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Effect of Folate Deficiency and Folate and B_{12} Excess on Memory Functioning in Young Chicks

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CROWE S. F. AND ROSS, C. K. *Effect of folate deficiency and folate and B_{12} excess on memory functioning in young chicks.* PHARMACOL BIOCHEM BEHAV **56**(2) 189–197, 1997.— The results of this series of experiments with chicks trained on a single trial, passive avoidance task, demonstrate that methotrexate-induced folate deficiency, and excess levels of folate and B_{12} lead to amnesia in these subjects. The amnesia appears only after 50 min following learning, leaving the earlier processing stages of memory formation unaffected. The application of methotrexate resulted in disruption of righting reflex in a dose dependent manner, however the ataxia did not appear to be the cause of the memory deficit. The deficit in memory induced by methotrexate-induced folate deficiency could be ameliorated with methionine. These studies suggest that cellular processes involving folate metabolism may play an important role in the memory formation of the young chick and that the observed disruption of memory may well occur due to its affect on protein synthesis mediated by alterations in methionine metabolism.

Methotrexate Folate-deficiency Memory Protein synthesis Methionine Chickens Vitamins Passive-avoidance learning Folate B_{12}

PREVIOUS research has demonstrated an important role for the B group vitamins in neuropsychiatric disturbance [see (4, 8) for reviews]. While much of this research has concentrated on thiamine, a growing body of literature implicates other B group vitamins, particularly folate and B_{12}, in neuropsychiatric disturbance (8).

Folate plays a fundamental role in a number of metabolic processes. These include: (i) the hydroxylation reactions necessary for neurotransmitter synthesis [e.g. serotonin and the catecholamines (1,5)]; (ii) deoxyribonucleic acid (DNA) synthesis (17), and (iii) the methylation cycle (36,41). Low levels of folate disrupt the balance between these pathways and, most importantly, deficiency of folate lowers the level of S-adenosylmethionine (SAM), producing a compensatory diversion away from neurotransmitter and DNA synthesis and into the methylation cycle (41). Despite the potential significance of folate deprivation as an aetiological factor in the development of dementia, few studies have systematically investigated the possible role of folate in memory disturbance. Reported memory impairments have been observed in psychiatric patients with a deficiency of one or more of the B group vitamins (8), and experimental investigations in animals have demonstrated nervous system damage as a result of folate deficiency. Rats born of folate-deficient mothers exhibit diminished learning capacity and electro-encephalogram (EEG) abnormalities (46), and the learning deficits observed in folate deficient rats can be reversed by the administration of folate or thiamine (2). The deficiency in folate caused by methotrexate in association with radiotherapy in sufferers of acute lymphoblastic leukemia also have been noted to produce memory deficits (29). Supplementation with folinic acid has also been noted to decrease perseverative responding and improve spatial memory in older rats (23).

To date, little data is available on the effects of excess B vitamins on memory and nervous system functioning. B vitamins are water soluble, and it has generally been thought that it was impossible to convert excess quantities of these vitamins into their coenzyme form and thus to raise the intracellulary active form above normal levels. This would appear not to be the case however, and toxicity of pyridoxine (B_6) (18,32), thiamine (B_1) (12), nicotinic acid (B_2) (27) and folate (7,20,27,38) have each been noted subsequent to the administration of high doses.

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The neurochemical basis of this effect remains largely unexplained. Of particular interest, however, is the observation that the symptoms produced by a number of these vitamins in excess are comparable to those observed in the relative deficiency states (7,18). This suggests that the degree of deficit resulting from vitamin imbalance may represent an inverted U-shaped function, with both the severe deficiency and excess of vitamin levels impairing normal functioning.

Observations concerning the toxicity of folate are inconsistent. Large doses (50-70 mg/kg) of folate have been reported to cause renal failure in the rabbit and the rat (37), and learning impairments in other animals (34). Nephrotoxic and neurotoxic effects of high doses (25 mg/kg) have also been observed in animal experiments (27). In humans receiving 15 mg of folate per day, mood swings, sleeplessness, irritability and gastrointestinal symptoms have all been observed (34,38).

It would thus appear that despite their water solubility, the administration of excess B vitamins may elevate intra-cellular coenzyme levels, resulting, in the cases of thiamine, folate and pyridoxine, in detrimental effects.

The aim in the present study was to examine the effect of methotrexate-induced folate deficiency and the application of high doses of folate on memory and nervous system functioning in the day-old chick. Because of its biochemical relationship to folate, the effect of B_{12} in excess was also investigated. The toxicity of B_{12} appears extremely low, and doses as high as 1,600 mg/kg have been administered to mice without harm (27). However, previous studies have examined only the effect of peripheral administration of excess B_{12} on animals with well developed blood–brain barriers (6) so any possible direct effect of the agent on the CNS has not yet been fully investigated.

METHOD

Animals

The subjects for all the experiments were male, Black Australorp x White Leghorn chickens obtained from a local hatchery. Chicks were housed in pairs in wooden boxes (20 × 25 × 20 cm). A white 25 Watt incandescent light globe was suspended above each box in order to maintain a constant temperature of 25°C–29°C. Water was available ad lib during each experiment except during the pre-training, training and testing trials. Food was not dispensed during the course of the experiments as this would have resulted in an uncontrolled intake of vitamins and hence a confounding factor. It should be noted, however, that the chick is provided with sufficient nutrients from the yolk sac to sustain it during the first 7 days of life (15,33). A pilot experiment was carried out on 160 chickens in order to determine whether lack of food per se had any effect on memory retention. No difference was found between the chicks administered food and those deprived of food in either chicks that had been injected with saline [$F(1, 75) = 0.21$, $p = 0.65$] or chicks that received no injection [$F(1, 75) = 0.20$, $p = 0.66$].

