CARBONIC ANHYDRASE INHIBITORS

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Inhibitors of carbonic anhydrase possessing remarkably high potency and specificity have been developed in recent years. The value of compounds of this group as pharmacologic tools for the investigation of reactions involving carbon dioxide and for the study of the role of carbonic anhydrase in biologic processes has been amply demonstrated. Their exact place as therapeutic agents, currently under intensive investigation, is yet to be established. The present review is an attempt to analyze the pharmacologic effects of carbonic anhydrase inhibitors with respect to the physiologic processes in which the enzyme is involved, and to summarize the information bearing upon the possible therapeutic usefulness of these compounds.

THE REACTIONS OF CO_2 IN SOLUTION

The only known action of carbonic anhydrase is upon the reactions of the CO_2-carbonic acid system. An understanding of the chemistry of this system is therefore essential to an insight into the actions of carbonic anhydrase and the effects of its inhibitors. In aqueous solution, dissolved CO_2 reacts reversibly with water to yield carbonic acid, which in turn dissociates to yield bicarbonate and hydrogen ions:

1) $CO_2 + H_2O \rightleftharpoons H_2CO_3$ 2) $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$

It is pertinent to consider the kinetics of the first reaction in the absence of enzyme. This is expressed by Roughton (203) as:

3) $$d[CO_2]/dt = \{-K_u[CO_2] + K_0[H_2CO_3]\}(1 + l[B^-])$$

Where K_u is the rate constant for hydration of CO_2, K_0 the rate constant for dehydration of carbonic acid, and l is the catalytic coefficient for an inorganic catalyst B, and $[B^-]$ is the concentration of the anion formed by the dissociation of B.

Roughton and Clark (204) give as the best estimates of the constants at 37°: $K_u = 0.11$ and $K_0 = 89.0$. Values of l range from 8 for $HPO_4^=$ to 30,000 for hypobromite. The reaction is catalyzed by a number of anions, but those of high activity are not likely to occur in biological systems (130, 203). At a pH above 8, the reaction is accelerated, by the direct reaction of CO_2 with hydroxyl ion or catalysis, by hydroxyl ion, of the reaction of CO_2 and water (203).

The ratio of the rate constants, K_0/K_u, is the ratio of dissolved CO_2 to carbonic acid at equilibrium and the high value of this ratio, 809, denotes a fact which is often not appreciated, namely, that the concentration of dissolved CO_2 vastly exceeds that of carbonic acid. Confusion often arises in this respect

because it is common practice to refer to the denominator of the fraction in the Henderson-Hasselbalch equation as "carbonic acid." Actually, the equation should be written:

$$4) \quad pH = pK' + \log \frac{[HCO_3^-]}{[H_2CO_3] + [\text{dissolved } CO_2]} = pK' + \log \frac{[HCO_3^-]}{810[H_2CO_3]}$$

Carbonic acid is therefore a relatively strong acid with a true pK less than its apparent pK (pK') of 6.3[1] by log 810. Roughton and Clark (204) give 3.39 as the best estimate of the true pK.

It is apparent that the bicarbonate-carbonic acid system, considered without the dissolved CO_2, is, at the pH of blood, a very poor buffer indeed. The system behaves as a buffer only insofar as the ready availability of the dissolved CO_2 acts as a source of or means of disposing of additional undissociated acid. In the face of rapid addition of acid or alkali to its solution, the system can act as an effective buffer only to the extent that the relatively slow interconversion of CO_2 and H_2CO_3 can be accelerated. It is in accelerating this interconversion, and thus rendering the CO_2 system an effective buffer, that carbonic anhydrase is believed to act in a number of secretory mechanisms and it is likely that carbonic anhydrase inhibitors exert their effects on such mechanisms by interfering with the buffering efficacy of the CO_2 system.

Although equilibrium with respect to reaction 2) is established virtually instantaneously, reaction 1) is relatively slow, requiring, at 38°C, approximately 200 seconds to come to within 10% of equilibrium. It is in accelerating this reaction that carbonic anhydrase is involved.

The enzyme is a zinc-containing protein (129) of molecular weight about 30,000 (188). Its only action is believed to be the acceleration in both directions of the reaction expressed in equation 1) (203, 204), although Smith (217) has proposed on the basis of a general hypothesis concerning the mode of action of metal peptidases that carbonic anhydrase accelerates the reaction:

$$5) \quad CO_2 + OH^- \rightleftharpoons HCO_3^-$$

Since the two mechanisms are not distinguished by available methods and since the overall results (because of the rapidity of equilibration in reaction 2)) are not distinguishable, the catalyzed reaction may, for most purposes, be considered that between CO_2 and water.

The enzyme, in common with other metalloprotein catalysts, is inhibited by such reagents as azide, cyanide and sulfide (152). The anions of many salts such as chloride, bromide, iodide and nitrate are mildly inhibitory, the concentration producing 50% inhibition being 10^{-1} to 10^{-2}M (204). Although inhibitory activity has been sought in a number of groups of organic compounds (160), only among sulfonamides unsubstituted on the sulfonamide nitrogen has significant activity been found. The pharmacology of carbonic anhydrase inhibitors therefore deals almost exclusively with this group of substances.

[1] The constants given are for dilute solution. The pK' for plasma at 37°C is generally accepted as 6.10.

The studies which led Mann and Keilin (152) to the discovery that sulfanilamide is a powerful inhibitor of carbonic anhydrase were suggested by the acidosis observed in patients treated with that drug. These observations and their interpretation will be considered in detail with the renal effects of carbonic anhydrase inhibitors. Mann and Keilin (152) showed sulfanilamide to be inhibitory at concentrations as low as 2×10^{-6}M. They also found that substitutions for one of the hydrogens of the amido group resulted in complete loss of activity. Although the latter finding has generally been corroborated (145, 155, 160, 237), Krebs (138) found N^1-substituted sulfonamides to have some activity but less than that of the unsubstituted compounds by a factor of 10^2 to 10^4. The inhibitory activity of numbers of sulfonamides has been examined by various investigators (138, 145, 152, 160, 169). Highest activity has generally been found among the heterocyclic compounds, but the structural requirements for maximum activity have not been clearly defined. Among those heterocyclic sulfonamides synthesized by Roblin and Clapp (201) were several, including the now commonly used acetazoleamide (Diamox®, # 6063, 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide), found by Miller *et al.* (169) to have activities several hundred to a thousand times greater than that of sulfanilamide and thus capable of 50% inhibition of carbonic anhydrase at concentrations as low as 10^{-8}M. Miller *et al.* (169) found that, within a series of compounds containing a particular heterocyclic ring structure, activity appeared to increase with increasing acid strength, but each type of ring seemed to constitute a separate series, since the correlation between activity and pKa did not carry over from one type of heterocycle to another.

The kinetics of the enzyme-inhibitor interaction have been studied by Davenport (59) and Krebs (138). Davenport found that the inhibition behaved as would be expected if there were a simple reversible reaction between carbonic anhydrase and the inhibitor expressed as:

6) $$E + I \rightleftharpoons EI$$

The equilibrium constants $K = [E][I]/[EI]$ were determined for sulfanilamide and thiophene-2-sulfonamide, and found to be 1.6×10^{-7} and 1.0×10^{-6} respectively at 0°C. Millichap *et al.* (172) have recently reported studies of the *in vitro* inhibition of carbonic anhydrase with acetazoleamide. Fifty per cent inhibition of enzyme activity was obtained when the acetazoleamide added was at a concentration of 9.1×10^{-9} mols per liter at 0°C and at 7.2×10^{-9} mols per liter at 10°C. The findings were unusual in two respects: 1) the activity of the inhibitor appeared to be greater at 10° than at 0°; 2) the relationship of inhibition to concentration of inhibitor appeared, at 0°C, to be that which would obtain if the enzyme were inhibited only when combined with two mols of acetazoleamide. At 10°C the relationship did not fit the usual curve for reaction with either one or two mols of the inhibitor, but appeared to fall somewhere between. In performing the calculations, the usual assumption was made that the amount of inhibitor bound to the enzyme was negligible with respect to the total. In the case of an inhibitor with such an extraordinary affinity for the enzyme this assumption may not be warranted, since partial inhibition may be obtained

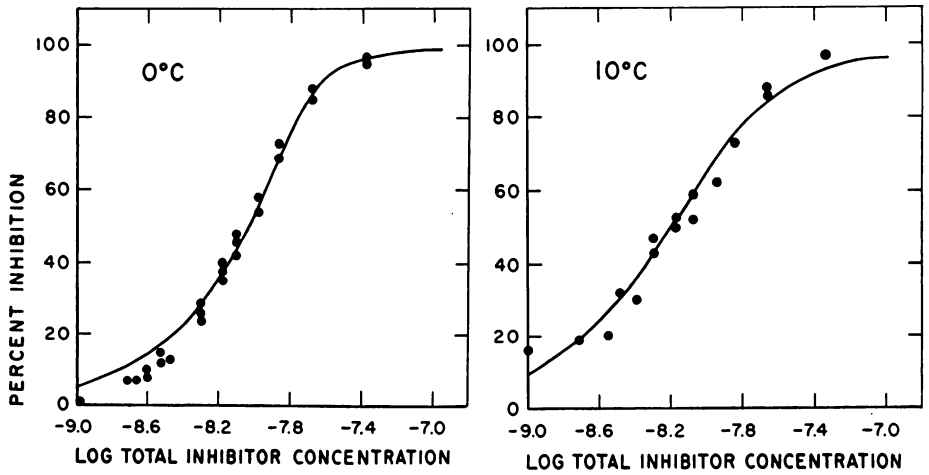

FIG. 1. Relationship between the concentration of acetazoleamide (free plus bound to enzyme) and inhibition of carbonic anhydrase activity. The data are those of Millichap *et al.* (172) and are replotted for comparison with curves calculated to take into account the amount of inhibitor bound to enzyme when the molar concentrations of inhibitor and enzyme are of the same order of magnitude. The enzyme-inhibitor complex is assumed to be composed of one molecule of each species. The total enzyme concentration used is assumed to be 16.2×10^{-9}M at 0°C and 6.3×10^{-9}M at 10°C, the constants for dissociation of the enzyme inhibitor complex 1×10^{-9} at 0°C and 3.15×10^{-9} at 10°C.

only when the concentrations of enzyme and inhibitor are of the same order of magnitude. If the curves are recalculated taking into account the possibility of drug bound to enzyme, the data fit reasonably well the curves calculated for reaction of one mol of enzyme with one of inhibitor—as shown in Figure 1. The calculation is somewhat complicated by the necessity of estimating the amounts of enzyme used, but only a relatively narrow range of concentrations is compatible with the data and with the procedure for preparing the enzyme.[2] When thus recalculated so that the concentrations considered are those remaining free, the inhibitor constants are even lower than previous estimates,—approximately 1×10^{-9} at 0°C and 3.15×10^{-9} at 10°C. In view of the uncertainties in estimating these figures, it does not seem warranted to extrapolate to 37°C. However, it is significant that the constant is now larger at the higher temperature, as would be expected. The higher constant at lower temperature in the original calculations (172) may have been due to the use of larger amounts of enzyme at 0°C. It would appear that some direct measurements of binding such as by equilibrium dialysis would be useful. Although present methods of analysis for acetazoleamide might not have sufficient sensitivity, it might be possible to use some other inhibitor of comparable activity for which methods are available.

