# Acceleration of Plasma Bicarbonate Conversion to Carbon Dioxide by Pulmonary Carbonic Anhydrase

Abstract. *In isolated rabbit lungs perfused with solutions containing little or no carbonic anhydrase activity, nearly complete equilibration between $H^{14}CO_3^-$ and $^{14}CO_2$ occurs during a single circulation. This equilibration can be inhibited by blocking pulmonary carbonic anhydrase with acetazolamide.*

Much of the carbon dioxide produced by the tissues of the body is buffered by the blood and delivered to the lungs in the form of plasma bicarbonate. Reconversion of bicarbonate to carbon dioxide in the pulmonary capillaries is accelerated by carbonic anhydrase (E.C. 4.2.1.1), an enzyme which is present within red cells but not plasma (*1*). Recent reports indicate that although the carbonic anhydrase promotes rapid formation of carbon dioxide from bicarbonate entering red cells, the absence of enzyme in the plasma is responsible for a slow decline in plasma hydrogen ion concentration which may require 20 seconds to reach 90 percent of completion (*2, 3*). It has been suggested that equilibrium is never reached in the body and that values of $P_{CO_2}$, $p$H, $HCO_3^-$, and $P_{O_2}$ obtained in collected blood may be misleading (*2–4*). The present study indicates that release of carbon dioxide from plasma bicarbonate stores is accelerated by pulmonary tissue carbonic anhydrase in a manner which may diminish the suggested disequilibrium.

Five White New Zealand rabbits (weighing 2 to 3 kg) were anesthetized with sodium pentobarbital (90 to 120 mg), ventilated, heparinized, and exsanguinated. Catheters were placed in the pulmonary artery and vein and the lungs were mounted in a box at 37°C. The lungs were ventilated 15 times per minute with 5 percent $CO_2$ in air at an inspiratory pressure of 10 cm-$H_2O$ and an end-expiratory pressure of 4 cm-$H_2O$. The first four lungs were perfused at a rate of 4 ml/sec with a solution which contained in each liter: 50 g of bovine serum albumin (fraction V, 96 to 99 percent pure, Sigma), 140 meq of $Na^+$, 2.5 mmole of $Ca^{2+}$, 1.8 mmole of $Mg^{2+}$, 120 meq of $Cl^-$, and 30 meq of $HCO_3^-$. The solution was adjusted to $p$H 7.4. By means of the radioactive bicarbonate procedure of Hodgen and Falk (*5*), it was possible to show that the carbonic anhydrase activity of this solution was less than a $5 \times 10^4$ dilution of rabbit red cells. The amount of enzyme in this concentration of red cells should not accelerate the uncatalyzed conversion of $H^{14}CO_3$ to $^{14}CO_2$ by more than 30 percent (*1*). During the 1-second transit time through the pulmonary capillaries (*6*) less than 1 percent of $HCO_3^-$ entering the capillaries (and a similar fraction of $H^{14}CO_3^-$) would be converted to $CO_2$ by this amount of enzyme. Enzyme concentration in the pulmonary venous outflow was also less than a $5 \times 10^4$ dilution of rabbit red cells. To be certain that enzyme activity within the perfusion fluid was not confused with tissue activity, we used a protein-free solution in the fifth study: each liter of solution contained 50 grams of polyvinylpyrrolidinone (molecular weight, 40,000, Matheson, Coleman and Bell), 1 g of glucose, 25 mmole of tris buffer, 140 meq of $Na^+$, 4 meq of $K^+$, 0.1 mmole of $Ca^{2+}$, 0.1 mmole of $Mg^{2+}$, 110 meq of $Cl^-$, and 30 meq of $HCO_3^-$ adjusted to $p$H 7.4.

The lungs were initially flushed with several hundred milliliters of perfusion fluid to diminish their red cell content. Recirculation of fluid to the lung was permitted only after carbonic anhydrase had been inhibited.