Drugs

All drugs were administered subcutaneously into a ventral skin fold just below the ribcage using a Becton Dickinson Tuberculin 1ml syringe with a 26.5 gauge needle. Unless otherwise stated all drugs were dissolved in 154 mM (0.9%) isotonic saline (NaCl), and each injection was administered in a volume of 100μl. Both Methotrexate (MTX) and Folate were initially prepared in dimethyl sulfoxide (DMSO) at a final concentration of 10% prior to dilution with saline. This volume of DMSO was also added to the saline to be used as a control. DMSO has been shown previously in our laboratory not to affect retention in chickens.

Neurological rating scale. Chicks were rated on time taken to right. Righting reflex was assessed by placing each chick on its back and timing how long it took to stand. Chicks not able to stand after 10 s were helped up and given a score of 10 s in order to prevent extreme scores from biasing the means.

Procedure. The general procedure employed was the same for each experiment. Upon arrival at the laboratory, the chicks were placed, in pairs, into their pre-warmed wooden boxes. One chick from each pair was marked on the head with an indelible black felt pen in order to distinguish one from the other. Chicks were pre-trained, trained and tested in pairs.

Unless otherwise stated, the chicks were given two subcutaneous injections, 24 and 48 h prior to the pre-training trial. The type of drug given varied according to the nature of the experiment. In each experiment one or more groups of chickens received saline as a control. All drugs and drug doses were administered blind, and drug codes were not broken until completion of the experiment.

On day three of each experiment, following a 24 hour lapse from the second drug administration, chicks were subjected to a single trial passive avoidance learning task (13).

Chicks were initially trained in two trials to peck at a chrome bead which had been dipped in water. Following an interval of approximately 20 min, each chick was presented with a red, and then a blue bead dipped in water. The number of pecks in each 10 second period were recorded on a computer using an electronic handset. After 30 min, chicks were then presented for 10 s with a red bead that was visually identical to the one used in the pre-training trial, but dipped in a chemical aversant, methyl anthranilate (MeA). Chicks pecking at this bead typically display disgust reactions such as head shaking, beak wiping and fleeing from the bead. The number of pecks in the 10 second period were again recorded. Chicks that failed to peck the bead during the training trial were excluded from later data analyses since they were deemed not to have been trained. Testing occurred at various times after learning, depending on the aims of the particular experiment. The testing trial involved presenting the red then the blue beads successively to each group, for 10 s each. During this trial the beads were presented dry, and again number of pecks were recorded. Memory was considered to be intact if chicks avoided the red bead but continued to peck at the blue bead. Chicks that failed to peck the blue bead during this trial were excluded from later data analysis for reasons outlined by Ng and Gibbs (30).

Retention was calculated as a discrimination ratio (DR) using the formula:

$$\mathrm{DR} = \frac{\text{number of pecks at the blue bead}}{\text{number of pecks at the blue bead} + \text{number of pecks at the red bead}}$$

Statistics. The data analyses were performed on the DRs of each group using analysis of variance (ANOVA) with appropriate post-hoc tests where relevant. All analyses were carried out on the unweighted means because sample sizes were unequal through factors not associated with the treatments.

Immediately following the test of memory functions, the chicks were assessed for righting reflex. Each chick was scored by two raters, both of whom were blind to the drug or dose administered to each chick. Inter-rater reliability was calcu-

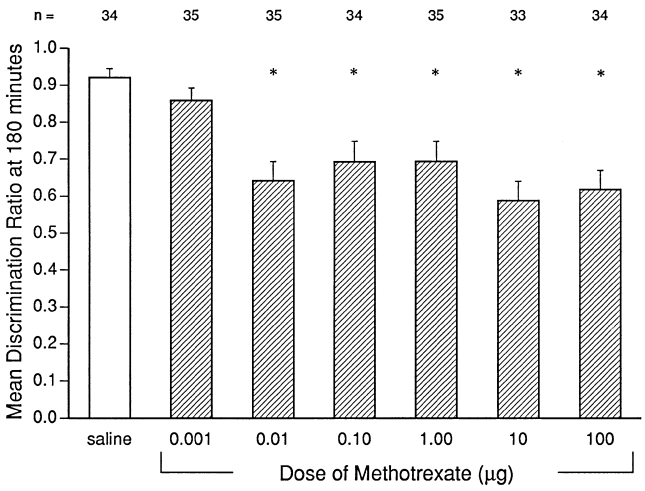

FIG. 1. The effect of various doses of methotrexate on mean discrimination ratio (±SEM). Chicks were injected twice, 24 and 48 h prior to pre-training, and tested at 180 min post-learning. (* indicate significant differences <0.05 between treatment and saline controls: doses quoted are the total dose given).

lated using the Pearson Product-Moment Correlation ($r = 0.80$, $p < 0.001$). Data analyses were performed on the mean of the two rater's scores. Righting reflex was analysed using one-way ANOVA followed by Dunnett's tests.

