Cerebral Acetylcholine in Thiamine Deficiency[1] (34932)

K. V. SPEEG, JR., D. CHEN, D. W. MCCANDLESS, AND S. SCHENKER[2]
(Introduced by J. D. Wilson)

Liver-GI Unit, Department of Internal Medicine, The University of Texas (Southwestern) Medical School at Dallas, Dallas, Texas 75235

Thiamine deficiency in man and in experimental animals may induce a dysfunction of the central nervous system (1–3). These neurologic signs, when of brief duration, are fully and rapidly reversible with administration of thiamine, and are, therefore, believed to have a metabolic, *i.e.*, biochemical basis (4, 5). The precise biochemical processes responsible for the cerebral disorder seen in thiamine deficiency have been extensively discussed (5, 6) but are not yet defined.

One postulated mechanism of the cerebral dysfunction seen with low thiamine is a decrease of brain acetylcholine (ACh). The evidence for the ACh depletion hypothesis is as follows. First, intracerebral synthesis of ACh requires the presence of Acetyl CoA and ATP, substances which, theoretically, might be decreased in brain with impaired activity of pyruvate decarboxylase, a thiamine-dependent enzyme (5, 7). Second, early, as well as some recent studies have suggested that thiamine-deficient brain *in vitro* has a decreased capacity to synthesize ACh (8, 9) and that the concentration of ACh-like compounds in the brain of thiamine-depleted animals may be decreased (7, 10). Finally, it is known that ACh is a synaptic transmitter in the autonomic nervous system, and recent data suggest that it may have a similar role in the central nervous system (12). On the other hand, all prior investigations have employed admittedly nonspecific bioassays for ACh, and one such study showed normal ACh levels in thiamine-deficient rat and pigeon whole brain (13). Furthermore, previous data refer only to whole brain despite the well-documented observations that the biochemical and later morphologic changes in thiamine deficiency are primarily confined to the brainstem and the cerebellum (3, 5). Finally, in the one study which assessed the correlation between ACh levels in the brain and neurologic dysfunction, no such parallelism was observed (7). It is evident that an evaluation of the role of cerebral ACh in thiamine deficiency is necessary. The present study assesses the concentration of ACh, employing a specific, accurate biochemical assay, in the cortex, brainstem, and cerebellum of thiamine-deficient rats and correlates these findings with the neurologic status of the animals.

Experimental Procedure. Materials. Sprague-Dawley female littermate rats, 60–80 g in weight, were placed singly in metabolic cages and pair-fed a synthetic diet with or without thiamine as has been previously described (5). A third group of female rats of equal weight was fed a regular Purina Chow diet *ad libitum* and served as normally fed controls. The pair-fed animals were felt to be the most comparable and meaningful controls. All animals were given free access to water.

The thiamine-deficient rats developed signs of neurologic dysfunction (ataxia, incoordination, and drowsiness) after about 4–5 weeks of the thiamine-deficient diet while the pair-fed and normally fed rats exhibited no alteration from normal behavior. An intraperitoneal injection of 25 μg of thiamine hydrochloride reversed the neurologic signs of the

[1] These studies were supported by U.S. Public Health Service Research Grant MH17363, U. S. Public Health Service, and were reported in part in abstract form to the Southern Federation for Clinical Research, New Orleans, January 1970.

[2] Recipient of Research Career Award 5 KO3 13746, U. S. Public Health Service.

thiamine-deficient rats within 16–36 hr.

The symptomatic thiamine-deficient rats had a cerebral thiamine concentration (mean ± SE) of 0.56 ± 0.07 μg/g while the thiamine brain levels in the normally fed and pair-fed controls, respectively, were 3.72 ± 0.08 and 3.56 ± 0.15 μg/g (5). Other biochemical parameters of the thiamine-deficient and control rats have been presented in detail elsewhere (5).

