Sulfate Potentiation of the Chloride Activation of Angiotensin Converting Enzyme†

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ABSTRACT: The angiotensin converting enzyme (ACE)-catalyzed hydrolysis of furanacryloyl-Phe-Gly-Gly is activated by monovalent anions, notably chloride. This activation is enhanced by sulfate; at pH 7.5, the effect is maximal at 0.8 M sulfate and is mediated through a specific interaction of the divalent anion with the enzyme, not through an increase in ionic strength. Sulfate decreases the apparent binding constant for chloride which manifests as a decrease of the apparent K_M value, but it does not change k_{cat}. Thus, at pH 7.5, sulfate solely affects substrate binding in accord with the ordered bireactant mechanism of chloride activation that pertains with this substrate [Bünning, P., & Riordan, J. F. (1983) *Biochemistry 22*, 100–116]. Increasing the pH from 6 to 9 in the absence of sulfate increases the apparent binding constant for chloride almost 60-fold from 3.3 to 190 mM. In the presence of 0.8 M sulfate, however, the change is only about 6-fold, from 0.7 to 4.2 mM. Over the same pH range, the apparent K_M for furanacryloyl-Phe-Gly-Gly obtained with saturating chloride concentrations shifts from 0.14 to 0.48 mM, while in the presence of 0.8 M sulfate about 3-fold lower apparent K_M values are obtained. Sulfate does not appear to affect the pK of a group on the enzyme that controls the mechanism of chloride activation but rather decreases the apparent K_M by reducing the apparent binding constant for chloride.

Angiotensin converting enzyme (ACE;[1] EC 3.4.15.1) is a zinc dipeptidyl carboxypeptidase that catalyzes the hydrolytic release of dipeptides from the carboxyl terminus of a broad range of oligopeptides (Soffer, 1976). Its best known physiological function is to convert the decapeptide angiotensin I to the vasoactive octapeptide angiotensin II, and, thus, it serves an important role in maintaining the homeostasis of peripheral vascular resistance as well as the volume and electrolyte composition of extracellular body fluids (Peach, 1977; Reid et al., 1978).

Chloride is an important modulator of ACE activity and is required for the ACE-catalyzed conversion of angiotensin I to angiotensin II (Skeggs et al., 1954). Detailed studies on the mechanism of the chloride activation of ACE have been carried out with a series of *N*-furanacryloyl-tripeptides (Holmquist et al., 1979; Bünning et al., 1983, Bünning & Riordan, 1983; Shapiro et al., 1983). These investigations have demonstrated that chloride functions mainly in mediating substrate binding. At neutral pH values, the activation process with Fa-Phe-Gly-Gly follows an ordered bireactant mechanism in which the anion must bind to the enzyme first to form an enzyme–anion complex which then binds the substrate to result in hydrolysis (Bünning & Riordan, 1981, 1983). Chloride also enhances the binding of other substrates by acting as a nonessential activator (Shapiro et al., 1983).

Several other monovalent anions also activate ACE though not as effectively as chloride. Studies using chloride and bromide in combination (Bünning & Riordan, 1983) demonstrated that these two anions compete for the same binding site on the enzyme. Reductive methylation has shown that this site involves a critical lysine residue (Shapiro & Riordan, 1983).

Not all anions are effective activators of ACE. The monobasic form of phosphate, for example, is an inhibitor (Holmquist et al., 1979), and acetate is a very poor activator, but it does compete with chloride. Sulfate, a divalent anion, is particularly interesting. By itself, it does not increase the activity of ACE, but it will activate the enzyme in the presence of chloride (Dorer et al., 1976). This effect has, in fact, been incorporated into a proposed method for measuring ACE activity in human serum (Neels et al., 1982). We have now investigated in greater detail the effect of sulfate on the chloride activation of the ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly. The principal effect of sulfate is to decrease the apparent binding constant for chloride, thereby increasing the amount of enzyme–activator complex capable of binding substrate. In addition, it decreases the apparent K_M of the chloride-saturated enzyme. Thus, the overall effect of sulfate is to potentiate substrate binding.

Materials and Methods

ACE was purified to homogeneity from rabbit lung acetone powder by a previously described procedure (Bünning et al., 1983). The final preparation was at least 98% pure as judged by polyacrylamide gel electrophoresis. Concentrations of ACE were determined by measuring the absorbance at 280 nm and expressed in molar concentrations using a molar absorptivity of 204 000 M^{-1} cm^{-1} (Bünning et al., 1983).

