Carbonic Acid Production and the Role of Carbonic Anhydrase in Decarboxylation in Brain

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Sudden oxygenation of a thin film of rat brain homogenate, suspended between the surface of a glass pH-sensitive electrode and a gas-permeable membrane, is accompanied by a fall in pH, which is greater when carbonic anhydrase is inhibited. The result suggests that oxidative decarboxylation yields carbonic acid (HCO_3^- and H^+), which dissociates to form molecular carbon dioxide. Brain carbonic anhydrase facilitates the formation of carbon dioxide from the decarboxylation products.

Severinghaus & Cotev (1968) observed that the pH of cerebrospinal fluid on the surface of the dog brain decreased rapidly after the intravenous administration of the carbonic anhydrase inhibitor acetazolamide (25 mg./kg. body wt.). This occurred despite prior nephrectomy and maintenance of a constant P_{CO_2} on the brain surface. Kjällquist & Siesjö (1966) demonstrated that whole-brain carbon dioxide content rose after the administration of acetazolamide. One possible explanation for these findings is that carbonic acid (or HCO_3^- and H^+) rather than carbon dioxide is the primary product of decarboxylation. If this is the case, when carbonic anhydrase is inhibited dehydration of carbonic acid is delayed, resulting in a rise of the intracellular concentration of carbonic acid, and of H^+ and HCO_3^- . The observed fall in pH in the extracellular fluid might be due to diffusion of carbonic acid or to transport of H^+ out of the cells. The experiments described here were accordingly designed to test whether carbonic acid (or HCO_3^- and H^+) or carbon dioxide is the direct product of aerobic metabolism in the brain.

MATERIALS AND METHODS

Adult rats were decapitated; their brains were removed within 30 sec., placed in an approximately equal volume of electrolyte containing (mequiv./l.): Na^+ (30), K^+ (100), Mg^{2+} (40), Cl^- (160) and HCO_3^- (10), under N_2 at 0° , and immediately ground at 0° to an acellular homogenate in a Teflon-glass homogenizer with 0.004–0.006 in. clearance. Samples (0.2 ml.) of homogenate were then transferred to glass test tubes (3 ml.) for mixing under N_2 with acetazolamide or various substrates or inhibitors. The mixture was

then pipetted under N_2 on to a 75 μm -thick Silastic membrane mounted on the electrolyte jacket of a Radiometer CO_2 electrode (Fig. 1); $\text{N}_2 + \text{CO}_2$ (95:5) was passed through the cuvette below the membrane.

The pH-sensitive glass electrode, wrapped by a spacer of single-thickness dry lens tissue (Joseph paper), was then inserted as shown in Fig. 1. The experiments were performed at room temperature. The pH was recorded in a control anoxic period and for several minutes after the change of the gas flowing below the Silastic membrane from $\text{N}_2 + \text{CO}_2$ (95:5) to $\text{O}_2 + \text{CO}_2$ (95:5). The procedure was repeated after the electrode had been dismantled, cleaned, dried, and refilled with homogenate containing 0.15 mM-acetazolamide. The pH-sensitive electrode was calibrated by replacing the homogenate with the electrolyte containing 10 mequiv. of $\text{NaHCO}_3/\text{l.}$ in which the pH was 7.0 at 25° , and the P_{CO_2} 35 mm. Hg. The wet cotton wick acted as a liquid junction between the homogenate and the AgCl reference electrode. The high permeability of Silastic to O_2 and CO_2 permitted the partial pressures of these gases in the homogenate to be essentially determined by the gas phase. The pH shift on oxygenation was corrected for the rise in pH (0.008) with oxygenation produced by the difference between the O_2 and N_2 mixtures, which contained 5.08% and 5.17% CO_2 respectively as recorded in the calibration solution. No correction was made for the Bohr effect of oxygenation of haemoglobin.

RESULTS

The initial exposure to oxygen caused a fall in pH greater than any subsequent shift from nitrogen to oxygen (Fig. 2). After two to five 1 min. exposures to oxygen, no further change in pH occurred on oxygenation. In 11 tests (control and with acetazolamide) in six rats, the mean decrease ($\pm$ s.d.) in pH in the absence of acetazolamide was 0.017 ± 0.007 and the mean decrease in the presence of acetazolamide was 0.039 ± 0.017 . This difference is significant ($0.005 < P < 0.01$) by paired

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analysis by Student's *t* test. The response to oxygen was totally abolished by addition to the homogenate of 2mM-sodium cyanide. It was neither augmented nor sustained longer by the addition of 1mM-ADP, 2mM-sodium pyruvate,

1mM-fructose 1,6-diphosphate, 2mM-fumarate, 1.6mM-phosphate, 55mM-glucose or 10mM-malonate. It was not inhibited by 0.1mM-iodoacetate, 0.01mM-dinitrophenol or 10mM-fluoroacetate.

