17084. Effects of Desoxypyridoxine and Vitamin B_6 on Development of the Chick Embryo.*

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The usefulness of metabolite analogs in nutritional and metabolic studies has been demonstrated by a number of workers. These studies have been summarizd by several authors.[1-3] It appeared logical, therefore, that vitamin analogs might be used in studies dealing with chemical embryology, and as an approach to this problem, a study of the effects of desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) on the development of the chick embryo was begun. Ott[4] has shown that this analog is markedly inhibitory to growth of chicks on diets which contain amounts of pyridoxine which limit growth, and that its effects are overcome by administration of additional pyridoxine.

While these studies were in progress, Ackermann and Taylor[5] reported the application of this technic to the study of the metabolism of chick embryos. The analog used by these workers, 3-acetylpyridine, was found to inhibit embryonic development and this inhibition could be prevented by nicotinamide. Nicotinic acid and tryptophan were much less active reversing agents.

Experimental. The procedure used in injecting the various compounds into the eggs was as follows: With the egg standing on its small end, the large end was swabbed with ethanol. A small hole was then drilled into the egg through which the compounds were injected. A tuberculin syringe and a No. 26 gauge needle approximately 1.5 cm in length were used for this purpose. The needle was inserted into the egg at such an angle that the solutions injected were deposited in the albumen in close proximity to the yolk. It is possible that occasionally the yolk was pierced by the needle but the data presented show that control injections of water did not result in a significant decrease in hatchability. In the first experiments the compounds were dissolved at concentrations which required injection of 0.2 cc of solution. This amount of water injected into eggs prior to incubation resulted in some decrease in hatchability. One-tenth cc appeared harmless, however, and in further experiments this volume of solution was used. For the injection of higher levels of the compounds at six days of incubation, volumes up to 0.5 cc could be used without harm. After the desired solutions were injected the hole was sealed with collodion and the eggs were immediately set in an incubator.

The eggs were candled at irregular intervals and the infertile eggs and dead embryos were removed. All eggs were broken out and examined to determine fertility and approximate age at death of the embryo. Any gross abnormalities were also recorded.

Results. The data of Table I show the effect of the injection of desoxypyridoxine prior to incubation on embryonic development. Approximately 500-1000 μg of the analog caused 100% mortality of the embryos. This inhibitory effect of the analog was largely prevented by simultaneous injection of 1.0 mg of pyridoxal hydrochloride. The "toxic" effect of desoxypyridoxine at these levels thus appears to result from its interference with normal utilization of vitamin B_6, and can be prevented by supplying additional quantities

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1 Roblin, R. O., Jr., *Chem. Rev.*, 1946, **38**, 255.

2 Woolley, D. W., *Advances in Enzymol.*, 1946, **6**, 129.

3 Wright, L. D., *J. Am. Dietet. Assn.*, 1947, **23**, 289.

4 Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 125.

5 Ackermann, W. W., and Taylor, A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 449.

TABLE I.
Effect of Desoxypyridoxine on Hatchability of Eggs.

Substance injected	Amt injected, μg	% Hatchability*	
		0.2 cc solutions	0.1 cc solutions
None		74	95
Water (H_2O)		35	90
Desoxypyridoxine	200	29	
"	500	11	21
"	1000	0	0
" + Pyridoxal • HCl	1000	13	83

* Not less than 14 eggs per group.

TABLE II.
Effect of Injection of Desoxypyridoxine at Different Stages of Incubation of Hatchability.

Time of injection†	% Hatchability*					
	Water	500 μg Desoxypyridoxine	Water	500 μg Desoxypyridoxine	Water	1000 μg Desoxypyridoxine
0 days	88	24	87	14		
68 hr						5
72 "	94	72	86	64	80	4
86 "						53
92 "						79
6 days	93	89	91	91		
9 "	95	88				
12 "	95	89				
15 "	100	95				
Control (uninj.)	95		96		84	

* 15-23 eggs per group.
† 0.1 cc of water or solution of inhibitor injected in all cases.

of the vitamin. The result demonstrates that vitamin B_6 is highly essential for normal embryonic development. It was observed that most of the embryos died at 2 to 4 days of development.

