THE TOXICITY OF VITAMIN B₆, 4-DESOXYPYRIDOXINE, AND 4-METHOXYMETHYLPYRIDOXINE, ALONE AND IN COMBINATION, TO THE CHICK EMBRYO*

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Ott (1, 2) has shown that desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) and methoxypyridoxine (2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine), analogues of pyridoxine, act as pyridoxine antagonists in the chick. Because of our interest in the effects of metabolic antagonists on the development of the chick embryo (3), and on the behavior of mouse tumors growing on the chorioallantoic membrane of the chick embryo (4), these substances, and various forms of vitamin B₆, were studied in the fertile chick egg.

**Materials and Methods**

Fertile white Leghorn eggs were obtained from a commercial source. The eggs were incubated at 38° and 75 per cent relative humidity. Pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride and its phosphate, and methoxy- and desoxypyridoxine hydrochloride were used in this study. These chemicals were dissolved in saline just before use, and introduced into the yolk sac through a hole drilled in the blunt end of the egg. The opening was then sealed with a drop of paraffin. The volume of solution injected into each egg ranged between 0.05 to 0.2 cc. Eggs varying from 0 to 13 days of incubation were used. Following injection, the eggs were candled daily, and the dead embryos were weighed and examined for gross developmental abnormalities. Observations were usually made for 10 days after injection, and embryos surviving beyond this period were often sacrificed. More than 1850 embryos were used in this study. The number of embryos used at each dosage of the injected drugs varied from six to twenty, and at critical dosages several experiments were run. It was not feasible to make accurate LD₅₀ determinations for each compound, or combinations of compounds, in embryos of various ages, and only approximate LD₅₀ values, sufficient to characterize

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each situation, were estimated by inspecting a graph of the pertinent data. In studying the protective value of various forms of vitamin B₆ against the antagonists, the ratio permitting 50 per cent survival of embryos was approximated.

Results

Toxicity of Vitamin B₆ and Related Compounds to Chick Embryo

The approximate LD₅₀ values of the compounds tested in the chick embryo after various periods of incubation are summarized in Table I. Cravens and Snell (5) tested some of these compounds in the 6 day embryo, and our values are in general accord. They found that pyridoxine was toxic between 5 and 10 mg. per egg, pyridoxal hydrochloride at 5 mg., desoxypyridoxine at 5 mg., and pyridoxamine was non-toxic at 10 mg. per egg, the highest dose tested. When injected at 0 days, desoxypyridoxine produced 100 per cent deaths at doses of 0.5 to 1.0 mg. per egg, a somewhat smaller dose than proved toxic in our experiments.

The most interesting toxicity data were obtained with methoxypyridoxine, and this compound was consequently studied in considerable detail. Only 0.04 mg. per egg of methoxypyridoxine was required to produce 50 per cent mortality at 0 days, but as the embryo became older, this amount became progressively greater, so that at 13 days it was 3.5 mg. per egg. This rise in the LD₅₀ dose roughly paralleled the increase in mass of the chick embryo. Embryonic deaths usually occurred within 2 to 5 days after the injection of the drug; the older embryos receiving a fatal dose tended to survive longer than the younger ones.

Embryonic abnormalities were not consistently found with these agents, but occasionally gross malformations were seen in embryos surviving for long periods, particularly following the injection of desoxypyridoxine, pyridoxal hydrochloride, or pyridoxal phosphate. In embryos intoxicated by methoxypyridoxine, apparently normal growth and development proceeded for the several days before they succumbed. The mechanism whereby methoxypyridoxine caused the death of the embryo is not known, but it did not appear to interfere with growth.

Counteraction of Effects of Antagonists by Vitamin B₆

Pyridoxine, pyridoxamine, pyridoxal hydrochloride, and pyridoxal phosphate gave clear cut protection to the 4 day chick embryo against methoxypyridoxine. The studies on the protective activity of each form of vitamin B₆ are described separately, and the results are summarized in Fig. 1.

Pyridoxine—The 4 day embryo was used in most of the experiments, but a few studies were carried out with 0 and 13 day embryos. In the 4
day embryo, 0.07 mg. per egg of pyridoxine protected 50 per cent of the embryos against 0.5 mg. per egg of methoxypyridoxine (3 × LD₅₀). As the dose of methoxypyridoxine was raised, a much smaller increase in pyridoxine was necessary to provide protection; at the highest dose of methoxypyridoxine tested, 40 mg. per egg (240 × LD₅₀), 0.5 mg. of pyridoxine protected 70 per cent of the embryos. It is of interest that, in the presence of pyridoxine, methoxypyridoxine has a lower intrinsic toxicity (>40 mg. per egg) than pyridoxine, pyridoxal hydrochloride, or pyridoxal phosphate.