The extent of binding of drug to enzyme is of considerable importance, as has

[2] We wish to express our gratitude to Dr. J. Gordon Millichap for making the original data available to us and to Dr. B. L. Horecker for helpful advice.

been pointed out by Hunter and Lowry (123a), in considering some of the apparent anomalies of distribution of acetazoleamide in the body.

EFFECT OF CARBONIC ANHYDRASE INHIBITORS ON RESPIRATION

The best recognized and understood function of carbonic anhydrase is that of accelerating the conversion of carbonic acid to CO_2 during passage of the blood through lung capillaries. The extent of catalysis required depends upon the time available for the liberation of CO_2 into the alveoli. Roughton (203) has estimated that if the blood spends one second in the pulmonary capillaries and if the liberation of CO_2 is to approach within 10% of equilibrium, the dehydration of carbonic acid must be accelerated some two hundred-fold. If the time spent by the blood in traversing the capillaries is greater, the catalysis required would be proportionately reduced and *vice versa*. It must be recognized that exactly the same process occurs in reverse in the tissue capillaries and also requires catalysis to an extent inversely proportional to the time the blood spends in transit.

The events in each area require that large amounts of CO_2 traverse the plasma between red cells and alveoli or tissue cells as the case may be. The absence of carbonic anhydrase from the plasma implies that in these regions this dissolved CO_2 is not in equilibrium with the plasma carbonic acid and bicarbonate. Roughton (202) suggested that this might have certain advantages for the organism in that it would tend to minimize the extremes of pH to which the capillaries would be subjected as CO_2 is taken up and unloaded. The presence of a normally-occurring carbonic anhydrase inhibitor found by Booth (34) in the plasma of a number of species seemed in accord with this view in that it would assure the absence of circulating carbonic anhydrase activity if red cells were destroyed. However, the inhibitor is not present in the serum of a number of animals, including man, and the pH changes which would occur if equilibrium of CO_2 and carbonic acid were established in the capillaries are relatively small compared to those which may occur physiologically.

The amount of carbonic anhydrase in human red blood cells, determined with hemolyzed cells, is sufficient to accelerate the conversion of CO_2 to H_2CO_3 some seven thousand and five hundred-fold, but there may be some question as to whether, when this enzyme is concentrated into the red cells, it is able to act with equal efficiency (203). In any case in the presence of high enzyme activity, it is doubtful that the rate of CO_2 uptake or liberation by a red cell suspension will be limited by the enzyme-catalyzed reaction, a circumstance which makes it difficult to evaluate the potentialities of the carbonic anhydrase under these conditions. For the liberation of most of the CO_2 from the blood, bicarbonate from plasma must exchange for red cell chloride, the bicarbonate becoming carbonic acid by interaction largely with hemoglobin, being then converted to CO_2. The exchange of chloride and bicarbonate, although rapid for the movement of ions across cell membranes, is not instantaneous. Roughton (202) has interpreted the findings of Dirken and Mook (78) to indicate that this exchange in ox blood is 90% completed in 1.3 seconds. If carbonic anhydrase activity in the cells is adequate to keep well ahead of this chloride shift, the ion exchange will become rate limiting in CO_2 uptake and release (202) and the overall rate will not yield information on the activity of carbonic anhydrase beyond a certain minimum of such activity. A possible additional factor which has not been fully

evaluated is the diffusion of CO_2 across the red cell membrane. The recent finding of Gibson *et al.* (100) that, contrary to previous supposition, the red cell membrane apparently constitutes an appreciable barrier to the entry of oxygen, raises the question as to whether it may similarly impede the diffusion of CO_2. However, since CO_2 is considerably more diffusible than oxygen, and since the permeation of CO_2 is probably much more rapid than the exchange of ions, it is unlikely that the diffusion of the gas would ever become rate-limiting.

An interesting phenomenon involving carbonic anhydrase and the application of carbonic anhydrase inhibitors to elucidation of its mechanism has been described by Jacobs and Stewart (126). These workers showed that the acceleration by bicarbonate of the swelling and hemolysis of red cells in solutions of ammonium salts was highly dependent on carbonic anhydrase and was almost abolished by cyanide or sulfanilamide. The mechanism involved was presumed to be as follows: in the absence of bicarbonate from the suspending medium ammonia diffuses into the cells and by hydrolysis yields ammonium and hydroxyl ions; hydroxyl ion then exchanges for chloride from the medium and the net result is entry of ammonium chloride into the cells. Because of the very low concentration of hydroxyl ions in the cells, the transfer proceeds very slowly. If bicarbonate is added to the medium, the dissolved CO_2 formed can enter the cell, be converted to carbonic acid and thence to bicarbonate ion, which now being present in the cell at much higher concentration than the hydroxyl ion can exchange much more rapidly for chloride. Red cell carbonic anhydrase greatly facilitates the process by increasing the rate of formation of bicarbonate in the red cells. Addition of carbonic anhydrase to the suspending medium further enhanced the rate of the overall process by providing for the rapid formation of CO_2 from bicarbonate so that the cycle could continue. Similar considerations were shown to apply to the shrinkage of red cells in an alkaline medium and in a sulfate containing medium. Jacobs and Stewart found detectable inhibition of red-cell swelling in solutions containing NH_4HCO_3 at remarkably low concentrations of sulfanilamide (2×10^{-6}M) corresponding to the lowest of those found to inhibit the isolated enzyme by Mann and Keilin (152). Sulfathiazole and sulfapyridine, both N^1-substituted sulfonamides, were inactive. The low concentrations of sulfanilamide at which inhibitory effects were observed are in striking contrast to levels required to inhibit measurably the CO_2 exchanges of whole blood (see below).

The consideration of which reaction is rate-limiting becomes important in evaluating the effects of inhibitors on the carbonic anhydrase activity of intact red cells (123a). For example, Davenport (59) found that the uptake of CO_2 by whole blood was only some seventeen times faster in the absence of inhibitor than in the presence of concentrations of thiophene-2-sulfonamide presumed to have eliminated carbonic anhydrase activity. At concentrations of inhibitor calculated to be adequate to inhibit the enzyme 99.97%, the CO_2 uptake was still accelerated three-fold above the lowest observed rate. This represented a reduction of some 87% of the rate of uptake in the absence of inhibitor. However, as Hunter and Lowry have pointed out (123a), this degree of inhibition is compatible with that predicted from the study of the isolated enzyme, provided it is assumed that 1) the enzyme activity in the cells is the same as that measured when the cells are lysed and 2) that in the uninhibited system some other step is rate-limiting.

The effect of carbonic anhydrase inhibition on CO_2 elimination. The immediate effect of inhibiting carbonic anhydrase would be expected to be a diminution of the elimination of CO_2, and the accumulation of CO_2 in the body until a new

steady state was reached. The amounts of CO_2 lost in the urine, even under the maximal effects of carbonic anhydrase inhibitors, are so small relative to respiratory CO_2 exchange as to make no significant contribution to the overall output. The distribution of the elevated CO_2 tension in the body when carbonic anhydrase is inhibited would be expected to be relatively complex. It is generally accepted that the CO_2 tension of pulmonary capillary plasma does not differ appreciably from the CO_2 tension of the alveolar air and this situation should not be disturbed by inhibitors of carbonic anhydrase. However, since most of the metabolic CO_2 is transported as bicarbonate, only a very small fraction of the CO_2 normally eliminated in the lung can be lost merely by equilibrating the dissolved CO_2 of the blood with that of alveolar air (202). The remainder must be derived from carbamino CO_2 and, predominantly, from conversion of bicarbonate to carbonic acid and thence to CO_2. If the last step were impeded by appreciable inhibition of carbonic anhydrase, it would be expected that the CO_2 tension of blood leaving the pulmonary capillaries, although at the same level as in the alveolar air, would be lower than that which would occur when equilibrium had been achieved by equilibration of the bicarbonate, carbonic acid and dissolved CO_2. The CO_2 tension would therefore rise progressively in the course of movement of the blood from pulmonary capillaries through the arterial tree (and presumably *in vitro* if an arterial sample is withdrawn for analysis). Conversely, in the tissue capillaries, the CO_2 tension of the blood would presumably become equal to that of the tissues, but because of the delay in conversion to bicarbonate, the blood might reach the venous system before equilibrium was achieved. Consequently, the CO_2 tension would fall during the course of movement from tissue capillary to pulmonary capillary as CO_2 was converted to bicarbonate. The net result would be elevation of tissue CO_2 tension to exceed alveolar CO_2 tension by considerably more than the usual increment and presumably a greater than normal fraction of the CO_2 would be transported in the carbamino form and liberated therefrom in the lungs (205). The effects of carbonic anhydrase inhibitors on CO_2 elimination would therefore be an immediate, though transient, decrease in CO_2 output and the appearance of a difference between alveolar CO_2 tension and that found in arterial blood. Both effects would be exaggerated by any circumstance which diminished the time spent in transit through the pulmonary capillaries in that less time would be available for the inhibited reaction to proceed while the loss of CO_2 was still possible.

The first experimental examination of these phenomena was that of Roughton *et al.* (205) who found that, in normal subjects receiving 2 to 3 g of sulfanilamide daily, there was a decrease in CO_2 elimination during severe exercise. In the subject showing the greatest effect on CO_2 output, the plasma sulfanilamide concentration was 4.2 mg% (2.4×10^{-4}M). During lesser degrees of exertion no abnormalities were detected. The authors also noted, in experiments not reported in detail, that in moderate exercise there was 1) an increase in the arteriovenous pCO_2 difference, a circumstance which would facilitate increased transport of carbamino-CO_2 and 2) a tendency for arterial pCO_2 to exceed alveolar CO_2 tension.

A number of investigators have examined the effects of the far more powerful inhibitor, acetazoleamide, on CO_2 elimination. In anesthetized dogs, Tomashefski *et al.* (227) found that doses of 5–100 mg/kg given intravenously caused a sharp drop in alveolar pCO_2 without a fall or with a slight rise in arterial pCO_2. Similar results are reported by Carter (42) in trained unanesthetized dogs given acetazoleamide intravenously; arterial pCO_2 was correlated with neither ventilation nor alveolar pCO_2. Both Carter and Tomashefski *et al.* (227) refer to a gradient of CO_2 tension from erythrocyte to plasma when carbonic anhydrase is inhibited. The existence of such a gradient is difficult to accept in view of the diffusibility of CO_2 and it seems virtually certain that the disequilibrium to which they refer is one step further back, in the conversion of carbonic acid to CO_2 in the red cells. Maren (154) has found that the administration of acetazoleamide intravenously or by mouth in doses as small as 10 mg/kg causes elevation of the plasma CO_2 tension. These effects are presumably also attributable to inhibition of red cell carbonic anhydrase although the measurements were made on venous blood and there are no estimates of alveolar pCO_2 to which they can be related. The rises in CO_2 tension were particularly marked in animals sufficiently acidotic to avoid a further decrease in plasma bicarbonate when acetazoleamide was administered, since the normal decrease in pCO_2 stimulated by metabolic acidosis tends to obscure the increase due to the acetazoleamide. In animals in which pCO_2 did not rise when the drug was administered, the effect was indicated by the further fall in pCO_2 in response to the existing metabolic acidosis as the acetazoleamide disappeared from the plasma.

