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Both Ethanol Toxicity and Thiamine Deficiency Are Necessary to Produce Long-Term Memory Deficits in the Young Chick

SIMON F. CROWE[1] AND STEPHANIE KEMPTON

School of Psychology, La Trobe University, Bundoora 3083, Australia

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CROWE, S. F. AND S. KEMPTON. *Both ethanol toxicity and thiamine deficiency are necessary to produce long-term memory deficits in the young chick.* PHARMACOL BIOCHEM BEHAV **58**(2) 461–470, 1997.—This series of studies revealed that a 2.5-mg dose of oxythiamine administered over 2 days yielded memory deficits from 10 min following passive avoidance learning. This result existed in association with slowing of righting reflex. Administration of thiamine reversed the memory deficit and the slowing of the righting reflex. A combination of oxythiamine and peripheral alcohol administration also resulted in memory deficits appearing from 10 min after training; however, in contrast to the effect of thiamine deficiency alone, the deficits induced by thiamine deficiency in association with acute administration of alcohol could not be reversed by thiamine resupplementation. The combination of alcohol and oxythiamine also slowed the righting reflex, but higher doses of thiamine also were unable to ameliorate the memory deficit caused by a combination of oxythiamine and alcohol; however, the slowing of righting reflex was reversed. The results suggest that preexistent thiamine deficiency may make the subject susceptible to the neurotoxicological effects of alcohol on memory function. © 1997 Elsevier Science Inc.

Thiamine Memory Wernicke-Korsakoff syndrome Oxythiamine Pyrithiamine Ethanol Day-old chick

THIAMINE deficiency has been implicated as the principal factor responsible for the classical triad of confusion, opthalmoplegia and ataxia characteristic of Wernicke's encephalopathy (28), and acute administration of thiamine appears to reverse most of these symptoms (21). However, the clinical application of thiamine in the treatment of alcoholism has not translated into dramatic decreases in the incidence of Korsakoff syndrome, as might have been expected, were these patients thiamine deficient alone.

Extensive studies carried out by Mair et al. (19) have defined a model of the Wernicke-Korsakoff syndrome (WKS) in the rat. Pyrithiamine (PT)-treated animals display the acute symptoms of aphagia, adipsia, ataxia, depressed levels of consciousness, alteration of posture, convulsions, opisthotonos and failure of righting reflex (19). Following a large intraperitoneal dose of thiamine, the animals generally recover, with the acute symptoms reversed within 1–2 h (19).

Rats that have recovered from PT-induced thiamine deficiency (PTD) exhibit lesions typical of WKS. Histological analysis has shown that PTD treatment produces a neuropathology characterised by bilaterally symmetrical lesions that are necrotic and haemorrhagic in nature. The lesions were typically in the thalamus, which encompasses the region of the internal medullary lamina (IML), and in the mamillary bodies, which were centred in the medial mamillary nuclei (20).

Homewood et al. (13) studied the effects of chronic alcohol consumption and thiamine deficiency on radial arm maze performance in the rat. Although the study differed in some ways from those conducted by Mair et al., the results showed no difference between alcohol-treated animals and the two control groups on delayed recall of the radial arm maze. The overall conclusion of this study was that long-term use of alcohol in the presence of good nutrition did not result in major memory impairment in rats.

This finding contrasts with the work carried out by Arendt et al. (2), who reported both working and reference memory impairment in alcohol-treated rats in the radial arm maze. Rats were subjected to high levels of alcohol intake for 28

[1]To whom requests for reprints should be addressed. E-mail: psysc1@lure.latrobe.edu.au

weeks. The ethanol-treated animals demonstrated a much higher error rate than did the control animals and an impairment of both working and reference memory. The memory deficit in the alcohol-treated animals showed no sign of recovery for 6 months after the end of the alcohol treatment. This result is consistent with the irreversibility of the memory loss seen in alcoholic WKS.

Subsequent testing of the animals indicated that the controls maintained normal performance. Rats in the sham-operation condition remained as impaired as before, and the rats that had received poor cholinergic transplantation did not differ from the sham-operated rats. Rats that had received cholinergic-rich transplants, however, improved performance (i.e., in up to 7 weeks, they were the same as controls), which is consistent with the transplant. Rats that received transplants into both neocortex and hippocampus performed better than rats receiving only one transplant. Thalamic lesions were absent in treated rats, suggesting that thalamic lesions might be due to thiamine deficiency rather than to the effects of alcohol. Histological study showed that the classic WKS lesions were not present because the animals were not malnourished, but significant cell loss was detectable in the cholinergic nuclei of the basal forebrain. Thus, alcohol itself appears to have been markedly detrimental to this region, with profound neurochemical and behavioural consequences.

Thus, there seems to be conflicting data in previous research as to the role of alcohol in the aetiology of WKS. It seems plausible to argue that alcohol interacts with thiamine to produce WKS and is not sufficient of itself to produce the clinical syndrome. The absence of diencephalic lesions in alcohol-treated rats that are not thiamine deficient supports this contention. Such a suggestion has been made by Langlais (14) who proposed that alcohol-induced thiamine deficiency is responsible for the anterograde amnesia via damage to the diencephalon, whereas the direct toxic effects of alcohol lead to global intellectual decline and cortical damage particularly of the frontal lobes.

The experiments in the present study are considered within the context of the model of memory formation proposed by Gibbs and Ng (8,22). This model relies on a single trial passive avoidance task (PAT) in young chicks. In this task, chicks are trained to avoid pecking at a coloured glass bead coated with an aversive substance, methyl anthranilate (MeA), and are tested for retention of the association between the nasty taste and the colour of the bead at different times after the training trial. The Gibbs and Ng model of memory formation based on this learning task suggests three sequentially dependent stages of memory, each with defined temporal parameters. The first of these is short-term memory (STM) and lasts for 5–10 min posttraining. The postulated mechanism for this stage is neuronal hyperpolarisation arising from increased potassium conductance across the neuronal membrane following tetanic stimulation. The second stage, intermediate-term memory (ITM), lasts for 20–50 min posttraining and is postulated to be dependent upon neuronal hyperpolarisation induced by Na^+/K^+ ATPase activity. The final stage, long-term memory (LTM), is effective 60 min posttraining and is thought to be protein-synthesis dependent (8,22). The three stages are separated behaviourally by transient retention deficits occurring at 15 min and 55 min after training (22) and are sequentially dependent upon each other in that disruption of an earlier stage leads to disruption of subsequent stages.