Scattered observations on embryos of different ages suggest that the protective ratio of pyridoxine to methoxypyridoxine is more closely re-
related to the LD₅₀ dose of methoxypyridoxine, rather than to the absolute dose of the drug. For example, 0.2 mg. per egg of pyridoxine failed to protect 0 and 4 day embryos against 5 and 20 mg. per egg, respectively (120 × LD₅₀) of methoxypyridoxine, whereas it protected 50 per cent of 4 day embryos against 5 mg. (30 × LD₅₀), and 0.1 mg. of pyridoxine protected 50 per cent of 13 day embryos against 20 mg. (6 × LD₅₀) of methoxypyridoxine.

Pyridoxine, injected several days prior to methoxypyridoxine, was still capable of protecting against the antagonist. Thus, when 0.5 mg. of pyridoxine was injected into 2 day embryos, it protected them against the injection of 5 mg. of methoxypyridoxine made 2 days later. Similarly, the injection of 1 mg. of pyridoxine at 4 days of incubation protected against 20 mg. of methoxypyridoxine injected at 10 days. Thus pyridoxine remains active for at least 6 days after injection into the egg.

The ability of pyridoxine to reverse the toxicity of methoxypyridoxine was tested in the 4 day embryo. When 5 mg. of methoxypyridoxine were followed 1, 2, 4, 8, 24, or 48 hours later with 0.5 mg. of pyridoxine, those embryos receiving pyridoxine within 24 hours showed appreciable protection, whereas all embryos injected at 48 hours died. The effects of a large dose of methoxypyridoxine (30 × LD₅₀) thus may be reversed by pyridoxine within 24 hours, but not at 48 hours. The great majority of deaths in the 4 day embryos treated with methoxypyridoxine occurred between 48 and 72 hours, so the failure of pyridoxine to reverse the toxicity of methoxypyridoxine at 48 hours is not surprising.

Pyridoxal Hydrochloride and Pyridoxamine—These compounds appeared to be as active as pyridoxine in counteracting the toxic effects of 0.5 mg. per egg of methoxypyridoxine. As the dosage of methoxypyridoxine was increased, the amounts of these agents necessary to provide protection rose promptly to higher levels, and pyridoxal hydrochloride showed considerably more protective activity than pyridoxamine (Fig. 1).

Pyridoxal Phosphate—This agent was found to have weak protective activity against methoxypyridoxine. It provided protection against 0.5 mg. per egg of methoxypyridoxine, but when the dose of the antagonist was increased above 1 mg. per egg, pyridoxal phosphate, given in doses up to its limiting intrinsic toxicity (3 to 5 mg. per egg), failed to protect the embryos.

Desoxypyridoxine—Cravens and Snell (5) found that vitamin B₆ would protect 0 day embryos against desoxypyridoxine, but in the 4 to 6 day embryo vitamin B₆ could not diminish the toxicity of desoxypyridoxine. Similarly, in our experiments with the 4 day embryo, desoxypyridoxine did not show any vitamin B₆ or antivitamin B₆ activity. The LD₅₀ of desoxypyridoxine in the 4 day embryo is 4 mg. per egg. At 5 mg. of
desoxypyridoxine, 5 and 3 mg. of pyridoxine and pyridoxal phosphate, respectively, did not provide any protection. Conversely, 2 mg. of desoxypyridoxine did not protect the chick embryo against 0.5 mg. of methoxypyridoxine.

DISCUSSION

A number of interesting differences between the chemically closely related compounds studied have been brought out by the use of the chick embryo. If the antivitamin B₆ activity of methoxypyridoxine is eliminated by adding pyridoxine, these agents show the following order of toxicity in the 4 day embryo: methoxypyridoxine, not toxic at 40 mg. per egg, the highest level tested, in the presence of a protective amount of pyridoxine; pyridoxamine, not toxic at 20 mg. per egg, the highest level tested; pyridoxine, approximate LD₅₀, 12 mg. per egg; pyridoxal hydrochloride and pyridoxal phosphate, LD₅₀ range, 3 to 5 mg. per egg; desoxypyridoxine, approximate LD₅₀, 4 mg. per egg.

