Seidel, J. C., Chopek, M., and Gergely, J. (1970), *Biochemistry 9*, 3265.
Seidel, J. C., and Gergely, J. (1971), *Biochem. Biophys. Res. Commun. 44*, 826.
Seidel, J. C., and Gergely, J. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37*, 187.
Seidel, J. C., Greaser, M. L., and Gergely, J. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1309 Abstr.
Silverman, R., Eisenberg, E., and Kielley, W. W. (1972), *Nature (London) New Biol. 240*, 207.
Stone, D. B. (1970), *Arch. Biochem. Biophys. 141*, 378.
Stone, D. B., Botts, J., Quinlivan, J. T., and McConnell, H. M. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 323.
Stone, D. B., Prevost, S. C., and Botts, J. (1970), *Biochemistry 9*, 3937.
Stone, T. J., Buckman, T., Nordio, P. L., and McConnell, H. M. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1010.
Takeuchi, K., and Tonomura, Y. (1971), *J. Biochem. 70*, 1011.
Tokiwa, T. (1971), *Biochem. Biophys. Res. Commun. 44*, 471.
Trentham, D. R., Bardsley, R. G., Eccleston, J. F., and Weeds, A. G. (1972), *Biochem. J. 126*, 635.
Viniegra-Gonzalez, G., and Morales, M. F. (1972), *Bioenergetics 3*, 55.
Young, M. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2393.
Young, D. M., Himmelfarb, S., and Harrington, W. F. (1964), *J. Biol. Chem. 239*, 2822.
Young, D. M., Himmelfarb, S., and Harrington, W. F. (1965), *J. Biol. Chem. 240*, 2428.
Yount, R. G., Babcock, D., Ballantyne, W., and Ojala, D. (1971a), *Biochemistry 10*, 2484.
Yount, R. G., Ojala, D., and Babcock, D. (1971b), *Biochemistry 10*, 2490.

Exchangeability of Bicarbonate Specifically Bound to Transferrin†

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ABSTRACT: The Fe(III)- and anion-binding functions of transferrin are strongly interdependent, with specific binding of either dependent on the presence of the other. Under physiologic conditions, bicarbonate is the anion preferentially bound by transferrin, although a variety of other anions are also capable of occupying the specific anion-binding site of the protein. Because of its possible role in the uptake of iron from transferrin by the reticulocyte, studies were undertaken of the exchangeability of transferrin-bound bicarbonate with bicarbonate free in solution. The rate of exchange depends on the anionic composition of the medium. At physiological pH and ambient p-CO_2, bicarbonate exchange is detectable but slow, with a half-time of about 20 days. The presence of millimolar concentrations of citrate or nitrilotriacetate increases the exchange rate by two orders of magnitude. Increasing the bicarbonate concentration also increases the exchange rate in an approximately proportional manner. The exchange of bicarbonate from monoferric transferrin prepared by isoelectric focusing is describable by a simple first-order plot. However, exchange from diferric transferrin is more complex and requires two exponential terms to fit the data satisfactorily. In every case studied the half-time for exchange in monoferric transferrin has a value intermediate to the two half-times for exchange in differric transferrin. These results point to an interaction between the two specific anion-binding sites of the protein. They may account, in part, for the observed difference in the rates at which iron is taken up from the two iron-binding sites of transferrin by the reticulocyte.

The transferrin molecule consists of a single polypeptide chain of mol wt ~80,000 (Greene and Feeney, 1968; Mann *et al.*, 1970), on which are disposed two specific metal-binding sites with identical, or nearly so, thermodynamic and spectroscopic properties (Aasa *et al.*, 1963; Aisen *et al.*, 1966). For each metal ion bound, an anion must also be bound. For a time it was thought that anion binding was facultative and that specific binding of ferric ions could occur in the absence of anions (Aasa and Aisen, 1968). However, recent studies indicate that for specific binding of Fe(III) to occur, binding of a suitable anion is obligatory (Price and Gibson, 1972). On the basis of present information, bicarbonate (or, possibly, carbonate (Williams and Woodworth, 1973)) seems to be the anion most favored by the protein. In its absence, however, specific binding of Fe(III) will occur if oxalate, malonate, EDTA, nitrilotriacetate, thiogylcollate, or other suitable anions are available to occupy the anion-binding site of transferrin (Aisen *et al.*, 1967; Aisen and Pinkowitz, 1973). The stoichiometries of specific anion binding and Fe(III) binding are identical, and neither metal ion nor anion is tightly bound in the absence of the other (Schade and Reinhart, 1966; Aisen and Leibman, 1973). Thus, the anion- and metal-binding functions of transferrin may be described as showing strong positive cooperativity.

