# Kinetic Analysis of the Recombination of NO with Ferrihemoproteins by the Flash Photolysis Method†

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ABSTRACT: The kinetic analysis of the recombination of NO with some ferric hemoproteins was performed by the use of flash photolysis and stopped-flow methods. The rate constants for recombination of NO with ferrimyoglobin obtained by the two methods were identical with each other in the whole pH range. The rate constants decreased with an increase in pH, giving a p$K$ value of 8.5 (cf. $5.2 \times 10^4$ $M^{-1}$ $s^{-1}$ at pH 6 and $1.3 \times 10^4$ $M^{-1}$ $s^{-1}$ at pH 10). The kinetic difference spectra of NO–ferrimyoglobin at 1 ms after flash were identical with the difference spectra of NO–ferrimyoglobin minus ferrimyoglobin at corresponding pHs. Unlike NO–ferrimyoglobin, NO–ferrihorseradish peroxidase gave different kinetics of NO binding for the two methods. Between pH 9.4 and 11.8, the velocity of NO recombination with the enzyme measured by flash photolysis remained constant, but that by the flow method decreased with increasing pH. Below pH 9.4, both methods gave an identical value of $1.9 \times 10^5$ $M^{-1}$ $s^{-1}$. The kinetic difference spectra showed that the acid form, but not the alkaline form, appeared first upon photolysis of NO–ferrihorseradish peroxidase even at alkaline pH. The acid form of peroxidase isoenzyme C re-formed the NO complex, while that of peroxidase isoenzyme A produced a mixture of the NO complex and the alkaline form. The data obtained here were compatible with the assumption that the formation of the alkaline form of the enzymes is the coordination of $OH^-$ at the sixth position, which is vacant at acidic pHs.

The photodissociability of various hemoproteins has been applied widely for the kinetic studies on ligand binding as the technique of flash photolysis. Applicability of this method, however, has been limited to the ferrous complexes of $O_2$, CO, and NO, since photodissociability is believed to be characteristic of the ferrous ligated states. Recently, photodissociation was also observed with the ferric NO complex (Tamura et al., 1978; Hoffman & Gibson, 1978). This finding opened the possibility of extending the various optical techniques to the ferric state as well as the ferrous state. Examples were flash photolysis studies at both ambient and cryogenic temperatures, where we demonstrated (Kobayashi et al., 1980) that the optical and electron paramagnetic resonance spectra of the photodissociated product of NO–ferric horseradish peroxidase (HRP)[1] trapped at 4.2 K were indistinguishable from that of native enzyme and proposed that HRP has a pentacoordinated structure in the ferric state. The present paper describes kinetic experiments on the flash photolysis of NO–ferrihemoproteins at room temperature; the results are compared with those obtained by stopped-flow experiments. A preliminary account of the present work has been given elsewhere (Tamura et al., 1978, 1981).

## Materials and Methods

HRP was purified from the crude material of Sigma (R.Z. = 0.1) by DEAE- and CM-cellulose column chromatography according to the method of Shannon et al. (1966). The isoenzymes used in this study were HRP-A and HRP-C according to the classification of Paul (1958) and Shannon et al. (1966). Sperm whale oxymyoglobin was prepared from meat by the method of Yamazaki et al. (1964). Ferrimyoglobin was obtained from the oxy form by treatment of ferricyanide, followed by DEAE-cellulose column chromatography. The NO complexes of ferrihemoproteins were prepared by two methods. In one method, nitrogen gas was passed over the surface of solutions of ferrihemoproteins in a small cuvette for 20 min with occasional stirring. The cuvette was evacuated, and then 1 atm of NO gas was introduced anaerobically. In the other method, a solution of 0.1 M phosphate buffer (pH 6–8), 0.1 M Tris-HCl (pH 8–10), or 0.1 M glycine–NaOH (above pH 10) was deaerated by extensive flushing with nitrogen and then was bubbled with NO gas anaerobically for at least 20 min. A small sample of concentrated hemoproteins was separately deoxygenated by repeated evacuation and flushing with nitrogen and added to the NO-saturated buffer solution. The concentration of NO in the solution was adjusted by mixing NO- and $N_2$-saturated buffer solutions. The value of 2 mM was used in the saturated NO solution. Optical absorption spectra were measured with a Cary 118 spectrometer. The concentration of HRP was determined spectrometrically by the use of an extinction coefficient at 403 nm of 107 $mM^{-1}$ $cm^{-1}$.