Enzyme activities were measured with Fa-Phe-Gly-Gly as substrate as previously described (Holmquist et al., 1979). Typically, assays were performed with 50 μM substrate in 50 mM Hepes buffer and 300 mM NaCl, pH 7.5, 25 °C. Under these conditions, hydrolysis is first order in substrate ([S] < K_M). Activities, expressed as $V_0/[E]$ in units of min^{-1}, were obtained either from initial velocities or from half-lives after complete hydrolysis. In the determination of the pH–rate

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[1] Abbreviations: ACE, angiotensin converting enzyme; Fa, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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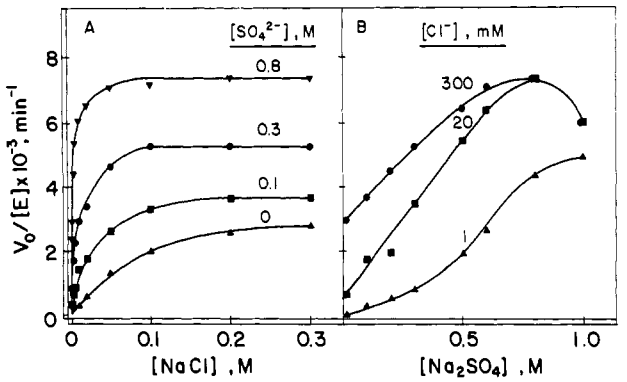


FIGURE 1: (A) Effect of sodium chloride concentration on the activity of ACE in the absence of sodium sulfate (▲) and in the presence of 0.1 (■), 0.3 (●), or 0.8 M (▼) sodium sulfate. (B) Effect of sodium sulfate concentration on ACE activity in the presence of 1 (▲), 20 (■), or 300 mM (●) sodium chloride. Enzyme activities were measured with 50 μM Fa-Phe-Gly-Gly as substrate in 0.05 M Hepes, pH 7.5.

Table I: Effects of Sulfate and pH on the Apparent Chloride Binding Constant (K_A') and K_M for the ACE-Catalyzed Hydrolysis of Fa-Phe-Gly-Gly[a]

	K_A' (mM)		K_M (mM)	
pH	−	+	−	+
6.0	3.3	0.7	0.14	nd[b]
7.5	80	3.2	0.3[c]	0.093[d]
9.0	190	4.2	0.48[e]	0.22[f]

[a] Determined in the absence (−) or presence (+) of 0.8 M sulfate. [b] Not determined. [c] Determined in the presence of 300 mM NaCl. [d] Determined in the presence of 20 mM NaCl. [e] Determined in the presence of 1 M NaCl. [f] Determined in the presence of 50 mM NaCl.

profiles, Mes (from pH 5.0 to 6.5), Hepes (from pH 6.5 to 8.5), and borate buffers (from pH 8.5 to 10.0) were used, all at 50 mM. These buffers have been shown to have little effect on ACE activity under standard conditions of assay (Bünning et al., 1983). Between pH 7.5 and 7.0, 10 μM zinc; between pH 7.0 and 6.0, 100 μM zinc; between pH 6.0 and 5.5, 1 mM zinc; and below pH 5.5, 10 mM zinc were added to assay mixtures to prevent spontaneous loss of zinc from the enzyme (Bünning et al., 1983).

The kinetic parameters K_M and k_{cat} were obtained from Lineweaver–Burk plots. Initial velocities were measured during the first 10% of hydrolysis. Apparent anion binding constants, K_A', were obtained from double-reciprocal plots of activity vs. anion concentration.

Results

The ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly exhibits an absolute dependence on the presence of monovalent anions, notably chloride. Activation by chloride is further enhanced by the presence of sulfate. The effects of 0.1, 0.3, and 0.8 M sulfate on the chloride activation of ACE at pH 7.5 and 50 μM substrate concentration are shown in Figure 1A. Under these conditions, i.e., [S] < K_M, first-order reaction kinetics are observed. Sulfate itself does not activate ACE, and with chloride alone, maximal activity is reached at a concentration of 300 mM. The presence of sulfate has two effects: it reduces the concentration of chloride required to attain maximal activity, and it increases the magnitude of the maximal enzyme activity. Thus, the apparent chloride binding constant, obtained from double-reciprocal plots of activity vs. anion concentration, decreases by a factor of 25 from approximately 80 mM in the absence of sulfate to 3.2 mM in the presence of 0.8 M sulfate (Table I). Furthermore, the activity of the chloride-saturated enzyme is increased 2.7-fold on addition