Methods. Cerebral ACh was measured in thiamine-depleted rats with overt neurologic signs, in animals whose neurologic dysfunction had been corrected by administration of thiamine, and in matched pair-fed and normally fed controls. Preliminary experiments established that ACh levels in brain of rats rapidly frozen in a mixture of Dry Ice and acetone yielded values similar to those obtained with fresh unfrozen brain, hence the latter brain preparation was used for all the assays. The rats were either decapitated without anesthesia, briefly anesthetized with diethyl ether prior to decapitation, or given eserine 0.175 mg/100 g body weight *ip* 10 min prior to decapitation. Ether anesthesia was used in one of the groups to ascertain that excitement immediately prior to decapitation did not selectively alter the brain ACh in the more vigorous pair-fed rats. Eserine was employed in another group since a recent study of brain ACh in thiamine deficiency used this procedure to decrease the breakdown of brain ACh during preparation of the tissue for assay (7). After the injection of the eserine, all rats exhibited tremulousness, increased salivation, and passivity. Cerebral cortex consisted of gray matter without specific localization, the brainstem comprised the medullopontine and caudal midbrain area, and the cerebellum was assayed *in toto*.

Acetylcholine was measured by a modification of the specific and sensitive fluorometric method of Fellman (14). Briefly, this procedure involves precipitation of ACh from solution as an iodine complex. The quarternary ester is absorbed on a resin which is treated with hydrazine and the acetyl hydrazide is eluted from the resin. The eluate is then treated with salicyladehyde to form an acetylhydrazyl salicylhydrazone which is intensely fluorescent. Certain modifications of the original procedure were necessary: (1) Instead of heating the sample in a 100° water bath for 10 min to remove the ether residue (which resulted in considerable loss of ACh in our experience), the sample was placed in a 47° water bath for 5 min and air was bubbled through the soultion for 1 min. After this the samples were cooled; (2) Five milliliters of resin was placed in standard 1 × 20-cm chromatographic tubes; (3) 2 *M* hydrazine (0.15 ml) was added to the ACh-loaded resin; (4) The acetylhydrazine was eluted with 7 ml of 0.07 *N* HCl, and the fractions known to contain the product from previous standardization of the resin were saved; (5) Standardization of each resin batch was achieved by carrying 12 mμmoles of radioactive acetylcholine iodide, labeled in the C_2 position, through the procedure. One-milliliter aliquots were collected and radioassayed. The elution pattern of each resin batch varied slightly, but the majority of ACh was found in the third and fourth milliliters of eluates. Using these modifications in 10 experiments we obtained a recovery (mean ± SE) of 91 ± 5.1% with known quantities of ACh added to brain. In 15 duplicate assays of the same brain area the mean variation was ± 4.1%.

Statistical analysis was carried out by the nonparametric Wilcoxson matched-pair test which takes into account the pair-feeding aspects and the sample size (15). Non-pair-fed animals were statistically evaluated by the nonparametric Mann–Whitney U Test (15).

Results. The cerebral regional ACh concentrations in thiamine-deficient and control rats are shown in Table I. It is evident that for each brain area studied and with every method of obtaining the brain, the cerebral ACh concentrations in thiamine-deficient rats exhibiting overt neurologic signs were similar to the values seen in the pair-fed controls ($p > .05$). Similar results were obtained in thiamine-deficient rats studied after reversal of their neurologic deficit (group 4, Table I). Except for the cerebellum, the ACh values in the normally fed rats paralleled closely those

TABLE I. Cerebral Regional Acetylcholine Concentration in Thiamine-Deficient Rats.[a]

Condition of thiamine-deficient rats at time of sacrifice	Method of obtaining brain[b]	Brain region	Normally fed controls	Pair-fed controls	Thiamine deficient
Thiamine-deficient rats used showed overt encephalopathy after 4–5 weeks of low-thiamine feeding	1) Decapitation	Cortex (10)	5.94 ± 0.89	6.69 ± 1.12	7.75 ± 1.04
		Brainstem (10)	13.52 ± 0.77	15.23 ± 1.92	13.63 ± 1.33
	2) Decapitation after prior administration of eserine	Cortex (6)	12.01 ± 1.31	11.12 ± 0.69	12.17 ± 0.34
		Brainstem (6)	19.17 ± 1.13	16.78 ± 1.04	16.58 ± 1.25
	3) Ether anesthesia	Cortex (10)	7.98 ± 0.58	8.18 ± 0.94	9.43 ± 0.78
		Brainstem (10)	15.91 ± 1.67	15.82 ± 1.46	13.91 ± 2.21
		Cerebellum (10)	3.51 ± 0.50	6.61 ± 1.72[c]	6.27 ± 1.33[c]
Thiamine-deficient rats used had fully recovered from encephalopathy after 3 daily doses of 25 μg thiamine ip	4) Ether anesthesia	Cortex (5)		11.04 ± 2.05	12.58 ± 2.58
		Brainstem (5)		14.33 ± 2.68	14.71 ± 0.81

[a] Acetylcholine is given in millimicromoles/g wet weight. All data are given as means ± SE of number of animals indicated in parentheses for each brain region. Statistical analysis is given in Results.