The photolysis experiment was carried out with a xenon flash lamp having a pulse width of 400 $\mu$s and an energy of 8 J and with an optical detection system. Temperature was thermostatically controlled at 20 ± 0.1 °C. The stopped-flow apparatus used was a Union Giken Model RA-401 spectrometer. A solution of ferrihemoprotein, which was prepared by careful equilibration with $N_2$ gas, was introduced into one of the reservoirs of the flow system.

## Results

*NO–Ferrimyoglobin.* Figure 1A shows the kinetic difference spectra in the Soret region obtained 1 ms after flash at different pHs. For comparison, the corresponding difference spectra of NO–ferrimyoglobin minus ferrimyoglobin are shown in Figure 1B. At pH 6, the kinetic difference spectrum had an absorption minimum at 420 nm and a broad peak around 400 nm and was similar to the difference spectrum of NO–ferrimyoglobin minus acid ferrimyoglobin at pH 6. Similarly, the kinetic difference spectrum at pH 10 equaled that of NO–ferrimyoglobin minus its alkaline form. The kinetic difference spectrum at pH 8.5 was intermediate between the two. The isosbestic point of 417 nm seen in Figure 1A cor-

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[1] Abbreviations: HRP, horseradish peroxidase; Tris, tris(hydroxymethyl)aminomethane.

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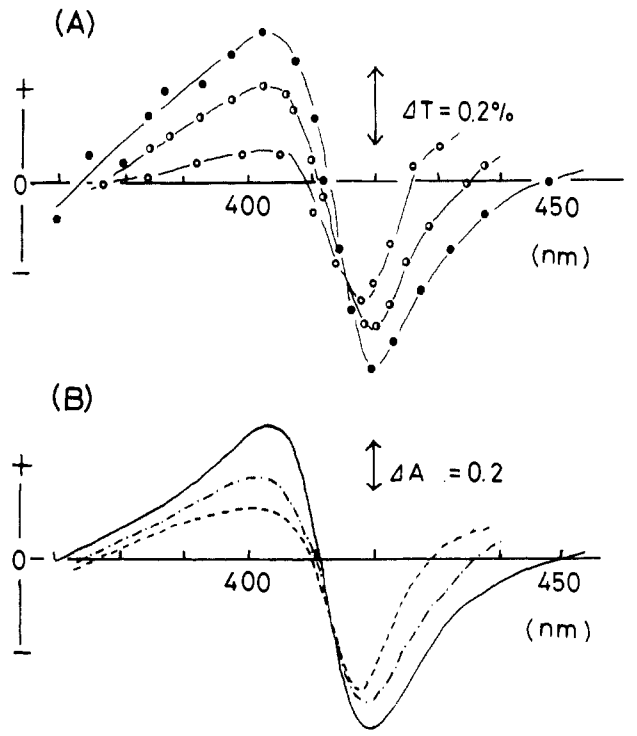



FIGURE 1: (A) Kinetic difference spectrum of flash photolysis of NO–ferrimyoglobin in the Soret region. The spectrum is taken at 1 ms after flash. (●) Phosphate buffer (pH 6.0), 0.1 M; (◑) Tris-HCl (pH 8.5), 0.1 M; (○) glycine–NaOH (pH 10.0), 0.1 M. (B) Difference spectra of NO–ferrimyoglobin minus ferrimyoglobin at pH 6.0 (—), 8.5 (·---), and 10.0 (- - -).