Recently, evidence has been presented indicating that the mechanism of iron transfer from transferrin to the reticulocyte involves the anion-binding site of the protein. It has been suggested that disruption of the anion–protein linkage must precede delivery of iron to the cell (Aisen and Leibman, 1973; Egyed, 1973). The present studies of the exchange of transferrin-bound bicarbonate were undertaken to gain understanding of some of the factors which affect the stability of the

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anion–protein bond and which may therefore have bearing on the physiologic function of transferrin.

Experimental Procedures

Preparation of Fe(III)–Transferrin-$H^{14}CO_3$. Transferrin was isolated from Cohn fraction IV-7 and apotransferrin was prepared from it by dialysis against a citrate–acetate buffer, by methods previously described (Aisen *et al.*, 1966). Because of the finding that dialysis of apotransferrin against distilled water is inadequate to remove residual citrate, the preparation was dialyzed overnight against 0.1 M perchlorate, as suggested by Price and Gibson (1972), before final dialysis against the working buffer, 0.1 M KCl–0.05 M *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonate (pH 7.5). This step ensured that the protein was virtually free of citrate (Aisen and Pinkowitz, 1973). Transferrin labeled with [^{14}C]bicarbonate was prepared under anaerobic conditions to minimize exchange of the label with atmospheric CO_2, as follows. The apoprotein at pH 4.5–5.0 was placed in a Thunberg tube, and an amount of iron sufficient to produce the desired degree of saturation of the iron-binding sites, as $Fe(NH_4)_2(SO_4)_2$ or as the Fe(III)–nitrilotriacetate complex (Aisen *et al.*, 1967), was pipeted into the bulb of the tube. The tube was alternately flushed with CO_2-free nitrogen and evacuated with an oil pump for ten cycles over the course of 2 hr. After mixing the apoprotein and iron the tube was then opened, and an approximately tenfold excess of $NaH^{14}CO_3$ (with respect to Fe(III)) was added to the preparation. The characteristic salmon-red color of Fe(III)–transferrin developed rapidly upon addition of the bicarbonate. The labeled protein was immediately placed to dialyze against a large excess of the working buffer in order to remove unbound labeled bicarbonate. The final preparation was stored at refrigerator temperatures for use within 3 days or frozen.

Labeled monoferric transferrin was isolated by isoelectric focusing from transferrin 30% saturated with iron (Aisen *et al.*, 1973). The LKB preparative column was used with 1% Ampholine, pH 5–7 (LKB Instruments, Inc.). Following recovery of the middle protein band, which had a pI near 5.6 corresponding to the monoferric species, residual Ampholine was removed by gel filtration using Sephadex G-25. On the basis of its optical absorbance at 470 and 280 nm, the monoferric transferrin contained about 1.1 g-atoms of iron/mol of protein.

Measurement of $H^{14}CO_3$ Exchange. The exchange of transferrin-bound $H^{14}CO_3^-$ with HCO_3^- free in solution was followed using the Crowe–Englander dialysis apparatus (Englander and Crowe, 1965) thermostated in a water bath at 25°. To simplify the sampling procedure, the dialysis bag was fitted with a length of polyethylene tubing so that aliquots of dialysand could be withdrawn without disassembling the apparatus. Initially, 7 ml of 6.2×10^{-5} M labeled transferrin was placed in the cellophane dialysis bag. This was dialyzed for 3 hr against two changes of buffer, to eliminate residual unbound $H^{14}CO_3^-$, before the final working buffer was placed in the 500-ml graduated cylinder. Samples of 0.5 ml were taken for determination of radioactivity and absorbancy at 280 nm. Approximately 0.1 ml of sample was pipeted in duplicate into 2 ml of 0.1 M KOH to ensure quantitative recovery of $H^{14}CO_3^-$ released from the protein. The exact quantity of protein solution used was determined by weighing the container of KOH solution before and after addition of protein. For determination of radioactivity by scintillation counting, 1 ml of the KOH–protein solution was pipeted into 10 ml of a scintillation mixture consisting of 7 parts of 0.4% Omnifluor (New England Nuclear) in toluene and 3 parts of Triton X-100 (Rohm and Haas Corp.). The measured activity was then normalized for the actual sample weight and the protein concentration as measured by the absorbancy at 280 nm. During the course of each experiment, the radioactivity of the dialysis bath was also measured to provide an estimate of the activity due to free $H^{14}CO_3^-$. This was usually negligible, except for the final points of each experiment. The results are presented as the average of the duplicate measurements for each point in time, which usually agreed to within 2%.

