The Immediate Product of Brain-Tissue Decarboxylations

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Enzymic decarboxylation accounts for most of the metabolically produced carbon dioxide in the living organism. Whether the initial product of the decarboxylation of substrate is bicarbonate or carbon dioxide has so far been of little interest, because of the rapid interconversion of these substances to a state of equilibrium as demanded by the buffer and gas composition of the milieu. Such interconversion is enhanced by carbonic anhydrase. Inhibitors of this enzyme cause diverse physiological effects when administered to respiring organisms (Berliner & Orloff, 1956). In an understanding of the mechanisms for producing these complex effects, especially in the central nervous system (Tschirgi, 1960), the necessity for the identification of the immediate end product of tissue decarboxylations has assumed importance. Direct studies on animal and plant tissue to determine the immediate end product of decarboxylations have not yet been made. Individual enzymes (Conway & O'Malley, 1953; Krebs & Roughton, 1948) have been studied by methods not applicable to homogenates and breis of respiring tissue for reasons discussed by Palmer (1961). Without any direct evidence, some authors have presumed that the end product of brain-tissue decarboxylations was bicarbonate, not carbon dioxide (Koch & Woodbury, 1960).

With the development of a method distinguishing between these two products and applicable to homogenates and breis producing carbon dioxide at physiological rates (Palmer, 1961), studies were undertaken on mouse brain. It is the purpose of this paper to demonstrate the application of the method to respiring tissue, and to show that carbon dioxide and not bicarbonate is the major end product of decarboxylation in brain.

MATERIALS AND METHODS

Animals. Male and female Swiss albino mice of the Webster strain, 4–11 weeks of age, were used.

Apparatus. The apparatus is essentially that of Palmer (1961), with a modification made in the collecting vessel. This is now a glass tube (2.5 cm. $\times$ 15 cm.), fitted with a sintered-glass air disperser through a rubber stopper. The rubber stopper may be fitted with a 5 ml. syringe, for the addition of solutions.

Medium. 'Phosphate-glucose-saline' medium, pH 7.4, as described by McIlwain (1951), was used.

Carbonic anhydrase. The dried enzyme was prepared from ox blood by the method of Meldrum & Roughton (1933), and contained 9 enzyme units/mg. In most experiments 100 units/ml. of homogenate were used. The enzyme activity was determined by the method of Maren, Ash & Bailey (1954). Where used, acetazolamide was added to the homogenate preparations to give a concentration (40 μ M) sufficient to inhibit more than 99.9% of the enzyme present (Maren, Parcell & Malik, 1960).

Method. Five mice were quickly decapitated (within 5 min.) and the entire brains (about 2 g.) removed, placed in cold medium and homogenized, in an ice bath, in 6 ml. of the medium with the Potter-Elvehjem homogenizer. The pH was 7.1. A portion (3 ml.) of the homogenate was placed in the reaction chamber (previously treated with a small amount of Dow-Corning Antifoam A Spray) from a 5 ml. syringe attached to the stop-cock. Pure air was passed through the reaction chamber containing the homogenate at a rate of 25–30 ml./min. After 2 min. the sintered-glass air disperser was placed in the collection tube containing 7 ml. of CO₂-free water and 3 ml. of 0.81 M-barium hydroxide. Gas was collected for three consecutive 2 min. periods in three separate collecting vessels. The amount of CO₂ collected in each 2 min. period was approximately equal in all experiments. Each value reported is an average of these three values. The final pH was 7.2. Two control collecting tubes were prepared, one a blank containing only the original starting barium hydroxide and water, the second containing barium hydroxide and water through which CO₂-free air was bubbled for 2 min. from the empty reaction chamber. The contents of these tubes were then titrated with 1.22 M-sulphuric acid to a yellowish-green end point, with 4 drops of 0.1% bromothymol blue as

Table 1. *Amount of carbon dioxide collected at various rates of air flow in the presence of acetazolamide*

Brain homogenate with acetazolamide (40 μ M) was placed in the reaction chamber. Six experiments at each of the six different flow rates were carried out; fresh homogenate was used in every case. The CO₂ output was calculated from titration of barium hydroxide in the collecting vessel; an individual experiment is the average of three 2 min. collection periods, which followed an initial flushing-out period of 2 min. The results are the means $\pm$ S.E. of six experiments.