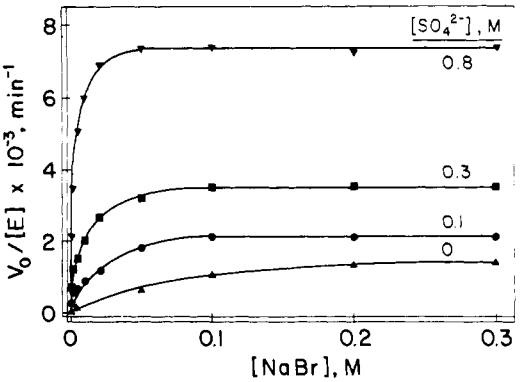


FIGURE 2: Effect of sodium bromide concentration on the activity of ACE in the absence of sodium sulfate (▲) and in the presence of 0.1 (●), 0.3 (■), or 0.8 M (▼) sodium sulfate. Enzyme activities were measured with 50 μM Fa-Phe-Gly-Gly as substrate in 0.05 M Hepes, pH 7.5.

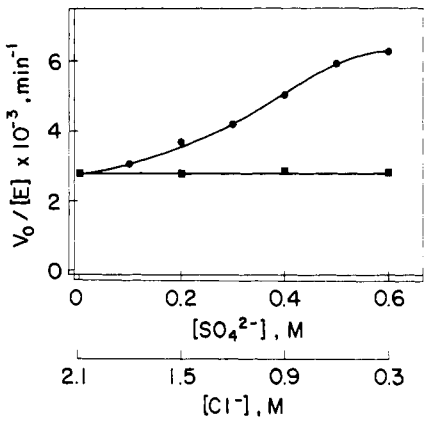


FIGURE 3: Activity measurements of ACE in the presence of varying sodium chloride and sodium sulfate concentrations at a constant ionic strength (●) and in the presence of varying chloride concentrations alone (■). Enzyme activities were measured with 50 μM Fa-Phe-Gly-Gly in 0.05 M Hepes, pH 7.5.

of 0.8 M sulfate, which is the optimal concentration. This dual effect of sulfate on the chloride activation of ACE is also observed when the sulfate concentration is varied in the presence of 1, 20, or 300 mM chloride (Figure 1B). At a concentration of 300 mM, the enzyme is saturated with chloride, and, thus, the rise in activity from 2750 to 7350 min^{-1} on addition of 0.8 M sulfate should only reflect the apparent activating effect of the divalent anion. In the presence of 20 mM chloride and increasing sulfate concentrations, ACE activity also reaches a maximum of 7350 min^{-1}. Under these conditions, the rise in activity is due to both the sulfate-mediated decrease of the apparent chloride binding constant and the increased maximal activity. However, in the presence of 1 mM chloride and 0.8 M sulfate, activity can only reach 70% of the optimal value since the enzyme does not become saturated with chloride under these conditions.

Bromide activation of ACE is similarly affected by sulfate (Figure 2). The apparent binding constant for bromide, like that for chloride, decreases from 80 to 3.2 mM in the presence of 0.8 M sulfate. In addition, the activity of the bromide-saturated enzyme rises from 1400 to 7350 min^{-1} on addition of sulfate, and, thus, it reaches the same activity observed with optimal chloride and sulfate. Hence, sulfate displays the same dual effect on the bromide activation of ACE that has been observed for the chloride activation.

The sulfate-mediated enhancement of halide activation of ACE may be due to either a specific effect of the divalent anion or an increase in ionic strength. To differentiate these pos-

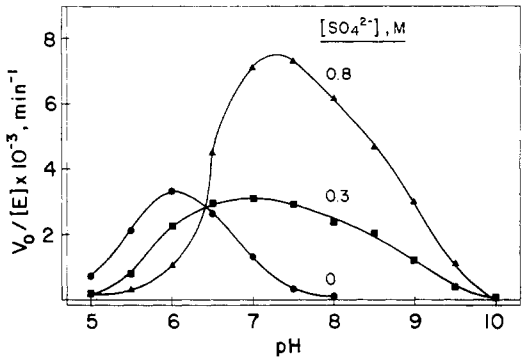


FIGURE 4: pH dependence of the ACE-catalyzed hydrolysis of 50 μM Fa-Phe-Gly-Gly in 20 mM sodium chloride (●) and 20 mM sodium chloride containing 0.3 (■) or 0.8 M (▲) sodium sulfate.