For experiments performed in an atmosphere of 5% CO_2, the 500-ml graduated cylinder, used to contain the dialysis bath and the Crowe–Englander apparatus, was fitted with a side arm at its base through which a 5% CO_2–air mixture (Matheson Gas Products) was bubbled. In these experiments the buffer solution was made 4.5×10^{-2} M in sodium bicarbonate to maintain the pH at 7.5 (Edsall and Wyman, 1958).

In order to demonstrate that the time for HCO_3^- to dialyze was not a limiting factor in the experiments, a control was carried out in which $NaH^{14}CO_3$ was added to a solution of transferrin in the Crowe–Englander apparatus. Within 15 min, which was negligible compared to the time course of the experiments, over 97% of the radioactivity was lost from the protein solution.

Buffers were extracted with dithizone to render them free of metal ions. Cellophane dialysis bags were boiled, washed in 10^{-4} M EDTA, and rinsed repeatedly with doubly distilled water before use.

Electron paramagnetic resonance (epr) spectroscopy was performed with a Varian E-9 spectrometer operating at X-band and equipped with a Heli-Tran variable temperature device (Air Products Corp.).

A nonlinear least-squares error program was used to find parameters which best fit the experimental data to functions of the form given in the text and figure legends (van der Waerden, 1969).

Results

Characteristics of Monoferric Transferrin. The optical difference spectrum of monoferric transferrin, read against an identical concentration of apotransferrin as a blank and normalized with respect to Fe(III) concentration, was the same as that of differic transferrin (Aisen *et al.*, 1967). Similarly, the epr spectrum of the monoferric preparation was essentially identical with diferric transferrin (Figure 1). The present results, therefore, do not provide evidence for a difference between the binding sites of transferrin such as has been observed for conalbumin (Aisen *et al.*, 1973).

Exchange of $H^{14}CO_3^-$ at Atmospheric p-CO_2 in the Absence of Complexing Anions. When no metal-complexing anion was present in the standard dialysis buffer, the half-time for $H^{14}CO_3^-$ exchange in differic transferrin was observed to be ~20 days. Experimental data were taken for about 17 days, and in this time interval it was not possible to determine whether the exchange behavior obeyed simple first-order kinetics. Because of the slow exchange rate, no attempt was made to study monoferric transferrin under these conditions.

Effect of Metal Complexing Anions at Atmospheric p-CO_2. The addition of 10^{-3} M citrate, nitrilotriacetate, or thioglycollate to the dialysis bath and protein sample greatly accelerated the rate of exchange of transferrin-bound bicarbonate with

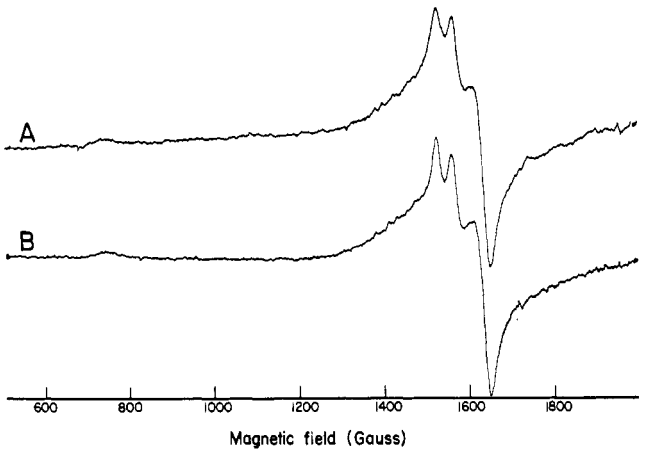

FIGURE 1: Epr spectra of (A) monoferric transferrin isolated by isoelectric focusing; (B) diferric transferrin; microwave frequency, 9.371 GHz; microwave power, 40 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; temperature, −190°.

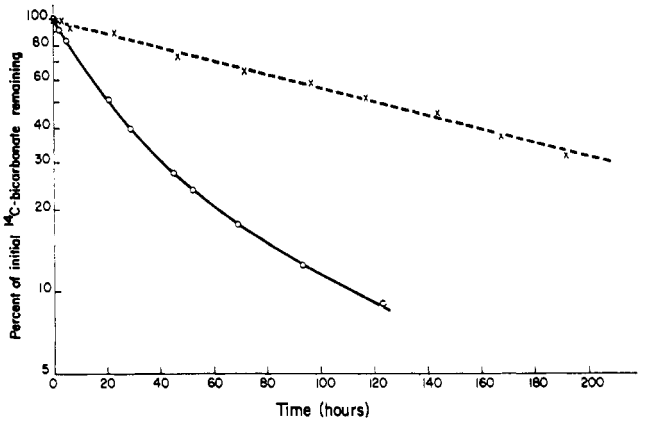

FIGURE 2: Exchange of transferrin-bound [^{14}C]bicarbonate at ambient p-CO_2 and in the presence of 10^{-3} M citrate: (×) monoferric transferrin; (○) diferric transferrin. The dashed line is a plot of the function $F = 99\%[\exp(-0.0058t)]$ and the solid line is a plot of the function $F = 50\%[\exp(-0.032t) + \exp(-0.0046t)]$.