We have been evaluating the effect of thiamine deficiency on memory function in the chick. The results suggest that in young chicks trained on a PAT, a dose of 2.5 mg of PT was sufficient to produce anterograde amnesia for the task (18). Disruption of memory occurred by 25 min following learning, and the PT-treated chicks displayed neurological abnormalities consisting of ataxia, altered level of consciousness and slowed righting reflex. Oxythiamine (OT), a competitive inhibitor of thiamine, also has proved to produce a similar effect.

A number of previous studies in this area have used PT rather than OT as the antimetabolite of thiamine (15–17). PT and OT differ in the mode of action. PT acts by inhibiting thiamine pyrophosphokinase, the enzyme that phosphorylates thiamine to the metabolically active form, thiamine pyrophosphate; OT acts as a competitive inhibitor of thiamine pyrophosphate after phosphorylation has been accomplished. The accumulation of PT in the central nervous system (CNS), with its consequent inhibition of thiamine activity, has been presumed to underlie the rapid development of the clinical and pathologic signs of the avitaminosis. A similar effect has not been reported to emerge with OT (25).

Mice treated with OT demonstrate a marked detrimental effect on weight and appetite but do not develop the typical symptoms characteristic of dietary vitamin deficiency (25). A similar observation has been made for the rat (29).

This apparent difference in effect has been attributed to the inability of OT to enter the brain in significant amounts (24). Harata and Iwasaka (11) suggested that the breakdown of the blood–brain barrier may not be a phenomenon secondary to the tissue destruction associated with the treatment with PT but may be directly involved in the pathogenesis of thiamine-deficiency encephalopathy itself. The day-old chick represents an excellent preparation for the study of the effects of antimetabolites of thiamine at this early stage of development because the blood–brain barrier in these subjects is in only a rudimentary form, thus affording a more direct insight into the effect of the antimetabolite on the unprotected CNS (3,26,30).

Deficits associated with OT (24,29) in rats and mice seem to be largely delimited by the blood–brain barrier (11) to the peripheral nervous system (20). Thus, in animals with well-developed blood–brain barriers, any effect of OT on cognitive functions that depend on CNS processes cannot be determined through peripheral administrations of the vitamins. Because young chicks possess only a rudimentary blood–brain barrier (26,30), any effect of OT would be expected to affect neurones of the central and peripheral nervous systems, allowing determination of any CNS dysfunction. Initial pilot testing in our laboratory comparing OT, PT and saline revealed that both antimetabolites produced similar effects on memory, motor coordination, level of consciousness and righting reflex at 180 min after initial learning, a finding consistent with the view that both agents act through a similar mechanism.

The experimental studies to be reported in the present paper investigated the relative roles of thiamine deficiency and alcohol intake on memory formation in the young chick. The specific aim of this study was to evaluate whether thiamine deficiency alone or thiamine deficiency coupled with the administration of alcohol impaired memory processing in an ongoing way, to ascertain which stage of the processing of memory was disrupted by either or both agents and to determine whether the impairment of movement caused by the thiamine deficiency acted as a confounding effect.

METHODS

Animals

Day-old White-Leghorn Black-Australorp cockerels were obtained from a local hatchery on the morning of each experi-

ment. Chicks were randomly placed in pairs into open-topped wooden boxes (20 × 25 × 20 cm); one chick from each pair was marked for identification. Each box was illuminated by a single white incandescent light bulb (25 W), which in combination with the ducted heating, maintained the temperature of the boxes at 25–29°C.

Each chick had ad libitum access to water at all times except during the pretraining, training and testing periods on the final day of the experiment. Food was not provided during any experiment because it would have produced a confounding variable. The neonate chick is provided with sufficient nutrients from the yolk sac to endure the first 7 days of life (10,23). Consistent with the findings of a pilot study, there were no group differences between those chicks deprived of food and the pair fed controls.

Drugs

All drugs used in this series of experiments were administered freehand by subcutaneous injection into a ventral skin fold just below the rib cage. One-millilitre syringes (Becton Dickinson Tuberculin) fitted with a 27.5-gauge needle were used for injection of test compounds and vehicle alike. OT (Sigma) and thiamine hydrochloride (TH; Sigma) were each dissolved in 154 mM (0.9%) isotonic saline (NaCl) to the required concentration and were injected in a 100-μl volume per chick. Saline was administered as a control in each experiment. In experiments in which ethanol (CSR Distillers) was used, it was diluted to 40% in double-distilled water and was injected subcutaneously in a volume of 300 μl per chick.

Procedure

The procedure used in this study was the same for each experiment. Specific modifications made to the general procedure are presented in the descriptions of each individual study.

The learning task employed in the experiments was an adaptation of Cherkin's original PAT (6) and modified by Gibbs and Ng (8), so that memory was indexed by colour discrimination of the pecking response. Essentially, on day 3 of each experiment, all chicks were given four pretraining trials, one training trial and one retention trial. In all trials (pretraining, training and retention), the beads were presented to the chicks for a 10-s interval.

In the first two pretraining trials, a chrome bead coated with water was presented to the chick with a view to reinforcing a pecking response. The next two pretraining trials involved a determination of the baseline pecking rates of each chick to a red and a blue bead. After an interval of at least 30 min, a bead similar in type to the red bead used during the latter pretraining trial was covered with the chemical aversant MeA. At different training tests intervals (TTI) following the training trial, the chicks were presented with red and blue beads identical to those used during the pretraining trials, and their differential level of pecking to the respective beads was used as the dependent variable. The index used to measure retention was the discrimination ratio (DR). The DR is defined as the number (n) of pecks on the blue bead (B) divided by the total number of pecks on the red bead (R) and blue bead:

$$\mathrm{DR} = \frac{n(B)}{n(B) + n(R)}$$

A DR of 1.0 represents perfect discriminated memory and a DR of 0.5 represents no discriminated memory of the aversive and nonaversive beads. The discrimination ratio provides an interval scale measure of retention level and allows for interval statistical methods to be applied. Chicks failing to peck at the blue bead on this trial were also excluded from later data analysis because failure to discriminate between aversive and nonaversive beads reflects a generalised response and is therefore not a true index of discrimination memory rendering the DR for chicks avoiding both beads indeterminately (22). No more than 10% of the birds in each subgroup were rejected on these grounds. The procedure for these experiments has been described in full by Gibbs and Ng (8).

Neurological Assessment

Prior to the administration of thiamine on day 3, all chicks were timed (in seconds) for the duration taken to stand upright once being placed on their back (righting reflex). Within 5 min of the completion of the PAT, all chicks were again timed for the duration taken to stand upright after being placed on their back. A ceiling level of 10 s was used in an attempt not to bias the results. Overall results are expressed as the mean time taken to stand (in seconds). This technique has proved useful in investigations of the effect of B vitamins on memory in the chick (7).