The experiment was conducted by simultaneously injecting 0.6 ml of acidified perfusion solution ($p$H 4.0) and 0.6 ml of dilute NaOH (adjusted so that the mixture would yield a $p$H of 7.4) within 1 second through a small Y-tube into the pulmonary artery line. The mixing of these solutions and the perfusion fluid was encouraged by a short irregular tube in the arterial line designed to promote turbulence. The delay required for arrival of the injected material at the pulmonary artery averaged 0.5 second. The $NaH^{14}CO_3^-$ (10 $\mu$c/ml) was anaerobically incorporated in either the acid solution (yielding $^{14}CO_2$) or the alkaline solution (yielding $H^{14}CO_3^-$) during preparation at least 30 minutes prior to the study. Tritiated water (10 $\mu$c/ml; used to estimate the transit time of water molecules) was added with the $H^{14}CO_3^-$, and $^{125}$I-labeled albumin (2 $\mu$c/ml; a vascular indicator) was always placed in the acid injection solution of each study in order to avoid denaturation by the alkali. In the last study and three additional control studies, $^{22}Na^+$ was used as the vascular indicator to ensure that there was no chance of introducing carbonic anhydrase activity. Previous studies have shown that the mean transit time of $^{22}Na^+$ is similar to that of $^{125}$I-labeled albumin (*7*).

Fluid was pumped from the outflow into a series of 40 glass syringes at 0.5- to 1.5-second intervals depending upon the flow rate through the lung. The mean transit time between the lung and syringes was 1.9 seconds. A custom-made anaerobic collector was used for this purpose (Altex). Portions of sample fluid (0.1 ml) were diluted in 1 ml of 0.1*M* tris buffer and mixed with 10 ml of Aquasol (New England Nuclear) in scintillation vials. The vials were than counted in gamma and beta counters. Crossover corrections of counts were calculated and indicator concentrations divided by the dose of each in the injection bolus, yielding fractional concentrations. Fractional concentrations were plotted on a logarithmic coordinate against time and extrapolated as indicated in Fig. 1, which shows a typical study. The relative recoveries ($R$) of $^{14}CO_2$ and $H^{14}CO_3^-$ were calculated from the ratio of the areas under the respective curves divided by that under the $^3H_2O$ curve rather than that of $^{125}$I-labeled albumin, because the former was always incorporated with the solution containing the $^{14}$C-labeled compound. Mean transit times ($\bar{t}$) were calculated as indicated elsewhere (*8*). The ratio $r = (\bar{t}_{^{14}C} - \bar{t}_{^{125}I})/(\bar{t}_{^3H_2O} - \bar{t}_{^{125}I})$ was used to predict the distribution of $^{14}$C and $^3H_2O$ that would prevail if the lung were perfused at a constant rate with these two indicators: $r = m_{^{14}C}/(am_{^3H_2O})$, where $m_{^{14}C}$ and $m_{^3H_2O}$ represent the total activity of these indicators in the extravascular space of the lung at steady state and $a$ is the ratio of activity of $^{14}$C to activity of $^3H_2O$ in each milliliter of the perfusion fluid (*9*). Slow entry of $^{14}$C into the tissue would decrease values of $\bar{t}_{^{14}C}$ calculated from single passage data, and the values calculated for $r$ would underestimate the steady-state distribution of $^{14}$C in the tissue. In those studies in which $^{22}Na^+$ was used, $\bar{t}_{^{125}I}$ was calculated on the basis of the previous observation that the extra-

Table 1. Extravascular distribution ($r$) and recovery ($R$) of $^{14}CO_2$ and $H^{14}CO_3^-$. Means and standard errors of the mean are provided. The control and acetazolamide studies were performed in each of five lungs. Paired $t$-tests showed that the $r$ values obtained for each condition differed from one another at the $P < .001$ level. The recovery of $^{14}CO_2$ with acetazolamide was significantly less at the $P < .001$ level than recovery of $^{14}$C under each of the other conditions.

| Condition | $r$ | $R$ |
|---|---|---|
| Control | | |
| $^{14}CO_2$ | 1.16 ± .10 | 0.99 ± .02 |
| $H^{14}CO_3^-$ | 0.92 ± .11 | 0.97 ± .01 |
| Acetazolamide | | |
| $^{14}CO_2$ | 2.28 ± .28 | 0.78 ± .03 |
| $H^{14}CO_3^-$ | 0.18 ± .06 | 0.97 ± .04 |


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vascular volume of $^{22}Na^+$ averages 12 percent that of $^3H_2O$ in a single circulation (*7*).

