglobin is increased by the addition of DPG to a degree similar to that of the normal hemoglobin. These results indicate that DPG has an influence on oxygen equilibrium properties including oxygen affinity and cooperativity of Hb Hiroshima equivalent to that of Hb A. Further, it is apparent that the C-terminal histidine residues of the β chains are not involved in the binding of DPG. The cooperativity of Hb Hiroshima, however, appears to be increased more markedly by the addition of DPG than does that of Hb A. Thus, the "intrinsic cooperativity," *i.e.*, the ΔF_1 in the absence of DPG, is only 450 cal/mole per site for Hb Hiroshima compared to 2040 cal/mole per site for Hb A. Consequently, the cooperativity is increased 3.8-fold for Hb Hiroshima but only 1.8-fold for Hb A by the addition of 0.3 mM DPG, although the absolute value of the increment $\Delta(\Delta F_1)$ is similar for the two hemoglobins (see Table I). Thus, it might be anticipated that the cooperativity of a hemoglobin with intermediate heme-

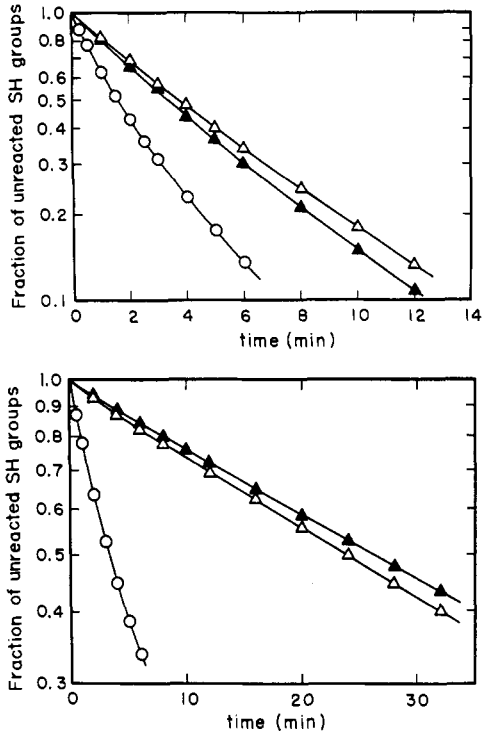

FIGURE 7: Pseudo-first-order rate plots of the reactions of the reactive sulfhydryl groups of oxyhemoglobin (A, top) and deoxyhemoglobin (B, bottom) with 4,4′-dipyridine disulfide (4-PDS). (○) Hb Hiroshima; (△) Hb A separated from Hb Hiroshima; (▲), Hb A from normal adult. Hb concentration, 4×10^{-5} M; initial concentration of 4-PDS, 1.6×10^{-4} M for oxyhemoglobin and 1.7×10^{-3} M for deoxyhemoglobin; in 0.1 M phosphate buffer, pH 7.0; temperature, 20°.

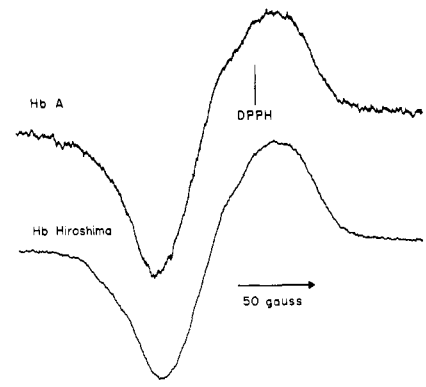

FIGURE 8: Electron paramagnetic resonance spectra of nitric oxide derivatives of Hb Hiroshima and Hb A. Microwave frequency, 9.532 GHz; modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 1.0 sec. Hb concentration, 1×10^{-3} M; in 0.1 M phosphate buffer, pH 7.0, at room temperature.

heme interaction would be strongly affected by DPG since the ratio of its "intrinsic" ΔF_I to the increment of ΔF_I caused by DPG is small. This has in fact been observed for *N*-ethylsuccinimide hemoglobin with an intermediate cooperativity ($n = 2$) (Benesch and Benesch, 1961; Riggs, 1961), that appears to be markedly diminished by stripping, though again, the decrement, $\Delta(\Delta F_I)$, is similar to that of unmodified hemoglobin A (K. Imai, unpublished observations).