RESULTS

Experiment 1: OT Time Course

Thiamine deficiency can produce memory dysfunction and neurological impairment in both humans and animals. Moreover, thiamine deficiency may be the principal aetiological factor underlying WKS (28). PT and OT, antimetabolites of thiamine, have been employed to induce thiamine deficiency and have been shown to yield symptoms of memory loss, ataxia, depressed level of consciousness and failure of righting reflex in the neonate chick (31).

Previous work in our laboratory has indicated that the optimal dose in producing the effects of acute thiamine deficiency is 2.5 mg OT per chick. The aim of this experiment was to determine at which stage of memory in the Gibbs and Ng (8) three-stage model of memory this effect of OT becomes apparent. In addition, it was aimed to determine the effect of OT on righting reflex.

The 360 chicks were divided into two equal groups: half received a total dose of 2.5 mg OT over 2 days and the other half received injections of 154.0 mM NaCl on days 1 and 2. All injections of OT and NaCl were delivered in a 100 μl volume. On the third day, all chicks were measured for righting reflex. They were then trained on the one-trial PAT. The timing of the retention trials were chosen to correspond with the different stages of memory as described by Gibbs and Ng (8). The times were as follows: 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 70 min, 180 min and 24 h. A total of 40 chicks were tested at each time point; one group of 20 chicks was pretreated with OT and the other with NaCl. Following the retention test, all chicks were measured for righting reflex.

The retention function for OT and saline treatment is given in Fig. 1A. A two-way analysis of variance (ANOVA) yielded a drug [$F(1, 301) = 92.45$, $p < 0.0005$] and a TTI [$F(8, 301) = 2.80$, $p < 0.005$] main effect. A significant drug × TTI interaction effect was also obtained [$F(8, 301) = 3.42$, $p < 0.001$]. Simple main effects analysis revealed significant differences at 20 min [$F(1, 309) = 18.08$, $p < 0.0005$], 30 min [$F(1, 309) =$

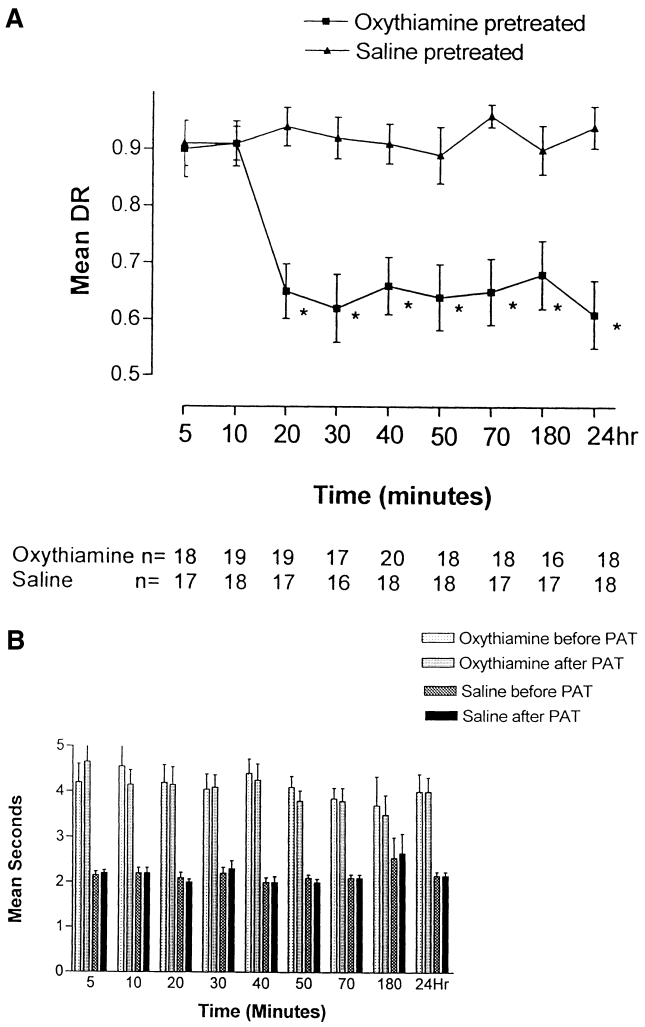

FIG. 1. A: The effect of OT on mean discrimination ratio (DR ± SEM) at different times following learning. Chicks were injected twice with either OT or saline, 24 and 48 h prior to pretraining and tested at the times indicated. B: The effect of OT on time taken to right (mean ± SEM s). Chicks were injected with OT twice, 24 and 48 h prior to pretraining. Chicks were measured for righting reflex twice on day 3: once before pretraining on the PAT and treatment with thiamine (OT/ethanol before thiamine, saline/ethanol before thiamine) and again after treatment with thiamine and completion of the retention task (OT/ethanol after thiamine, saline/ethanol after thiamine).

18.31, $p < 0.0005$], 40 min [$F(1, 309) = 14.20$, $p < 0.0005$], 50 min [$F(1, 309) = 13.59$, $p < 0.0005$], 70 min ($F(1, 309) = 18.62$, $p < 0.0005$], 180 min [$F(1, 309) = 9.13$, $p < 0.003$], and 24 h [$F(1, 309) = 22.74$, $p < 0.0005$]. No significant differences were found at 5 min [$F(1, 309) = 0.01$, $p = 0.912$] or at 10 min [$F(1, 309) = 0.01$, $p = 0.914$] following training. Furthermore, a significant difference was found for TTI within OT [$F(8, 302) = 4.64$, $p < 0.0005$] but not for TTI within saline [$F(8, 302) = 0.15$, $p = 0.996$].

The data for righting reflex can be seen in Fig. 1B. A three-way (drug × TTI × pre-post) ANOVA with repeated measures on the last variable yielded a significant drug main effect [$F(1, 342) = 271.27$, $p < 0.0005$], with no other significant main effects or interaction. These results suggest that OT treatment alone increases the time for the righting reflex at all TTIs measured.

The results of this experiment indicate that OT disrupts memory at a dose of 2.5 mg, with amnesia first appearing after 10 min postlearning and lasting for at least 24 h after the initial training experience. Although the behavioural response in the experiment requires a passive avoidance, with chicks pecking at the previously nonaversive bead, the effects of the OT on the motor functions and level of consciousness cannot be definitively dismissed as the cause of this response. Thus, the results of this experiment may have been undermined to a degree due to the effect of the thiamine deficiency on the motor response and level of consciousness of the chicks.