The effects of injecting desoxypyridoxine at different stages of incubation on embryonic development are shown in Table II. Whereas 500 μg of the analog injected prior to setting resulted in approximately 75% mortality of the embryos, a similar quantity injected at 3 or more days of incubation was non-toxic. Injecting 1000 μg at 68 or 72 hours of incubation resulted in approximately 95% embryonic mortality. This inhibitory effect was almost immediate since the bulk of the embryos were recorded as dying at 3 or 4 days of development. However, this same amount injected at 86 or 92 hours had little, if any, effect on embryonic development. The inhibitory effects of this analog are thus most marked during the first 3 to 4 days of embryonic development; subsequent to this time, the compound has greatly reduced toxicity.

In view of this fact, the toxic level of the analog at 6 days of incubation was determined. In a preliminary experiment, 5 mg of the analog proved almost completely inhibitory at this time, but this inhibition could not be overcome by simultaneous injection of any of the 3 forms of vitamin B_6. Further experiments, summarized in Table III, indicate that pyridoxal hydrochloride is also toxic at these high levels, while pyridoxine hydrochloride is somewhat less toxic and pyridoxamine dihydrochloride proved non-toxic at the highest level tested (10 mg per egg). Inhibition at this period by these high concentrations of desoxypyridoxine, therefore, is not specifically related to an interference in utilization of vitamin B_6 for essential metabolic purposes, as is the inhibition by smaller amounts of

TABLE III.
Comparative Toxicities of Desoxypyridoxine, Pyridoxal, Pyridoxamine, and Pyridoxine When Injected at 6 Days of Incubation.

Mg of compound injected	% Hatchability†			
	Desoxypyridoxine	Pyridoxal hydrochloride	Pyridoxamine dihydrochloride	Pyridoxine hydrochloride
Uninj. control	100	100	100	100
Inj. control*	70	70	70	70
1.0	80	90	70	100
2.5	70	50	50	90
5.0	0	20	90	80
10.0	0	0	90	10

* 0.5 cc of water or solutions injected in all cases.
† 10 fertile eggs per group.

TABLE IV.
Comparative Potencies of Pyridoxal, Pyridoxamine, and Pyridoxine in Overcoming the Inhibitory Action of Desoxypyridoxine.

Substance injected*	Amt injected, μg								
	0	2	5	10	20	50	70	100	1000
	% hatchability†								
Desoxypyridoxine									0
Desoxypyridoxine, 1000 μg									
+ Pyridoxal • HCl		0	0	0	18	30	56	63	47
Pyridoxamine • 2HCl		0	14	27	45	71	67	71	57
Pyridoxine • HCl		0	15	50	74	71	83	62	92

* 0.1 cc of water or solution injected in all cases. Control groups run at the same time gave the following per cent hatchability: water, 75; 1000 μg pyridoxal hydrochloride, 83; 1000 μg pyridoxamine dihydrochloride, 62; 1000 μg pyridoxine hydrochloride, 60; uninjected, 83.
† All groups contained 12 fertile eggs or over; at critical levels (20 and 50 μg of vitamin plus 1000 μg of desoxypyridoxine) 23 to 40 fertile eggs were used per group.

desoxypyridoxine which occurs when this compound is injected before the start of incubation.

It has been shown[6] that pyridoxal and pyridoxamine are the predominant forms of vitamin B_6 present in both the egg and the chick; pyridoxine, if present at all, occurs only in relatively small amounts. It was of interest, therefore, to compare the relative activities of these three compounds in counteracting the inhibitory action of desoxypyridoxine. Results of such an experiment are summarized in Table IV. The ratios of vitamin to inhibitor which permitted approximately 50% of the embryos to produce live chicks were for pyridoxal hydrochloride 1:20; for pyridoxamine dihydrochloride 1:50; and for pyridoxine hydrochloride 1:100.

