Methylazoxymethanol-Acetate Induced Neurotoxicity of Chick Embryos[1] (37202)

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Cycasin was suspected as the etiology of a human neurological disease, amyotrophic lateral sclerosis, which is characterized by gradual motor system dysfunction (1). The compound is a glucoside found in *Cycas circinalis* and *C. revoluta* and perhaps other species of the cycadaceae family. *C. circinalis* and *C. revoluta* are important sources of carbohydrate in Guam and Imami, Japan (2). Cycasin requires deglucosylation to form methylazoxymethanol (MAM) in order to exert its toxic effects. Neurological disorders consisting of destruction of the cerebellar differentiating microneurons and clinical symptoms of tremor and hind-legs paralysis were observed in mice and hamsters when they were injected at one day of age with cycasin (3). Further investigation by Jones *et al.* (4) and Sanger *et al.* (5) revealed that cycasin or methylazoxymethanol-acetate[3] (MAM-acetate) destroyed the external differentiating cell layers of the cerebellum of the Swiss albino mice and mongrel puppies.

Objectives of the present research were to determine motor function and cerebellar adenyl cyclase activity, histology and weight of chicks treated with MAM-acetate when an embryo. Adenyl cyclase, an enzyme in high concentration in the cerebellum (6) has been suggested by Bloom *et al.* (7) as possibly related to neurotransmission in synaptic endings. Since granule cell-purkinje contacts predominate in the cerebellum it was hypothesized that adenyl cyclase activity will be decreased in the chick previously treated with MAM-acetate. Furthermore, because of the suggested role of the enzyme in synaptic transmission we determined whether changes in the enzyme activity may accompany neurological lesion and motor dysfunction.

Procedures. Six-day-old white leghorn embryos were treated with 2 or 4 μl of MAM-acetate or 4 μl of physiological saline by introducing the compound or saline into the yolk sac and eggs returned to the incubator until hatching. Beginning 24 hr after hatching, the ability of the chick to perch on a dowel was determined for five consecutive days. In a second experiment, chick embryos were similarly treated with either 4 μl of MAM-acetate or saline for histological examination and adenyl cyclase assay. For these purposes, 1-, 3-, and 5-day-old chicks from the MAM-acetate or saline treated embryos were perfused intracardially via the left ventricle with physiological saline and then neutral buffered formalin (NBF) and brains finally post-fixed in NBF, embedded in paraffin and stained with hematoxylin-eosin as well as Luxol fast blue-Nissl. Chick embryos at 14-days of embryonation were decapitated and the entire head fixed in Bouin's solution. Brains were also obtained from decapitated chick embryos (14th day) and chicks (1, 3, and 5 days post hatching) and cerebellums determined for adenyl cyclase using the method developed by Krishna *et al.* (8). The weight of the cerebral hemisphere and cerebellum was determined. The number

[1] This research was partially supported by National Institutes of Health Grant CA-07052. Published with the approval of the Director of the Michigan Agricultural Experimental Station as Journal Article Number 6225.

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[3] MAM-acetate was purchased from Mann Research Laboratory, New York. It is an acetate ester of methylazoxymethanol.

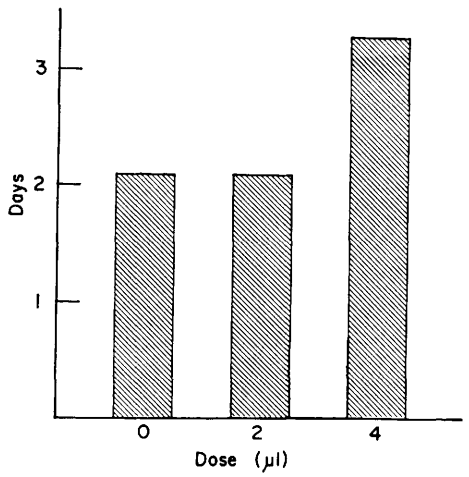

FIG. 1. Average age (days) of chicks to perch 15 sec on a dowel. Those that received 4 μl took significantly more days to perform the task than those treated with 2 μl or control ($p < 0.01$). The numbers of chicks tested were respectively 10, 8, and 9.

of chicks used for various determinations is indicated in appropriate tables and figures. All data were analyzed by means of analysis of variance.