RESULTS

Experiment 1A: Methotrexate Dose Response Function

Folate deficiency can be induced through the inhibitory action of methotrexate on the reduction of dihydrofolate to tetrahydrofolate (16). Chicks were administered either 154 mM saline or differing doses of methotrexate (0.001, 0.01, 0.1, 1.0, 10 or 100 μg/per chick) with the aim of determining whether the administration of methotrexate would result in memory impairment. These data are presented in Fig. 1. Each treatment was administered to 40 chicks, and chicks were tested at 180 min post-learning, by which time protein synthesis-dependent long-term memory is thought to be well established (13).

A one-way ANOVA on the discrimination ratios as a function of dose of methotrexate revealed a significant dose effect [$F(6, 233) = 6.94$, $p < 0.001$] of methotrexate on memory, and post hoc Dunnett's t tests indicated significant differences between each dose of methotrexate from 0.01 μg upwards and the saline controls. The data indicated that methotrexate at doses 0.01 μg or higher impaired memory processing 3 h after passive avoidance training.

Data on time taken to right were submitted to a one-way ANOVA, revealing a significant dose effect [$F(6, 233) = 5.08$, $p < 0.001$]. Post hoc Dunnett's tests indicated a significant difference in time taken to right between each dose of methotrexate except the 0.001 μg dose and saline.

It was also of interest to determine the level of correlation between the neurological index of righting reflex with the level of discriminated memory. It is possible that the movement disorder per se may be contributing to the impairment of response on the behavioural test. The results indicated that there was a significant correlation between righting reflex and discrimination ratio only for the saline controls ($r = -0.47$, $p = 0.004$), and for the chick given 0.1 ($r = -0.41$, $p = 0.015$) and 10.0 ($r = -0.48$, $p = 0.004$) μg/per chick of methotrexate. The inconsistency of this pattern in not affecting all subjects, nor affecting them in a dose responsive way; in association with the fact that the saline controls produced the highest level of disruption of righting reflex, seem to indicate that the effect on memory is not produced as a direct consequence of motor disturbance in the chicks.

These results indicate that methotrexate-induced folate deficiency significantly inhibits memory retention in the three day-old chick. All doses in the range used except the lowest dose of 0.001 μg MTX induced substantial decrements in recall. Folate deficiency also appears to cause neurological impairment in the chicks as evidenced by the effect of methotrexate on time taken to right.

Whilst the impairment in the index of neurological functioning in the chick may act to undermine the specific effect of methotrexate on memory formation, it appears that amnesic effect is specific to the agent. The chicks included in the analysis were only those which demonstrated discrimination on the passive avoidance task used. If there were an effect of the agent on general behavioural functioning it would be anticipated that there would be a consequent decline in the number of chicks pecking at the beads overall. Due to the manner in which the discrimination ratio is calculated, chicks which failed to peck at the blue bead would have been excluded from the analysis. As such it would be anticipated that the number of subjects in the neurologically impaired groups would decrease proportionately. Examination of both the subject numbers and the number of pecks on the blue bead indicated that there was no systematic bias to this effect. This suggestion is further supported by the inconsistent pattern of correlation between the level of impairment of righting reflex and the discrimination ratio.

Experiment 1B: Methotrexate Time Course

Following the establishment of the fact that methotrexate impairs memory formation in the chick, it appeared of interest to determine at which stage of memory formation, the effect of methotrexate becomes apparent.

A distinct advantage of this paradigm is that the time of learning is known, and the stage at which memory formation is disrupted can be easily determined. The paradigm is thus a valuable one in which to study the modulation of memory by folate, both because the stage at which memory is disrupted can be precisely determined and also because of the relatively rudimentary development of the blood–brain barrier in these animals (44, 45), thus affording more direct insight into the effect of this vitamin on the unprotected central nervous system.

The aim of the present study was to determine the stage in memory formation at which these changes occurred. It is hoped that this may provide a useful starting point from which to ascertain the possible mechanism of folate deprivation in inducing memory impairments.

Chicks were administered either 154 mM saline or a total dose of 10 μg/per chick of methotrexate. This dose was chosen having found it to yield the lowest DR in the previous experiment (see Experiment 1a). Chicks were tested at various training-test intervals (TTIs). The times were chosen to correspond with different stages of memory formation as proposed by the Ng and Gibbs three stage model (30). For each time point, 20 chicks were administered methotrexate and 20 chicks were administered saline. This allowed a direct comparison at each

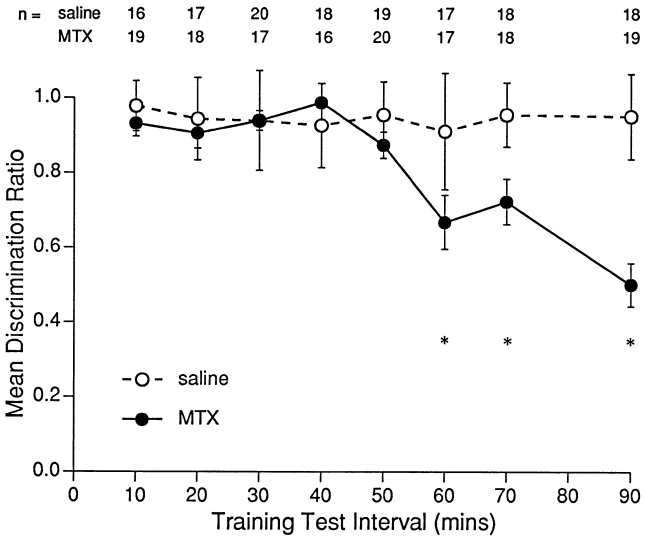

FIG. 2. The effect of a 10μg total dose of methotrexate on mean discrimination ratio (±SEM) at various times following learning. Chicks were injected twice, 24 and 48 h prior to pre-training, and tested at the times shown (* indicate significant differences <0.05 between treatment and saline controls).