Discussion. These experiments show clearly that development of the chick embryo is inhibited by the injection of desoxypyridoxine when this is made prior to the start of the incubation period, and that this inhibition is prevented by simultaneous injection of any of the three forms of vitamin B_6. Vitamin B_6 is an essential catalyst for a variety of reactions involved in protein metabolism, as well as in the synthesis of nicotinic acid from tryptophan. Which of these reactions first fails the embryo because of the blocking action of the inhibitor is not known. Since nicotinic acid may arise from tryptophan in metabolism and since nicotinic acid is synthesized by the chick embryo at an increasing rate as embryonic development progresses,[7] this compound was injected together with desoxypyridoxine to determine whether the inhibitor simply blocks its formation from tryptophan. Neither nicotinic acid nor its amide affected the inhibitory properties of the des-

[6] Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, 1948, **176**, 1157.

[7] Snell, E. E., and Quarles, E., *J. Nutrition*, 1941, **22**, 483.

oxypyridoxine. Similar negative results were obtained when DL-alanine was injected either alone or with nicotinic acid. DL-alanine was tested since Holden and Snell[8] have shown that D-alanine will replace vitamin B_6 for growth of certain microorganisms.

Umbreit and Waddell[9] have offered an explanation for the mode of action of desoxypyridoxine which may explain our observations that the amount of desoxypyridoxine required to inhibit embryonic growth is increased when injection of the inhibitor is postponed until later in the incubation period. These workers, who used cell preparations containing tyrosine decarboxylase as their test system, showed that desoxypyridoxine exerts its inhibitory effect by being phosphorylated and then competing with pyridoxal phosphate for the apoenzyme. Desoxypyridoxine phosphate was found to be only slightly inhibitory if the pyridoxal phosphate had first been allowed to combine with the tyrosine decarboxylase protein. It is suggested, therefore, that desoxypyridoxine, when injected prior to the start of embryonic development, is phosphorylated and thus is able to compete with pyridoxal phosphate for combination with some newly-formed apoenzyme with which this coenzyme functions, and whose function is necessary for continued development of the embryo. If injection of the inhibitor is delayed beyond about three days, this apoenzyme has already been saturated with pyridoxal phosphate, thus making it impossible for desoxypyridoxine phosphate, even if formed, to exert its inhibitory action.

The toxicity of high levels of desoxypyridoxine when injected at six days of embryonic development was not due to a competitive antivitamin action of the inhibitor since it could not be prevented by any of the 3 forms of vitamin B_6. Pyridoxal hydrochloride proved to be toxic at similarly high levels while pyridoxine hydrochloride was somewhat less toxic and pyridoxamine dihydrochloride was nontoxic at the highest level tested.

The role of vitamin B_6 in development of the chick embryo is not readily determined directly, since hens fed diets deficient in this vitamin stop laying before sufficient eggs are obtained to determine its effect on embryonic development.[10] Our experiments show that vitamin B_6 is essential for embryonic development and also that the need for this vitamin becomes critical early in embryonic growth. It is thus possible to use antimetabolites to study problems dealing with the nutrition of the avian embryo which cannot be studied directly.

Summary. Desoxypyridoxine (1000 μg) injected into eggs prior to the start of the incubation period resulted in 100% mortality of the embryos; this inhibition was prevented by simultaneous injection of any of the three forms of vitamin B_6. The ratio of vitamin to inhibitor which permitted approximately 50% of the embryos to produce live chicks was 1/20 for pyridoxal, 1/50 for pyridoxamine and 1/100 for pyridoxine.

When injected after 4 or more days of incubation, 1000 μg of desoxypyridoxine proved non-toxic. Higher levels injected at 6 days of incubation proved toxic but this toxicity could not be prevented by any of the 3 forms of vitamin B_6. A possible explanation for this observed variation in toxicity of desoxypyridoxine at different stages of incubation is offered. Pyridoxal hydrochloride and pyridoxine hydrochloride also proved toxic at high levels while pyridoxamine dihydrochloride was not toxic at the highest level tested.

Neither nicotinic acid nor its amide affected the inhibitory action of desoxypyridoxine; negative results were also obtained when DL-alanine was injected alone or with nicotinic acid.

These experiments demonstrate that vitamin B_6 becomes essential for embryonic growth very early in the incubation period.

[8] Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, 1949, **178**, 799.

[9] Umbreit, W. W., and Waddell, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 293.

[10] Cravens, W. W., Sebesta, E. E., Halpin, J. G., and Hart, E. B., *Poultry Sci.*, 1946, **25**. 80.

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