These limited data, and those contained in Table I, suggest that the toxicity of these agents, in the presence of vitamin B₆, shows relatively little change in the chick embryo from 0 to 13 days of age, particularly as contrasted with the marked decrease in the toxicity of methoxypyridoxine with the increase in the age of unprotected embryos. The mechanisms of the lethal action of these agents, as apparently dissociated from their vitamin B₆ activity of antagonism, present an interesting problem.

The embryo can be protected against the toxic effects of methoxypyridoxine (and this was studied particularly in the 4 day embryo) by vitamin B₆, and considerable differences were found in the protective activity of the different vitamin B₆ compounds. The protective dose of pyridoxine

TABLE I

| Approximate LD₅₀ Doses of Vitamin B₆ and Related Compounds for Chick Embryos of Different Ages |

<table>
<thead>
<tr>
<th>Age of embryo when injected</th>
<th>Pyridoxine</th>
<th>Pyridoxamine</th>
<th>Pyridoxal</th>
<th>Pyridoxal phosphate</th>
<th>Desoxypyridoxine</th>
<th>Methoxypyridoxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td>&gt;3.0</td>
<td></td>
<td>1.0-2.0</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>&gt;20.0</td>
<td>5.0</td>
<td>3.0-5.0</td>
<td>0.07</td>
</tr>
<tr>
<td>4</td>
<td>12.0</td>
<td>&gt;20.0</td>
<td>5.0</td>
<td>3.0-5.0</td>
<td>4.0</td>
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<td></td>
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</tr>
<tr>
<td>13</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>5.0</td>
<td>3.0-5.0</td>
<td>12.0</td>
<td>3.50</td>
</tr>
</tbody>
</table>

The values are expressed as mg. per egg.

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was shown to increase relatively little with large increases in methoxy-
pyridoxine. Pyridoxal hydrochloride and pyridoxamine showed protec-
tive activity approximately equal to that of pyridoxine at 3 × \( \text{LD}_{50} \)
doses of methoxypyridoxine, but as the dose of methoxy pyridoxine was
increased, these compounds appeared less active than pyridoxine, and
pyridoxamine was less active than pyridoxal hydrochloride. Pyridoxal
phosphate was the least active of the vitamin \( B_6 \) compounds tested against
3 × \( \text{LD}_{50} \) doses of methoxy pyridoxine, and it did not protect against
larger doses. On the basis of the relative protective plateaus in dosage
that these agents (excluding pyridoxal phosphate) achieve against 5 mg.
and higher doses of methoxy pyridoxine, their relative protective activ.
ities against methoxy pyridoxine are estimated as 30 (pyridoxine) to 3
(pyridoxal hydrochloride) to 1 (pyridoxamine) (Fig. 1).

Our results with desoxypyridoxine are in accord with those of Cravens
and Snell (5). In their experiments with the 0 day eggs, desoxypyridoxine
was lethal at doses of 1.0 mg. per egg. 50 per cent of the embryos was
protected against this dose by approximately 0.01 mg., 0.02 mg., and
0.05 mg. of pyridoxine, pyridoxamine, and pyridoxal hydrochloride, re-
spectively. In 4 and 6 day embryos, however, larger amounts of des-
oxypyridoxine were required to show toxicity, and none of these agents
protected against these increased amounts of desoxypyridoxine; they con-
clude that this toxic action is not due to the antivitamin \( B_6 \) activity of
desoxypyridoxine. Our data support this view, and it seems probable
that desoxypyridoxine can intoxicate the chick embryo by two different
mechanisms. The first mechanism is unrelated to its antivitamin \( B_6 \)
activity, and may be referred to as its intrinsic toxicity. The intrinsic
toxicity of desoxypyridoxine rises from about 2 mg. per egg at 0 day to
12 mg. per egg at 13 days, and this toxic activity may be related to those
of pyridoxine, pyridoxal hydrochloride, and pyridoxal phosphate (Table
I).

The second mechanism by which desoxypyridoxine is toxic is due to
its antivitamin \( B_6 \) activity. At 0 day, desoxypyridoxine is toxic at 1.0
mg. per egg, and this action can be prevented by vitamin \( B_6 \). As the
embryo becomes older, a larger amount of antivitamin \( B_6 \) activity is nec-
essary to produce embryonic death. For example, the \( \text{LD}_{50} \) dose of meth-
oxypyridoxine increases from 0.04 mg. per egg at 0 day to 0.17 mg. per
egg at 4 days, a 4-fold increase. If it is assumed that the dose of desoxy-
pyridoxine must be increased proportionally in order to produce a lethal
antivitamin \( B_6 \) effect in the 4 day embryo, the estimated dose of desoxy-
pyridoxine necessary will produce death because of its intrinsic toxicity.
Methoxypyridoxine is, thus, far superior to desoxypyridoxine as an an-
tagonist of vitamin \( B_6 \) in the chick embryo, since it has a much lower in-
trinsic toxicity, and from the above considerations it is estimated to be at least 25 times more active than desoxypyridoxine as a vitamin B₆ antagonist.