sibilities, activity measurements were carried out at increasing sulfate concentrations and constant ionic strength (Figure 3). While the sulfate concentration of the assay mixture was increased, the chloride concentration was simultaneously reduced such that the ionic strength was kept constant. This decrease of the chloride concentration from 2.1 to 0.3 M alone does not affect the activity of ACE. However, increasing the sulfate concentration at a constant ionic strength activates the enzyme. Thus, the activation by sulfate is the result of a specific effect of this divalent anion and is not due to a change in ionic strength.

In the neutral pH region, halides activate the ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly by decreasing the K_M but without changing the k_{cat} (Bünning & Riordan, 1983). The effect of sulfate on the kinetic parameters of the FA-Phe-Gly-Gly hydrolysis was studied at pH 7.5 in the presence of 20 mM sodium chloride. At 20 mM chloride alone, only 30% of the optimal activity is obtained whereas in the presence of 0.8 M sulfate this chloride concentration is almost able to saturate the enzyme (Figure 1). Lineweaver–Burk plots demonstrate that the apparent K_M decreases from 1.3 mM in the presence of 20 mM chloride alone to 0.093 mM on addition of 0.8 M sulfate (data not shown). The k_{cat} value, 19 000 min^{-1}, is not affected by the addition of sulfate and is identical with that observed for chloride alone.

The effect of sulfate on the pH dependence of ACE catalysis was studied in the presence of 20 mM chloride over the pH range 5–10 (Figure 4). Throughout this range, the hydrolysis of Fa-Phe-Gly-Gly at a concentration of 50 μM follows first-order reaction kinetics. The pH–rate profile in the presence of 20 mM chloride exhibits an optimum centered at pH 6.0. Addition of sulfate increases ACE activity in the alkaline pH region, and the pH optimum is shifted from pH 6.0 to 7.0 by 0.3 M sulfate and to pH 7.5 by 0.8 M sulfate. Furthermore, the maximal turnover rate increases from 3400 min^{-1} at pH 6 in the presence of 20 mM chloride alone to 7350 min^{-1} at pH 7.5 on addition of 0.8 M sulfate. Below pH 6.5, the addition of sulfate inhibits the enzyme.

The effect of sulfate on the apparent binding constant for chloride, K_A', and on the apparent K_M was studied at pH 6.0, 7.5, and 9.0 (Table I). In the absence of sulfate, K_A' increases with pH ranging from 3.3 mM at pH 6.0 to 190 mM at pH 9.0. On addition of 0.8 M sulfate, K_A' decreases to 0.7 mM at pH 6.0, 3.2 mM at pH 7.5, and 4.2 mM at pH 9.0. The apparent K_M obtained at saturating chloride concentrations but in the absence of sulfate increases from 0.14 mM at pH 6.0 to 0.48 mM at pH 9.0. At pH 6.0, a value for K_m' in the presence of sulfate was not obtained since sulfate inhibits the enzyme at this pH. However, at pH 7.5, K_M' decreases to 0.093 and at pH 9.0 to 0.22 mM on addition of 0.8 M sulfate. Thus, sulfate decreases K_A' by a factor from 5 to 50 and K_M' by about 3-fold over the pH range 6–9.

Discussion

Angiotensin converting enzyme, a zinc dipeptidyl carboxypeptidase, catalyzes the release of dipeptides from a broad range of oligopeptide substrates. One of its characteristic features is a requirement for monovalent anions, notably chloride, for catalytic activity (Skeggs et al., 1954). In spite of its significance, chloride activation has only recently become the subject of extensive investigation (Bünning & Riordan, 1983; Shapiro et al., 1983; Shapiro & Riordan, 1984). The present studies have employed Fa-Phe-Gly-Gly, a substrate which has proved to be particularly suitable for kinetic characterization of ACE (Holmquist et al., 1979; Bünning et al., 1983; Bünning & Riordan, 1983).