[b] Detailed explanation of the procedure for obtaining the brain in each group is given under the Methods.

[c] Six animals were assayed.

noted in the other two groups ($p > .05$). The cerebellar ACh concentration in the thiamine-deficient rats was comparable to values noted in pair-fed control cerebellum ($p > .05$), but both of these exceeded, though not significantly ($p > .05$), the cerebellar ACh levels in the normally fed controls. Although the results are expressed in terms of tissue wet weight, prior studies in our laboratory have shown that the brain water content is not altered in thiamine deficiency (5). Thus, wet weight is a proper reference base in these experiments.

As anticipated from prior data using a bioassay technique (16), in the normally fed rats brainstem ACh concentration was higher than that of the cortex ($p < .01$) and the latter exceeded the values in the cerebellum ($p < .01$). Eserine premedication in the normally fed controls enhanced the ACh concentration both in the cortex and the brainstem, as compared to decapitated normal rats (group 1, Table I) ($p < .01$), but the increase was more marked in the cortex. In the other two groups of rats, eserine increased cortical ACh significantly ($p < .05$), but the rise in brainstem ACh was only slight as compared to the appropriate decapitated groups ($p > .05$). The ACh concentrations in brainstem and cortex of animals anesthetized briefly prior to sacrifice (group 3, Table I) were not significantly higher ($p > .05$) than the corresponding values in the rats decapitated without prior anesthesia.

Discussion. The present study demonstrates that thiamine-deficient rats have a normal cerebral ACh concentration. These observations are especially valid since the ACh assay employed is both specific and accurate (14), strict pair-feeding of control animals was carried out to rule out any effect of decreased food intake *per se* on brain ACh, and those areas of the brain were analyzed (brainstem and cerebellum) which are known to exhibit the most severe biochemical derangement in thiamine deficiency (5). Furthermore, since the thiamine-deficient rats showed overt neurologic signs while their brain ACh concentration was normal, and similar cerebral ACh values were found in thiamine-depleted rats studied after reversal

of the encephalopathy with thiamine, it is evident that a depletion of total ACh cannot account for the neurologic dysfunction of these animals.

Consideration of available information about the biochemical derangements present in brain in thiamine deficiency supports this demonstration of normal brain ACh. As mentioned earlier, the main theoretical arguments in favor of possible depletion of brain ACh in thiamine deficiency was the possibility of decreased formation of cerebral ATP and Acetyl CoA (used in the synthesis of ACh) due to diminished activity of pyruvate decarboxylase, a thiamine-dependent reaction (5, 7). Recent studies in our laboratory indicate that the maximal depression of pyruvate decarboxylase activity observed in symptomatic thiamine-deficient rats is in the brainstem and cerebellum but is only of the order of about 30% (5). This small decrease in pyruvate decarboxylase activity is apparently not sufficient to depress the cerebral synthesis of ATP via the Krebs' cycle, and, indeed the ATP concentration is normal in both of those areas (5). Normal brain ATP was also observed in mice showing neurologic signs after the administration of the thiamine antagonist, pyrithiamine (17). In renal medulla, which like the brain subsists primarily on glucose as substrate, a 60% fall in pyruvate decarboxylase activity after thiamine depletion results in only a 20% decrease in ATP concentration (18). There are no data on the regional cerebral Acetyl CoA concentration in thiamine deficiency but it has been suggested that the rate of ACh synthesis in the brain is only about 6% of the rate of Acetyl CoA formation (19). This suggests that, unless there occurs a significant compartmentation of the Acetyl CoA precursor pool for ACh, a major depression of cerebral Acetyl CoA would be needed to depress the synthesis of ACh. The small decrease in activity of pyruvate decarboxylase and the normal brain ATP concentration in severe thiamine deficiency suggest that cerebral Acetyl CoA formation is not likely to be sufficiently decreased to impair the generation of ACh. These arguments therefore support our direct findings of normal ACh in thiamine-deficient brain.