H. F. Bunn (personal communication) found that the oxygen affinity of Hb Hiroshima in 0.05 M Bis-Tris buffer (pH 7.2) containing 0.1 M NaCl was decreased by DPG in the same manner as that of Hb A, but Hill's constant for Hb Hiroshima was increased slightly from 2.3 to 2.5 by the addition of 1 mM DPG, in contrast to our results where *n* decreased almost to 1. This is probably due to differences in experimental conditions. According to Benesch *et al.* (1969) chloride can also reduce oxygen affinity although the extent of the effect is not as marked as DPG; further, DPG no longer exerts an effect on oxygen affinity in the presence of 0.5 M NaCl. Apparently 0.1 M NaCl masks the effect of DPG on oxygen equilibrium by weakening the specific bonds between DPG and hemoglobin. Thus the insensitivity of Hill's constant to DPG in the presence of 0.1 M NaCl reported by Bunn is probably due to the large ionic strength of the solvent and his results are compatible with ours.

The "full cooperativity" of Hb Hiroshima, *i.e.*, that in the presence of DPG, even though increased to a greater extent over the stripped form than Hb A, is still less than normal. Briehl and Hobbs (1970) showed that the narrow banded uv spectra in the range from 280 to 295 mμ are form dependent, being attributable to perturbations of aromatic chromophores, and suggested that the tryptophans at C3β were probably involved. Bolton and Perutz (1970) demonstrated from their analysis of X-ray data that these C3β residues have contacts with five residues of the partner α chains in oxyhemoglobin which decrease to four in deoxyhemoglobin. Chemically modified Hb A from which two residues, the penultimate tyrosine and the C-terminal histidine of the β chain, are removed has no heme–heme interaction (Antonini *et al.*, 1961) and its form-dependent difference spectra are greatly diminished (Nagel *et al.*, 1966). Genetically modified Hb Bethesda (HC2β, tyrosine → histidine) has reduced heme–heme interaction (Hayashi *et al.*, 1971) and its form-dependent difference spectra are likewise diminished (K. Imai, unpublished observations). This evidence strongly suggests that the form-dependent difference spectra are related to the conformational changes at the $\alpha_1\beta_2$ contacts which accompany the cooperative reactions of oxygen binding. The dimin shed narrow-banded spectra of Hb Hiroshima imply that the conformational change at the $\alpha_1\beta_2$ contact during oxygenation is impaired, resulting in turn in the impaired cooperativity of oxygen binding of Hb Hiroshima.

The proposed mechanism to explain impaired cooperativity of hemoglobin variants such as Hb Chesapeake (Nagel *et al.*, 1967), Yakima (Novy *et al.*, 1967), Kempsey (Reed *et al.*, 1968), and Kansas (Bonaventura and Riggs, 1968), which have amino acid substitutions at the sites of $\alpha_1\beta_2$ contacts, is a direct inhibition of the conformational changes at the $\alpha_1\beta_2$ contacts which are essential for rearrangement of the subunits in cooperative oxygen binding. In Hb Hiroshima the inhibition would probably be indirect because the amino acid substitution is remote from the region of $\alpha_1\beta_2$ contacts. According to Perutz's stereochemical model (Perutz, 1970) the C-terminal histidine residues of the β chains are free to rotate in oxyhemoglobin but in the deoxy form the histidine's imidazole side chain and α-carboxyl group form salt bridges with γ-carboxyl groups of FG1 aspartates of the same β chains and with the ε-amino groups of the C-5 lysines of the partner α chains, respectively, thereby tending to constrain the quaternary structure in the deoxy conformation. A plausible explanation for the diminished cooperativity of Hb Hiroshima was recently suggested by Perutz *et al.* (1971): Hb Hiroshima, with an aspartate replacing the β-terminal histidine, has no salt bridges between the Cβ termini and the FG1β aspartates, as a consequence of which the salt bridges with the C5α lysines are

probably rendered unstable, resulting in an impaired interaction between the α_1 and β_2 subunits.