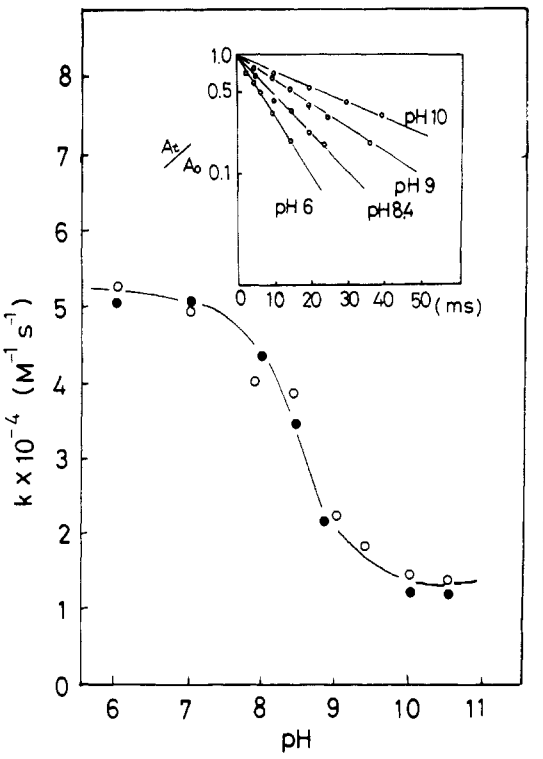



FIGURE 2: pH dependence of the rate constant of recombination of NO with ferrimyoglobin. (●) Stopped-flow method; (○) flash photolysis method. Inset: First-order plots of the absorption change at 403 nm by flash photolysis at different pHs. $A_t$ and $A_0$ are the absorption change at any time and the total absorption change, respectively. Buffers used are 0.1 M phosphate (pH 6–8), 0.1 M Tris-HCl (pH 8–10), and 0.1 M glycine–NaOH (above pH 10).

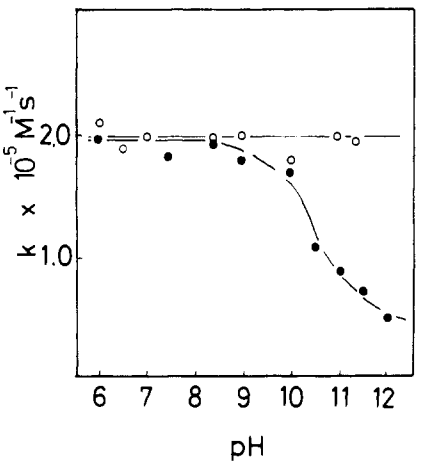



FIGURE 3: pH dependence of the rate constant of recombination of NO to HRP-C. The data obtained by the flash photolysis (○) and the stopped-flow (●) methods are plotted against pH.

responded with that between acid and alkaline forms of ferrimyoglobin (cf. Figure 1B). This indicates that the acid and alkaline forms or a mixture of the two forms of ferrimyoglobin appeared 1 ms after photolysis. Therefore, it is concluded that the absorption change after photolysis was due to photodissociation of the NO complex into ferrimyoglobin and NO, followed by the recombination process.

The recombination reaction of NO with ferrimyoglobin obeyed the pseudo-first-order kinetics in the whole pH range (inset of Figure 2) because of the high concentration of NO. This shows that the equilibrium was practically attained between the acid and alkaline forms at pH 8.4 and 9 during the recombination process. Figure 2 shows the pH–rate constant curves for the recombination of NO with ferrimyoglobin obtained by the flash photolysis and the stopped-flow methods. The rate constants obtained were not different between the two methods in the whole pH range (pH 6–10.5). The values decreased with the increase of pH, and its pH dependence gave a p$K$ value of 8.3. This corresponded to the p$K$ value for the acid–alkaline transition of ferrimyoglobin. From the figure, the second-order rate constants for NO binding to the acid and alkaline forms of ferrimyoglobin were calculated as $5 \times 10^4$ and $1.5 \times 10^4$ $M^{-1}$ $s^{-1}$, respectively.

*NO–HRP-C.* Figure 3 shows pH–rate constant curves for the recombination of NO with HRP-C obtained by flash photolysis and stopped-flow experiments. The rate constants obtained by the two methods were identical ($1.9 \times 10^{-5}$ $M^{-1}$ $s^{-1}$) in the pH range between 5 and 9. Above pH 9, however, the values determined by the stopped-flow method decreased with the increase of pH, giving a p$K$ value of 10.8. This value corresponded to the p$K$ value for the acid–alkaline transition of this enzyme. On the other hand, the values determined by the flash photolysis method remained constant until pH 11.8. This shows that the ferric enzyme which appeared immediately after photolysis differed from the alkaline form above pH 8, recombining with NO at the same velocity as with the acid form. This is consistent with the fact that the kinetic difference spectra by flash photolysis at alkaline pHs were identical with those at neutral and acidic pHs and were assigned to the difference spectrum of NO–ferric enzyme minus the acid form, but not the alkaline form (Tamura et al., 1978).