As shown in Fig. 1A and Table 1, injections of $^{14}CO_2$ and $H^{14}CO_3^-$ in the control studies yielded rather similar indicator dilution curves which are also similar to those of $^3H_2O$. Nevertheless the mean transit times of $^{14}CO_2$ were consistently somewhat greater than those of $H^{14}CO_3^-$. This indicates that the apparent volume of distribution available to $^{14}CO_2$ was larger than that accessible to $H^{14}CO_3^-$. This is more precisely indicated by values of $r$ for $^{14}CO_2$ which in each study exceeded those of $H^{14}CO_3^-$ by an average of 29 percent. The inequality of $r$ values indicates that complete equilibration was not attained between the two forms of $CO_2$ during transit through the lungs. However, when carbonic anhydrase was inhibited with acetazolamide (20 mg per liter of perfusion solution) the differences between the $H^{14}CO_3$ and $^{14}CO_2$ curves and $r$ values became much more marked (Fig. 1B and Table 1). The $^{14}CO_2$ curve became considerably prolonged compared to that of water, whereas the $H^{14}CO_3^-$ curve much more closely resembled that of the vascular indicator. After enzyme inhibition, $^{14}CO_2$ had access to a volume which exceeded that of $^3H_2O$ and presumably included alveolar gas. Indeed, $^{14}CO_2$ loss appeared to increase after administration of acetazolamide. Under the same conditions, $H^{14}CO_3^-$ was confined to a very small extravascular compartment, only slightly larger than the volume accessible to $^{125}$I-labeled albumin. In the acetazolamide studies (Fig. 1B), the extravascular volume available to $^{14}CO_2$ ranged from 5 to 70 times as great as that available to $H^{14}CO_3^-$.