DISCUSSION

The formal alternative sequences examined in this experiment are given in Scheme 1.

The mitochondrial membrane is presumably permeable to either carbon dioxide or carbonic acid, but not necessarily to H^+ or HCO_3^- . Brain mitochondria are reported to contain carbonic anhydrase (Karler & Woodbury, 1960). The effect on H^+ in the homogenate of inhibition of carbonic anhydrase with acetazolamide depends on which sequence is followed. In sequence (1) (Scheme 1), inhibition of carbonic anhydrase would decrease the amount of H^+ generated by delaying the hydration of carbon dioxide while carbon dioxide escaped through the Silastic membrane. In sequence (2), metabolically produced H^+ would accumulate until the uncatalysed rate of dehydration to carbon dioxide equalled the rate of metabolic production of carbonic acid. The experiment suggests that sequence (2) is followed in rat brain.

This analysis assumes that the only relevant action of acetazolamide is to inhibit carbonic anhydrase. In an exhaustive review of carbonic anhydrase, Maren (1967) stated that this is the only known action of acetazolamide. More specifically, there is considerable evidence that it does not stimulate oxidative metabolism in man or animals (Coppen & Mezey, 1961; Thangamani, 1965).

This problem has been studied by several other investigators. Krebs & Roughton (1948), examining the actions of the isolated enzyme urease on urea and of carboxylase on pyruvate, with a Warburg apparatus, concluded that carbon dioxide and not carbonic acid or HCO_3^- was the primary product of decarboxylation. Conway & O'Malley (1953), studying the latter system, concluded that both carbon dioxide and carbonic acid (or HCO_3^-) are primary products, the total experimental conditions determining which one will predominate.

Fig. 1. P_{CO_2} electrode as modified to measure the pH of a thin film of brain homogenate at the instant of oxygenation at constant P_{CO_2} .

Fig. 2. Response of the pH of a 50% brain homogenate to the addition of O_2 in the presence and absence of acetazolamide. The response to a second addition of O_2 is also shown for the homogenate with acetazolamide.

Scheme 1.

Hall & Vennesland (1967), after studying the action of glyoxylate carboligase on glyoxylate, concluded that carbonic acid (or HCO_3^- and H^+) was the product. Natour & Palmer (1962) studied mouse whole-brain homogenate by collecting carbon dioxide in a purging gas stream. They concluded that carbon dioxide (non-hydrated) was the primary product. Analysis of their method suggests that washout of HCO_3^- from the homogenate by dehydration of carbonic acid to carbon dioxide during the preliminary 2 min. elution would have been less complete in the presence of acetazolamide, leaving more HCO_3^- to be collected later.

The evidence that carbonic acid (or bicarbonate) is the primary product of decarboxylation in the brain implies that carbonic anhydrase in brain (Giacobini, 1962; Nishimura, Taninukai & Nishinuma, 1963; Karler & Woodbury, 1960) facilitates the dehydration of metabolically produced H^+ , HCO_3^- and carbonic acid to carbon dioxide for removal by the bloodstream. Acetazolamide *in vivo* may be expected to induce intracellular carbonic acidosis if it gains access to the intracellular carbonic anhydrase.

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REFERENCES

- Conway, E. J. & O'Malley, E. (1953). *Biochem. J.* **54**, 154.
Coppen, A. J. & Mezey, A. G. (1961). *J. appl. Physiol.* **16**, 367.
Giacobini, E. (1962). *J. Neurochem.* **9**, 169.
Hall, R. L. & Vennesland, B. (1967). *Fed. Proc.* **26**, 1083.
Karler, R. & Woodbury, D. M. (1960). *Biochem. J.* **75**, 538.
Kjällquist, Å. & Siesjö, B. K. (1966). *Acta physiol. scand.* **68**, 225.
Krebs, H. A. & Roughton, F. J. W. (1948). *Biochem. J.* **43**, 550.
Maren, T. H. (1967). *Physiol. Rev.* **47**, 595.
Natour, R. M. & Palmer, R. F. (1962). *Biochem. J.* **85**, 110.
Nishimura, T., Taninukai, H. & Nishinuma, K. (1963). *J. Neurochem.* **10**, 257.
Severinghaus, J. W. & Cotev, S. (1968). *Scand. J. Lab. clin. Invest.* suppl. 102, section I, E.
Thangamani, A. (1965). *Biochem. Pharmacol.* **14**, 89.