*NO–HRP-A.* HRP-A has a p$K$ value of 8.4 for the acid–alkaline transition. The value resembles that of ferrimyoglobin. The time course of the absorption change at 420 nm after photolysis is shown in the inset of Figure 4. The absorption change at pH 6.0 obeyed first-order kinetics, from which the second-order rate constant for NO recombination was estimated to be $2.0 \times 10^5$ $M^{-1}$ $s^{-1}$. Above pH 9.4, however, the absorption change after photolysis deviated from first-order

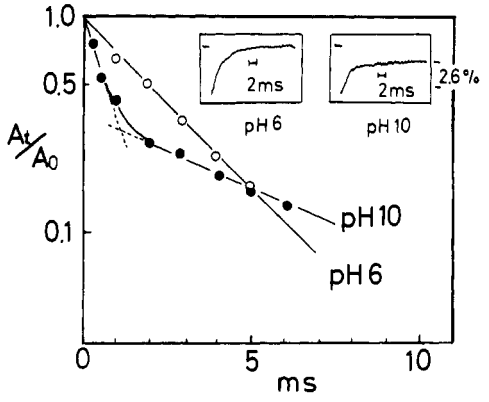



FIGURE 4: First-order plots of the absorption change at 420 nm from the traces of the inset of Figure 4. Inset: Oscilloscope traces of the absorption change after photolysis of NO–HRP-A. HRP-A, 10 $\mu$M, phosphate buffer (pH 6.0), 0.1 M, and glycine–NaOH (pH 10.0), 0.1 M, were present.

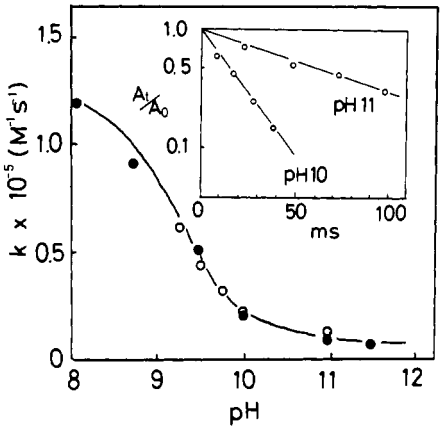
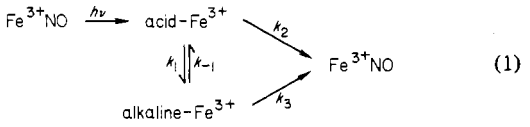



FIGURE 5: pH dependence of the rate constants of recombination of NO with HRP-A. (●) Stopped-flow method; (○) flash photolysis method. Inset: First-order plots of the absorption change in the slow process at 441 nm by photolysis of NO–HRP-A.

kinetics and consisted of fast and slow phases (Figure 4). At 441 nm, an isosbestic point between NO–HRP-A and acid HRP-A, the absorbance remained unchanged in the reaction at pH 6.0, but at pH 10.0, it increased with a half-time of 2 ms and then decreased slowly. The increase of the absorption at 441 nm was attributable to the formation of the alkaline form during the reaction. The slow decrease obeyed first-order kinetics, as shown in the inset of Figure 5. With the assumption that this absorption change represents the recombination of NO with the alkaline form of HRP-A, the rate constant can be calculated. Figure 5 shows the pH dependence of the values obtained by the above procedure and the flow method. The values were identical with those obtained by the flow method. This indicates that the slow process is due to the recombination of NO and the alkaline form of HRP-A. The fast phase seen in Figure 4 was thus concluded to involve an acid–alkaline transition and recombination of NO with the acid form. The disappearance of acid HRP-A was followed at 432 nm, an isosbestic point between alkaline HRP-A and NO–HRP-A. This absorption change obeyed first-order kinetics in the whole pH range. The reaction paths can be schematized as follows:




(1)

With the assumption that $k_{-1}$ and $k_3$ are much smaller than

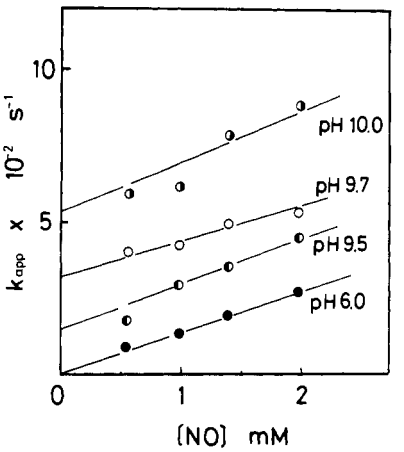



FIGURE 6: Correlation between NO concentration and the first-order rate constant determined at 432 nm.