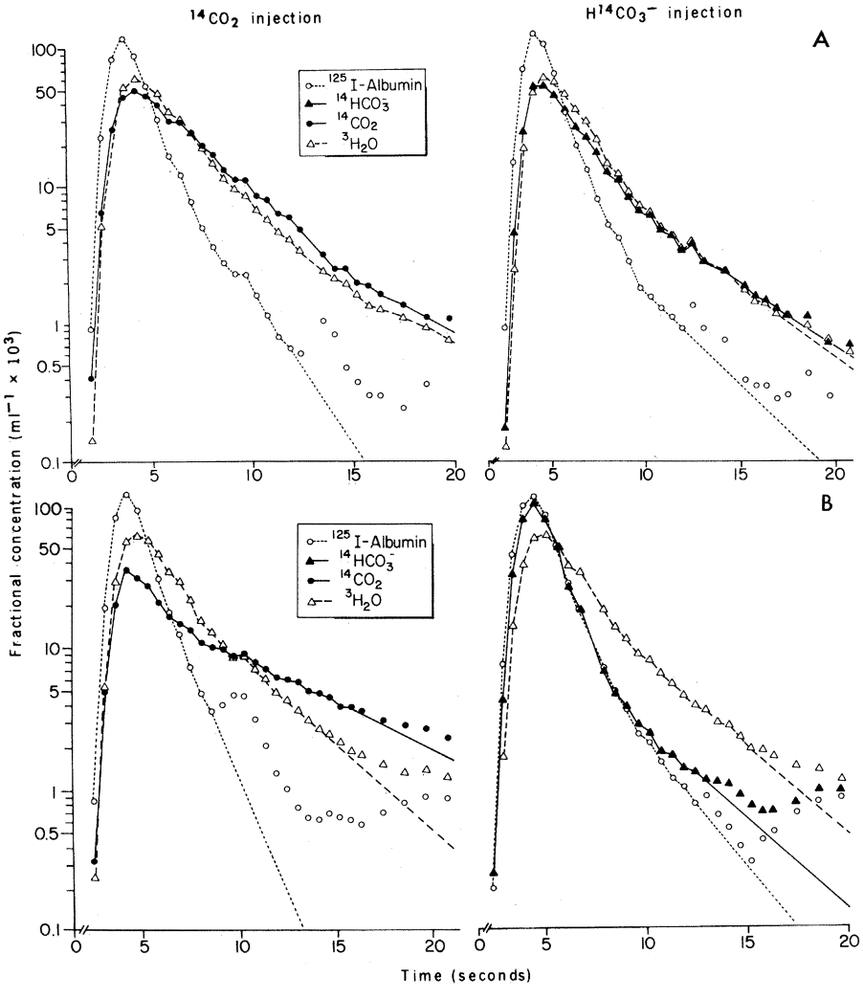


Fig. 1. (A) Control studies. The solid line in each graph represents the $^{14}C$-labeled compound. Although the curves are somewhat similar, the downslope of $^{14}CO_2$ follows, whereas that of $H^{14}CO_3^-$ precedes, the downslope of $^3H_2O$. There was no recirculation in these studies but some scatter occurred in the data for $^{125}$I-labeled albumin at very low activities. (B) Acetazolamide studies. The peak of the $^{14}CO_2$ curve is less than that of $^3H_2O$ and the downslope is prolonged. In contrast, the peak of $H^{14}CO_3^-$ exceeds that of $^3H_2O$ and the curve resembles that of $^{125}$I-labeled albumin. Recirculation is evident in this study but is attenuated by the large amount of perfusion fluid. Choice of alternative downslopes had little effect on calculated $r$ and $R$ values. Recirculation during the collection interval was eliminated in subsequent studies.

Since carbonic anhydrase activity of the perfusion fluid was either negligible or absent and since none was added to the pulmonary outflow, the observed enzyme activity was presumably associated with the lung itself. It is improbable that the enzyme activity was attributable to trapped intact red cells since the present studies indicate that enzyme is accessible to most of the exchange surface (*10*). It would be necessary for red cells to remain in the majority of capillaries even after the blood volume of the lung had been washed out with approximately 50 times as much perfusion fluid. In addition, the trapped red cells would have to be accessible to the perfusion fluid without obstructing its flow. Microscopic examination of these lungs revealed only occasional red cells.

Convincing evidence has been obtained that the pulmonary parenchyma contains carbonic anhydrase, but the location and function of the enzyme remain uncertain (*11*). Chinard *et al.* (*12*) and Feisal *et al.* (*13*) showed that there is at least some equilibration between $HCO_3^-$ and $CO_2$ in the lungs of anesthetized dogs, but this could represent the action of red cell carbonic anhydrase. The present study indicates that in the absence of red cells, $HCO_3^-$ reaching the lung is rapidly converted into $CO_2$ which readily diffuses into the tissue. When the enzyme is inhibited, $HCO_3^-$, like $Na^+$, $Cl^-$, and other ions, remains confined to a volume only slightly greater than that of albumin during the 1-second transit time through the pulmonary capillaries (*6, 14*). The observation that ionic indicators do not have access to a large portion of the extravascular water of the lung during a single transit suggests that they remain largely extracellular during this brief interval. Conversion of $HCO_3^-$ to $CO_2$ may therefore reflect extracellular enzyme activity, perhaps because of carbonic anhydrase bound to cellular membranes. If the reaction is extracellular, hydrogen ions in the plasma will be rapidly consumed and plasma $p$H will promptly stabilize. Tissue carbonic anhydrase activity may therefore serve to diminish the slow changes in $p$H which have been documented in red cell suspensions by Forster and Crandall (*3*).

Fain and Rosen (*15*) have recently reported the presence of carbonic anhydrase in the pulmonary endothelium of frogs, toads, and turtles, but the specificity of the histochemical procedures has been challenged (*16*) and the accessibility of enzyme to plasma $HCO_3^-$ could not be determined. The pulmonary endo-

thelium has been implicated in the rapid uptake and degradation of a variety of metabolites, and angiotensin-converting enzyme has been found in vesicles lining the plasma surface of the endothelium (*17*). It is possible that carbonic anhydrase is also associated with the plasma surface of the endothelial cells. The presence of additional enzyme within the cells or interstitium is suggested by the finding that $^{14}CO_2$ tends to be lost following enzyme inhibition. Presumably, once $^{14}CO_2$ has entered the tissue, enzyme inhibition hinders equilibration with tissue $HCO_3^-$ and the gas escapes into the alveoli.