In contrast to the relatively consistent effects of acetazoleamide on CO_2 elimination in the dog, the results of administration in man have differed in the hands of different investigators. Becker *et al.* (20) administered 50 mg/kg orally to three trained subjects and found no change one and two hours later in ventilation, oxygen consumption, respiratory exchange ratio, and arterial and alveolar CO_2 tension. Mild exercise, sufficient to double oxygen consumption, induced no evidence of impaired elimination of CO_2. A similar lack of effect in two normal subjects given 25 mg/kg by mouth was reported by Tomashefski *et al.* (227). Shepard *et al.* (216) found no significant effect of acetazoleamide in resting subjects, but during severe exercise two subjects, given approximately 12 mg/kg by mouth, showed arterial-alveolar pCO_2 gradients of 5–12 mm Hg and changes in the elimination of CO_2 which suggested that CO_2 production exceeded elimination. On the other hand Cranston *et al.* (54) describe a marked rise in arterial pCO_2 thirty to sixty minutes after the oral administration of 25 mg/kg of acetazoleamide. At this time there was no change in alveolar ventilation or alveolar pCO_2 and CO_2 output fell only slightly. The reasons for the discrepancies among the results obtained by these groups of investigators are not clear, but even more striking is the relatively slight effect observed in man and the difference between the results obtained in man and in the dog. In all of the reported studies in man the acetazoleamide has been given by mouth and in most of those in the dog the intravenous route has been used. However, Maren *et al.* (156) have found little difference after the first hour in the concentration of acetazoleamide in plasma and red cells when the drug was given by mouth and when it was administered

intravenously. Furthermore, Maren (154) has found what appear to be effects on CO_2 elimination after doses of only 10 mg/kg orally in the dog.

The relatively modest effects, particularly in man, of amounts of inhibitor which greatly exceed those required to produce complete inhibition *in vitro* remain essentially unexplained. Maren *et al.* (156) record concentrations of acetazoleamide in the order of 30 μg/ml in plasma and red cells in patients given doses of only 7–10 mg/kg. These concentrations (15×10^{-5}M) are to be compared with those (approximately 10^{-6}M) found by Miller *et al.* (169) to yield virtually complete inhibition of carbonic anhydrase *in vitro*. It is true, as pointed out by Hunter and Lowry (123a), that concentrations of acetazoleamide as high as 20 μg/ml in red cells may represent only one molecule of inhibitor per molecule of enzyme and so could yield only partial inhibition, but higher concentrations have been found with moderate doses of drug. That a large part of the acetazoleamide is bound to enzyme in red cells is suggested by the observations of Maren *et al.* (156) that the concentration in the cells is largely independent of plasma concentration when the latter is low and that appreciable amounts may remain in the red cells when the drug has virtually disappeared from the plasma. This would be predicted from the extremely low dissociation constant of the enzyme inhibitor complex ((172) and see above).

The effect of sulfanilamide on the ability of rabbits and dogs to withstand simulated high altitudes was studied by Lawson (140). The animals were able to tolerate an altitude some 10% greater when given large doses of sulfanilamide. In the unanesthetized rabbits, this was associated with a higher terminal concentration of CO_2 in the blood. In anesthetized dogs, no difference between blood and alveolar CO_2 tensions was found in what the author termed "the more successful experiments" and the plasma CO_2's were not higher in those given sulfanilamide. The results in the dogs do not suggest appreciable interference with CO_2 elimination. The effect in rabbits is compatible with such interference, but enough data could not be collected to warrant a definite conclusion and the observations could be explained if hyperventilation were suppressed by some other mechanism. Using a reduction of barometric pressure to 350 mm Hg, Carter (43) found that acetazoleamide-treated dogs (100 mg/kg intravenously) had a lower arterial CO_2 content than did control dogs, an observation in accord with the results obtained by Lawson in the dog (140).

The effects of acetazoleamide in patients with pulmonary emphysema. There have been a number of studies of the effect of acetazoleamide in patients with respiratory acidosis due to chronic pulmonary disease. Nadell (179) administered 8–10 mg/kg/day to two patients with emphysema and respiratory acidosis. The fall in plasma bicarbonate as a result of the renal effect was anticipated, but the rather surprising effect was a return of the plasma pH to normal over a period of several days to weeks. This represented not only the usual compensation for the decreased plasma bicarbonate but also considerable improvement of ventilatory elimination of CO_2 beyond that before the drug was administered. There was marked symptomatic improvement in these patients as well as a striking, though transient, clearing of the mental symptoms in another patient with emphysema.

The studies which have subsequently been reported generally confirm the

findings of Nadell (179). Lyons *et al.* (149) examined the effects of 250 mg of acetazoleamide twice daily in nine patients with emphysema. A decrease in arterial pCO_2 occurred at some time during the course in six of the nine, although in several of these the pCO_2 fluctuated rather erratically. Two of the three patients who showed no response did not have elevated CO_2 tensions before treatment.

Bell *et al.* (22) found decreases of arterial CO_2 tension in five of seven patients with respiratory acidosis although in these instances the plasma pH was lowered and did not return to normal during continued therapy. Marked but transient symptomatic improvement occurred in four patients. Lukas (148) has reported similar results. Galdston (94, 95, 96), in five patients with respiratory acidosis, found the lowering of pCO_2 to be smaller than the drop which occurred in patients without pulmonary disease. The arterial pCO_2 fluctuated erratically and arterial pH did not rise to normal. Fishman *et al.* (87) also describe a lowering of arterial CO_2 tension in emphysematous patients treated with acetazoleamide.

It seems reasonably well-established that acetazoleamide does tend to cause a lowering of arterial CO_2 tension in patients with respiratory acidosis and in many instances produces marked symptomatic improvement (47, 87, 114, 148, 149, 179). The mechanisms by which these effects are produced are not clear. Patients with respiratory acidosis would appear to have sufficient stimulus for increased ventilation were they able to respond and were their respiratory centers sensitive to such stimuli. As a matter of fact, it is well known that their responsiveness to acidosis and increased CO_2 tension is markedly depressed, the major stimulus to respiration being arterial oxygen unsaturation. Restoration of sensitivity to CO_2 might, therefore, be an adequate reason for the fall in pCO_2. However, there is no evidence for such a restoration of sensitivity: Fishman *et al.* (87) found the poor ventilatory response of emphysematous patients to inhaled CO_2 was not restored when acetazoleamide was administered even for prolonged periods and Cranston *et al.* (54) report no change in the ventilatory response to inhaled CO_2 when acetazoleamide was given to normal subjects. Cohn *et al.* (47) believe the respiratory effects are the consequence of the diuretic effects of the drug. This interpretation follows from the view of Riley (199) that no substantial lowering of alveolar pCO_2 can be accomplished by the emphysematous patient except by diminishing the work of breathing—the energy cost of respiration is considered to be such that more CO_2 is produced in the effort of ventilation than can be dispelled in the process. That this view is not strictly correct, however, is indicated by the fact that salicylates, by increasing the sensitivity of the respiratory center to CO_2 (1), caused a decrease in arterial pCO_2 in patients with emphysema and chronic respiratory acidosis (232). If pulmonary vascular and interstitial volumes were to be reduced and lead to facilitated ventilation, the CO_2 tension might be expected to fall, but it seems unlikely that the relatively mild and short-lived diuretic effect of acetazoleamide would produce this result, particularly in the absence of cardiac failure. The thesis should, however, be susceptible to experimental test by the use of other more effective diuretics.

The lowering of plasma bicarbonate which results from the renal effects of acetazoleamide has also been suggested as responsible for the improvement of the emphysematous patient (87, 148). It would be of interest to see whether the effects of acetazoleamide could be reproduced if the decreased plasma bicarbonate were produced by some other means such as the administration of acidifying salts. The only conclusion which would appear justified at this time is that the administration of carbonic anhydrase inhibitors results, in some patients with respiratory acidosis, in improvement of the mental state and lowering of arterial CO_2 tension, and that the mechanisms by which these effects are produced remain to be established.

THE EFFECT OF CARBONIC ANHYDRASE INHIBITORS IN ORGANS OF SECRETION

Although the presence of carbonic anhydrase in a number of organs of secretion has been recognized for some time, in many of these instances the involvement of the enzyme in the secretory functions of the organ had been subject to considerable question (61). The effects of the new, more powerful inhibitors have firmly established the necessity of carbonic anhydrase for the *normal* operation of the secretory processes, without, however, defining the role played by the enzyme in the mechanisms. In general it has not been possible to demonstrate complete inhibition of secretion in the presence of large amounts of inhibitor, either because the enzyme activity is not completely abolished at the concentrations attained, or, which seems more likely, because the secretory processes do operate, albeit less efficiently, in the absence of carbonic anhydrase activity.

In most of the organs concerned, secretion is associated with the development of a demonstrable hydrogen ion gradient across a cellular membrane. At the same time, a gradient of bicarbonate concentration in the opposite direction is established,—a corollary of the gradient of hydrogen ion concentration in any system permeable to and containing CO_2. Such processes can be looked upon as the transport of hydrogen ion in one direction and of hydroxyl ion in the other, the hydroxyl ion, in the presence of CO_2, appearing as bicarbonate. In each instance, for the maintenance of electroneutrality, there must be movement of some other cation in the direction opposite to that of the hydrogen ion or some anion, other than hydroxyl, in the same direction as the hydrogen ion. The sum of all the steps involved is the splitting of water or of carbonic acid.

The commonly used schema which shows CO_2 combining with water under the influence of carbonic anhydrase to yield carbonic acid which in turn provides the hydrogen ion secreted across the cell membrane is probably a misleading oversimplification. Actually, the most acceptable view of the role of carbonic anhydrase in such processes is in maintaining the buffering efficacy of the CO_2-carbonic acid-bicarbonate system in the secreting cells as proposed by Davies and Roughton (73). A cell extruding hydrogen ion at any appreciable rate will be left with an excess of hydroxyl ions and will undergo a rise in cell pH to an extent dependent upon the effectiveness of its buffers. The provision of carbonic acid to neutralize this alkali and minimize the rise in pH is a likely function of the car-

bonic anhydrase.[3] Inhibition of the enzyme would result in a rise in cell pH and depression of the secretion of hydrogen ion. If, on the other hand, the secretory process is considered to be the primary extrusion of hydroxyl ion, exactly the reverse would be anticipated, the carbonic anhydrase providing for rapid conversion of H_2CO_3 to CO_2 so as to minimize the depression of cell pH from the accumulation of hydrogen ion. In either case, the more rapid the secretion, the more necessary the activity of carbonic anhydrase would be to minimize the departure of the cell pH from the equilibrium value. However, since the carbonic anhydrase is not directly involved in the transport process itself, even complete suppression of enzyme activity need not be associated with total interruption of secretion.

Kidney

Soon after the introduction of sulfanilamide in the treatment of bacterial infections, Southworth (219) noted that patients receiving the drug showed a consistent drop in the plasma CO_2 combining power. Although some observers (111) attributed the decreased plasma bicarbonate to respiratory alkalosis, several groups of investigators showed that upon the administration of sulfanilamide, there was an increase in urine pH and bicarbonate excretion and that these changes preceded the changes in plasma bicarbonate (21, 159, 163, 164, 220). It was thus apparent that sulfanilamide produced acidosis through an effect on the renal regulation of acid-base balance, by interfering with the acidification of the urine. The discovery by Mann and Keilin (152) that sulfanilamide is an inhibitor of carbonic anhydrase and the demonstration of high concentrations of the enzyme in the kidney by Davenport and Wilhelmi (65) provided the basis for an understanding of the effects of the drug.