Other findings reported here are likewise explicable in terms of the loss of the salt bridges between the Cβ termini and the FG1β aspartates in Hb Hiroshima, and were predicted recently by Perutz *et al.* (1971). Thus, Hb Hiroshima and Hb A have similar intrinsic microscopic equilibrium constants, k_4, for the fourth stage of oxygenation, but the constant for the first stage, k_1, for Hb Hiroshima is much larger than for Hb A. The former finding suggests that Hb Hiroshima and Hb A assume similar conformations after binding the third oxygen molecule. During oxygenation the C-terminal histidine residues of the β chains in Hb A are freed by rupture of their salt bridges, the constraints maintaining the molecule in the deoxy form are lost, and the molecule assumes the oxy form (Perutz, 1970). During oxygenation the salt bridges with C5α lysines in Hb Hiroshima will be ruptured and the C termini freed as in Hb A. Thus, Hb Hiroshima will assume an unconstrained conformation similar to that of Hb A. Epr spectroscopy of NO Hb Hiroshima showed that no detectable distortion of globin around the hemes of the β chains occurs. This is consistent with the observation that the k_4 for Hb Hiroshima and Hb A are similar, and with the proposal that the fourth oxygen molecule probably binds to the heme of one of the β chains (Perutz, 1970), an assumption supported by data of Tyuma *et al.* (1971). That k_1 for Hb Hiroshima was much larger than for Hb A is probably because, as discussed above, the deoxy form of Hb Hiroshima is less constrained than deoxy A, due to the absence of the salt bridges between the residues HC3β and FG1β. This tends to shift the equilibrium between the oxy and deoxy conformations toward the oxy form, thereby increasing the oxygen affinity of Hb Hiroshima.

Almost all sulfhydryl reactivity of hemoglobin is attributable to the sulfhydryl groups of the F9β cysteines. The reactivity of the sulfhydryl groups of Hb A is greatly decreased by deoxygenation (see Table II). This is accounted for by the salt bridge, described above, between HC3β and FG1β (94β), which limits accessibility to the adjacent F9β (93β) cysteine (Perutz, 1970). The reactivity of the sulfhydryl groups of the F9β cysteines of Hb Hiroshima is somewhat increased in the oxy form, but is much greater than Hb A in the deoxy form. There is no salt bridge in Hb Hiroshima to inhibit the SH reagent from reacting with the sulfhydryl groups. Similarly, the absence of this same salt bridge which, in Hb A, is responsible for half of the alkaline Bohr effect (Perutz *et al.*, 1969), clearly accounts for the halved Bohr effect of Hb Hiroshima (Pertuz *et al.*, 1971).

Nagel *et al.* (1966) suggested on the basis of experimental evidence from spectrophotometric titration and uv difference spectra that oxy- and carbonmonoxyhemoglobin A have eight normal tyrosine residues with pK's of 10.6 which decrease to six on deoxygenation, probably because of environmental changes around the HC2(145)β tyrosine residues. This is supported by Perutz's (1970) stereochemical model of hemoglobin. That the uv difference spectra of CO-Hb Hiroshima and A have almost identical ΔOD at 245 mμ suggests that they have an equal number of normal tyrosine residues (*i.e.*, eight), when they are in the carbonmonoxy and also probably in the oxy form. Further, the similarity of the pH dependent ΔOD at 242 mμ may be taken to indicate that the Hb Hiroshima HC2β tyrosine residues adjacent to the substituted aspartates at the C termini undergo environmental changes similar to those which occur in Hb A during oxygenation. From this it follows that the expulsion of the penultimate tyrosine residues from the pockets between the F and H helices of the β chains during oxygenation (Perutz, 1970) is probably not inhibited in Hb Hiroshima. On the other hand, the expulsion of the penultimate tyrosines seems to be inhibited in Hb Chesapeake (Nagel *et al.*, 1967) which is almost noncooperative, and moreover hemoglobins which have lost the penultimate tyrosine residues, such as Des-His (146β)-Tyr (145β) hemoglobin (Antonini *et al.*, 1961), Hb Rainier (HC2β, tyrosine → cysteine) (Hayashi *et al.*, 1971), and Hb Bethesda (HC2β, tyrosine → histidine) (Hayashi *et al.*, 1971), exhibit almost completely diminished cooperativity (A. Hayashi, 1971, personal communication). These data indicate that expulsion of the penultimate tyrosine seems to be essential to cooperative oxygen binding. That the cooperativity of Hb Hiroshima is not diminished completely may be attributed in part to the lack of any apparent constraints on the movement of the penultimate tyrosine.

It is worth noting that the oxygen equilibrium functions of Hb Hiroshima are quite similar to those of *N*-ethylsuccinimide hemoglobin (Benesch and Benesch, 1961; Riggs, 1961) and Des-His (HC3β) hemoglobin (Kilmartin and Wootton, 1970) with regard to increased oxygen affinity, halved Bohr effect, and intermediate cooperativity. The similarities seem to result from interference with the formation of, in the case of *N*-ethylsuccinimide hemoglobin, or in the absence of the salt bridges between the γ-carboxyl groups of the FG1β aspartates and the Cβ termini in Des-His hemoglobin and Hb Hiroshima. These three hemoglobins, two man made, the other a naturally occurring mutant, aptly illustrate the importance of the β terminal in the allosteric functions of hemoglobin and offer convincing support for Perutz's stereochemical model of hemoglobin.