Experiment 2: Thiamine Dose Response

This experiment aimed to determine the effects of resupplementation of thiamine on thiamine-deficient chicks. Following thiamine administration to thiamine-deficient rats, neurological impairments were reversed within an hour (19). This effect also has been seen in human subjects suffering from WKS (28). In contrast, memory deficits often have been reported to linger.

Having established that OT can induce amnesia 10 min following learning and produce impaired righting reflex in the chick, we wanted to establish whether administration of thiamine restored the righting reflex, memory or both. In this experiment, the aim was to determine the range of thiamine doses that might reverse the deficits accompanied with thiamine deficiency.

The 200 chicks used received either a total dose of 2.5 mg per chick of OT (dissolved in saline) over 2 days in 100 μl volume per dose or two injections of 154.0 mM NaCl alone. Prior to thiamine injection on day 3, all chicks were measured for righting reflex. Subsequently, chicks were divided into experimental groups containing 40 chicks each, 20 of which had been pretreated with OT and 20 with NaCl. There were 5 groups in total, and all chicks were given a 100-μl injection of one of the following treatments: 154.0 mM NaCl, 0.025 mg thiamine, 0.05 mg thiamine, 0.5 mg thiamine or 1.0 mg thiamine. Two hours later, the chicks were trained on the PAT. Three hours afterward, they were all tested for memory retention. Righting reflex was measured after completion of the memory retention test.

The results of experiment 2 are shown in Fig. 2A. A two-way (drug × dose) ANOVA with unweighted means revealed a nonsignificant main effect for drug [$F(1, 140) = 2.16$, $p = 0.144$]. However, a significant dose main effect for thiamine was obtained [$F(4, 140) = 3.34$, $p = 0.012$]. Furthermore, there was a significant interaction effect between drug and thiamine dose [$F(4, 140) = 4.92$, $p < 0.001$]. Simple main effects analysis showed a significant difference between OT and NaCl pretreatment within the zero thiamine dose (NaCl) [$F(1, 144) = 18.39$, $p < 0.0005$]. This result is consistent with the results from experiment 1. No significant differences were found between OT and NaCl pretreatment for any of the doses of thiamine. The effects of thiamine doses differed significantly in OT pretreated groups [$F(4, 141) = 7.01$, $p < 0.0005$] but not in saline pretreated groups [$F(4, 141) = 1.07$, $p = 0.375$].

The neurological data for righting reflex are illustrated in Fig. 2B. Three-way (drug × dose of thiamine × pre-post) ANOVA with repeated measures on the last variable yielded

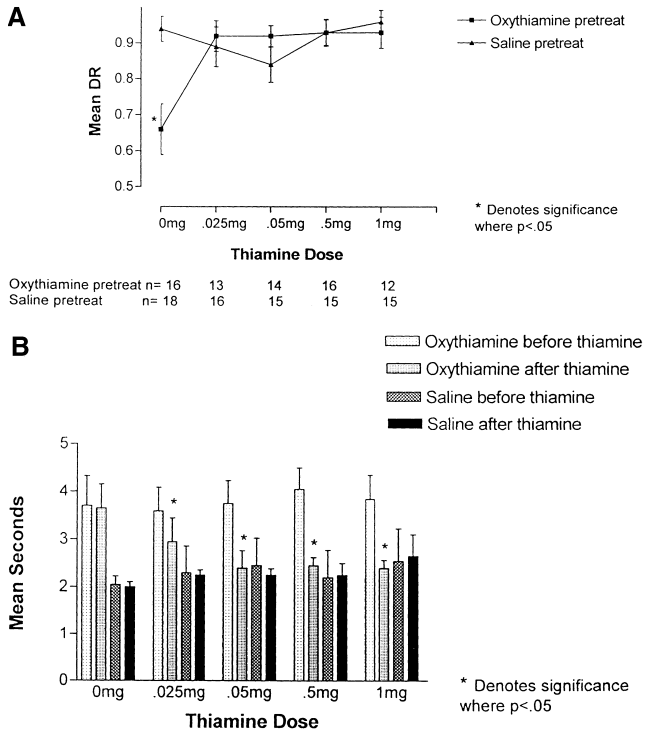

FIG. 2. A: The effect of different doses of thiamine on mean discrimination ratio (DR ± SEM). Chicks were pretreated with OT or NaCl injected 24 and 48 h prior to pretraining. Thiamine was injected once on day 3, 2 h prior to pretraining and tested at 180 min after training. B: The effect of different doses of thiamine on time taken to right (mean ± SEM s). Chicks were pretreated with either OT or saline injected 24 and 48 h prior to pretraining. Chicks were measured for righting reflex twice on day 3: once before pretraining on the PAT and treatment with thiamine (OT/ethanol before thiamine, saline/ethanol before thiamine) and again after treatment with thiamine and completion of the retention task (OT/ethanol after thiamine, saline/ethanol after thiamine). The * indicate significant differences between the pretest and the posttests.

significant drug [$F(1, 190) = 30.80$, $p < 0.001$] and pre-post [$F(1, 190) = 35.76$, $p < 0.001$] main effects and a significant drug × pre-post [$F(1, 190) = 31.83$, $p < 0.001$], dose of thiamine × pre-post [$F(4, 190) = 2.42$, $p < 0.05$] and drug × dose of thiamine × pre-post [$F(4, 190) = 2.88$, $p < 0.025$] interaction effects.

The results of experiment 2 indicate that even small doses of thiamine may overcome the memory deficit induced by OT. Thiamine itself had no effect on memory retention. Thiamine administration also appears to reverse the slowing of righting reflex. The analysis of the neurological data suggests that OT alone yields a significant deterioration in righting reflex, and this effect of OT is counteracted by thiamine at all doses used. Thiamine alone has no effect on righting reflex.

This behavioural observation confirms the observation by Mair et al. (19) that thiamine reverses many of the neurological symptoms, including impairment of righting reflex induced by thiamine deficiency.

Experiment 3: Alcohol Dose Response

Following the reversal of memory and righting reflex in OT pretreated animals with administration of thiamine, the aim of this experiment was to explore the interaction effect between alcohol and thiamine deficiency. In the human WKS, alleviation of the neurological symptoms occurs quite rapidly following administration of thiamine; however, memory impairment has been reported to persist (28). This finding promotes the speculation that thiamine deficiency in combination with other factors such as alcohol can cause the more severe impairments evident in Korsakoff's psychosis (12). Previous experiments by Taaffe (27) have employed 1–2 g/kg ethanol to neonate chicks to induce retention deficits. Although these deficits were transient, a slightly higher dose was adopted (although given in a larger volume) in the present study, with the total amount of ethanol determined by the number of doses administered.