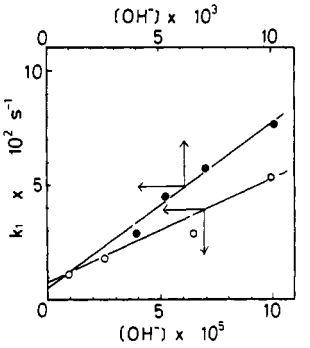



FIGURE 7: $k_1$ vs. pH plot in the acid–alkaline conversion of HRP-A (○) and HRP-C (●).

$k_1$ and $k_2$, the rate of disappearance of the acid form is expressed by eq 2 where $k_2$ is the rate constant for the recombination of NO with the acid form and $k_1$ is the rate constant for the conversion from the acid form to the alkaline form. The apparent first-order rate constant ($k_{app}$) determined at 432 nm can be expressed as

$$-\frac{d[\text{acid form}]}{dt} = \{k_2[NO] + k_1\}[\text{acid form}] \quad (2)$$

$$k_{app} = k_2[NO] + k_1 \quad (3)$$

Figure 6 shows the dependence of $k_{app}$ upon the concentration of NO at different pHs, giving the straight lines. From the slope and intercept in Figure 6, $k_2$ and $k_1$ can be estimated. Here, the $k_2$ values are almost constant within experimental error, which supports the reasonable basis of eq 3. Since eq 3 of the NO-saturated solution becomes $k_{app} = 4 \times 10^5\ s^{-1} - k_1$ for HRP-A, and $k_{app} = 3.8 \times 10^5\ s^{-1} - k_1$ for HRP-C, $k_1$ values were calculated. The $k_1$ value increased with the increase of pH, in both cases of HRP-A and HRP-C. The plot of $k_1$ vs. [$OH^-$] exhibited a linear relationship as shown in Figure 7.

## Discussion

The photodissociation of NO complexes into the ferric state can provide a unique approach for the kinetic studies of ligand binding. The reaction scheme is proposed in Figure 8. The water molecule is taken up into the pentacoordinated form within 1 ms after dissociation of NO by photolysis. This is confirmed by the fact that the kinetic difference spectrum is identical with that of acid ferrimyoglobin, but not the pentacoordinated form which has a broad absorption maximum at 380 nm, when trapped at 4.2 K (Kobayashi et al., 1980).

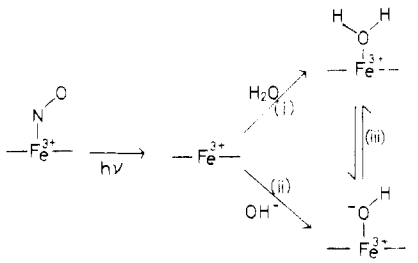


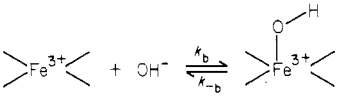
FIGURE 8: Schematic presentation of the photodissociation of NO–ferrimyoglobin.

This shows that the coordination of $H_2O$ to the pentacoordinated ferrimyoglobin is much faster than the recombination of NO with ferrimyoglobin. If the half-time for the coordination of the water molecule is less than 100 $\mu$s, the rate constant of coordination of $H_2O$ is greater than $10^2$ $M^{-1}$ $s^{-1}$.

The formation of the alkaline form is also much faster than the recombination of NO with ferrimyoglobin. The alternative pathways for the formation of the alkaline form after photolysis are shown in Figure 8. First, a water molecule is taken up into a pentacoordinated form to yield the acid form, and then the water molecule dissociates a proton to form the alkaline form. Second, the direct coordination of a hydroxylate ion occurs at the sixth position of the pentacoordinated form. At the moment, we cannot discriminate between these two pathways. The proton transfer in the acid–alkaline transition (iii) is very fast, i.e., on the order of $10^6$ $s^{-1}$. If the reaction occurs via path i, the rate constant should be larger than $10^6$ $M^{-1}$ $s^{-1}$.