Rate of air flow (ml./min.)	CO ₂ collected (μ mole/g. wet wt./min.)
5	0.19 $\pm$ 0.034
10	0.50 $\pm$ 0.097
20	0.80 $\pm$ 0.082
25	0.97 $\pm$ 0.038
30	0.91 $\pm$ 0.053
35	0.86 $\pm$ 0.063

indicator. All experiments and titrations were performed at room temperature (24–26°). The amount of CO₂ collected in each of the experimental tubes was calculated by using the titration average of the two control tubes (which usually agreed to within 0.1 μ mole) as blank value.

Control experiments were done with the homogenate of heated brain tissue, medium alone, medium exposed to room air, and medium through which CO₂ had been bubbled.

RESULTS

If the brain were producing carbon dioxide, the presence of carbonic anhydrase in the homogenate should retard the rate of evolution of carbon dioxide as collected, by accelerating the hydration to bicarbonate, thereby 'trapping' it in the medium. If bicarbonate were being produced, carbonic anhydrase should accelerate the rate of evolution of carbon dioxide from the reaction chamber (Palmer, 1961).

To achieve an experimental differentiation, a flow rate had to be selected that was neither too rapid nor too slow. For if the rate of air flow were too high, all the carbon dioxide would be blown off as it was formed despite the presence of carbonic anhydrase; if it were too low, all the carbon dioxide would be hydrated, even in the absence of carbonic anhydrase, by the uncatalysed reaction. A flow rate of 25–30 ml. of air/min. provided the experimental differentiation (Palmer, 1961). Table 1 shows that, in the present system, this flow rate is optimum for collecting carbon dioxide in the absence of carbonic

anhydrase. Table 2 shows the proportionality between weight of brain and carbon dioxide collected.

Table 3 shows that the results satisfy the theoretical criteria for the primary production of carbon dioxide and not bicarbonate by mouse brain. Brain homogenate and brain homogenate plus carbonic anhydrase give the same rate of evolution of carbon dioxide, indicating that endogenous enzyme is adequate for available substrate. Acetazolamide increases the evolution of carbon dioxide by specifically inhibiting carbonic anhydrase, and slowing the conversion of carbon dioxide into bicarbonate. Interpretation of these results, and their verification by the appropriate controls, show that mouse-brain homogenate produces primarily carbon dioxide as its immediate form under these conditions.

Table 2. *Amount of carbon dioxide collected from various amounts of brain in the presence of acetazolamide*

Different weights of brain were homogenized and each homogenate was diluted to 3 ml. The homogenates contained acetazolamide (40 μ M). Air at a flow rate of 25–30 ml./min. was passed through the homogenate in the reaction chamber, and CO₂ was collected in 2 min. periods as described in Table 1. The results are the means $\pm$ s.e. of six experiments.

Amount of brain used (g.)	CO ₂ collected (μ mole/min.)
0.27	0.29 $\pm$ 0.022
0.54	0.49 $\pm$ 0.022
0.81	0.78 $\pm$ 0.050

Table 3. *Amount of carbon dioxide collected from brain treated in various ways and from controls*

Homogenates of brain were variously treated: with acetazolamide (40 μ M) or carbonic anhydrase (100 units/ml.) or both; or heated for 5 min. at 100°. Medium containing no brain, and medium containing carbonic anhydrase and gassed with 100% CO₂ were also tested. The procedure was as noted in the text and in Table 2. Each now contains a series of replicate values; each value is the average of three 2 min. collection periods after the initial 2 min. flushing-out period.

CO₂ collected (μ moles/g. wet wt./min.)