The 320 chicks used were divided into groups of 40 for each of conditions 1–3 with 2.72 g/kg doses of alcohol and groups of 20 for the control conditions. Groups of 40 chicks were used to guard against possible subject losses on the test and to maximise the power of the analysis. Another group of 20 chicks, which received 4 doses of alcohol, was included in this study to ensure that a ceiling effect of alcohol administration was obtained. All injections of ethanol and corresponding NaCl control injections were administered in 300-μl volumes, and OT and corresponding NaCl and TH were administered in 100-μl volumes. Overall, for days 1 and 2, 5 groups of chicks were pretreated with OT and 5 groups with NaCl. One group in each treatment condition received either saline or 1, 2, 3 or 4 2.72-g/kg doses of alcohol. An interval of 2.5 h was allowed between alcohol doses, and no chick received more than 2 doses of alcohol in any one day. Preliminary experiments indicated that these time intervals between injections provided minimal disruption to the apparent physiological function of the animals. At least 24 h elapsed between the administration of the last alcohol dose and the collection of the behavioural or memory observations.

On day 3, all chicks were measured for righting reflex prior to any other treatment. Subsequently, they were then given an injection of thiamine 2 h prior to pretraining on the PAT. Memory retention was tested 3 h after the training procedure. All chicks were then measured for righting reflex.

The results of experiment 3 are presented in Fig. 3. A two-way ANOVA (drug × alcohol dose) with unweighted means indicated a significant main effect for drug [$F(1, 232) = 31.93$, $p < 0.0005$], a significant main effect for dose of alcohol [$F(4, 232) = 2.61$, $p = 0.036$] and a significant interaction effect between drug and dose of alcohol [$F(4, 232) = 4.22$, $p = 0.003$].

Simple main effects analysis displayed a significant difference for drug within alcohol dose 2 [$F(1, 236) = 20.57$, $p < 0.0005$], alcohol dose 3 [$F(1, 236) = 22.91$, $p < 0.0005$] and alcohol dose 4 [$F(1, 236) = 8.13$, $p < 0.005$]. Nonsignificant differences were found between drug within the zero alcohol dose groups (NaCl) [$F(1, 236) = 0.08$, $p = 0.784$] and within alcohol dose 1 [$F(1, 236) = 1.92$, $p = 0.167$]. A significant main effect was seen amongst doses of alcohol within OT pretreatment [$F(4, 233) = 6.15$, $p < 0.0005$] but not within saline pretreatment [$F(4, 233) = 1.02$, $p = 0.399$].

The neurological data for righting reflex can be seen in Fig. 3B. A three-way (drug × dose of alcohol by pre-post) ANOVA with repeated measures on the last variable indicated significant drug [$F(1, 190) = 41.51$, $p < 0.001$] and pre-post [$F(1, 190) = 82.73$, $p < 0.001$] main effects and a significant drug × pre-post interaction effect [$F(1, 190) = 65.86$, $p < 0.001$]. No other effects were significant.

Simple main effects analysis of the drug × pre-post interaction showed that OT-treated chicks differed significantly in

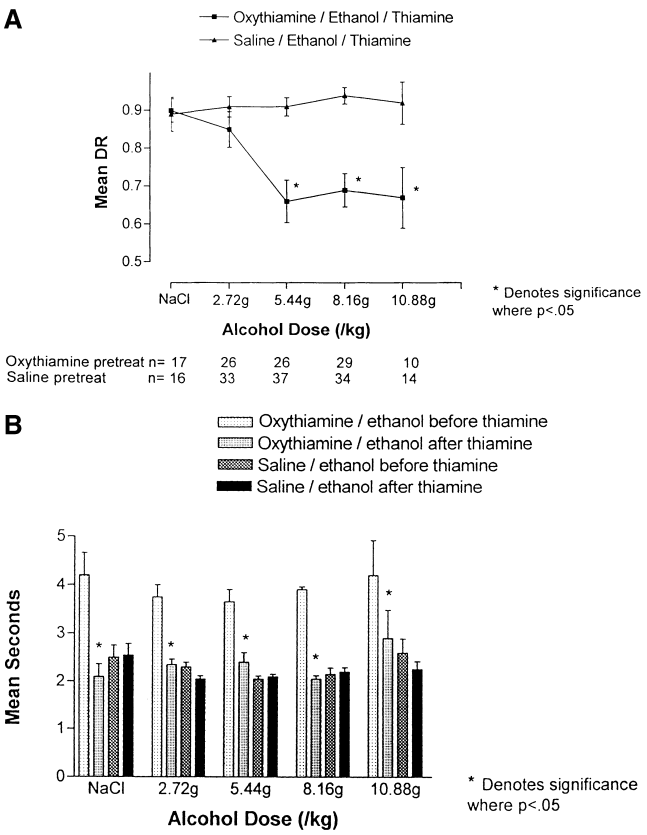

FIG. 3. A: The effect of different total doses of ethanol on mean discrimination ratio (DR ± SEM). Chicks were pretreated with either OT or NaCl and given 1, 2, 3 or 4 2.72-g/kg doses of ethanol over 2 days. Thiamine was administered 2 h prior to pretraining on the third day. Retention was measured at 180 min. B: The effect of different doses of ethanol on time taken to right (mean ± SEM s). Chicks were pretreated with either OT or NaCl and given 0, 1, 2, 3 or 4 2.72-g/kg doses of ethanol over 2 days. Chicks were measured for righting reflex twice on day 3: once before pretraining on the PAT and treatment with thiamine (OT/ethanol before thiamine, saline/ethanol before thiamine) and again after treatment with thiamine and completion of the retention task (OT/ethanol after thiamine, saline/ethanol after thiamine). The * indicate significant differences between the pretest and the posttests.

righting reflex from saline-pretreated chicks in the pretest [$F(1, 380) = 97.67$, $p < 0.001$] but not in the posttest [$F(1, 380) = 0.63$, $p = 0.4$]. Conversely, pretest righting reflex scores were higher than posttest righting reflex scores in OT-treated groups [$F(1, 190) = 148.11$, $p < 0.001$] but not in saline-treated groups [$F(1, 190) = 0.45$, $p = 0.4$].

The results of experiment 3 indicate that alcohol in the presence of thiamine does not impair memory formation at any dose used because groups pretreated with saline did not show retention deficits with any dose of alcohol. However, OT combined with administration of alcohol impaired memory when tested 180 min postlearning. Furthermore, it appeared to do so in a dose-dependent manner. Thus, a single injection of 2.72 g/kg alcohol did not significantly impair memory, whereas 2, 3 and 4 injections did. One milligram of thiamine administered to the animals failed to reverse the memory deficit observed with the higher doses of ethanol. The fact that the groups treated with saline or a single dose of ethanol yielded the same retention levels as the saline pretreated groups confirms the previous findings that thiamine overcomes OT-induced retention loss. Thus, ethanol in association with thiamine deficiency apparently results in a memory deficit that is unaffected by subsequent thiamine administration. This result occurs after the ataxia associated with thiamine deficiency has been reversed, indicating that the effect is not attributable to a motor problem.