The present results demonstrate that unlike ferrimyoglobin the conformation of HRP which appeared after photolysis of NO–HRP was identical with the acid form of HRP in the whole pH range. This is verified by the kinetic difference spectra and by the velocity of recombination of NO with the enzyme. The deviation from first-order kinetics in the NO recombination at pH 10 can be explained in terms of the competition between the NO recombination with the acid form and the transformation from the acid form to the alkaline form. The rate constant for the formation of the alkaline form calculated from eq 3 agreed with the values obtained from the acid–alkaline transition of HRP-C and -A (Araiso & Yamazaki, 1978; Kihara et al., 1978; Epstein & Schejter, 1972). Morishima et al. (1977) have suggested that this slow rate is ascribed to the direct exchange of the sixth ligand on the ferric heme. It is obvious, however, that the slow rate of alkaline ionization does not necessarily mean an involvement of a conformation change. If an amino acid residue deprotonates in alkaline solution with a conformation change in the protein, a saturation effect should occur as the hydroxide ion concentration increased. Such a saturation effect was not observed, and the rate increased exponentially with the increase of pH as shown in Figure 7. Similar values for the acid–alkaline conversion were obtained by the pH jump method of Araiso & Yamazaki (1978), who proposed that this process is due to abstracting a hydrogen ion from the ligand water molecule, and the slow rate was explained by assuming that the dissociable proton of the water molecule at the sixth position is hydrogen bonding strongly. The results shown in Figure 7 might be explained by this mechanism, but this proposal is based on the assumption that $H_2O$ coordinates at the sixth position of the acid form of HRP.

Previously (Kobayashi et al., 1980), we proposed that acid HRP holds a pentacoordinated structure. Therefore, the formation of the alkaline form of HRP should be explained simply by the direct coordination of $OH^-$ at the sixth position, which is vacant at acidic and neutral pHs:



The values of $k_b$ and $k_{-b}$ are $6.8 \times 10^5$ $M^{-1}$ $s^{-1}$ and 65 $s^{-1}$ for HRP-A and $7.3 \times 10^4$ $M^{-1}$ $s^{-1}$ and 46 $s^{-1}$ for HRP-C, respectively (Figure 7). So far, examination by laser photolysis revealed that the difference spectrum appearing 100 ns after photodissociation of NO–HRP was indistinguishable from that of the native enzyme. There was no appreciable spectral indication, suggesting the presence of intermediates at any wavelength in this time scale. Therefore, even if a water molecule coordinates at the sixth position of HRP, the recombination of a water molecule occurs in less than 100 ns.

Acknowledgments

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References

Araiso, T., & Yamazaki, I. (1978) *Biochemistry 17*, 942.
Epstein, N., & Schejter, A. (1972) *FEBS Lett. 25*, 46.
Hoffman, B. M., & Gibson, Q. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2.
Kihara, H., Saigo, S., Iizuka, T., & Ishimura, Y. (1978) *Biochim. Biophys. Acta 533*, 112.
Kobayashi, K., Tamura, M., Hayashi, K., Hori, H., & Morimoto, H. (1980) *J. Biol. Chem. 255*, 2239.
Morishima, I., Ogawa, S., Inubushi, J., Yonezawa, T., & Iizuka, T. (1977) *Biochemistry 16*, 5109.
Paul, K. G. (1958) *Acta Chem. Scand. 12*, 1312.
Shannon, L. M., Kay, E., & Lew, T. Y. (1966) *J. Biol. Chem. 241*, 2166.
Tamura, M., Kobayashi, K., & Hayashi, K. (1978) *FEBS Lett. 88*, 124.
Tamura, M., Kobayashi, K., & Hayashi, K. (1981) *Oxidase and Related Redox Systems* (Mason, H. S., King, T. E., & Morrison, M.) Vol. III, Academic Press, New York.
Yamazaki, I., Yokota, K., & Shikama, K. (1964) *J. Biol. Chem. 239*, 4151.