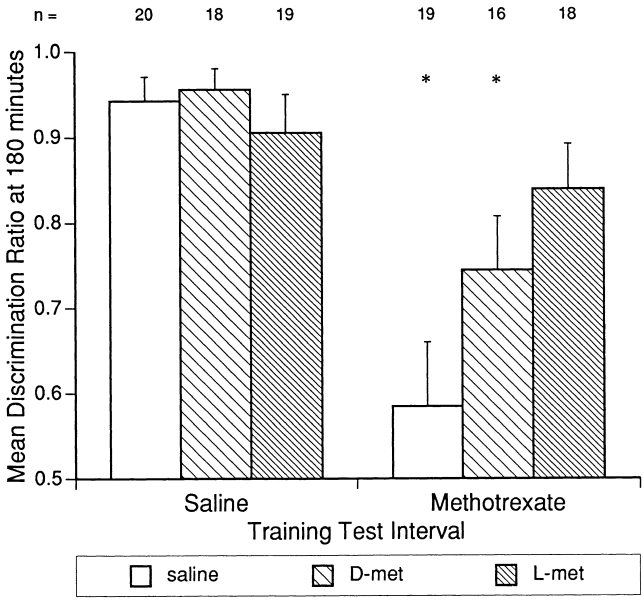

FIG. 3. Effect of a challenge of a 250mM dose of D and L methionine to chicks pre-treated with saline or a 10μg total dose of methotrexate as measured by mean discrimination ratio at 180 min after training (±SEM). Chicks were injected twice, 24 and 48 h prior to pre-training and challenged immediately following training with methionine or saline. They were tested at 180 min post-learning. (* indicate significant differences <0.05 between groups).

time point. Chicks were tested at 10, 20, 30, 40, 50, 60, 70 and 90 min post-learning.

A two-way ANOVA [drug (2) x TTI (8)] was performed (see Fig. 2). This revealed a significant effect for drug [$F(1, 271) = 49.59$, $p < 0.001$], a significant effect for time [$F(7, 271) = 19.27$ $p < 0.001$] and a significant drug by time interaction effect [$F(7, 271) = 9.78$, $p < 0.001$]. Tests of simple main effects illustrated non-significant drug differences within TTIs of 10 [$F(1, 271) = 0.31$, $p = 0.58$], 20 [$F(1, 271) = 0.34$, $p = 0.56$], 30 [$F(1, 271) = 0.02$, $p = 0.88$], 40 [$F(1, 271) = 0.73$, $p = 0.40$] and 50 [$F(1, 271) = 1.92$, $p = 0.17$] min post-learning, but significant drug differences within TTIs of 60 [$F(1, 271) = 15.67$, $p < 0.001$], 70 [$F(1, 271) = 15{,}16$, $p < 0.001$] and 90 [$F(1, 271) = 60.86$, $p < 0.001$] min post-learning. These results suggest that methotrexate disrupts memory after 50 min post-training.

It is apparent that in the presence of methotrexate, the earlier stages of memory formation, STM and ITM remain intact, while LTM formation is impaired.

Experiment 1C: Methionine Challenge to Methotrexate

Folate is involved in the synthesis of methionine from homocysteine. A deficiency state of this vitamin thus results in depleted levels of methionine (41), and a number of investigators (10,41) have suggested that decreased methionine is the principal cause of the nervous system dysfunction associated with folate deficiency. As methionine plays a critical role in the biochemical pathways that contribute to RNA, DNA and protein synthesis (41), it is possible that depletion of endogenous methionine may contribute to the impairment of LTM formation observed in the folate deficient chicks.

If methionine deficiency is implicated, it should be possible to challenge the effects of methotrexate through the administration of methionine. If the decreased methionine levels brought about by methotrexate cause memory impairment, then the pharmacological supplementation of methionine should attenuate the methotrexate-induced memory impairment. This hypothesis was examined in Experiment 1c.

Sixty chicks were administered 154 mM saline and sixty chicks were administered 10 μg/per chick methotrexate. On day 3 of the experiment, 24 h following the second injection of methotrexate or saline, chicks received a third injection (the challenge) immediately following training (+0 min). Chicks were injected with either D-methionine (250 mM/per chick), L-methionine (250 mM/per chick) or saline. Twenty chicks that had previously been injected with saline received D-methionine, 20 received L-methionine and 20 received saline. The same procedure applied for chicks that had previously been administered methotrexate. Chicks were then tested at 180 min post-learning (Fig. 3).

This dose (250 mM) of methionine was chosen as it has been shown previously not to affect memory retention in the chick (14). The administration of methionine has been observed to disrupt responding in a delayed-shock delivery task (3), so in order to attribute the results of this experiment to the effect of methionine on depleted folate levels, rather than to the effect of methionine per se, it was necessary to use a dose that did not, in itself, influence retention.