The mechanism of urine acidification and bicarbonate reabsorption. The first experimental exploration of the effect of carbonic anhydrase inhibitors on renal function was that of Höber (118) who showed that sulfanilamide and a series of other sulfonamides unsubstituted on N^1 caused the urine of the perfused frog kidney to become alkaline. In accord with the view commonly held at the time that the urine was made acid by the reabsorption of bicarbonate salts, Höber concluded that carbonic anhydrase was involved in the reabsorption of bicarbonate by the kidney. The theory of urine acidification by bicarbonate reabsorption implied that the acid excreted is derived largely from the "carbonic acid" in the glomerular filtrate. Pitts and Alexander (190) subsequently showed that in acidotic dogs infused with and excreting large amounts of buffer, the amount of titratable acid excreted was several times that which could be derived from the filtered "carbonic acid" (*i.e.*, carbonic acid plus dissolved CO_2) by reabsorption of the filtered bicarbonate. They therefore concluded that the urine must be acidified by the secretion of acid and proposed that this secretion was actually an

[3] In the steady state of secretion, the essential role of carbonic anhydrase and CO_2 in this system can be considered that of providing a means by which hydroxyl ions can more easily escape from the cells (as bicarbonate). In this respect the function of CO_2 and carbonic anhydrase is considered similar to that proposed by Jacobs and Stewart (126) for ion exchanges in red blood cells (see above).

exchange of hydrogen ions from the tubule cells for sodium ions from the tubule lumen. Demonstrating that the excretion of titratable acid could be partially suppressed by sulfanilamide in mildly acidotic dogs, they concluded that carbonic acid was the immediate source of the secreted hydrogen ion and that carbonic anhydrase was involved in the conversion of metabolic CO_2 to carbonic acid.

An alternative mechanism which would account for the observations of Pitts and Alexander (190) without requiring secretion of hydrogen ion has recently been pointed out by Brodsky (38). If bicarbonate were to be reabsorbed, the resulting decrease in the pH of the tubule fluid would cause carbonic acid to interact with other urinary buffers to yield more bicarbonate. At the same time the CO_2 tension of the fluid in the tubule would fall as the dissolved CO_2 was converted to carbonic acid and thence to bicarbonate and more CO_2 would diffuse in from the surrounding tissues and blood. By a continuation of such a cycle any amount of acid excretion could be accounted for by bicarbonate reabsorption. However, Berliner (27) has shown that the uncatalyzed rate of hydration of CO_2 would be inadequate to supply bicarbonate at the rate required to explain the data of Pitts and Alexander (190) and therefore the mechanism suggested by Brodsky (38) could operate only if carbonic anhydrase were present in the luminal surface of the tubule cells. The latter is rendered unlikely, though not excluded, by the observation of Ochvadt and Pitts (182) that when carbonic anhydrase is administered intravenously so as to cause its appearance in the urine, it causes a decrease in the CO_2 tension of alkaline urine—an effect which would not be expected if carbonic anhydrase were normally in contact with the fluid in the tubules.

Pitts and Lotspeich (191) found that when bicarbonate was infused into acidotic dogs, there was a decrease in the excretion of titratable acid and that when sulfanilamide was administered in large doses there was a decrease not only in titratable acid, but a consistent although small decrease in bicarbonate reabsorption by the tubules. The authors concluded that a part of the reabsorption of bicarbonate by the tubules was effected by a carbonic anhydrase dependent mechanism in the distal tubule segment, which, by exchanging hydrogen ion for sodium ion, converted bicarbonate to carbonic acid in the renal tubule. Upon formation of CO_2 from the carbonic acid, the CO_2 returned to the blood by diffusion. The major fraction of bicarbonate reabsorption, not susceptible to inhibition by sulfanilamide, was presumed to be effected by an anion transport process in the proximal tubule. However, with the far more powerful inhibitor, acetazoleamide, Berliner, Kennedy and Orloff (26, 28) found much larger amounts of bicarbonate excreted after administration of the inhibitor, with as much as 33 to 51 % of the filtered bicarbonate appearing in the urine of acidotic dogs. Since these amounts of bicarbonate are larger than those which may be assumed to reach the distal tubule under these conditions, it was concluded that either the proximal tubule transport mechanism was a separate one but also catalysed by carbonic anhydrase or, which seemed much more reasonable, that the same mechanism, an exchange of hydrogen and sodium ions, was responsible for bicarbonate reabsorption throughout the tubule. From observations on the effect of varying arterial CO_2 tension and the administration of acetazoleamide, Dorman *et al.* (79) and Brazeau and Gilman (36) have likewise concluded that hydrogen-sodium exchange is the mechanism responsible for all of the bicarbonate reabsorption by the tubule. These investigators, as well as Relman *et al.* (197)

have shown that with acute changes, the rate of reabsorption of bicarbonate is directly related to arterial CO_2 tension. Dorman *et al.* (79) and Brazeau and Gilman (36) picture carbonic acid as the immediate source of the secreted hydrogen ion and attribute the effects of pCO_2 to the increased formation of carbonic acid in the secreting cells. However, the changes in intracellular hydrogen ion concentration are presumably parallel to changes in pCO_2, and the observations are just as readily interpreted as due to changes in hydrogen ion concentration in a cell which secretes hydrogen ion at a rate dependent upon its concentration (29, 197).

In this view, changes in pCO_2 would exert their effect in the same way as changes in potassium concentration (2, 28, 29, 93, 185, 200) and a low CO_2 tension would have an effect similar to the inhibition of carbonic anhydrase. Although it has been suggested that the effect of elevated CO_2 tension may be through an increase in carbonic anhydrase activity (197), there is no evidence for such an occurrence (154) and since, in the presence of the large amounts of carbonic anhydrase normally in the kidney cells, it seems reasonably certain that the CO_2-carbonic acid system is virtually at equilibrium, it seems unlikely that additional carbonic anhydrase would have any effect.

Thus it is fairly generally agreed, though by no means incontrovertibly established, that bicarbonate reabsorption throughout the renal tubule is effected by exchange of the sodium, by which it is balanced, for hydrogen ion and that the carbonic acid thus formed escapes by diffusion in the form of CO_2. The effects of conditions which presumably modify cell pH are strongly in support of the view that extrusion of hydrogen ion at the luminal border is a primary event, rather than the extrusion of bicarbonate or hydroxyl ion at the cell surface facing the interstitial fluid. The reported effects of carbonic anhydrase inhibitors are entirely compatible with this view and with the assumption that carbonic anhydrase is involved primarily in maintaining intracellular buffering.

Effects of carbonic anhydrase inhibitors on renal function. The rise in urine pH and increase in bicarbonate excretion which follow the administration of sulfanilamide have been referred to above (21, 111, 118, 159, 163, 164, 190, 191, 220). With the use of acetazoleamide, similar but more striking effects are observed. These have been studied in detail in the dog (26, 28, 158), the rat (158), the rabbit (123) and man (39, 53, 81, 89, 223) as well as in several species of fish (113, 119), the alligator (115, 184), and the chicken (184, 236). In the mammals which have been studied, the results are qualitatively uniform,—there is, in the normal animal, a marked increase in the excretion of sodium, potassium, and bicarbonate, an increase in urine flow, and a decrease in the excretion of ammonia and titratable acid. Except for the increase in potassium excretion, these changes are immediately predictable from an interference with hydrogen-sodium exchange. The direct effects of the diminished exchange are the decrease in the reabsorption of sodium and bicarbonate and consequently the failure to acidify the urine. Urine flow rises as a result of the increased solute excretion, while ammonia excretion, normally being inversely related to urine pH (189), falls. The effect on potassium excretion can be inhibited with mercurial diuretics (28)

and is therefore an enhancement of the secretion of potassium by the tubules in exchange for sodium. This enhancement of potassium secretion has been attributed to the suppression of hydrogen ion transport since the rates of secretion of these two ions have been found, under a number of conditions, to be inversely related (28, 29). The immediate effects of intravenous administration of acetazoleamide in the dog are not appreciably altered by an increase in the dose from about 5 mg/kg up to 100 mg/kg (28, 158). This strongly supports the view that carbonic anhydrase activity is effectively eliminated by doses in this range. Although with the rapid intravenous administration of 500 mg/kg of the sodium salt of acetazoleamide, effects on electrolyte excretion of another order of magnitude (excretion of up to 90% of the filtered bicarbonate and half of the filtered sodium and chloride) have been reported (211), further studies have indicated that the additional effect is not related to the inhibition of carbonic anhydrase since the injection of similar doses of analogues of acetazoleamide rendered inactive as carbonic anhydrase inhibitors by substitution on the sulfonamide nitrogen, produce virtually indistinguishable effects (155, 212). The exact mechanism of this phenomenon is not clear but it has been attributed (155) to the alkalinizing effects of the excess of sodium with which the drug is injected. The solutions of sodium acetazoleamide used for injection contain 1.6 mequiv. of sodium per millimol of drug and have a pH above 9. This must be neutralized by the blood buffers yielding approximately one mequiv. of Na^+ per mmol injected, since the pK values of acetazoleamide are 7.3 and 9.1.

Except for this unusual action of large doses of the sodium salt, there is little room for question that the effects of acetazoleamide on renal function are due to inhibition of carbonic anhydrase. Aside from the very small doses and the extremely low concentrations in the plasma (2 mg/l (158)) at which the effects are observed, identical results are produced by other active inhibitors differing in their chemical and physical characteristics (30). The effects of the considerably weaker inhibitor, *p*-sulfamylbenzoate (Dirnate), have been reported by several investigators (31, 55, 56, 144) and are qualitatively similar to those of acetazoleamide. The interpretation of the results with *p*-sulfamylbenzoate is complicated by the cation excretion obligated by the rapid renal excretion of the drug, a fairly strong acid secreted by the tubules (31).

The results obtained when doses of acetazoleamide large enough to produce maximal effects were administered have differed somewhat in the hands of different investigators, apparently because of the use of different experimental conditions. Maren (154) using fasted, non-infused dogs and collection periods of the order of an hour or two has found large doses of acetazoleamide to cause the excretion of 20–25% of the filtered bicarbonate and much less in acidotic dogs. Similar results are reported in man by Counihan *et al.* (53). Berliner (26) has reported that in animals given saline infusions the effects are much greater, with up to 50% of the filtered bicarbonate excreted, 30% or more being excreted even when the plasma bicarbonate was as low as 12 mequiv./l. The exact explanation of the difference is not certain, but it probably is related, at least in part, to the sustainment of glomerular filtration rate and extracellular fluid volume in the

face of the diuresis in the infused dogs, since, even in the latter, the data suggest that the fraction of the filtered bicarbonate excreted rises as glomerular filtration rate increases.