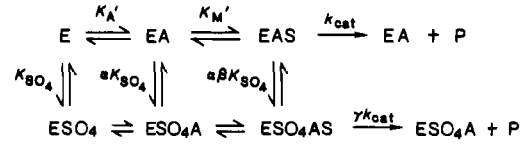
The monovalent anion activation of ACE is characteristically modulated by sulfate (Figures 1 and 2). Whereas sulfate itself does not activate the ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly, it enhances the chloride activation of the enzyme. At pH 7.5 and under first-order reaction conditions, the maximal effect of sulfate is observed at a concentration of 0.8 M and a (minimal) chloride concentration of 20 mM. This activation enhancement by sulfate is the composite of a decrease of the apparent chloride binding constant, K_A', and a 3-fold activation of the chloride-saturated enzyme. At pH 7.5, for both chloride and bromide, K_A' is shifted from 80 to 1.6 mM on addition of 0.8 M sulfate. Although the optimal activities of ACE for chloride, 2750 min^{-1}, and bromide, 1400 min^{-1}, differ almost by a factor of 2, they become identical in the presence of sulfate, i.e., 7350 min^{-1}. This enhancement of the halide activation of ACE by sulfate is a specific effect of the divalent anion (Figure 3) and is not due to an increase in ionic strength as has been suggested (Dorer et al., 1976). Hence, it appears likely that ACE contains at least two specific anion binding sites, one for monovalent anions, in particular chloride, and a second that binds sulfate.

The Lineweaver–Burk plots for the Fa-Phe-Gly-Gly hydrolysis obtained at pH 7.5 in the presence of 20 mM chloride and increasing sulfate concentrations show that the effect of sulfate is solely mediated through a decrease in K_M'. This is in accord with the ordered bireactant mechanism of chloride activation established for this substrate in which a decrease in K_A' manifests as a decrease in K_M' (Bünning & Riordan, 1983). The 3-fold activation of the chloride-saturated enzyme by sulfate is the result of an additional decrease in K_M' itself. Thus, at pH 7.5, sulfate modulates ACE activation through an effect on K_M', and, hence, the mechanism of the chloride activation of ACE remains unaltered.

The effect of sulfate on ACE activity was studied in the presence of 20 mM chloride over the pH range 5–10 (Figure 4). In the presence of 20 mM chloride alone, activity increases to an optimum of 3400 min^{-1} at about pH 6.0 but then falls off to less than 5% of this value at pH 9.0. This behavior is a consequence of the fact that the apparent chloride binding constant increases from 3.3 to 190 mM as the pH is raised from 6 to 9 (Table I). Thus, a chloride concentration of 20 mM is well in excess of K_A' at pH 6.0 but is well below it at pH 9.0. Above pH 6.5, sulfate increases activity and shifts the pH optimum from pH 6.0 to 7.5. The activity observed at pH 7.5 in the presence of 0.8 M sulfate is 7350 min^{-1}, compared to 2750 min^{-1} for the optimal activity in the presence of chloride alone, and, thus, reflects the 3-fold activation of the chloride-saturated enzyme by sulfate.

The effects of sulfate on ACE activity are the result of changes in both K_A' and K_M' (Table I). As shown previously,

Scheme I



K_A', the apparent binding constant for chloride to the free enzyme, increases markedly on going from pH 6.0 to pH 9.0 (Bünning & Riordan, 1983). At the pH studied, sulfate reduces the K_A' by up to about 50-fold. Similarly, it reduces K_M' by 2–3-fold at pH 7.5 and 9.0. Because of the inhibition by sulfate at pH 6.0, it was not possible to obtain a valid K_M' at this pH value. For this reason, a complete description of the effect of sulfate on the pH dependence of K_M' cannot be made. Nevertheless, it does not seem likely that sulfate induces a shift in the pK of a group on the enzyme that governs the pH dependence of K_A' since the magnitude of this change is not a function of pH.

Chloride activation of the ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly at pH 7.5 has been shown to follow an ordered bireactant mechanism in which anion binds to the free enzyme prior to the binding of substrate (Bünning & Riordan, 1981, 1983). At pH 6.0, however, the mechanism of activation follows a random rather than an ordered pathway, but chloride remains an essential activator (Bünning & Riordan, 1983). Sulfate does not appear to change the mechanism of activation of ACE by chloride at pH 7.5. Increasing concentrations of sulfate only affect K_M' and do not alter k_{cat} as they would if the reaction mechanism changed to a random bireactant process. This is consistent with the fact that sulfate does not shift the pK of a group on the enzyme that controls the mechanism of chloride activation.