Homogenate + acetazol- amide	Homogenate + acetazol- amide + carbonic anhydrase	Homogenate + carbonic anhydrase	Homogenate (only)	Homogenate from dead brain + acetazolamide	Homogenate from dead brain + carbonic anhydrase	Medium* exposed to atmosphere	Medium* + carbonic anhydrase saturated with CO ₂
1.18	1.02	0.73	0.62	—	—	—	—
0.92	0.92	0.50	0.78	0.30	0.16	0.18	0.02
0.90	0.70	0.64	0.45	—	—	—	—
0.90	0.84	0.44	0.43	0.45	0.09	0.08	0.05
1.08	1.00	0.88	0.36	—	—	—	—
0.80	0.76	0.36	0.74	0.02	0.03	-0.02	0.01
1.03	1.33	0.74	0.68	—	0.15	—	—
0.91	0.88	0.48	0.62	0.10	0.01	0.14	0.21
1.12	0.88	0.42	0.48	0.06	0.08	—	—
0.75	0.75	0.51	0.32	0.08	0.13	0.20	0.08
1.06	0.90	0.10	0.54	0.10	0.17	—	—
0.98	0.81	0.94	—	0.12	0.06	0.20	0.04
Mean $\pm$ s.e.							
0.97 $\pm$ 0.038	0.89 $\pm$ 0.049	0.56 $\pm$ 0.078	0.54 $\pm$ 0.042	0.15 $\pm$ 0.050	0.10 $\pm$ 0.020	0.14 $\pm$ 0.030	0.10 $\pm$ 0.025

* CO₂ collected from 4 ml. of medium that did not contain brain (μ mole/min.).

DISCUSSION

Interest in the immediate product of tissue decarboxylations was aroused by the attempt of Conway & MacDonnel (1945) to explain a membrane-transport theory in yeast on the assumption that bicarbonate was the immediate end product of yeast respiration. Attempts to demonstrate this assumption directly by manometric methods yielded equivocal results (Conway & O'Malley, 1953).

In man and experimental animals, including mice, acetazolamide, a specific carbonic-anhydrase inhibitor, is an anticonvulsant, both in human clinical trials (Bergstrom, Garzoli, Lombroso, Davidson & Wallace, 1952; Merlis, 1954) and experimental electric shock (Millichap, Woodbury & Goodman, 1955; Gray, Maren, Sisson & Smith, 1957). The present results together with other recent work yields evidence on the mechanism of anticonvulsant activity of carbonic-anhydrase inhibitors. It seems clear that acetazolamide causes an immediate retention of carbon dioxide in venous capillaries, due to inhibition of erythrocyte carbonic anhydrase. This effect is prominent in the brain (Maren & Robinson, 1960), and the present work supports the idea that carbon dioxide is produced in the brain and would require transport (as bicarbonate) by erythrocytes, as for other organs. Such transport would be slowed by acetazolamide. The anticonvulsant activity could then be essentially that of carbon dioxide, which agrees with the experiments of Koch & Woodbury (1958).

The explanation has been put forward that carbonic-anhydrase inhibitors act as anticonvulsants by inhibition of brain enzyme (Millichap *et al.* 1955), but the supporting results are equally consistent with inhibition of erythrocyte enzyme. 'Brain enzyme', in the theory of Koch & Woodbury (1960), would be in neurones, to catalyse the intracellular conversion of bicarbonate into carbon dioxide. But if carbon dioxide is the immediate product of decarboxylation reactions in neurones, there is no obvious role for carbonic anhydrase in these cells, and it is significant that Giacobini (1961) found that the enzyme was virtually absent from neurones but present in glia.

SUMMARY

1. The application of a method to tissue homogenates producing carbon dioxide at physiological rates, that distinguishes between carbon dioxide and bicarbonate as the immediate respiratory product, is presented.

2. The results fit the theoretical criteria for the immediate production of carbon dioxide as the decarboxylation product in mouse-brain homogenates.

3. The significance of this finding in the interpretation and theory of the anticonvulsant effect of carbonic-anhydrase inhibitors is discussed.

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