That, in the normal dog or man, the effect of acetazoleamide in causing the loss of sodium and bicarbonate is self-limited on continued administration, has been repeatedly observed (10, 39, 53, 81, 141, 154, 158). In part, this is attributable to the acidosis produced by such losses (53, 128, 141, 150, 154, 157, 158) since acidosis produced by other means also markedly diminishes the renal effect. This is to be expected and does not indicate that there is a diminution of the intimate effect of the inhibitor on the kidney, nor that some other mechanism has necessarily taken over or is playing an augmented role (154, 158, 215). If it is accepted that bicarbonate reabsorption is effected by exchange of hydrogen ion for sodium ion, then the hydrogen ion *secretion* is not to be measured by the net acid excretion (titratable acid plus ammonia) but by the *bicarbonate reabsorbed plus* the net acid excretion. Although it may seem paradoxical, this interpretation leads to the conclusion that hydrogen ion secretion is greatest when the urine is alkaline because of the administration of large doses of bicarbonate salts, since it is at this time that the tubules are reabsorbing the most bicarbonate. Thus, in severe acidosis the urine may remain acid despite inhibition of renal carbonic anhydrase, not because of a compensatory increase in hydrogen ion secretion by some other mechanism (215) nor because of increased noncatalyzed formation of carbonic acid, but because even the limited capacity to secrete hydrogen ion is adequate to dispose of the limited quantity of bicarbonate delivered to the tubules in the glomerular filtrate. The increased excretion of bicarbonate after acetazoleamide despite low bicarbonate concentrations in respiratory alkalosis (158) is not pertinent to this problem since cell pH almost certainly rises when pCO_2 is reduced and this will, itself, impair bicarbonate reabsorption.

Two other possible factors should be mentioned. It might be presumed that with acidosis of the extracellular fluid, the pH of the tubule cells is lowered and consequently hydrogen ion secretion enhanced, an effect which would tend to diminish dependence on carbonic anhydrase. However, there is no evidence that the pH of intracellular fluid does vary with that of extracellular space and the assumption that it does has led to misinterpretations in the past (28). Actually it has been shown that extracellular fluid may undergo acid-base changes opposite to those of the rest of the body (33, 51, 97, 185) and that the apparent pH of kidney slices is normally independent of the pH of the medium in which they are immersed (2). A second possibility worthy of note in the resistance which develops on continued administration of acetazoleamide is that there may be a contributory effect of potassium depletion, an occurrence which would increase renal acid secretion presumably by lowering the pH of kidney cells (2, 93, 185, 200). This may, to some extent, be a factor but the potassium depletion due to acetazoleamide is generally mild and its contribution to resistance must be small at best.

Reduction of the plasma bicarbonate concentration is presumably the major cause of resistance to acetazoleamide in acidosis, but is not the only factor. Another may be reduction of glomerular filtration rate. However, Maren (154) has found that the severity of base depletion has an influence beyond reduction

in the total amount of bicarbonate filtered. This is probably analogous to the resistance to any diuretic which develops when extracellular volume is sufficiently diminished. The mechanism is not established.

That the inhibitors continue to produce their renal effects despite acidosis is also indicated by the fact that losses of potassium continue after acetazoleamide has ceased to cause appreciable loss of sodium (53, 158). These findings suggest that the drug, even under these conditions, causes the usual rise in cell pH. The contributions of sodium and potassium to the total cation loss vary considerably with the experimental conditions. The administration of potassium salts and conditions which tend to cause sodium retention increase both the relative and absolute rate of potassium excretion (10, 53, 80, 89, 141, 158, 168). The cessation of potassium loss on continued administration of acetazoleamide is presumably the result of some degree of potassium depletion. Fatal potassium depletion apparently occurred in two dogs given 1000 mg/kg daily for three days (156). Here, however, one must consider the demand for cation excretion with the daily excretion of approximately 5 mM/kg of acetazoleamide, which as an anion would require that considerable amounts of cation be excreted with it. With smaller doses, severe grades of potassium depletion have not generally occurred.

Depression of ammonia excretion when carbonic anhydrase inhibitors are administered was first reported by Ferguson (86) who gave *p*-sulfamylbenzoate to rats. The effect of inhibitors in the rat differs somewhat from that in dog or man, since, although in association with the rise in urine pH there is an immediate decrease in ammonia excretion, there is at the same time stimulation of ammonia production by the kidneys which returns ammonia excretion to or above control levels despite continued elevation of urine pH. After a period of several days this increased ammonia synthesis is associated with a demonstrable rise in the glutaminase activity of the rat kidney as shown by Rector *et al.* (195). In addition, Leonard and Orloff have found, in the rat, an increase in ammonia excretion not due to changes in urine pH before there has been a rise in glutaminase (142). In the dog and in man, however, there is no evidence for the occurrence of these changes. Acutely, ammonia excretion falls as urine pH rises in the same way when acetazoleamide is administered as when bicarbonate salts are given (53, 183). With chronic administration, ammonia excretion returns to control levels as the urine pH also reverts to control values in the face of acidosis (158). These findings suggest that, in these species, the acidosis due to carbonic anhydrase inhibition is not associated with the adaptive change, presumably in glutaminase activity, which accounts for the usual slow phase of increased ammonia excretion in response to acidosis. When the stimulus of ammonium chloride administration was added to acetazoleamide acidosis, Maren (158) found ammonia plus titratable acid output could be increased to five times normal. Whether this was due to a lowering of urine pH or to an increased capacity to form ammonia is not certain.

The changes in urine flow with carbonic anhydrase inhibition are attributable to the solute diuresis and not to any detectable effect on the transport of water, since Maren (158) has shown that dogs respond normally to pitressin during

acetazoleamide diuresis. The finding of Welt *et al.* (233) that acetazoleamide, administered during water loading, causes an increase in the "free water clearance" (234) is of interest for the light it may shed on the site of the inhibitor effect on the tubule. The observation has been confirmed by Counihan *et al.* (53) and Orloff and Walser (186). The latter have found that the increase in free water clearance when acetazoleamide is given is not greater than occurs when equivalent sodium excretion is produced by the infusion of saline solutions in the hydrated subject. If, as is generally held, "free water" results from the reabsorption of sodium salts in the distal tubule, the fact that the "free water clearance" is not lower than with equal solute excretion from other causes would imply that little of the acetazoleamide depression of sodium reabsorption is attributable to an effect in the distal segment.

A fall in glomerular filtration rate frequently occurs when acetazoleamide is administered intravenously to dog or man (28, 150, 156, 186) but this effect is absent if the drug is given by mouth (53, 156). The decrease, which has been reported to be associated with a diminished blood pressure (150), may be unrelated to carbonic anhydrase inhibition.

A few miscellaneous observations on the effects of acetazoleamide on electrolyte excretion are worthy of mention. No specific effect on chloride excretion has been observed, small and irregular changes being the usual occurrence (28, 53, 156). The same is true of phosphate excretion (28, 108, 109, 156, 179). It has no effect on the excretion of calcium (45). Harrison and Harrison (108, 109) have found that the administration of acetazoleamide to rats on a high calcium and phosphate intake produces a striking depression of citrate excretion and the production of renal calculi. The mechanism of the decreased citrate excretion is not clear; alkalinization of the urine by the administration of bicarbonate produces an increase rather than a decrease in citrate excretion. Several investigators have noted the general resemblance of the renal effects of carbonic anhydrase inhibitors to the clinical syndrome of renal tubule acidosis (28, 108, 125), but in view of the number of agents which depress the renal secretion of hydrogen ion without affecting carbonic anhydrase activity (26), the resemblance may be more apparent than real. Satran *et al.* (209) have reported a decrease in urine pH without appreciable change in bicarbonate excretion when acetazoleamide was administered to bicarbonate-loaded dogs. Anything which decreases bicarbonate concentration in the urine will lower its pH and it may be that these observations indicate a diminished capacity to concentrate the urine. The latter would be in accord with the marked decreases (30–50%) in glomerular filtration rate reported in these studies. Other observers have not found bicarbonate excretion to decrease when acetazoleamide was administered to alkali-loaded dogs (36, 79, 93, 222).

The effects of acetazoleamide in some non-mammalian vertebrates merit brief description. In the chicken the results resemble those in the mammal (184, 236). It is of some interest that acetazoleamide infused via the renal portal circulation at the rate of 3–4 μg/kg min will cause alkalinization of the urine on the infused side only and within five minutes (184). This indicates that the drug need not reach the tubules via the glomerular

filtrate, and, since there is no evidence that the tubules secrete the drug into the urine, does not support the hypothesis that the concentration of the inhibitor in the urine of the distal tubule is critical for the renal effects (31). Hernandez and Coulson (52, 115) have reported the very interesting observation that, in the alligator, acetazoleamide produces a *decrease* in bicarbonate and an increase in *chloride* excretion, suggesting interference with a bicarbonate (or hydroxyl) for chloride exchange. The alligator is unique among animals which have been studied in normally excreting very large amounts of ammonium and bicarbonate ions simultaneously.

In fresh water fish, Heineman and Hodler (113) and Hodler *et al.* (119) have reported that acetazoleamide has an effect not unlike that in mammals, increasing urine pH and the excretion of sodium and potassium. In marine fish (sculpin and dogfish), however, there is no detectable effect on urine pH which remains at its usual acid level. These fish normally regulate their electrolyte balance by transport processes in the gills; and it was shown that, when acetazoleamide is administered, there is marked impairment of the capacity to excrete bicarbonate, the equivalent of the effect on the mammalian renal tubule if the gill epithelium is presumed to secrete hydrogen ion into the blood in exchange for sodium ion. There is an additional effect on the respiratory loss of CO_2 in the dogfish reflected by an elevation of CO_2 tension, presumably due to inhibition of red cell carbonic anhydrase.

Carbonic anhydrase inhibitors as diuretics. The possible usefulness of carbonic anhydrase inhibitors as diuretics was first recognized by Schwartz (210) who treated three cardiac patients with large doses of sulfanilamide. Although toxic side-effects were marked, some increase in the excretion of sodium and potassium and loss of weight were noted in all. It was on the basis of these observations and in the hope of finding a diuretic useful on oral administration that Roblin and Clapp (201) synthesized a series of highly active heterocyclic sulfonamide inhibitors of carbonic anhydrase, among which was acetazoleamide.

Maren (158) has reported extensive studies of the diuretic effects of acetazoleamide in normal dogs. The pattern and extent of electrolyte loss depend upon the size and spacing of doses. Small daily doses (5–10 mg/kg) cause a brief period of diuresis each day until the concentration of drug in the body has fallen to ineffective levels. During the remainder of the 24-hour period, the animal is able to make up the losses from intake. As the dosage is increased or when multiple daily dosage is used, the period of inhibition is extended, greater losses of electrolyte are produced, the animal is unable to reconstitute its electrolyte composition between doses and, because of the development of acidosis and body-fluid depletion, no longer has a diuretic response to the drug. Maximum net sodium losses were of the order of 5–8 mequiv./kg.

Although the studies of Maren (158) define the pattern of the renal effects of acetazoleamide, they do not permit an evaluation of its potential usefulness as a diuretic. The animal given small amounts of drug once daily will respond to each dose with a diuretic response, but there is no overall negative balance of fluid and electrolyte. With larger doses, there is a modest, although definite negative balance which can be maintained, but not increased, as long as the drug is administered. The true value of a diuretic can be evaluated only in the presence of the accumulations of excess extracellular fluid which characterize the conditions in which diuretics have therapeutic usefulness. Unfortunately, the clinical conditions which are associated with such expansions of extracellular fluid are

often extremely variable, not only from one patient to another, but from day to day in the same patient. The inherent variability of the test object makes the evaluation of the effectiveness of acetazoleamide particularly difficult since it has proven to be relatively ineffective in the seriously ill edematous patient, in whom some degree of stability is present, while it is widely reported to be useful in the mildly decompensated patient whose spontaneous course is most variable.