bicarbonate free in solution. When citrate was present, the half-time for exchange in monoferric transferrin fell to 120 hr (Figure 2). The experiment was continued for 192 hr, during which time 68% of the $H^{14}CO_3^-$ initially bound to the protein was lost in a simple first-order manner.

The exchange of bicarbonate in diferric transferrin under similar conditions was more complicated. In a semilogarithmic plot of residual activity as a function of time a substantial curvature was present (Figure 2). Using a nonlinear least-squares curve fitting program (van der Waerden, 1969), it was possible to find rate constants for a function of the form $F = 50\%[\exp(-k_1t) + \exp(-k_2t)]$, in which each of the two sites was assumed to have one-half of the total radioactivity at zero time. The rate constants found for this experiment, 0.033 hr^{-1} and 0.0046 hr^{-1}, correspond to half-times of 21 and 151 hr, respectively.

Comparable results were observed when 10^{-3} M nitrilotriacetate was present, except that exchange rates were faster (Figure 3). In the case of monoferric transferrin, the exchange of bicarbonate followed first-order kinetics, with a half-time of 7 hr. Exchange in diferric transferrin could be described by a sum of two exponential decays, each accounting for half of the initial radioactivity. The rate constant for the more rapidly exchanging bicarbonate was 0.17 hr^{-1}, for a half-time of 4.1 hr, while the more slowly exchanging bicarbonate gave a rate constant of 0.021 hr^{-1} corresponding to a half-time of 33 hr.

Bicarbonate exchange from monoferric transferrin in the presence of 10^{-3} M thioglycollate also followed first-order kinetics with a half-time of 56 hr.

In none of these experiments was there net loss of specifically bound Fe(III) from transferrin, since the optical absorbancy at 470 nm of the protein did not decrease. It would appear, then, that in diferric transferrin, when both anion-binding sites of the protein are occupied by bicarbonate ions, one of these exchanges more rapidly and the other more slowly than does the bicarbonate of monoferric transferrin.

Effect of Free Bicarbonate on Exchange of Transferrin-Bound Bicarbonate. In an atmosphere of 5% carbon dioxide, when the equilibrium concentration of bicarbonate in solution at pH 7.5 is 4×10^{-2} M (Edsall and Wyman, 1958), or about 120-fold greater than at ambient p-CO_2, the exchange of bicarbonate from monoferric transferrin again showed simple first-order behavior with a half-time of 11.2 hr (Figure 4A). In accord with the previous results, diferric transferrin showed more complex exchange behavior which could be fitted by a sum of two exponentials, each accounting for half of the initial $H^{14}CO_3^-$ activity (Figure 4B). The half-times for the exchanging bicarbonate ions were 4.2 and 15 hr, respectively. Addition of 10^{-3} M citrate under these conditions produced no further effect in either the monoferric or diferric preparations.

Effect of Saturating Labeled Monoferric Transferrin with Unlabeled Bicarbonate. Monoferric transferrin labeled with $H^{14}CO_3$ was isolated with the isoelectric focusing column and then brought to ~95% saturation with Fe(III) and unlabeled bicarbonate. The exchange behavior of this preparation in an atmosphere of 5% CO_2 required at least two exponential terms for satisfactory fit; a better fit was obtained by also taking into account the 10% of radioactivity still present in

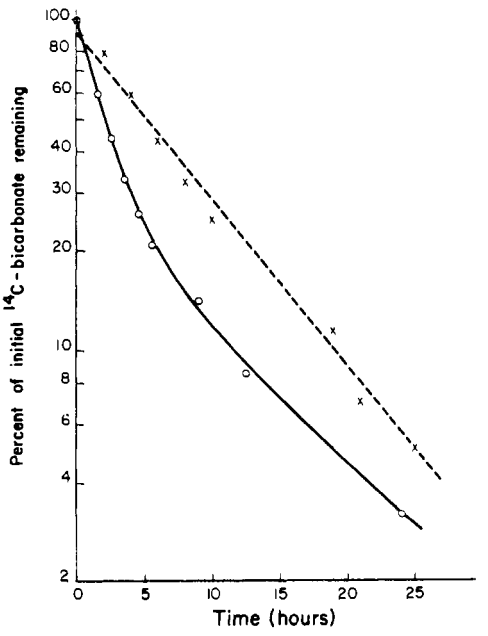

FIGURE 3: Exchange of transferrin-bound [^{14}C]bicarbonate at ambient p-CO_2 and in the presence of 10^{-3} M nitrilotriacetate; (×) monoferric transferrin; (○) diferric transferrin. The dashed line is a plot of the function $F = 100\%[\exp(-0.093t)]$ and the solid line is a plot of the function $F = 50\%[\exp(-0.17t) + \exp(-0.022t)]$.