The analysis of righting reflex revealed ethanol did not have any effect at any dose. Thus, the loss of righting reflex may be attributed entirely to OT treatment and, hence, to thiamine deficiency, an effect that is overcome by thiamine. Alcohol treatment did not lead to a deterioration in righting reflex, independent of whether or not the chicks were thiamine deficient. Taken together, these results indicate that impairment of memory, independent of motor deficit, can be induced in the chicks by the application of ethanol in association with OT-induced thiamine deficiency.

Experiment 4: Alcohol Time Course

Having established that OT pretreated chicks in association with an alcohol intake displayed irreversible memory deficits while displaying reversible neurologic symptoms such as the righting reflex, it was necessary to establish at which stage of memory the combined effects of thiamine deficiency and alcohol administration on memory becomes apparent.

Based on the previous experiment, the 2 2.72-g/kg doses of alcohol dose were adopted for future experiments. Overall, 360 chicks were used. Injections of alcohol were administered in 300-μl volumes, and OT, NaCl and TH were administered in 100-μl volumes. On day 3, all chicks were measured for righting reflex prior to any further treatment. All chicks were then administered thiamine, and another 2 h elapsed before commencing pretraining on the PAT. Retention of memory was measured at one of the following TTIs: 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 70 min, 180 min and 24 h posttraining. A total of 40 chicks were measured at each time point, 20 of which had been pretreated with OT and alcohol and 20 with NaCl and alcohol. Following retention testing, all chicks were scored for righting reflex. The results of experiment 4 are presented in Fig. 4.

The effects of OT and alcohol or the saline vehicle and alcohol in the presence of pretraining thiamine supplementation on memory retention tested at various times is shown in Fig. 4A. A two-way (drug × TTI) ANOVA revealed a significant drug main effect [$F(1, 297) = 111.16$, $p < 0.0005$], a significant TTI main effect [$F(8, 297) = 4.84$, $p < 0.0005$] and a significant drug × TTI interaction effect [$F(8, 297) = 5.76$, $p < 0.0005$]. Simple main effects analysis revealed significant differences between drug groups at 20 min [$F(1, 305) = 19.08$, $p < 0.0005$], 30 min [$F(1, 305) = 18.86$, $p < 0.0005$], 40 min [$F(1, 305) = 22.35$, $p < 0.0005$], 50 min [$F(1, 305) = 16.73$, $p < 0.0005$], 70 min [$F(1, 305) = 17.18$, $p < 0.0005$], 180 min [$F(1, 305) = 16.73$, $p < 0.0005$] and 24 h [$F(1, 305) = 28.45$, $p < 0.0005$] posttraining. No significant differences were found at 5 min [$F(1, 305) = 1.11$, $p = 0.29$] and 10 min [$F(1, 305) = .17$, $p = 0.68$] posttraining. Furthermore, significant effects of TTI was found within the OT/alcohol/thiamine groups [$F(8, 298) = 7.34$, $p < 0.0005$] but not for time within the saline/alcohol/thiamine groups [$F(8, 298) = .23$, $p = 0.99$].

The data for righting reflex are illustrated in Fig. 4B. A three-way (drug × dose of thiamine × pre-post) ANOVA with repeated measures on the last variable showed significant drug [$F(1, 342) = 63.35$, $p < 0.0005$] and pre-post [$F(1, 342) =$

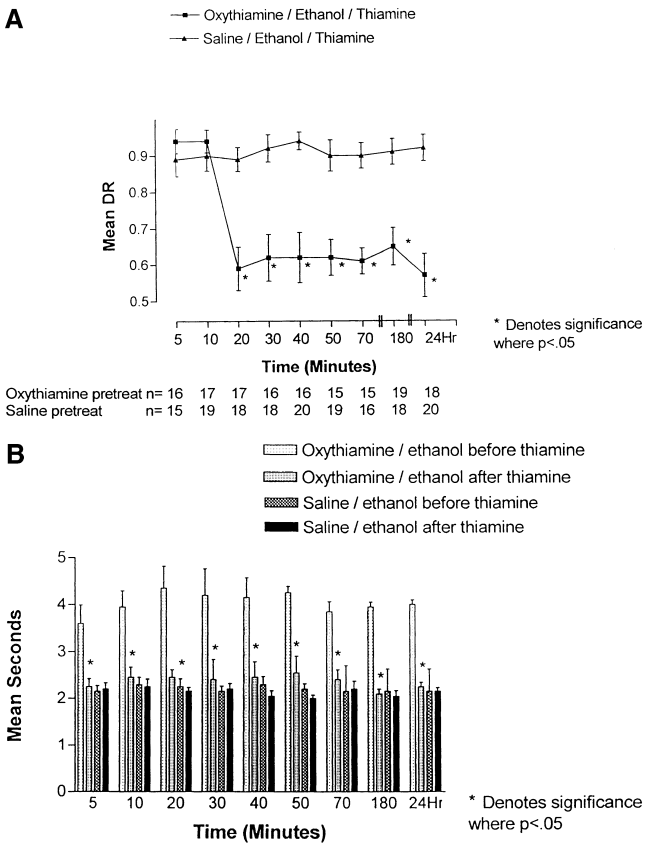

FIG. 4. A: The effect of ethanol on mean discrimination ratio (DR ± SEM) at different times following learning. Chicks were injected 24 and 48 h prior to pretraining with either OT or NaCl and given 2 2.72-g/kg doses of ethanol. Prior to pretraining on day 3, all chicks were given an injection of thiamine. B: Time taken to right (mean ± SEM s). Chicks had received either OT or NaCl and 2 doses of ethanol (2.72 g/kg/dose) over 2 days. Chicks were measured twice, once before administration of thiamine on day 3 and again after administration of thiamine and the retention task.

64.10, $p < 0.0005$] main effects and a significant drug × pre-post interaction effect [$F(1, 342) = 55.33$, $p < 0.0005$]. No other main or interaction effects were significant.