In patients with severe cardiac failure, the administration of acetazoleamide has, with a few exceptions, resulted in little or no useful diuresis (23, 41, 53, 90, 141, 143, 198, 206, 213). It is not surprising that the same is true when the less active inhibitor, *p*-sulfamylbenzoate, is used (144). Although the urine has been observed to become alkaline in such patients and potassium excretion increased, there was often little or no increased excretion of sodium (53, 141). In those patients who did lose sodium during the administration of acetazoleamide, there was essentially no increase in chloride excretion either during the administration of the drug or when its effect had been dissipated. This, as has been pointed out by Counihan *et al.* (53) and Leaf *et al.* (141), is the essence of the failure of acetazoleamide as an effective diuretic for the maintenance of such patients. Since bicarbonate and its covering cation represent only about 20 % of the extracellular electrolyte, the extent to which the volume of extracellular fluid can be diminished by the loss of bicarbonate only is sharply limited. The effectiveness of carbonic anhydrase inhibitors is markedly diminished by the acidosis which results from loss of bicarbonate, so that their continued effect will depend on the elimination of the acidosis. Since administration of more bicarbonate as the salt of fixed base is out of the question, the relief of the acidosis must depend on the excretion of chloride. This might occur during the period of acetazoleamide effect by excretion of chloride covered by additional sodium ion, or, during drug-free intervals, by an output of chloride balanced by ammonium ions. Apparently neither of these possibilities is realized in the seriously ill, edematous cardiac patient, although one or both must occur in those less resistant patients who do appear to respond favorably to acetazoleamide.

Although, in normal animals and in a few less severely ill patients, the diuretic effects of acetazoleamide have been found to compare favorably with those of mercurial diuretics (41, 105, 175), those investigators who have tried acetazoleamide in seriously ill cardiac patients have generally found it far less effective than the mercurial diuretics (53, 55, 143, 198). In such resistant patients, whose response to mercurials may also be poor, some success has been reported in the use of acetazoleamide to potentiate the mercurial effect (98, 106, 206, 215) Rubin *et al.* (206) administered both ammonium chloride and acetazoleamide to mercurial-resistant patients and found that good responses to Mercuhydrin® could be obtained, particularly if administration of the carbonic anhydrase inhibitor were discontinued two days before the Mercuhydrin was given. It is difficult to assess the role of the acetazoleamide in this phenomenon since no observations on the effect of ammonium chloride alone were made and in the only instance where acetazoleamide alone preceded the mercurial no potentiation

occurred. These investigators report that when the mercurial and acetazoleamide were given on the same day, optimal diuresis did not occur; Seldin (215) states that under these conditions the diuretic effect of mercurial diuretics is inhibited. Maren (153) reported that when the two types of diuretic were given simultaneously to normal dogs, the immediate response was less than the sum of the responses to the individual drugs given separately, but that during the third and fourth hours after the combined drugs potentiation was observed. No suppression of the diuresis due to mersalyl (Salyrgan®) was observed by Berliner *et al.* (26, 28) when the mercurial was administered to normal *dogs* shortly before or after acetazoleamide. However, Orloff and Walser (186) have observed that injection of Thiomerin® in human subjects undergoing acetazoleamide diuresis may result in little or no increase in the excretion of sodium and chloride, despite a depression in potassium excretion. The latter effect, depression of potassium secretion, but no chloride diuresis, is also observed when mercurial diuretics are injected in bicarbonate-infused dogs (30). The mechanism by which acetazoleamide suppresses mercurial diuresis when they are administered together and the way in which the acetazoleamide may potentiate the effect of a mercurial diuretic given subsequently are not established. They may well be related to the mechanism by which acidifying salts potentiate and alkalinizing salts suppress mercurial diuresis generally, but the nature of this phenomenon is not known—at least these effects do not appear necessarily to be directly related to the pH and bicarbonate content of extracellular fluid (117, 177). There is a place for a carefully controlled study of the value of acetazoleamide in the potentiation of mercurial diuretics and of the nature of mercurial potentiation in general.

When we turn to the application of acetazoleamide to the management of cardiac patients with mild to moderate sodium retention and edema, a host of publications attest to its usefulness (23, 24, 41, 77, 82, 90, 98, 99, 106, 112, 116, 124, 143, 151, 161, 162, 173, 207, 221). There can be little question that many such patients have been adequately maintained for long periods and many have been able to dispense with mercurial diuretics. In some instances, there may be room for question as to whether any diuretic was required. Unfortunately detailed data are presented in few of the reports. Rates of sodium excretion as high as 125, 144 and 162 mequiv./day *before* acetazoleamide was administered were reported in one of the few papers giving such data (90) and do not suggest much need for the use of a diuretic. However, it would appear that acetazoleamide may be a useful adjuvant in those patients in whom any degree of restriction of sodium intake is virtually impossible.

The diuretic effectiveness of acetazoleamide has also been assayed in other states associated with retention of sodium and water. Several of the reports concerning its use in hospitalized cardiac patients also describe its use in patients with cirrhosis and ascites (24, 74, 77, 124, 151). In general the response has been poor, as might be expected since such patients are often more resistant to the effect of diuretics. Moseley (174) has described the successful use of acetazoleamide in the treatment of potassium retention in patients with an adequate urine flow.

Since the widest use of acetazoleamide is as a diuretic, it seems appropriate to mention its toxic effects here. In animals the drug is remarkably non-toxic (156) with LD_{50}'s ranging up to 3000 to 6000 mg/kg. In man, definite, although rarely serious, toxic symptoms occur quite frequently at doses of 12–15 mg/kg. Paresthesias and drowsiness have been regularly encountered at doses of the order of 1 g/day. Gastrointestinal symptoms are not uncommon. Of these, at least the paresthesias seem to be related to carbonic anhydrase inhibition since they have been reported after *p*-sulfamylbenzoate (144). One instance of drug fever (214) and one of agranulocytosis (187) attributed to acetazoleamide have been reported.

Stomach

In 1938, Davenport and Fisher (63) reported the presence of carbonic anhydrase in gastric mucosa. In subsequent work, Davenport (57) showed that the distribution of the enzyme in the mucosa of rats and cats was highly correlated with the distribution of parietal cells. The concentration of enzyme in the parietal cells was estimated to be three to six times that found in the red blood cells. It has since generally been assumed that carbonic anhydrase has some function related to the gastric secretion of hydrochloric acid. However, views as to the importance of the enzyme have varied widely since a role was first proposed and various hypotheses as to the nature of its function have been suggested. Davenport (58) found that thiocyanate markedly and reversibly inhibits the secretion of acid by the dog stomach and that thiocyanate inhibits carbonic anhydrase. The two effects were assumed to be related and it was proposed that carbonic anhydrase was a part of the secretory mechanism, providing carbonic acid from which the secreted hydrogen ion was derived. However, Feldberg *et al.* (85) found that although thiocyanate markedly depressed gastric acid secretion, it inhibited carbonic anhydrase only weakly, while sulfanilamide, at concentrations which they believed to inhibit the enzyme completely, did not inhibit, or even increased, the secretion of hydrochloric acid. It was therefore concluded that the effect of thiocyanate was through some other action and that carbonic anhydrase did not catalyze the secretion directly, although the possibility of some other involvement of the enzyme in the secretory process was conceded. Davenport and Jensen (64) found that thiophene-2-sulfonamide and sulfanilamide at concentrations sufficient to yield 99.98% inhibition of carbonic anhydrase did not inhibit secretion by the mouse stomach *in vitro* and, although doubling the concentration of thiophene-2-sulfonamide (to 25 mg %) did produce some inhibition, the effect was considered non-specific. They concluded, as Davenport had previously (60, 61), that the necessity for carbonic anhydrase for gastric secretion of hydrochloric acid had not been demonstrated. Anderson and Wilbur (3) were unable to inhibit the gastric acid secretion of turtles with massive doses of sulfanilamide.

The first clear demonstration that gastric secretion of acid could be affected by inhibitors of carbonic anhydrase was that of Davies and Edelman (69) who found, using isolated frog and toad stomach mucosae, that high concentrations

of *p*-toluenesulfonamide (1.7×10^{-2}M), *p*-sulfonamidobenzoate (10^{-2}M), thiophene-2-sulfonamide (10^{-2}M) and prontosil soluble (10^{-3}M) completely abolished acid secretion after a lag period. It was assumed that failure to demonstrate inhibition previously in the intact animal had been due to the fact that the amounts of inhibitor administered had been inadequate. It was also concluded that the amounts required to produce the effect would have been incompatible with survival of the animal because of the inhibition of red cell carbonic anhydrase.

The secretion of acid by the frog gastric mucosa *in vitro* has been found by Hogben (122) to be inhibited by acetazoleamide at a concentration of 10^{-2}M but not at 10^{-3}M. There was a time delay in the onset of the effects similar to that described by Davies and Edelman (69), but complete inhibition was not obtained, hydrogen ion secretion being depressed only some 40% while the transport of chloride ion and the spontaneous electrical potential of the mucosa were more markedly reduced.

That gastric acid secretion could be depressed in the intact animal by carbonic anhydrase inhibitors was first shown by Janowitz *et al.* (127) who administered acetazoleamide to Heidenhain pouch dogs. There was a depression of both volume and hydrochloric acid concentration in the gastric secretion so that total acid secretion was depressed 85%, on the average, by doses ranging from 20 to 120 mg/kg. Over this range there was no significant increase in effect with dosage. In one of three dogs, similar effects were obtained with doses of only 5 and 10 mg/kg. As in the isolated mucosa preparations, there was a delay in the onset of the inhibition, the latent period of from twenty to eighty minutes being generally shorter in the most rapidly secreting stomachs and not related to the size of the dose. In most instances the extent of inhibition increased progressively over a period of several hours. Rehm *et al.* (196) have also observed marked depression of acid secretion by exteriorized gastric segments in dogs given 40 to 200 mg/kg of acetazoleamide. It is apparent that the activity of acetazoleamide in reducing the gastric secretion of dogs *in vivo* is far greater than would have been anticipated from the concentrations required to produce effects in the isolated amphibian mucosa preparations. The doses which produce effects in the intact dog yield concentrations in the plasma of the order of 10^{-5}M while 10^{-2} to 10^{-3}M concentrations are necessary in the bathing medium *in vitro*. The reason for this difference is not certain. It has been suggested that it might be due to more rapid penetration of the drug by way of the circulation than is obtained when the drug must diffuse the longer distances from the *in vitro* bathing medium, but it seems that the long periods of observation used in the *in vitro* systems should have been more than adequate for penetration. It may be that the more rapid secretion of acid by the intact animal makes the catalysis provided by carbonic anhydrase more essential.

Some depression of gastric acid secretion in man following the administration of acetazoleamide has been reported by McGowan and Stanley (165), by Kinzlmeier and Kimbel (134), and by Texter and Baborka (224). Effects have been reported with doses as small as 14 mg/kg per day in divided doses (224).