The relatively small differences found between the $^{14}CO_2$ and $H^{14}CO_3^-$ curves under control conditions appear to be due in part to failure of some of the perfusion fluid to be adequately exposed to tissue carbonic anhydrase. Evidence for this was obtained in three additional studies in which bovine erythrocyte carbonic anhydrase (5 mg/100 ml, 4000 Wilbur-Anderson units per milligram; Sigma) was added to the perfusion fluid after the control runs. This served to reduce $r$ value differences by 22 to 73 percent. Residual differences after addition of enzyme may be related to incomplete mixing of the injection solutions. A simple model calculation suggests that no less than 86 percent of the plasma flow through the pulmonary vasculature has access to carbonic anhydrase (*18*).

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14. Both $^{22}Na^+$ and $^{36}Cl^-$ have access to a small portion of the extravascular volume accessible to $^3H_2O$ during a single transit through the lung: $r_{22_{Na^+}}$ 0.10 ± .02 (standard error of the mean) and $r_{36_{Cl^-}}$ 0.11 ± .02 in a series of five runs in five lungs in which both isotopes were incorporated.
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18. It was assumed that a fraction of the capillaries have no carbonic anhydrase and therefore have $r$ values for $^{14}CO_2$ and $H^{14}CO_3^-$ corresponding to these $r$ values after the administration of acetazolamide. The remainder of capillaries have $r$ values equal to the average of control values for $^{14}CO_2$ and $H^{14}CO_3^-$.
19. The anaerobic collector was funded by the California Lung Association. There was additional support from NIH grants HL-18606 and 5K04 HL-00132-04.

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## Inherited Medullary Thyroid Carcinoma: A Final Monoclonal Mutation in One of Multiple Clones of Susceptible Cells

Abstract. *Inherited medullary thyroid carcinomas contain one form of glucose-6-phosphate dehydrogenase (G6PD) in black female patients who are mosaic in normal tissues for G6PD types A and B. The same individual may have several tumors each containing either G6PD A or G6PD B. The data suggest that the inherited defect is an initial mutation producing multiple clones of defective cells; each tumor then arises as a final mutation in one clone of these cells.*

Recently we reported that medullary thyroid carcinoma and pheochromocytoma tissue obtained from a black female patient heterozygous for glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49; G6PD) forms A and B contained only the B form of the enzyme (*1*). These data gave biochemical evidence that the final mutation in formation of these inherited tumors was a clonal event. In Knudson's theory of genetic tumor formation (*2*) at least two mutational events must occur; the first mutation is the inherited defect which renders the involved cells susceptible to neoplastic change. The second event is a somatic mutational change which completes tumor formation. Our above data for G6PD types define only the nature of the final mutational event for inherited medullary thyroid carcinoma. In the present report, we give biochemical evidence that the initial inherited mutation in this disease produces multiple clones of cells susceptible to neoplastic change; each tumor formed is then the result of one or more mutational changes in one clone of the susceptible cells.

To continue our studies of the A and B forms of G6PD in black female patients with inherited medullary thyroid carcinoma we have used electrophoresis as in (*1*) according to the method of Ellis and Alperin (*3*) with the following modifications. The normal and tumor tissues were homogenized in 0.1*M* tris-HCl buffer (*p*H .8) containing 2 m*M* NADP (*4*) and centrifuged at 4°C, 2000 rev/min; the supernatant was centrifuged again. The final clear supernatant gave discrete bands of G6PD types A and B with a minimum of trailing during electrophoresis. Tissues obtained at surgery were either used immediately or stored at −70°C for no longer than 1 month.

Four patients have now been studied for the clonal origin of inherited medullary thyroid carcinoma. The first patient was described previously (*1*) and the three others are from another black kindred. In patients 1, 2, and 3 (Fig. 1), the normal tissues studied (red blood cells in all patients and thyroid in two) contained equal amounts of both forms of G6PD while all the tumor tissues sampled contained either the A or B G6PD. In two of these three patients, only one thyroid tumor was available for study while patient 3 had small lesions in both lobes of the thyroid gland. In each case, the entire available tumor tissue was used for the analysis of G6PD forms. The data confirm our earlier suggestion that the final mutation in the formation of inherited medullary thyroid carcinoma occurs as a clonal event (*1*).

In patient 4 (Fig. 2), larger amounts of tumor tissue from both sides of the thyroid gland were available for study. This patient was also important because studies of her red cells (Fig. 2) indicated an unequal heterozygosity for the A and B forms of G6PD, with a predominance of the B form. This type of the hetero-


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