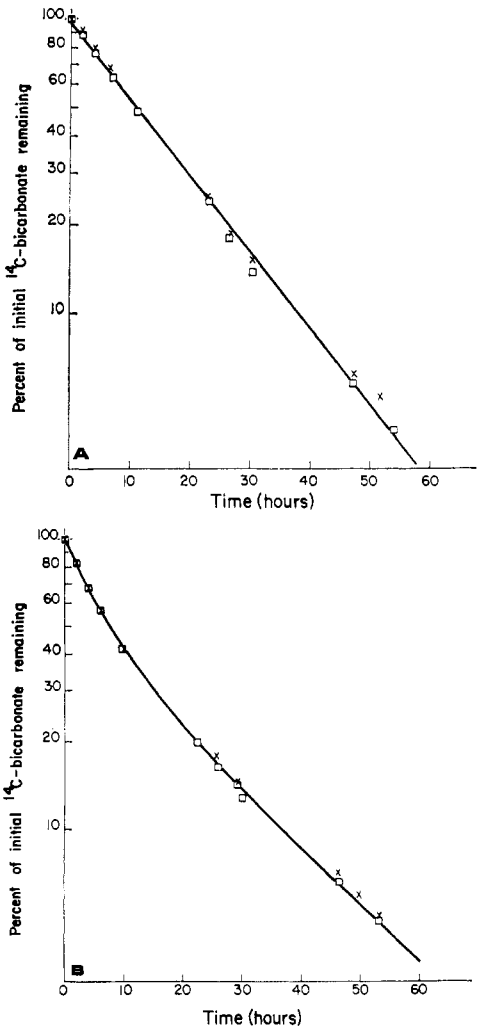

FIGURE 4: (A) Exchange of [^{14}C]bicarbonate from monoferric transferrin at a p-CO_2 of 0.05 atm: (□) buffer; (×) buffer + 10^{-3} M citrate. The solid line is a plot of the function $F = 99\%[\exp \times (-0.062t)]$. (B) Exchange of [^{14}C]bicarbonate from diferric transferrin at a p-CO_2 of 0.05 atm: (□) buffer; (×) buffer + 10^{-3} M citrate. The solid line is the function $F = 50\%[\exp(-0.16t) + \exp(-0.046t)]$.

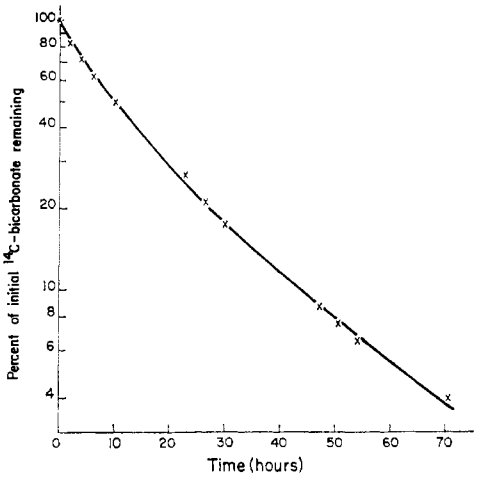

FIGURE 5: Bicarbonate exchange from monolabeled transferrin brought to 90% saturation with iron and unlabeled bicarbonate. The experimental points are represented by X and the solid line is the function $F = 45\%[\exp(-0.12t) + \exp(-0.036t)] + 10\%[\exp \times (-0.062t)]$.

molecules bearing only one ferric ion (Figure 5). The two half-times for exchange in the diferric species were found to be 5.7 and 19 hr, close to the values of 4.2 and 15 hr observed for randomly labeled diferric transferrin.

Effects of Anions on Epr Spectra. The epr spectra of the transferrin complexes reported on in this study resembled that of native Fe(III)–transferrin–bicarbonate (Aisen *et al.*, 1967), even when complexing anions were present. The perturbations produced by these anions, if any, were too small to be detected. On this basis, we feel confident that almost all of the Fe(III)–transferrin at any given time was in the form of the bicarbonate complex, even in the presence of other anions capable of occupying the anion-binding site of the protein.