It is thus fairly conclusively established that carbonic anhydrase has some

function related to acid secretion by the stomach mucosa. The nature of this function, as well as the nature of the secretory mechanism, remain unestablished although it is becoming more generally accepted that the effect of carbonic anhydrase is to help maintain a normal intracellular pH during the active extrusion of hydrogen ion (73). Other functions have been proposed. The idea that carbonic anhydrase supplied carbonic acid which was the immediate source of secreted hydrogen ion (58) has generally been discarded (60, 61). Bull and Gray (40) suggested a mechanism for the secretion of hydrochloric acid which involved the secretion of an organic acid at the head of the intracellular canaliculus. As the secretion passed down the canaliculus the organic anion was believed to be exchanged for chloride. The anion was then rapidly metabolized to CO_2 and hydrated to yield bicarbonate ion under the influence of carbonic anhydrase, the bicarbonate ion then being returned to the blood in exchange for chloride. However, as pointed out by Davies (67), the hypothesis implies a higher ratio of oxygen utilized to hydrochloric acid produced than is observed experimentally.

Obrink (181) suggested that the function of carbonic anhydrase is that of maintaining a barrier to the diffusion of bicarbonate from blood to gastric lumen by catalyzing conversion of carbonic acid to CO_2. When acid secretion was inhibited by thiocyanate there was a rise in the bicarbonate concentration of gastric juice. However, it is doubtful, as indicated above (85), that the pertinent effect of thiocyanate is on carbonic anhydrase, and a rise in bicarbonate concentration is the inevitable result of an increase in pH in a CO_2-containing system (121, 122).

Davies has stressed the importance of CO_2 for the process of gastric secretion (67). Intact, tied tubes of frog and toad gastric mucosa produced HCl faster than they produced CO_2 and therefore needed added CO_2 for the neutralization of the alkali formed in the process of secretion which eventually appeared in the medium as bicarbonate. In bags which secreted acid at a rapid rate in the absence of exogenous CO_2, Davies and Longmuir (72) observed the development of ulcers and perforations which they attributed to the formation of alkali which, in the absence of adequate supplies of CO_2, remained unneutralized. Similar lesions were observed by Davies and Edelman (69) when carbonic anhydrase inhibitors were used. Davies (67, 68) believes the secreted hydrogen ion to be derived ultimately from water by the utilization of energy from glucose oxidation and high-energy phosphates, and that the extrusion of hydrogen ion leaves the cell with excess alkali which is neutralized either by reaction with carbonic acid or by combination directly with CO_2 to yield bicarbonate (217). Davies and Roughton (73) have calculated the extent of catalysis by carbonic anhydrase required to maintain intracellular pH within various limits in the face of acid secretion. For the assumed rate of secretion by mammalian parietal cells a four hundred-fold increase in reaction rate would be required if the pH were to rise only 0.01 pH unit; a forty-fold catalysis would be needed for a rise of 0.1. A ten-fold magnification of the reaction of CO_2 and water would be required no matter how high the intracellular pH (69). The carbonic anhydrase in mammalian parietal cells is sufficient for a catalysis of some fifteen thousand-fold at 38°C (73). Davies

attributes the very high concentrations of carbonic anhydrase inhibitors required to suppress secretion to the very high turnover number of the enzyme, so that extremely high degrees of inhibition are required to reduce its activity below an effective level (68). Conway (48), although in disagreement with Davies concerning the mechanism of acid secretion, accepts the view that carbonic anhydrase is involved in the maintenance of intracellular buffering.

Although the absence of exogenous CO_2 had quite marked deleterious effects on the tied mucosal bags used by Davies, the gastric mucosa is able, under other conditions, to secrete acid over long periods, without added CO_2 and despite the fact that more hydrogen ion than CO_2 is produced (62, 120). It would appear from such studies that, if the role of carbonic anhydrase in gastric secretion is to assure the neutralization of hydroxyl ion pending its escape from the secreting cell as bicarbonate, there must, under some conditions, be a means for the hydroxyl ion equivalent to leave the cell without the intermediary formation of bicarbonate, unless some unsuspected means exists for retaining CO_2 in the parietal cell and recycling it in the process.

Eye

In the eye, carbonic anhydrase has been found present in high concentration in the lens and retina (11, 231) and in significant, although lower concentrations in the iris and ciliary processes (235). Wistrand (235) found the concentration of carbonic anhydrase in rabbit iris and ciliary processes to be approximately one seventh that of red blood cells. More recent observations of Ballintine and Maren (13) indicate a somewhat lower concentration in the anterior uvea, not greater than one twentieth of that in red cells and most of it confined to the ciliary processes. The function of carbonic anhydrase in the lens and retina remains undetermined. Recent work with acetazoleamide justifies the belief that in the ciliary processes, the enzyme is involved with the secretion of aqueous humor.

Aqueous humor arises in the posterior chamber (behind the iris) by a secretory mechanism, the nature of which is not entirely established, and flows through the pupil into the anterior chamber whence it leaves by a process of filtration in the angle formed by cornea and iris (91, 131). The unique feature of the aqueous humor is its bicarbonate concentration which, in the posterior chamber, is of the order of 50% greater than that of plasma; the pH is also correspondingly higher. The chloride concentration is lower than that of plasma ultrafiltrate to an extent equivalent to the elevation of the bicarbonate. As the fluid flows through the anterior chamber, the bicarbonate concentration falls toward that of plasma with a corresponding increase in chloride concentration, these alterations presumably resulting from diffusional exchange with the blood vessels of the iris (132).

Views as to the mechanism by which the fluid is secreted have generally been based on the hypothesis of Friedenwald (91) who proposed a redox pump mechanism with the formation of hydroxyl ions in the ciliary epithelium and, by an electron transport process, a corresponding formation of hydrogen ions in the ciliary stroma. The hydroxyl ions were considered to be neutralized by combina-

tion with CO_2 to form bicarbonate under the influence of carbonic anhydrase in the epithelial cells while the hydrogen ions were neutralized by blood bicarbonate in the stroma, to yield CO_2, also under the influence of carbonic anhydrase (92). It was suggested that ascorbic acid, which is present in uniquely high concentration in the eye, might be a link in the redox chain (92). Extending the hypothesis of Friedenwald, Kinsey (131) has proposed that extrusion of bicarbonate from the ciliary epithelium into the aqueous humor is the primary event in secretion. The electrical potential gradient would then cause the entry of sodium and the osmotic effects the entry of water to maintain essential isotonicity. Although none of the evidence is directly opposed to these views, the following are worthy of note. 1) The rate of secretion in this particular system has not been shown to exceed four hydroxyl (or hydrogen) ions per molecule of oxygen, the theoretical maximum for a redox mechanism, but in other systems (stomach, frog skin) this maximum is exceeded (68, 239). Although there is no necessity for assuming sodium transport mechanisms to be the same throughout the body, it seems a reasonable working hypothesis. 2) The transport of electrons from cell to stroma is a process difficult to visualize in a system with the low conductivity of biological material. 3) Sodium transport mechanisms are virtually universal in the animal body, while specific strong electrolyte anion transport has rarely been identified. Determination of the electrical potential gradient across the ciliary epithelium would probably provide an answer to the question of whether sodium or bicarbonate is the ion primarily transported. The data of Kinsey and Palm (133) suggesting that sodium enters the posterior chamber by secretion while anions appear to enter by diffusion favors the view that it may be the potential generated by the transport of sodium which provides the force for the entry of the anions.

The similarity of the process of aqueous humor secretion to transport by the renal tubule cells is obvious if the luminal fluid in the kidney is considered to correspond to the blood of the ciliary process and the peritubular blood of the kidney to correspond to the aqueous humor. Although the exact nature of the transport process in the kidney is not established, it would seem a useful working hypothesis that the process is the same in renal tubule cell and ciliary epithelium. As noted earlier, the process in the kidney is generally believed to be an exchange of hydrogen ions derived from the cells for sodium ions from the lumen and the function of carbonic anhydrase to provide buffering for the residual hydroxyl ion in the cell. Such a view as to the function of carbonic anhydrase in the ciliary process is compatible with the effects of inhibitors on the formation of aqueous humor. In this connection it would be of interest to determine the effects of varying CO_2 tension on the rate of formation of aqueous humor and on the effects of acetazoleamide.

The oral or parenteral administration of acetazoleamide results in a sharp decrease in the intraocular pressure in patients with glaucoma (14, 37, 101) and a lesser fall in subjects with normal eyes. The fall in pressure was shown (14) to be due to a decrease in the rate of secretion of aqueous humor without appreciable change in the facility of outflow. The failure of local application of acetazole-

amide to produce the same effects (16, 88) has raised the question of whether the observed effects are due to a direct action of the inhibitor on the ciliary processes or are attributable to the renal effects of the drug. The latter possibility seems to have been excluded by the experiments of Becker (16) which showed persistence of the ocular effects in rabbits even though the diuretic effect was prevented by the administration of ammonium chloride or the acidosis and renal effects prevented by nephrectomy. In nephrectomized rabbits effects were produced with as little as 4–8 mg/kg of acetazoleamide. Although it is by no means established with certainty, the most acceptable explanation of the ineffectiveness of local application seems to be failure to reach the site of action.

Considerable confusion has arisen concerning the mechanism of action of acetazoleamide because of failure of some rabbits to respond with a fall in intraocular pressure. Whereas the animals studied by Becker (16) have consistently responded with a drop in pressure, Ballintine and Maren (13) found that of forty-six rabbits only six had consistent large responses and nineteen consistent small responses. The differences in response were not related to differences in carbonic anhydrase activity or acetazoleamide concentration, and it was found that the concentration of acetazoleamide in the ciliary processes was adequate to yield almost complete inhibition of the enzyme activity. Although Green *et al.* (104) had found earlier that the intravenous administration of acetazoleamide virtually eliminated carbonic anhydrase activity from the anterior uvea and suggested the enzyme was involved in the formation of the bicarbonate of aqueous humor, subsequent failure to obtain *a reduction in pressure* on local application or on systemic administration of acetazoleamide or *p*-sulfamylbenzoate led Green *et al.* (102) to conclude that carbonic anhydrase was *not* involved in the secretion of bicarbonate in the aqueous and in the maintenance of intraocular pressure. This conclusion appeared to be supported by a failure to observe a change in the concentration of bicarbonate in the anterior aqueous following local and intravenous sulfonamide (103).

However, the elegant data of Becker (17) and of Becker and Constant (18) would definitely appear to invalidate the conclusions of Green *et al.* (103) while providing an explanation for the failure of the latter to find a fall in aqueous bicarbonate concentration and for the apparent differences among rabbits in responsiveness to acetazoleamide. Becker (17) measured bicarbonate concentration in both anterior and posterior chambers in the rabbit and in the anterior chamber in guinea-pigs. The excess of bicarbonate in the posterior chamber was reduced quite consistently by approximately 50%, that in the anterior chamber by about two thirds after the administration of acetazoleamide. (The initial excess in the anterior chamber was considerably smaller than that in the posterior chamber.) It was shown that the time required for appreciable changes in concentration to occur in the anterior chamber was such that no significant depression of bicarbonate concentration was to have been anticipated at the time used by Green *et al.* for the sampling of the anterior chamber. From the change in bicarbonate concentration it was estimated that the rate of secretion of aqueous humor had been depressed 64% on the average, while from the rise of ascorbic

acid concentration, assuming no change in the rate of ascorbic acid secretion, depression of aqueous secretion by 60% was indicated. At the same time tonographic tracings indicated an average depression of 63% in the rate of aqueous flow (18). The figures for the three independent estimates are in impressive agreement. It was also found that massive doses of acetazoleamide failed to give further suppression of the rate of secretion. In a few eyes of both patients and rabbits when there was a low spontaneous rate of flow, no decrease followed the administration of acetazoleamide, a finding compatible with the view that the spontaneous reaction rate of CO_2 and water may be adequate to maintain secretion without carbonic anhydrase activity when the rate of secretion is low enough. Most instances of non-responsiveness in rabbits, however, were shown to be due to a compensatory increase in resistance to outflow so that, although secretion could be markedly decreased with acetazoleamide, there was little drop in intraocular pressure. The compensatory increase in resistance was found to be prevented by 9-alpha fluorohydrocortisone.