One probable explanation for the effect of sulfate on ACE activity is given in Scheme I. In the absence of sulfate, chloride, the activating anion, binds to the free enzyme, E, to form the enzyme–anion complex, EA. Binding of substrate gives the ternary EAS complex which goes on to form product. Sulfate interacts with all three forms of the enzyme; E, EA, and EAS. Since K_A' is up to 50-fold lower in the presence of sulfate, α, which is a measure of the effect of chloride on the binding of sulfate and vice versa, must have a value of between 0.02 and 0.2. This implies that binding of sulfate to the free enzyme, or at least to a specific activation site on the free enzyme, is very weak. Binding of sulfate to EA will lower the concentration of this complex, shift the equilibrium to the right, and, hence, decrease the apparent K_A. In addition, binding of sulfate to ESA will similarly decrease the apparent K_M. Since maximal activity is not affected by sulfate, the rates of product formation by either pathway will be the same, and γ must have a value of 1.

The physiological relevance of a sulfate-mediated enhancement of the chloride activation of ACE is at present unknown. The intracellular sulfate concentration is 10 mM while the plasma concentration is 0.5 mM. A major locus of ACE activity is the lumenal side of the plasma membrane of vascular endothelial cells (Ryan et al., 1975; Caldwell et al., 1976). Thus, ACE is exposed to the sulfate concentration in plasma, i.e., 0.5 mM, which appears to be too small to fully exploit the enhancement of the chloride activation of ACE. Therefore, it is unlikely that sulfate significantly affects ACE activity in vivo. However, it could be that other substances present in plasma display an effect on the chloride concentration of ACE similar to that observed by sulfate and, thereby, regulate the activity of ACE. Heparan sulfate, a constituent of endothelial cell membranes, should also be considered from this point of view. Moreover, ACE in other tissues may be exposed to different concentrations of sulfate or related substances and, hence, be subject to local control. It might also be that the response of membrane-bound ACE to sulfate may not be the same as that observed with the enzyme in solution.

Finally, Fa-Phe-Gly-Gly is characteristic of class I substrates of ACE which at pH 7.5 follow an ordered bireactant mechanism where chloride binds prior to substrate. Class II substrates, in contrast, are hydrolyzed by a nonessential activator mechanism (Shapiro et al., 1983) and require much less chloride for maximal activation. There is, in addition, a third class of substrates whose mechanism of activation has not been established but may also be nonessential. These three classes have been distinguished by the effect of pH on activation, the relative activating potencies of various anions, and the apparent activation constant for chloride as well as the kinetic mechanism (Shapiro & Riordan, 1983). The effect of sulfate on the monovalent anion activation of ACE with each of these substrate classes has not yet been examined but may well provide an additional characteristic. It may also provide an understanding of this complex mechanism for anion regulation of ACE activity.

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REFERENCES

Bünning, P., & Riordan, J. F. (1981) *Isr. J. Chem. 21*, 43–47.
Bünning, P., & Riordan, J. F. (1983) *Biochemistry 22*, 110–116.
Bünning, P., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 103–110.
Caldwell, P. R. B., Seegal, B. C., Hsu, K. C., Das, M., & Soffer, R. L. (1976) *Science (Washington, D.C.) 191*, 1050–1050.
Dorer, F. E., Kahn, J. F., Lent, K. E., Levine, M., & Skeggs, L. T. (1976) *Biochim. Biophys. Acta 429*, 220–228.
Holmquist, B., Bünning, P., & Riordan, J. F. (1979) *Anal. Biochem. 95*, 540–548.
Neels, H. M., Scharpe, S., van Sande, M., Verkerk, R. M., & van Acker, K. J. (1982) *Clin. Chem. (Winston-Salem, N.C.) 28*, 1352–1355.
Peach, M. J. (1977) *Physiol. Rev. 57*, 313–370.
Reid, I. A., Morris, B. J., & Ganong, W. F. (1978) *Annu. Rev. Physiol. 40*, 377–410.
Ryan, J. W., Ryan, U. S., Schultz, D. R., Whitaker, C., Chung, A., & Dorer, F. E. (1975) *Biochem. J. 146*, 497–499.
Shapiro, R., & Riordan, J. F. (1983) *Biochemistry 22*, 5315–5321.
Shapiro, R., Holmquist, B., & Roirdan, J. F. (1983) *Biochemistry 22*, 3850–3857.
Skeggs, L. T., Marsh, W. H., Kahn, J. R., & Shumway, N. P. (1954) *J. Exp. Med. 99*, 275–282.
Soffer, R. L. (1976) *Annu. Rev. Biochem. 45*, 73–94.