Discussion

The binding of iron by transferrin is dependent upon the concomitant binding of a suitable anion. The cooperativity between metal ion and anion binding is complete: neither is bound in substantial amounts in the absence of the other (Price and Gibson, 1972; Aisen and Pinkowitz, 1973). A variety of metal-complexing anions other than bicarbonate have been shown to be capable of occupying the anion-binding site when bicarbonate is absent (Aisen *et al.*, 1967; Aisen and Pinkowitz, 1973). As far as is known, however, bicarbonate is most favored by the protein and will displace other anions.

At physiological pH and ambient p-CO_2, when the equilibrium bicarbonate concentration in solution is $\sim 3 \times 10^{-4}$ M (Edsall and Wyman, 1958), exchange of ferric ions among transferrin molecules does not occur in the absence of a metal-complexing agent (Aisen and Leibman, 1968). Under these circumstances, bicarbonate exchange is detectable but slow, with a half-time of about 20 days. When millimolar concentrations of citrate, nitrilotriacetate, or thioglycollate are present the exchange rate increases by as much as two orders of magnitude. The mechanism for this effect is, presumably, a displacement of bicarbonate by the competing anion. From our experiments, it cannot be determined whether, when metal-complexing agents are present, bicarbonate exchange is accompanied by exchange of metal ions among binding sites in transferrin molecules. It should be emphasized, however, that at any given time the fraction of anion-binding sites occupied by the competing anions is too small to be detected by epr spectroscopy and there is no net loss of Fe(III) from protein to anion. The bicarbonate ion itself is an effective promoter of bicarbonate exchange. In an atmosphere of 5% CO_2, when the equilibrium bicarbonate concentration is 4×10^{-2} M, the half-time for bicarbonate exchange falls to as little as 4.2 hr (Figure 4B). At this concentration of bicarbonate, the addition of 10^{-3} M citrate produces no further effect on the observed exchange. Presumably, then, the mechanisms of the exchange induced by bicarbonate and other anions are similar. The mechanism we propose, then, would be

$$\text{Fe–transferrin–H}^{14}\text{CO}_3 + \text{anion} \longrightarrow \text{Fe–transferrin–anion} + \text{H}^{14}\text{CO}_3^- \quad (1)$$

$$\text{Fe–transferrin–anion} + \text{HCO}_3 \longrightarrow \text{Fe–transferrin–HCO}_3 + \text{anion} \quad (2)$$

The species Fe–transferrin–anion is short-lived and present in only trace amounts at any time since it is not detectable

by epr spectroscopy. This appears to be in substantial agreement with kinetic studies of the binding of ferric chelates by transferrin (Bates *et al.*, 1967). The formation of the Fe–transferrin–bicarbonate complex from apotransferrin and mononuclear ferric citrate or ferric nitrilotriacetate most likely concludes with the displacement of the anion by bicarbonate, as in reaction 2. The rates of formation of the Fe–transferrin–bicarbonate complex from apotransferrin and ferric citrate on ferric nitrilotriacetate would then place a lower limit on the rate of radioactive bicarbonate exchange from Fe–transferrin–bicarbonate in the presence of these anions, as given by reactions 1 and 2. The measured rates of formation of the bicarbonate complex from ferric chelates are faster than the measured rate of bicarbonate exchange in the presence of the chelating anions. This suggests that reaction 2 is fast and that the steady-state concentration of Fe–transferrin–anion must be small.

When exchange is observed in monoferric transferrin it appears to follow a simple first-order scheme. This is true whether the exchange is initiated by citrate, nitrilotriacetate, thioglycollate, or bicarbonate itself. Equilibrium dialysis and electrophoretic studies (Aasa *et al.*, 1963; Aisen *et al.*, 1967) have indicated that the binding constants for Fe(III) of the two sites are identical or nearly so when corrected for a statistical factor. Both sites of the protein should be equally populated, then, in the monoferric preparation unless there is a kinetic effect favoring one of them. Since monoferric transferrin is not distinguishable from diferric transferrin by spectroscopic means, in contrast to what has been found to be the case in conalbumin (ovotransferrin) (Aisen *et al.*, 1973), it would seem that the sites are identical or that neither site is kinetically favored. In the latter case, the iron and therefore the bicarbonate would be randomly distributed between binding sites. The simple first-order exchange of bicarbonate in monoferric transferrin indicates that the metal-binding sites are not distinguishable by the exchange behavior of their associated anion-binding functions.