Results and Discussion. The motor function test using a perch proved to be a sensitive method to detect early motor skills of chicks. The chicks treated when embryos with 4 μl of MAM-acetate required one day longer than those treated with 2 μl of MAM-acetate or saline to perch 15 sec or longer ($p < 0.01$; Fig. 1). No significant differences were observed between the control and those treated with 2 μl of MAM-acetate. During the first day post-hatching both groups of MAM-acetate treated chicks had motor dysfunction as indicated by the shorter perching time when compared to the perching time of control chicks. However, during the second day and thereafter the 2 μl chicks performed as well as the controls (Fig. 2). The 4 μl chicks persisted in poorer performance for Days 2 and 3 and were significantly poorer than the control or 2 μl chicks ($p < 0.05$). On the 4th and 5th day of testing, no significant differences were observed among the 3 groups of chicks.

When chick embryos were treated with 4 μl of MAM-acetate the cerebral weight was significantly less ($p < 0.05$) than control weight at 14-days of embryonation. The micrencephaly, however, did not persist since the weight was not statistically different from controls at 1-, 3-, and 5-day post hatching. Micrencephaly has also been reported for rats which were treated *in utero* with methylazoxymethanol derived from cleavage of cycasin (9). In rats, however, the decreased cerebral size was still evident several months after treatments. Furthermore, cerebellar weights determined at four different periods were not significantly affected by MAM-acetate treatments ($p > 0.05$; Table I).

Histologically, no lesions were observed in the cerebellums or cerebrums of 5 embryos or 5 chicks each at any of the 4 periods after MAM-acetate treatment. This was surprising since motor dysfunction was noted in the MAM-acetate treated chicks during the first 3 days post hatching. Furthermore, cerebellar lesions and motor dysfunction are characteristic of MAM-acetate treated post-natal mice and puppies (4, 5). In addition, specific and total activities of adenyl cyclase in the cerebellum were decreased when the chicks were 1-day of age ($p < 0.005$; Table II). The enzyme activities were similar for the

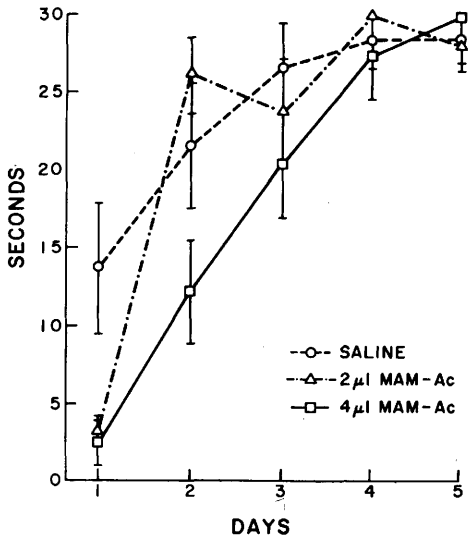

FIG. 2. Average ± SE length (in sec) of perching time of chicks over a 5-day testing period. The numbers of chicks tested were respectively 10, 8, and 9 for 4 and 2 μl of MAM-acetate and control.

TABLE I. MAM-Acetate Effect on Cerebellar and Cerebral Weights of Chicks and Chick Embryos After Injection into Yolk Sacs of 6-day Embryos.