It would appear from these results that the deleterious effects of OT and alcohol on righting reflex did not depend on time of measurement after training. Simple main effects analysis of the drug × pre-post interaction shows that the OT/alcohol-treated groups differed significantly overall from the saline/alcohol-treated groups in prethiamine measures of righting reflex [$F(1, 684) = 118.68$, $p < 0.001$] but not in postthiamine measures [$F(1, 684) = 0.55$, $p = 0.45$]. Pretest measures were significantly different from posttest measures for the OT/alcohol-treated groups [$F(1, 342) = 119.27$, $p < 0.001$] but not for the saline/alcohol-treated groups [$F(1, 342) = 0.16$, $p = 0.65$]. The absence of a significant third-order interaction suggests that the differences are independent of the TTI at which the measures were taken. These findings confirm those of experiment 2. OT-induced thiamine deficiency results in impairment of the righting reflex, an impairment that is overcome by thiamine replacement.

The results of experiment 4 indicate that in the combination of thiamine deficiency and alcohol administration, STM remains intact. However, memory impairment is apparent at 20 min after training, suggesting that ITM formation and subsequent consolidation into LTM formation are affected. Furthermore, memory is still impaired 24 h after learning, suggesting that memory may not have been consolidated. However, a 1-mg dose of thiamine may have been insufficient to reverse the memory deficit despite being adequate to reverse the slowed righting reflex in this experiment. To examine this possibility, the effect of increasing doses of thiamine in the presence of OT and alcohol were examined.

Experiment 5: Thiamine Dose Response

Having shown that memory is impaired in thiamine deficient/alcohol-treated chicks after 10 min postlearning and that this memory deficit could not be reversed by 1.0 mg thiamine supplementation, the possibility that higher doses of thiamine may have a counteracting effect was tested by using a dose range of 1.4 mg per chick to 3.0 mg per chick of thiamine. The maximum thiamine dose of 3.0 mg per chick was chosen because it has been established in our laboratory that doses exceeding this amount are toxic to chicks, resulting in cardiac failure (18).

The 200 chicks used were divided into two groups: one group received a total dose of 2.5 mg OT over 2 days and the other received NaCl. All chicks were given 2 doses of ethanol on day 2. The injection volumes of alcohol, NaCl, OT and thiamine were the same as those used previously. On day 3, all chicks were measured for righting reflex prior to any further treatment. Subsequently, chicks were divided into experimental groups containing 40 chicks, 20 of which had been pretreated with OT and 20 with NaCl; all chicks had been administered 2 doses of alcohol. There were 5 groups in total, and all were given a 100-μl injection of one of the following treatments: NaCl, 1.4 mg thiamine, 1.8 mg thiamine, 2.4 mg thiamine or 3 mg thiamine. Two hours later, the chicks were trained on the PAT. Retention was measured 3 h after the training trial. Righting reflex was measured subsequent to completion of the previous task.

Results of experiment 5 can be seen in Fig. 5. A two-way ANOVA (pretreatment × dose of thiamine) revealed a significant pretreatment main effect [$F(1, 165) = 159.43$, $p < 0.0005$]. There was no significant thiamine dose main effect [$F(4, 165) = 0.83$, $p = 0.506$] and no significant pretreatment × thiamine dose interaction [$F(4, 165) = 0.30$, $p = 0.876$].

The results of the righting reflex are illustrated in Fig. 5B. A three-way (drug × dose of thiamine × pre-post) with repeated measures on the last variable yielded a significant drug [$F(1, 190) = 99.90$, $p < 0.0005$], dose of thiamine [$F(4, 190) = 4.73$, $p < 0.001$] and pre-post [$F(1, 190) = 101.45$, $p < 0.0005$] main effects. All second-order interactions were also significant for drug × dose of thiamine [$F(4, 190) = 3.44$, $p < 0.010$], drug × pre-post [$F(1, 190) = 61.98$, $p < 0.0005$] and dose of thiamine × pre-post [$F(4, 190) = 11.04$, $p < 0.0005$] as were third-order (drug × dose of thiamine × pre-post) interactions [$F(4, 190) = 7.83$, $p < 0.0005$]. Because third-order interactions were significant, interpretations are best based on examination of cell means by using simple main effects analysis. These post hoc analyses show that significant changes occurred in the righting reflex from the prethiamine treatment to postthiamine treatment for those groups of animals pretreated with OT and alcohol, independent of the dose used (all ps $<.001$). There was no significant difference between the prethiamine and postthiamine measures of righting reflex in groups given saline instead of thiamine [$F(1, 190) = 214$,

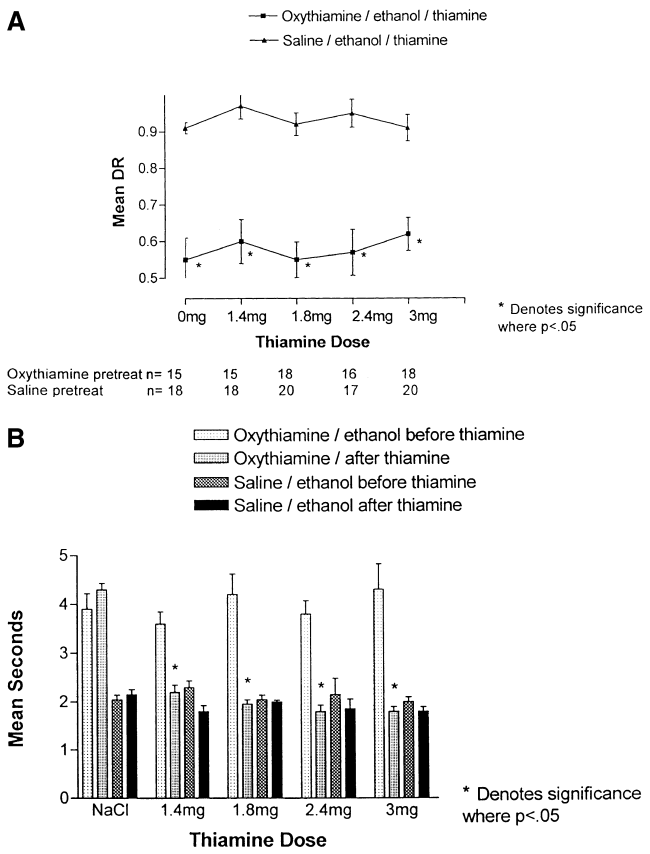

FIG. 5. A: The effect of different doses of thiamine on mean discrimination ratio (DR ± SEM). Chicks were pretreated with OT or NaCl over days 1 and 2. All chicks also were given 2 doses of ethanol (2.72 g/kg/dose) on day 2. Thiamine was administered 2 h prior to pretraining on day 3. B: The effect of different doses of thiamine on time taken to right (mean ± SEM s). Chicks were pretreated with OT or NaCl over days 1 and 2. All chicks also were given 2 doses of ethanol (2.72 g/kg/dose) on day 2. Chicks were measured for righting reflex twice on day 3: once before pretraining on the PAT and treatment with thiamine (OT/ethanol before thiamine, saline/ethanol before thiamine) and again after treatment with thiamine and completion of the retention task (OT/ethanol after thiamine, saline/ethanol after thiamine). Retention was measured at different times after completion of the training task. The asterisk indicates significant differences between the pretest and the posttest.