The resistance of the embryo to methoxypyridoxine as it increases in age prompts speculation. This may mean that the embryo, as it grows, is forming vitamin B₆, or, as suggested by Cravens and Snell (5), that the vitamin B₆-dependent systems, as they develop, become resistant to the antagonist. The suggestion, in our data, that the dose of pyridoxine protecting embryos against methoxypyridoxine is more closely related to the LD₅₀ dose of the antagonist at a given age than to the absolute amount of the drug does not clarify this problem. The vitamin B₆ content of the egg must be considered as a factor in these experiments, particularly since Rabinowitz and Snell (6) have reported that each gm. of dried egg contains 0.0056 mg. of pyridoxal hydrochloride, 0.0012 mg. of pyridoxamine, and a negligible amount of pyridoxine. This may be roughly estimated as about 0.08 mg. of vitamin B₆ per whole egg, the significance of which in relation to our observations is not clear.

The mechanism whereby methoxypyridoxine antagonizes vitamin B₆ is unknown. Umbreit and Waddell (7) have studied the antagonistic action of desoxypyridoxine in vitro. They conclude that desoxypyridoxine is phosphorylated and competes with pyridoxal phosphate, the active form of vitamin B₆. In the chick embryo, however, the facts that pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate are effective against methoxypyridoxine in a descending order, and that pyridoxine, once it achieves a certain level, will protect against increasing doses of methoxypyridoxine, suggest that pyridoxine is the most active form of vitamin B₆ of those tested.

The exact rôle of vitamin B₆ in the metabolism of birds and mammals has not been demonstrated. It is known, however, to play an important rôle as a coenzyme in transamination, decarboxylation, and possibly in carboxylation reactions, and Bonner and Bonner (8) have stated that, "In its possible rôle as a coenzyme in transamination, in particular, it may occupy a key position in the synthesis... of the proteins of the plant." It is of interest, therefore, that chick embryos treated with lethal doses of methoxypyridoxine continue to differentiate and enlarge for the 2 to 5 day period before death, presumably due to the continued synthesis of protein and the formation of new cells. It appears likely that the vitamin B₆ deficiency induced by methoxypyridoxine in the chick embryo produces death by means of a physiological derangement, not immediately related to the processes of tissue growth, or by the accumulation, in the closed system of the egg, of lethal concentrations of the by-products of a vitamin B₆ deficiency. One of these limiting factors, in producing early embryonic
death, may mask the development of more complete or profound evidences of a vitamin B₆ deficiency. At the moment, it can only be concluded that the effects of methoxypyridoxine in the chick embryo may permit a further exploration of the mechanism of action of vitamin B₆ and supply a new method for assaying compounds for vitamin B₆ activity.

**SUMMARY**

Methoxypyridoxine is toxic to the chick embryo because of its anti-vitamin B₆ activity. Its toxicity decreases with the age of the embryo, the LD₅₀ increasing from 0.04 mg. per egg at 0 day to 0.17 mg. per egg at 4 days and 3.5 mg. at 13 days. The lethally intoxicated embryos may survive for several days and continue to show grossly normal growth and development. Pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate will protect the chick embryo against methoxypyridoxine, and their activity decreases in the order listed. A relatively constant amount of pyridoxine will protect the embryo against a wide range of concentrations of methoxypyridoxine, whereas, at the other extreme, pyridoxal phosphate protects against only a small amount of methoxypyridoxine.

Desoxypyridoxine is a weak vitamin B₆ antagonist in the 0 day chick embryo, as shown by Cravens and Snell (5). In older embryos its intrinsic toxicity proves lethal at levels which do not apparently produce a severe vitamin B₆ deficiency, as evidenced by the failure of vitamin B₆ to protect the embryo against its action. It is estimated that methoxypyridoxine is at least 25 times more active than desoxypyridoxine as a vitamin B₆ antagonist in the 0 to 4 day chick embryo.

**BIBLIOGRAPHY**

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