D and L are different isomers of methionine, D-methionine being the biologically inactive form (47). D-methionine was used in this experiment to control for any non-specific effects associated with methionine administration.

The results of Experiment 1c are illustrated in Fig. 3. A two way ANOVA [drug (2) by challenge(3)] was performed on 110 cases. The analysis revealed a significant effect for drug [$F(1, 104) = 26.78$, $p < 0.001$] and a significant interaction effect [$F(2, 104) = 4.13$, $p = 0.02$]. No significant effect for the challenge was found [$F(2, 104) = 2.62$, $p = 0.08$]. Tests for simple main effects revealed a significant drug within challenge

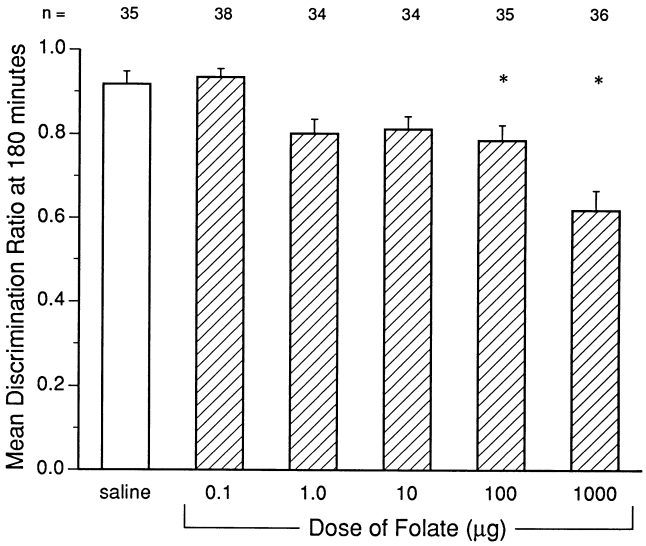

FIG. 4. The effect of various doses of folate on mean discrimination ratio (±SEM). Chicks were injected twice, 24 and 48 h prior to pre-training, and tested at 180 min post-learning. (Doses quoted are the total dose given: * indicate significant differences <0.05 between treatment and saline controls).

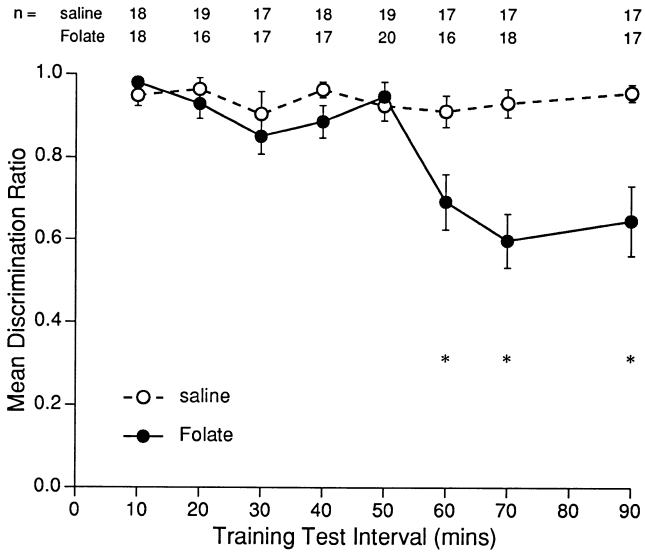

FIG. 5. The effect of a total dose of 1000 μg/chick of folate on mean discrimination ratio (±SEM) at various times following learning. Chicks were injected twice, 24 and 48 h prior to pre-training, and tested at the times shown. (* indicate significant differences <0.05 between treatment and saline controls).

effect for D-methionine [$F(1, 104) = 9.62$, $p < 0.002$] and saline [$F(1, 104) = 25.38$, $p < 0.001$], but not for L-methionine [$F(1, 104) = 0.93$, $p < 0.34$]. This demonstrates that L-methionine attenuated methotrexate-induced amnesia, while saline and D-methionine had no significant effect on the memory deficits of chicks rendered amnesic through methotrexate administration.

Experiment 2A: Folate Dose Response

A number of the B group vitamins, including folate, have been found to exert deleterious effects on the nervous system when administered in high doses (27), and it has been suggested that these effects are produced through the same mechanism as that which produces symptoms of the relative deficiency states (7,18). The lesions associated with excess B vitamins, however, are observed to be largely limited by the blood–brain barrier to the peripheral nervous system (18). Thus, in animals with well developed blood–brain barriers, any effect of excess B group vitamins on cognitive functions which depend on CNS processes, cannot be determined through peripheral administrations of the vitamins, such as occurs in dietary intake (6). Since young chicks possess only a rudimentary blood–brain barrier (44,45), any effect of excess folate would be expected to affect neurones of the central as well as the peripheral nervous system, allowing determination of any central nervous system dysfunctioning. The aim of this experiment was thus to determine whether folate would impair memory at high doses, and to determine the dose at which amnesia was most apparent.

Forty chicks were administered either 154 mM saline or differing doses of folate (0.1, 1.0, 10, 100, 1000 μg/per chick). Chicks were tested at 180 min post-learning. The results of Experiment 2a are presented in Fig. 4.