The exchange bicarbonate in diferric transferrin is not a simple exponential. At least two exponential terms are required to achieve a formal expression which satisfactorily fits the experimental data. It is important to note that in fitting experimental data to a function which is the sum of two exponentials it is difficult to obtain parameters which uniquely give "best fits" (Lanczos, 1956). We would emphasize, however, that in our experiments it was always possible to obtain a satisfactory fit using only two exponential terms, each of which contributed equally to the radioactivity at zero time. When this condition was imposed, the rate constant for one term was faster, while the rate constant for the other term was slower than the single rate constant observed for monoferric transferrin under similar conditions. On this basis, it appears that each site of the protein is associated with an exchangeable bicarbonate ion, and a site–site interaction occurs when each of the anion-binding sites of transferrin is occupied. This view is strengthened by the observation that $H^{14}CO_3^-$-labeled monoferric transferrin, which gives a simple first-order exchange, produces a complex behavior when saturated with Fe(III) and unlabeled HCO_3^-. It is also in accord with the observation by Young and Perkins (1968) that the two bicarbonate ions of diferric transferrin show a difference in their susceptibility to displacement by oxalate.

The role of transferrin in providing iron for hemoglobin synthesis by the reticulocyte has long been appreciated, but the mechanism by which iron is transferred from protein to cell is still only poorly understood (Morgan, 1971). The metal–protein bond, under physiological conditions, is so strong that simple dissociation is excluded on both theoretical and experimental grounds (Aisen and Liebman, 1968). Since the protein is conserved during the transferrin–reticulocyte interaction, a specific mechanism for effecting release of iron from the protein to the cell must exist. Recently, it has been suggested that this mechanism entails a specific attack by the reticulocyte on the specifically bound bicarbonate of the transferrin (Aisen and Leibman, 1973; Egyed, 1973). The present studies, which demonstrate that the bicarbonate–protein bond is susceptible to disruption under physiological conditions, lend support to this view. The mechanism by which the bicarbonate–protein bond is broken by the reticulocyte remains unknown.

Fletcher and Huehns (1967, 1968) and, more recently, Awai *et al.* (1972) have produced evidence to show that the two sites of diferric transferrin are not equivalent in their ability to donate iron to the reticulocyte for the synthesis of hemoglobin. This effect may be due to an intrinsic difference between the two sites (Aisen *et al.*, 1969; Aasa, 1972). However, the spectroscopic findings for monoferric transferrin do not support, but also do not exclude, such a difference. Since the anion-binding function of transferrin appears to be involved in the mechanism by which iron is removed by the reticulocyte (Aisen and Leibman, 1973), the basis for the Fletcher–Huehns effect may lie in a site–site interaction between bound bicarbonate ions of diferric transferrin such as we have described.

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References

Aasa, R. (1972), *Biochem. Biophys. Res. Commun. 49*, 806.

Aasa, R., and Aisen, P. (1968). *J. Biol. Chem. 243*, 2399.

Aasa, R., Malmström, B. G., Saltman, P., and Vänngard, T. (1963), *Biochim. Biophys. Acta 75*, 203.

Aisen, P., Aasa, R., Malmström, B. G., and Vänngard, T. (1967), *J. Biol. Chem. 242*, 2484.

Aisen, P., Aasa, R., and Redfield, A. G. (1969). *J. Biol. Chem. 244*, 4628.

Aisen, P., Lang, G., and Woodworth, R. S. (1973), *J. Biol. Chem. 248*, 649.

Aisen, P., and Leibman, A. (1968), *Biochem. Biophys. Res. Commun. 32*, 220.

Aisen, P., and Leibman, A. (1973), *Biochim. Biophys. Acta 304*, 797.

Aisen, P., Leibman, A., and Reich, H. A. (1966), *J. Biol. Chem. 241*, 1666.

Aisen, P., and Pinkowitz, R. A. (1973), *Ann. N. Y. Acad. Sci.* (in press).

Awai, M., Chipman, B., and Brown, E. B. (1972), *Clin. Res. 20*, 784.

Bates, G. W., Billups, C., and Saltman, P. (1967), *J. Biol. Chem. 242*, 2810.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, New York, N. Y., Academic Press, pp 555–561.