Falbriard *et al.* (84) have found a significant decrease in the potassium concentration of aqueous humor after the administration of acetazoleamide (100 mg/kg) to rabbits. However, the changes in plasma potassium concentration were not reported and since such doses produce marked potassium losses in the urine a decrease would have been expected. There are few data on the potassium content of aqueous humor (132) and little information concerning the mechanism by which it enters.

Acetazoleamide has proved of definite value in the clinical handling of patients with glaucoma (15, 19, 37, 76, 101, 137, 178, 192, 193, 226). Detailed analysis of the circumstances in which it appears to be useful is beyond the scope of this review and the competence of the reviewers. It may be worthy of note that Becker and Middleton (19) found it possible to maintain thirty-one of fifty patients with otherwise uncontrolled glaucoma for periods of six to fifteen months by the administration of around-the-clock acetazoleamide and that most of the failures were due to side-effects. Only 6% became resistant to the drug. The major side-effect necessitating discontinuance of therapy was loss of appetite.

Pancreas

The pancreas produces a secretion of high pH and bicarbonate concentration and contains large amounts of carbonic anhydrase (230). The natural assumption that the two circumstances are related has passed through the usual history of failure of support because of lack of demonstrable effects of sulfanilamide *in vivo* (229) and validation with the application of more powerful inhibitors (32). Tucker and Ball (229) administered up to 500 mg/kg of sulfanilamide to dogs and found neither the rate of secretion nor the bicarbonate concentration affected although the sulfanilamide concentration in the secretion was as high as 32 mg% (approximately 1.5×10^{-3}M). Birnbaum and Hollander (32) have more recently shown, however, that pancreatic secretion and bicarbonate output can be markedly depressed with acetazoleamide. Definite effects were observed three hours after 10 mg/kg; at lower levels of dosage the results were unimpres-

sive. Bicarbonate output was depressed approximately 85%, on the average, by maximally effective dosage and in no instance was completely eliminated.

The necessity of carbonic anhydrase for normal pancreatic secretion is therefore established. Lacking more information on the nature of the secretory process the exact role of the enzyme is difficult to assess. The concentration of bicarbonate in the secretion is very high, approaching approximately 140 mequiv./l as the rate of secretion increases (110, 218). This has generally been interpreted to indicate the secretion of bicarbonate ion, but it could represent secretion of sodium ion if the transport of sodium ion were linked with transport of hydrogen ion in the opposite direction. Whether or not there is chloride in the pancreatic juice as it is formed is not certain, since the chloride concentration rises as the rate of secretion decreases and it could represent diffusional exchange of chloride and bicarbonate in a subsequent process. The data of Ball *et al.* (12) on the rate of secretion as compared to oxygen consumption make it certain that CO_2 from the blood is required for the process. However, the fact that radioactive bicarbonate appears in the secretion with the same specific activity as in the blood (12) does not indicate that the bicarbonate in the pancreatic juice is derived directly from the blood bicarbonate as such, since, particularly in the presence of carbonic anhydrase, the CO_2 and bicarbonate are presumably in equilibrium. It seems more likely that the bicarbonate of the secretion is formed in the cells from CO_2 derived largely from the blood and that the cell is the site of action of the carbonic anhydrase.

Primary extrusion of bicarbonate would imply a tendency of intracellular pH to fall with secretion and such a decrease in pH has been inferred from the findings of Hammarsten and Jorpes (107). However, it is doubtful that these experiments could have yielded information on changes in intracellular pH during the process of secretion. In this connection it would be interesting to know whether the decreased secretion of pancreatic juice observed in animals exposed to low oxygen tensions by Chardon and Gross (44) was attributable to anoxia or to low CO_2 tensions secondary to hyperventilation.

Formation of Cerebrospinal Fluid

Depression of the secretion of spinal fluid in cats after the intravenous administration of acetazoleamide has been demonstrated by Tschirgi *et al.* (228). This has been confirmed by Kister (135) who found the rate of secretion to be depressed approximately 70%, an effect independent of dose from 0.5 to 150 mg/kg. Analogues of acetazoleamide, inactive as carbonic anhydrase inhibitors, were without effect. Although changes in arterial CO_2 tension are known to cause marked changes in cerebrospinal fluid pressure, these are the result of changes in intracranial vascular volume and Tschirgi *et al.* (228) found that 30% CO_2 inhalation had no effect on the rate of secretion either before or after acetazoleamide. The nature of the process resulting in the formation of cerebrospinal fluid is unknown and even its site is not established with certainty; probably the parenchymal vasculature, as well as the choroid plexus, is involved. Although the similarity of cerebrospinal fluid and aqueous humor as to composition and

electrolyte exchanges has been considered to indicate that the primary process is the same, transport of sodium from the plasma (75), the high bicarbonate concentration of the aqueous humor has not been shown to have a counterpart in the cerebrospinal fluid. It may be that fluid for analysis has not been collected in sufficient proximity to the site of secretion to obtain fluid with elevated bicarbonate concentration before the gradient was dissipated by diffusional exchange, if, indeed, such elevation of bicarbonate concentration does exist.

Salivary Glands

The saliva contains bicarbonate in concentrations considerably in excess of the plasma and the salivary glands contain carbonic anhydrase (208). The secretory process appears to be similar to that in the pancreas, although beyond flows of 1.5 ml/min, there is no increase in bicarbonate concentration with flow (225). The immediate effects of carbonic anhydrase inhibitors on salivary flow and bicarbonate concentration have not been reported. A decrease in the sodium and bicarbonate content of saliva on continued administration of acetazoleamide has been reported by Niedermeier *et al.* (180) but it is not clear whether or not this is due to a local effect or is secondary to metabolic acidosis which has been reported by Sand (208) to produce similar changes in saliva bicarbonate content. Carbonic anhydrase has also been reported to be present in the salivary secretion (194). Whether or not this has any physiologic significance has not been determined.

Sweat Glands

The presence of carbonic anhydrase in the glands of human skin has been detected by a histochemical technique (35). Kleeman *et al.* (136) observed no effect of acetazoleamide on the composition of thermal sweat in man.

EFFECT OF CARBONIC ANHYDRASE INHIBITORS ON THE BRAIN

Although the distribution of carbonic anhydrase in the brain has been extensively studied (4, 5, 6, 7, 8, 9), the nature of its function in this tissue has not been clarified. It has been suggested that it may accelerate the loss of CO_2 from cells during activity and, since the nervous system is depressed by high CO_2 tensions, thus maintain a normal level of excitability. How the carbonic anhydrase would have such an effect is not clear. If the enzyme is located within the cells it might be expected to have just the reverse effect tending to produce a more gradual loss of CO_2, if, as is most generally accepted, CO_2 rather than carbonic acid is the immediate product of metabolism. (The effects observed by Krebs and Roughton (139) with urease and yeast decarboxylase strongly support this view, although Conway (49, 50) believes carbonic acid may be directly produced.) If, on the other hand, the enzyme is located outside the cells, loss of CO_2 from the cells might be accelerated, but it is not clear why the blood carbonic anhydrase would not suffice. Davies and Krebs (71) and Davies *et al.* (70) have reported that carbonic anhydrase inhibitors, in high concentration, reduce potassium uptake and sodium extrusion in brain (and kidney) slices. The significance

of these findings is uncertain since the effects were observed only at concentrations (10–17 mM) of *p*-toluenesulfonamide which markedly depressed oxygen consumption. Lower concentrations have not had these effects (166, 176). Even if this effect does prove to be one mediated through an effect on carbonic anhydrase, it can not be assumed that the role of the enzyme is similar to that which has been proposed as involved in other electrolyte transporting organs such as stomach and kidney since the latter are involved in the formation of a secreted fluid differing in acid content from its precursors. Even if one were to consider the electrolyte transport of brain cells to involve exchange of hydrogen ion, there is, in the steady state, no net output of acid or alkali and hence no excess of one of these which might require neutralization with CO_2 in the cell to maintain the integrity of the latter.

Despite the lack of adequate hypotheses concerning the function of carbonic anhydrase in the central nervous system (beyond that involved in the formation of cerebrospinal fluid), the effects of inhibitors appear to indicate that it is in some way involved in the activity of the brain. The paresthesias and somnolence which occur in individuals receiving acetazoleamide have been noted by virtually every investigator who has administered it to patients. The well-known effect of metabolic acidosis in epilepsy led to the early trial of sulfanilamide in epileptic patients (46) and to the investigation of acetazoleamide soon after the drug was introduced. Although no definitive studies have been published, a number of preliminary reports have suggested that acetazoleamide is effective in many instances in reducing the incidence of seizures (25, 66, 146, 167, 170).

In clinical studies, it is difficult to dissociate the direct effects of acetazoleamide on brain carbonic anhydrase from the effects of the metabolic acidosis it produces, but the results in experimental animals appear to indicate that the effect is a direct one on the brain. Falbriard and Gangloff (83) found that acetazoleamide decreased diencephalic excitability in rabbits and thought that the rapidity of the effect suggested a local action rather than one due to metabolic acidosis. Millichap *et al.* (172) have reported that both acetazoleamide and sulfanilamide exert an anticonvulsant action in mice subjected to electroshock seizures and that the effect of acetazoleamide can be produced in the nephrectomized animal. The maximal protection against seizures was reported to occur at the time of the maximum degree of inhibition of brain carbonic anhydrase and to be proportional to the local effects on carbonic anhydrase. Thus, despite the much greater activity of acetazoleamide *in vitro*, it was only twice as active as sulfanilamide on a weight basis *in vivo* because the latter appeared at relatively higher concentration in brain. Preliminary experiments are said to indicate that acetazoleamide causes a decrease in total brain sodium and an increase in the ratio of intracellular to extracellular potassium. It is interesting that these are the effects directly opposite to those reported to occur in brain slices incubated with large amounts of inhibitors (70). They are effects which might occur in animals treated with a large dose of a diuretic which also causes loss of potassium in the urine.

The action of acetazoleamide in mice is potentiated by high CO_2 tension (238) and by the administration of ammonium chloride (171). In animals given aceta-

zoleamide over a period of six days there was a sixteen-fold increase in the dose of acetazoleamide required for a given degree of protection against electroshock convulsions. The development of such tolerance was minimized by the administration of ammonium chloride (171). No information concerning the possible nature of this tolerance has been adduced; it is not associated with a change in the degree of inhibition of brain carbonic anhydrase (171).

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