A one-way ANOVA indicated a significant dose effect [$F(5, 206) = 11.58$, $p < 0.001$] of folate, and a post hoc Dunnett's test demonstrated significant differences between the two highest doses of folate (100 and 1000 μg/chick) and saline. The retention levels for the three lower doses of folate did not differ significantly from those for saline, suggesting that the effect was dose dependent.

Following testing, the chicks were assessed for righting reflex. A one-way ANOVA demonstrated a significant effect of dose for time taken to right [$F(5, 234) = 3.57$, $p = 0.004$], and Dunnett's tests indicated that time taken to right was significantly slowed in chicks administered the three highest doses of folate (10, 100 and 1000 μg) as compared to saline, but not in those receiving 0.1 or 1.0 μg folate. Correlations between the level of impairment in righting reflex and the discrimination ratios revealed no significant association ($\alpha < 0.05$).

These results corroborate previous findings (7,20,27) which demonstrate the adverse effects of high levels of folate administration. The effect on memory was dose dependent, with the lowest dose of folate actually yielding a higher mean discrimination ratio than the saline control, and the highest doses severely impairing retention. High doses of folate also appear to influence righting reflex. The effect of folate on the neurological index also appeared to be dose dependant.

Experiment 2B: Folate Time Course

Having established that excess folate impairs retention in the young chick, the aim of this experiment was to determine the stage of memory formation at which this effect becomes apparent.

Chicks were administered either 154 mM saline or a total dose of 1000 μg/chick of folate. This dose of folate was selected on the basis that it produced the greatest amnesic effect in Experiment 2a. Chicks were tested at various training-test intervals (TTIs). For each time point, 20 chicks were administered folate and 20 chicks were administered saline. Testing occurred at 10, 20, 30, 40, 50, 60, 70 and 90 min post-learning. The results of Experiment 2b are presented in Fig. 5.

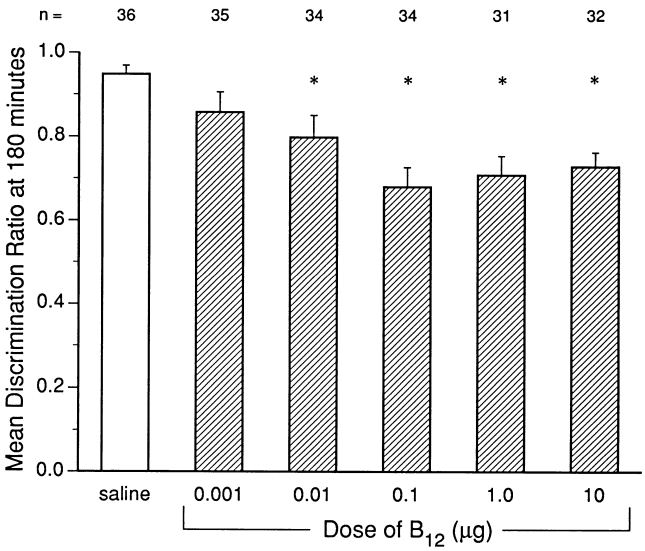

FIG. 6. The effect of various doses of vitamin B_{12} on mean discrimination ratio (±SEM). Chicks were injected twice, 24 and 48 h prior to pre-training, and tested at 180 min post-learning. (Doses quoted are the total dose given: * indicate significant differences <0.05 between treatment and saline controls).

A two-way ANOVA [drug (2) by time (8)] revealed a significant main effect for drug [$F(1,265) = 31.78, p < 0.001$] and time [$F(7,265) = 6.47, p < 0.001$], and a significant drug by time interaction effect [$F(7,265) = 5.70, p < 0.001$]. Tests for simple main effects yielded significant differences between saline and folate at 60 [$F(1,265) = 10.19, p < 0.002$], 70 [$F(1, 265) = 27.09, p < 0.001$] and 90 [$F(1, 265) = 21.90, p < 0.001$] min post-learning, but non-significant effects at 10 [$F(1,265) = 0.23, p = .64$], 20 [$F(1,265) = 0.51, p = .48$], 30 [$F(1,265) = 0.65, p = .42$], 40 [$F(1,265) = 1.46, p = .23$] and 50 [$F(1,265) = 0.17, p = .68$] min post-learning.

These results indicate that memory is impaired after 50 min post-learning following excess folate administration. It would appear that folate in excess exerts no effect on STM or ITM but interferes with the formation of LTM.

Experiment 2C: Vitamin B_{12} Dose Response

Vitamin B_{12} and folate interact to a large extent in terms of their biochemical mechanisms. B_{12} appears to be rather innocuous in high doses (27), but previous studies have examined only the effect of peripheral administration of excess B_{12} on animals with a mature blood–brain barrier (6), as such any possible effect of the agent on the CNS has not yet been fully investigated. Such investigation would be of interest not only to determine whether high doses of B_{12} do exert a central effect, but also as a means of comparing the effect of B_{12} excess and B_{12} deficiency, on the central nervous system. This may provide insight into the possible mechanism of action of B_{12} on cognitive functions which depend on CNS processing. The aim of this experiment was to examine the effect of excess B_{12} on memory functioning, and on the neurological index of righting reflex.

Chicks were administered either 154 mM saline or differing doses of vitamin B_{12} (0.001, 0.01. 0.1, 1, 10 μg/per chick). Chicks were tested at 180 min post-learning. These results are presented in Fig. 6.