Egyed, A. (1973), *Biochim. Biophys. Acta 304*, 805.
Englander, S. W., and Crowe, D. (1965), *Anal. Biochem. 12*, 579.
Fletcher, J., and Huehns, E. R. (1967), *Nature (London) 215*, 584.
Fletcher, J., and Huehns, E. R. (1968), *Nature (London) 218*, 1211.
Greene, F. C., and Feeney, R. E. (1968), *Biochemistry 7*, 1366.
Lanczos, C. (1956), Applied Analysis, Engelwood Cliffs, N. J., Prentice-Hall, pp 272–280.
Mann, K. G., Fish, W. W., Cox, A. C., and Tanford, C. (1970), *Biochemistry 9*, 1348.
Morgan, E. H. (1971), *Biochim. Biophys. Acta 244*, 103.
Price, E. M., and Gibson, J. F. (1972), *Biochem. Biophys. Res. Commun. 46*, 646.
Schade, A. L., and Reinhart, R. W. (1966), *Protides Biol. Fluids, Proc. Colloq. 14*, 75.
van der Waerden, B. L. (1969), Mathematical Statistics, Heidelberg, Germany, Springer-Verlag, pp 127–133.
Williams, S. C., and Woodworth, R. C. (1973), *J. Biol. Chem.* (in press).
Young, J. W., and Perkins, D. J. (1968), *Eur. J. Biochem. 4*, 385.

Proton Magnetic Resonance Study of Angiotensin II (Asn^1Val^5) in Aqueous Solution†

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ABSTRACT: All the resolved resonances in the 220-MHz proton magnetic resonance (pmr) spectrum of angiotensin II (Asn^1Val^5) (AII′) in D_2O have been assigned to specific hydrogens. In H_2O additional assignments were made of resonances originating from peptide NH hydrogens of Phe and Arg, primary amide NH hydrogens of Asn, and the four equivalent guanidino NH hydrogens of Arg. Conformational transitions associated with titration of the α-amino and/or the imidazole group(s) ($pK_a = 6.6 \pm 0.2$) and with titration of the phenol group ($pK_a = 10.2 \pm 0.2$) have been confirmed. Pmr determined pK_a values in H_2O at 23° and in D_2O at 4° (shown in parentheses) are all normal: carboxyl 3.07 ± 0.03, imidazole 6.26 ± 0.04 (6.82 ± 0.02), α-amino (6.98 ± 0.04), and phenol 10.2 ± 0.2 (10.5 ± 0.5). The peptide NH–αCH coupling constants in acidic solution are: 6.5 ± 0.3 (Arg), 6.0 ± 0.5, 7.2 ± 0.5, 7.3 ± 0.3 (Phe), 7.0 ± 0.3, and 8.0 ± 0.4. Various classes of labile hydrogens were defined on the basis of their exchange rates as determined from broadening of their respective resonances. The pmr data are consistent either with a rapid equilibrium between various conformations or with a unique conformation of the hormone, but additional evidence is required to definitively determine the structure(s) significantly contributing to the equilibrium. Previously proposed structures excluded by these data include the α helix, the conventional β turn, the γ turn, and a structure stabilized by a salt bridge.

Angiotensin II (AII)[1] is a potent natural pressor agent derived from an α_2-macroglobulin by renin hydrolysis. The present study deals with a more readily available synthetic congener, angiotensinamide (AII)′ (Asn^1-Arg^2-Val^3-Tyr^4-Val^5-His^6-Pro^7-Phe^8), which elicits similar biological responses to AII but differs chemically from it only in the replacement of the N-terminal Asp residue of AII by Asn.

It has long been recognized that the conformation of the hormone may be related to its biological activity (Bumpus *et al.*, 1961). Even though it is the conformation of the hormone at the receptor site which is of primary significance (Marshall and Bosshard, 1972), the free solution orientation of this hormone has received the most attention because it is more easily monitored by available methods. In free solution AII has been variously described as an α helix (Smeby *et al.*, 1962), random-coil (Paiva *et al.*, 1963), antiparallel pleated sheet (Fermandjian *et al.*, 1972a, 1972b; Printz *et al.*, 1972a), and a number of more complex conformations (Weinkam and Jorgensen, 1971a; Glauser *et al.*, 1970). The effect of pH on the conformation of the hormone in free solution has also been debated. Thus, Paiva *et al.* (1963) report no conformational transitions between pH 2.5 and 8.5, but others have associated conformational perturbations with titration of the carboxyl group (Weinkam and Jorgensen, 1971a, 1971b), the imidazole group (Craig *et al.*, 1964), and the phenol group (De Fernandez *et al.*, 1968).

Clarification of this controversy is desirable not only in order to delineate the structure–activity relationship of AII, but also in order to define the capabilities and limitations of various methods employed in studying the conformation of peptides in solution. Toward this end we have undertaken a detailed proton magnetic resonance (pmr) study of the conformation of AII′. A preliminary report of our observations has been presented (Glickson *et al.*, 1972). Here we present a more detailed description of the method of resonance assignment, the effects of pH on conformation, analysis of peptide NH–αCH coupling constants, and estimation of proton exchange rates from line-broadening data.

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[1] Abbreviations used: angiotensin II (AII), and angiotensin II (Asn^1Val^5) (AII′).