Acknowledgment

The authors express their gratitude to Professor I. Tyuma, Dr. A. Hayashi, and Dr. T. Suzuki for their helpful comments. We also thank Dr. T. Shiga for his kind advice on the techniques for epr measurements.

References

Adair, G. S. (1925), *J. Biol. Chem. 63*, 529.

Ames, B. M., and Dubin, D. T. (1960), *J. Biol. Chem. 235*, 769.

Ampulski, R. S., Ayers, V. E., and Morell, S. A. (1969), *Anal. Biochem. 32*, 163.

Antonini, E., Wyman, J., Zito, R., Rossi-Fanelli, A., and Caputo, A. (1961), *J. Biol. Chem. 236*, PC60.

Benesch, R., and Benesch, R. E. (1961), *J. Biol. Chem. 236*, 405.

Benesch, R., Benesch, R. E., and Yu, C. I. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 526.

Benesch, R., Macduff, G., and Benesch, R. E. (1965), *Anal. Biochem. 11*, 81.

Benesch, R. E., Benesch, R., and Yu, C. I. (1969), *Biochemistry 8*, 2567.

Bolton, W., and Perutz, M. F. (1970), *Nature (London) 228*, 551.

Bonaventura, J., and Riggs, A. (1968), *J. Biol. Chem. 243*, 980.

Briehl, R. W., and Hobbs, J. F. (1970), *J. Biol. Chem. 245*, 544.

Bunn, H. F., and Briehl, R. W. (1970), *J. Clin. Invest. 49*, 1088.

Edsall, J. T., Felsenfeld, G., Goodman, D. S., and Gurds, F. R. N. (1954), *J. Amer. Chem. Soc. 76*, 3054.

Gordy, W., and Rexroad, H. N. (1961), *in* Free Radicals in Biological Systems, Blois, Jr., M. S., Brown, H. W.,

Lemmon, R. M., Lindblom, R. O., and Weissbluth, M., Ed., New York, N. Y., Academic Press, p 263.
Hamilton, H. B., Iuchi, I., Miyaji, T., and Shibata, S. (1969), *J. Clin. Invest. 48*, 525.
Hayashi, A., Stamatoyannopoulos, G., Yoshida, A., and Adamson, J. (1971), *Nature New Biol. 230*, 264.
Imai, K. (1968), *Arch. Biochem. Biophys. 127*, 543.
Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M. (1970), *Biochim. Biophys. Acta 200*, 189.
Kilmartin, J. V., and Wootton, J. F. (1970), *Nature (London) 228*, 766.
Nagel, R. L., Gibson, Q. H., and Charache, S. (1967), *Biochemistry 6*, 2395.
Nagel, R. L., Ranney, H. M., and Kucinskis, L. L. (1966), *Biochemistry 5*, 1934.
Novy, M. J., Edwards, M. J., and Metcalfe, J. (1967), *J. Clin. Invest. 46*, 1848.
Paul, K. G., Theorell, H., and Åkeson, Å. (1953), *Acta Chem. Scand. 7*, 1284.
Perutz, M. F. (1965), *J. Mol. Biol. 13*, 646.
Perutz, M. F. (1970), *Nature (London) 228*, 726.
Perutz, M. F., del Pulsinelli, P., Ten Eyck, L., Kilmartin, J. V., Miyaji, T., Iuchi, I., Shibata, S., and Hamilton, H. B. (1971), *Nature New Biol. 232*, 147.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219*, 131.
Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J., and Kilmartin, J. V. (1969), *Nature (London) 222*, 1240.
Reed, C. S., Hampson, R., Gordon, S., Jones, R. T., Novy, M. J., Brimhall, B., Edwards, M. J., and Koler, R. D. (1968), *Blood 31*, 623.
Riggs, A. (1961), *J. Biol. Chem. 236*, 1948.
Shiga, T., Hwang, K.-J., and Tyuma, I. (1968), *Arch. Biochem. Biophys. 123*, 203.
Shiga, T., Hwang, K.-J., and Tyuma, I. (1969), *Biochemistry 8*, 378.
Tyuma, I., Shimizu, K., and Imai, K. (1971), *Biochem. Biophys. Res. Commun. 43*, 423.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.