$p = 0.145$], and no pre-post thiamine differences were significant for animals pretreated with saline and alcohol and given the various doses of thiamine. Simple main effects analysis showed that animals given alcohol and either OT or saline differed significantly in mean righting reflex scores prior to the administration of thiamine (all $ps < 0.001$). Not surprisingly, the only instance of a postthiamine difference in righting reflex occurred for the control group given saline instead of thiamine. These results suggest that thiamine at each of the doses used was effective in overcoming the OT-induced impairment of righting reflex.

From the results of experiment 5, no dose of thiamine in the range tested seemed to overcome the memory deficit induced by the combined treatments of OT and alcohol. This finding is consistent with the results obtained with 1 mg of thiamine (experiment 4) and the inability of ethanol alone to impair memory (experiments 3 and 4).

In the present experiment, ethanol alone had no effect on retention measured 180 min posttraining because the group of chicks given saline pretreatment, ethanol treatment and 0 mg thiamine showed normal retention levels. Thus, assuming OT induces thiamine deficiency, a deleterious effect of ethanol on memory must be promoted under the thiamine-deficiency conditions. Alternatively, both ethanol and thiamine deficiency may adversely affect cell processes associated with the formation of ITM.

DISCUSSION

The results of these five experiments demonstrate that thiamine deficiency induced by OT produces amnesia after 10 min following learning. The interruption to the memory process observed was reversed by the subsequent administration of thiamine. Interestingly, even small doses of thiamine were able to counteract the memory impairment induced by OT. Furthermore, OT when combined with the administration of 2 doses of alcohol led to amnesia, which became apparent after 10 min following learning. Thiamine administration in even larger doses failed to ameliorate the memory deficit. Finally, alcohol treatment alone, in the absence of thiamine, produced normal memory retention.

One possible explanation for the observed differential effect of alcohol and thiamine deficiency may be that they both work by a similar mechanism but that in the present experimental series neither was given sufficient time, dosage or both to produce the effects independently. This explanation seems implausible given that the effects of the two agents could be dissociated from each other at the dosage levels used. Ethanol neurotoxicity of itself did not result in permanent impairment of memory or righting reflex, thiamine deficiency of itself did not result in permanent impairment of memory or righting reflex but a combination of both did. This is not to undermine previous observations in the literature of the ability of both agents of themselves to produce permanent memory impairments but rather to underline the fact that in the clinical realm both insults generally coexist.

The results of these experiments support the previous findings of the effects of thiamine deficiency in rats (13–17) and clearly indicate that a similar phenomenon can be induced in the chick. The possibility of the use of the chick model in this area is useful because it provides a rapid, reliable and robust means of inducing the deficits associated with acute thiamine deficiency in an animal with a relative poorly developed blood–brain barrier, which allows the determination of the effects of these agents on the unprotected CNS. The possibility of precisely determining the time of learning provided by PAT provides a useful index of when such changes are taking place in the memory processing of a time delimited training experience.

It would be premature based on our current state of knowledge to draw any firm conclusions regarding the mechanisms of memory dysfunction in thiamine deficiency in association with alcohol on the basis of the data presented in this paper. Nonetheless, the possibility that ethanol neurotoxicity may be mediated by NMDA receptor activation (1) and that thiamine deficiency has effects on NMDA receptor expression (15–16) and on cell energy production clearly warrant serious consideration.

In this latter respect, studies using the central thiamine antagonist, PT, suggest that the activity of α-ketoglutarate dehydrogenase (αKGDH) in rat brains is decreased (9). Decreases of αKGDH would be expected to result in diminished glucose (and pyruvate) oxidation because αKGDH is rate limiting in

brain (4). Gibson et al. (9) suggested from in vivo studies that even moderate decreases of αKGDH could result in diminished glucose oxidation. Furthermore, decreases in αKGDH in cerebral cortex of symptomatic PTD rats may be associated with a parallel reduction of cerebral cortical glucose oxidative capacity. This decrease in αKGDH in the brainstem of PTD rats is accompanied by increases in alanine (5), which is consistent with decreased entry of pyruvate into the TCA cycle.

These investigators have shown that administration of thiamine to PTD animals results in complete reversal of the neurological symptoms such as nystagmus and loss of righting reflex, a situation similar to that observed in the acute treatment of Wernicke's encephalopathy. Decreases in αKGDH levels as a consequence of thiamine deficiency may produce symptoms such as loss of righting reflex, which promptly reverses following thiamine resupplementation.

In the experiments reported here, alcohol alone did not affect the righting reflex of the chicks, but OT alone and OT in combination with alcohol did. Unlike the amnesic effects, doses of thiamine from 1 mg per chick to 3 mg per chick overcame the effects of these treatments on the righting reflex. These results suggest that disturbances to the righting reflex obtained in the present study are probably due to the thiamine deficiency alone.

The explanation of the memory deficit, however, remains elusive. If indeed both thiamine deficiency and ethanol neurotoxicity are necessary to produce WKS, then the interactive effects of these agents or the separate nature of their actions must be described.

The results of this series of experiments suggests several avenues for further investigation. The effect of thiamine deficiency seems to be the intermediate stage of memory formation proposed by Gibbs and Ng (8). This notion raises the possibility that agents that specifically target Na^+/K^+ ATPase activity may act to ameliorate this deficit. Preliminary investigations in our laboratory indicate that the effects of OT-induced thiamine deficiency can be overcome by the action of diphenylhydantoin, a facilitator of the sodium pump. The further exploration both pharmacologically and in vitro of Na^+/K^+ ATPase activity under conditions of thiamine deficiency seems very promising.

The observations arising out of the present series of experiments indicate that the combined treatment of OT and alcohol leads to a relatively permanent impairment of memory (i.e., at least 24 h) but a reversible impairment of the righting reflex. The effects of thiamine deficiency may be impermanent and may apply to both the righting reflex and to the level of memory function. In the clinical situation, the effects of chronic alcohol administration may only impair memory functions in those individuals who are predisposed to damage in the memory circuitry by prior thiamine deficiency. The orthodoxy (28) that thiamine deficiency leads to WKS may be misleading because the results of the present series of studies indicate that thiamine-deficient subjects become susceptible to the toxic effects of alcohol, thus leading to the permanent memory disorder.

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