Further studies will be needed to determine the effect of thiamine deficiency on cerebral ACh turnover. Although our data refer only to net ACh levels in the brain, the observation that these values in thiamine-deficient rats with neurologic signs are almost identical to data in pair-fed controls implies that any decrease in ACh synthesis in the symptomatic animals would require an equal decrease in cerebral ACh utilization. Since there is at present no evidence for diminished brain ACh utilization in thiamine deficiency, a significant alteration in ACh turnover in these animals seems unlikely. In addition, it has now been convincingly established that ACh is concentrated in synaptosomes and that different fractions of ACh can be found in cerebral subcellular pools (11, 20). Subsequent investigations are required to ascertain that the present findings with total ACh reflect the ACh levels at the nerve endings and the behavior of the individual ACh fractions.

Summary. This study assesses the hypothesis that severe thiamine deficiency may lead to a depletion of cerebral ACh, which, in turn, may be responsible for the cerebral dysfunction seen in this condition. Acetylcholine assays were carried out in rats with diet-induced thiamine deficiency resulting in overt neurologic signs and in asymptomatic pair-fed and normally fed controls. The brainstem and cerebellum were assayed, in addition to the cortex, since the former sites exhibit the most significant biochemical alterations in thiamine deficiency. Acetylcholine was assayed by a modification of a specific and accurate fluorometric procedure. For every brain area studied the cerebral ACh concentrations in thiamine-deficient rats exhibiting overt neurologic signs were comparable to values seen in asymptomatic pair-fed controls. This study indicates that thiamine deficiency does not cause an alteration in cerebral regional ACh stores and that alternate mechanisms for the neurologic dysfunction will have to be sought.

The authors thank Mrs. Martha Cook for technical assistance and Mrs. Margaret Gignac for help with the manuscript.

1. Victor, M., and Adams, R. D., Amer. J. Clin. Nutr. **9**, 379 (1961).

2. Church, C. F., Amer. J. Physiol. **111**, 660 (1935).

3. Dreyfus, P. M., J. Neurochem. **8**, 139 (1961).

4. Phillips, G. B., Victor, M., Adams, R. D., and Davidson, C. S., J. Clin. Invest. **31**, 859 (1952).

5. McCandless, D. W., and Schenker, S., J. Clin. Invest. **47**, 2268 (1968).

6. Wolstenholme, G. E. W., and O'Connor, M., eds, Ciba Found. Study Group Pap. No. 28. (1967).

7. Cheney, D. L., Gubler, C. J., and Jaussi, A. W., J. Neurochem. **16**, 1283 (1969).

8. Mann, P. J. G., and Quastel, J. H., Nature (London) **145**, 856 (1940).

9. Bhagat, B., and Lockett, M. F., J. Pharm. Pharmacol. **14**, 37 (1962).

10. Lissak, K., Kovaks, T., and Nagy, E. K., Pflugers Arch. Gesamte Physiol. **247**, 124 (1943).

11. Beani, L., Biachi, C., Megazzini, P., Ballotti, L., and Bernardi, G., Biochem. Pharmacol. **18**, 1315 (1969).

12. Comis, S. D., and Davies, W. E., J. Neurochem. **16**, 425 (1969).

13. Hosein, E. A., Chabrol, J. G., and Freedman, G., Rev. Can. Biol. **25**, 129 (1966).

14. Fellman, J. H., J. Neurochem. **16**, 135 (1969).

15. Siegel, S., "Nonparametric Statistics for the Behavioral Sciences." McGraw-Hill, New York (1956).

16. Crossland, J., and Merrick, A. J., J. Physiol. **125**, 56 (1954).

17. Holowach, J., Kauffman, F., Ikossi, M. G., Thomas, C., and McDougal, D. B., Jr., J. Neurochem. **15**, 621 (1968).

18. Schenker, S., Qualls, R. M., Butcher, R., and McCandless, D. W., J. Nutr. **99**, 168 (1969).

19. Schuberth, J., Sollenberg, J., Sundwall, A., and Sorbo, B., J. Neurochem. **13**, 819 (1966).

20. Chakrin, L. W., and Whittaker, V. P., Biochem. J. **113**, 97 (1969).

Received Feb. 26, 1970. P.S.E.B.M., 1970, Vol. 134.