Treatment	14-day embryos	Age of chicks (days) 1	3	5
		Cerebellum (mg)		
Control	26 ± 1 (7)[a]	104 ± 4 (6)	115 ± 3 (5)	124 ± 5 (5)
MAM-acetate	25 ± 2 (7)	103 ± 2 (6)	105 ± 3 (5)	111 ± 4 (5)
Change, %	−2	−1	−9	−11
		Cerebrum (mg)		
Control	338 ± 4 (7)	669 ± 12 (6)	723 ± 7 (5)	748 ± 9 (5)
MAM-acetate	293 ± 14 (7)	644 ± 11 (6)	676 ± 14 (5)	694 ± 15 (5)
Change, %	−13[b]	−4	−7	−7

[a] Average ± SE; number in parentheses indicates number of animals.
[b] Cerebral weight was significantly smaller for MAM-acetate treated embryos ($p < 0.05$).

MAM-acetate treated embryos and controls at Day 14 of embryonation. No significant differences were observed for the chicks at either 3- or 5-days of age. The occurrence of the decreased cerebellar adenyl cyclase at 1-day of age thus only corresponds partially to the age when the chicks showed dysfunction in perching.

Our data indicate that a simple test such as perching performance may be superior to histological examination in detecting subtle neurological changes produced by MAM-acetate treatment. Chicks are particularly useful for this purpose because of the suitability for motor testing soon after hatching. However, a major drawback has to be overcome as yet and this is the uncertainty of the amount of MAM-acetate absorbed into the embryos via the yolk sac. Presumably a direct relationship exists between absorption and perching ability since only the higher level affected the chicks. Further evidence to substantiate this relationship came from preliminary studies to determine the proper level of MAM-acetate for the present chick studies. When 5 μl of saline or 1, 2, 3, 4, or 5 μl of MAM-acetate were introduced into each yolk sac of 6-day-old embryos, the respective % hatches were 82, 88, 74, 75, 66, and 17. These data suggest that there is a direct relationship between dose and effect. The determination of MAM-acetate remaining in embryonic tissue and yolk sac to ascertain the quantity of the compound enter-

TABLE II. Specific and Total Cerebellar Adenyl Cyclase Activities of Chicks and Chick Embryos After Injection of MAM-acetate into Yolk Sacs of 6-day Embryos.[a]

Treatment	14-day embryos	Age of chicks (days) 1	3	5
	Specific activities (picomoles cyclic AMP/min/100 mg wet tissue)			
Control	182 ± 13 (5)[b]	1216 ± 62 (4)	1078 ± 49 (5)	1304 ± 59 (4)
MAM-acetate	201 ± 5 (5)	764 ± 14 (4)	1088 ± 69 (5)	1433 ± 57 (4)
p values[c]	0.15	0.005	0.5	0.25
	Total activities (picomoles cyclic AMP/min/cerebellum)			
Control	46 ± 3 (5)	1264 ± 65 (4)	1240 ± 57 (5)	1612 ± 73 (4)
MAM-acetate	51 ± 1 (5)	785 ± 15 (4)	1142 ± 72 (5)	1585 ± 63 (4)
p values[c]	0.15	0.005	0.5	0.25

[a] 5–7 cerebellums were pooled for each determination of adenyl cyclase activities.
[b] Average ± SE; number in parentheses indicates number of determinations.
[c] p values indicate levels of statistical significance.

ing the embryo would be helpful but presently no chemical assay method is available.

Summary. Six-day-old chick embryos were treated with either methylazoxymethanol acetate (MAM-acetate) or saline and allowed to hatch. When 4 μl of the neurotoxic compound were introduced into the yolk sacs of the embryos, motor function was impaired when the chicks were tested for their perching ability at 1, 2, and 3 days of age. No statistical differences were observed when the chicks were 4- or 5-days of age. Treatment with 2 μl of MAM-acetate produced motor impairment only during the 1st day post hatching. The higher level of MAM-acetate was also accompanied by a significantly reduced adenyl cyclase activities 1-day post hatching. The activity, however, became normal when the chicks reached 3 and 5-days of age. No histological lesions were detected in the brain of the chick. It was concluded that chicks are useful subjects for detection of subtle neurological changes produced by MAM-acetate using a simple perching test.

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Received Jan. 26, 1973. P.S.E.B.M., 1973, Vol. 143.