One-way ANOVA revealed a significant dose effect for B_{12} [$F(5, 196) = 6.22, p < 0.001$], and post hoc Dunnett's tests indicated significant differences between saline and every dose of B_{12} with the exception of the lowest dose. The results demonstrate that vitamin B_{12} impairs retention in a dose dependent manner.

Following memory testing the chicks were assessed by two raters for level of righting reflex. A one-way ANOVA revealed that righting reflex did not differ significantly between the groups [$F(5, 234) = 1.32, p = 0.26$].

The results of this experiment demonstrate that B_{12} in excess impairs retention in the young chick. This result is somewhat surprising in view of the low toxicity of vitamin B_{12} (27). However, all toxicity studies of B_{12} to date have employed humans or other animals with well developed blood–brain barriers, so no possible effect on the CNS has been investigated. Use of the young chick which possesses only a rudimentary blood–brain barrier suggests that high doses of B_{12} may well have an adverse effect on neurones of the CNS.

B_{12} in excess does not appear to impair righting reflex. This result is consistent with previous findings of the lack of adverse effects associated with high doses of B_{12}, but is unexpected given the memory impairment observed in this experiment. It appears that excess B_{12} has a particular effect on the cellular processing underlying memory formation in the young chick. This is further suggested by the finding that neurological status did not correlate with memory impairment.

Experiment 2D: Vitamin B_{12} Time Course

Having observed that Vitamin B_{12} in excess can impair memory, the aim of this experiment was to determine when, in the course of memory formation, B_{12} exerts its effect. Chicks were administered either 154 mM saline or a total dose of 1.0 μg/chick of vitamin B_{12}. Chicks were tested at various times following learning. For each time point, one group of chicks were administered vitamin B_{12} and one group of chicks were administered saline. Testing occurred at 10, 20, 40, 50, 60, and 80 min post-learning. The results of Experiment 2d are presented in Fig. 7.

A two-way ANOVA [drug(2) by time (6)] was performed and yielded significant main effects for both drug [$F(1, 216) = 23.55, p < 0.001$] and time [$F(5, 216) = 3.05, p < 0.01$] and a significant drug x time interaction effect [$F(5, 216) = 3.59, p < 0.01$]. Tests for simple main effects demonstrated significant drug effects at TTIs of 60 [$F(1, 216) = 18.52, p < 0.001$] and 80 [$F(1, 216) = 18.37, p < 0.001$] min post-learning, but not at TTIs of 10 [$F(1, 216) = 0.31, p = 0.58$], 20 [$F(1, 216) = 2.03, p = 0.16$] or 40 [$F(1, 216) = 1.01, p = 0.32$] min post-learning. The results indicate that excess B_{12} exerts its effect on memory after 50 min post-learning.

These results demonstrate that the administration of B_{12} disrupts LTM. This result is comparable to that of Experiment 2b where excess folate was also found to interfere with LTM formation.

DISCUSSION

The results of this series of experiments with chicks trained on a single trial passive avoidance task, demonstrate that methotrexate-induced folate deficiency leads to amnesia after 50 min following learning. This effect seems to be independent of the effect of the drug on motor functioning. Furthermore, a folate/B_{12} derivative, methionine, was observed to attenuate methotrexate-induced amnesia, suggesting the possibility that

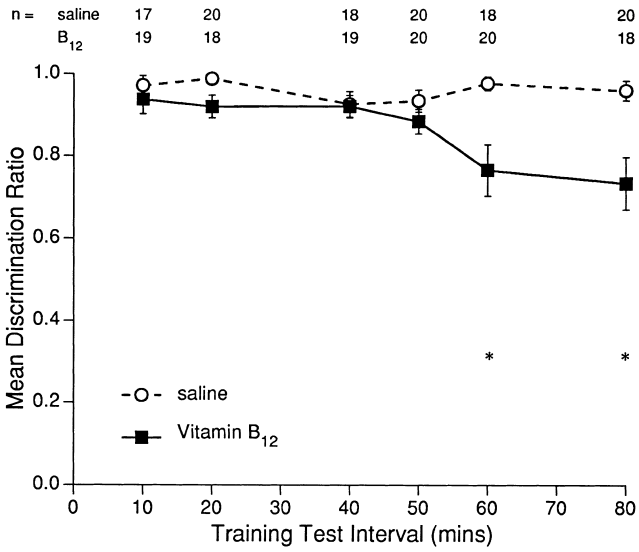

FIG. 7. The effect of a total dose of 1.0 fg/chick of vitamin B_{12} on mean discrimination ratio (±SEM) at various times following learning. Chicks were injected twice, 24 and 48 h prior to pre-training, and tested at the times shown. (* indicate significant differences <0.05 between treatment and saline controls).

methotrexate may impair memory through lowering methionine levels.

The administration of both folate and B_{12} in excess, also lead amnesia. The interruption to memory processes was apparent after 50 min post-learning. According to the Ng and Gibbs (30) model of memory formation, long-term memory is dependent on protein synthesis. The postulation that protein synthesis underlies permanent consolidation of memory is in fact one of the most enduring hypotheses relating to memory formation (9). The interruption in memory processes after 50 min post-training indicates that both the short and intermediate term memory stages are unaffected by the manipulation of the folate levels, while the formation of the long-term memory stage is disrupted.

While the mechanism of action of methotrexate-induced folate deficiency and excess cannot be definitively elucidated from the behavioural data obtained in these experiments, the wealth of data indicating that antibiotics are capable of impairing protein synthesis (9,13,19,31,39,42), and the observed behavioural impairment of memory at a time similar to that observed with antibiotics, invites the speculation that this may well be the mediating mechanism.

A deficiency of folate interferes with the synthesis of RNA and DNA. This has direct implications for protein biosynthesis, and suggests that the disruption of long-term memory by methotrexate may be mediated by interference with this process.

The hypothesis that it is the impairment of the biochemical pathways involving DNA synthesis that interferes with protein synthesis is further supported by the results of Experiment 1c in this series of studies, which demonstrates that the administration of methionine ameliorates the methotrexate-induced deficit in long-term memory. This result suggests that the memory impairment associated with folate deficiency may be the result of decreased methionine synthesis, rather than the result of interference with the other biochemical pathways on which folate exerts an effect. The production of methionine, a derivative of folate, is markedly decreased when folate is depleted (41). Adequate levels of methionine are necessary for the synthesis of purines and the pyrimidine, thymidylate (21). Hence, low levels of methionine may contribute to the disruption in protein synthesis by virtue of its role in DNA synthesis. In addition, as methionine is an amino acid, low levels may contribute directly to impaired protein synthesis as a result of insufficient incorporation into structural proteins.

While, investigations specifically examining the role of folate in memory have been limited, there is some evidence in the literature to support the hypothesis that the observed memory dysfunction occurs as a result of decreased production of methionine. L-ethionine, the ethyl analog of methionine, interferes with the activity of methionine, disrupting methylation of transfer RNA (28), DNA replication (24,35) and protein synthesis (10,11,40). If disruption of these processes underlies deficits in the consolidation of memory, then administration of ethionine should interfere with memory formation. This has been reported to be the case in rats, which displayed impaired performance on a learned passive avoidance task subsequent to ethionine administration near the time of training (26). The effect persisted after the drug was eliminated from the blood stream, and occurred without concomitant alterations in gross motor activity. These factors led the authors to conclude that the effect obtained was due to a permanent effect on memory, rather than activity changes or a dependency on the continuing presence of the drug. The results of the present study support this notion.

There are no previous studies that have examined the effect of high doses of folate or B_{12} on memory. The observation that excess B_{12} impaired memory was surprising given the previous findings that B_{12} is innocuous in high doses (27). It may be that high doses of vitamin B_{12} do have adverse effects on the central nervous system, but that this is usually prevented by the blood–brain barrier. In young chicks which possess only a rudimentary blood–brain barrier, excess B vitamins would be expected to exert their effect on CNS neurones as well as neurones of the peripheral nervous system. Knowledge concerning the relationship between serum B_{12} and brain B_{12} concentration is sparse, but evidence suggests that B_{12} penetrates the blood–brain barrier at a slow but measurable rate. The vitamin is usually present in the brain at high concentrations, particularly in the choroid plexus (25), and it may be that further penetration in the presence of excess quantities occurs at a very slow rate. More is known about the relationship between serum and brain concentrations of folate. Alterations in serum folate concentrations are accompanied by a corresponding alteration in brain concentrations (43). This relationship is a direct one, with a concentration gradient between serum and CSF folates. However, this gradient may only be operative at low doses of folate: when humans are administered high doses of folate, serum folate levels increase rapidly, while CSF concentrations rise slowly or not at all (43). This explains why excess folate produces lesions delimited to the peripheral nervous system in humans and other animals with well-developed blood–brain barriers. Results of this study suggest that both of these vitamins may exert effects on the unprotected CNS when administered in high doses.

Folate in excess also caused neurological impairment as evidenced by the impaired righting reflex. This corroborates previous findings of adverse neurological effects associated with the administration of high doses of folate. Neurological impairment was not apparent in the chicks administered high doses of B_{12}. This is consistent with previous research demonstrating a lack of adverse effects of B_{12} in high doses. B_{12} has not been found to cause nervous system dysfunction, even

when administered in doses as high as 1,600 mg/kg to mice (27). This result, however, is surprising in view of the fact that B_{12} has such a dramatic effect on retention.

The results of the present research on passive avoidance learning in the young chick support the hypothesis that both folate and B_{12} play a critical role in the memory process. The finding that the observed memory impairment was invariably a deficit in the LTM stage, with STM and ITM stages remaining intact, is indicative of a disruption to protein synthesis.

While the results of this study provide an indication of the effect of excess folate and B_{12} on memory, many questions remain unanswered, not least of which, the mechanism associated with the effects of these excess vitamins on memory functioning. Excess levels of both folate and B_{12} lead to an ensuing increase in the amino acid methionine. A number of amino acids, when given in high doses, have been observed to alter cerebral amino acid uptake (22,34). The fact that this has direct implications for protein synthesis, invites the speculation that the increased concentrations of methionine that result from the administration of folate or B_{12}, may competitively interfere with the uptake of other amino acids, and hence disrupt protein synthesis. We thus hope to undertake an analysis of the incorporation of amino acids into protein following treatment with methotrexate with a particular interest in those areas of the chick brain previously observed to be significant in memory formation. The present study also throws some light on the substrate of the neuropsychiatric impairments, notably memory disturbance, observed in elderly patients with B_{12} and folate deficiency, as a possible protein synthesis-mediated impairment of memory consolidation.

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