THE LATHYROGENIC EFFECT OF ISONICOTINIC ACID HYDRAZIDE (INAH) ON THE CHICK EMBRYO AND ITS REVERSAL BY PYRIDOXAL*

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PLATE 79

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Evidence has been previously presented to show that lathyrogenic agents increase the solubility of collagen and the fragility of the connective tissues in the chick embryo (1). Assay systems for the detection of lathyrogenic agents have hitherto relied upon loss of weight and morphological changes in the rat as criteria of activity. In the chick embryo however, skeletal deformity can occur in the absence of changes in collagen solubility, and *vice versa* (1). Since the changes in the functional properties of collagen were considered to be more fundamental than changes in morphology, an assay system has been used based on collagen solubility and tissue tensile properties in the chick embryo (2).

The main interest in lathyrism stems from its usefulness as a tool with which to study mechanisms by which collagen, once laid down, may be mobilized, but so far studies on the lathyrogenic nitriles, such as β-aminopropionitrile (BAPN), have failed to elucidate such mechanisms. During an investigation into the nature of chemical groups possessing lathyrogenic activity using the chick embryo assay method, it was repeatedly noted that isonicotinic acid hydrazide ("isoniazid" or INAH) a compound effective against tuberculosis, possessed marked lathyrogenic activity. This report discusses its mode of action, comparing it with that of BAPN, the reversal of the INAH effect by pyridoxal, and the possible relevance of these findings to mechanisms of collagen mobilization.

Materials and Methods

Fertile eggs of the White Leghorn variety were injected *via* the chorio-allantoic membrane with isonicotinic acid hydrazide (INAH)[1] or β-aminopropionitrile (BAPN)[2] in distilled water

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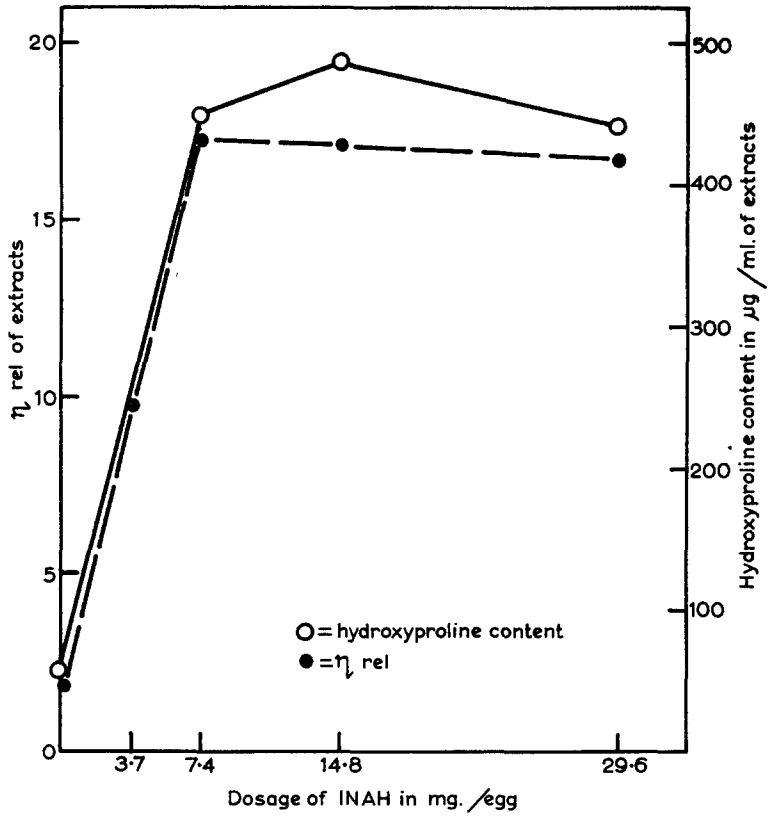
[1] Kindly supplied by Dr. H. L. Morrison of Roche Products, Welwyn Garden City, England.

[2] Kindly supplied by Abbott Laboratories of North Chicago.

through a pinhole drilled into the shell, the hole being then covered with Scotch tape and the egg reincubated at 37°C. The bulk of the experiments on collagen solubility were performed on 14 day embryos and the survivors harvested 2 days later at 16 days. An absolute minimum of 15 embryos was injected for each experiment, the usual dose being 0.054 mM



TEXT-FIG. 1. Dosage effect of INAH on relative viscosity and hydroxyproline content of 1 M sodium chloride extracts of bones from 16 day embryos treated 2 days earlier.

per egg dissolved in 0.1 ml. sterile distilled water. Comparisons between the effects of different compounds were always on an equimolar basis.

Fragility Measurements.—Changes in the tensile properties of the whole embryo were determined as previously described (2) by measuring the average time necessary for a constant stretching load to separate the head from the body of 15 to 20 embryos aged 16 days, which had been injected 2 days earlier with 0.054 mM of the compound being tested. The "fragility index" used was:

$$\frac{\text{Average time for separation, in seconds}}{\text{Average embryo weight in grams}}$$

Comparison of fragility indices was made on eggs of the same batch only, using the same stretching load.

Extraction Procedure.—As previously described (1) tibiae and femora from a minimum of 9 embryos aged 16 days which had been injected 2 days earlier, were carefully dissected on ice, pooled, minced with fine scissors, and extracted with 2 volumes of cold 1 M sodium chloride buffered with phosphate, pH 7.6, ionic strength 0.02, at 5°C., with constant shaking for 24 hours. The extract was separated from the residue by centrifugation in the Spinco model L preparatory ultracentrifuge at 20,000 R.P.M. for 30 minutes and the supernatant passed through a sintered glass filter in the cold.

The relative viscosity of each extract, which gave an index of the amount of collagen in solution (3) was measured in an Ostwald viscometer at 5°C. Occasionally as a check, the hydroxyproline content of the extract was measured since hydroxyproline in the bound form, forms approximately 14 per cent of the dry weight of collagen to which protein it is virtually peculiar.

TABLE I

Table Showing Residual Collagen after Extraction of Bones of 16 Day Embryos Treated 2 Days Earlier with Various Doses of INAH

INAH dose	η rel of bone extracts	Hydroxyproline content of bone residues
mg./egg		*per cent dry weight*
0	1.9	1.97
3.7	11.9	1.78
7.4	21.2	1.43
14.8	24.5	1.93

Analytical Method.—Extracts and residues were analyzed for hydroxyproline content after hydrolysis in sealed tubes at 138°C. for 3 hours in an oil bath, using a modification of the method of Neuman and Logan (4) with a blank omitting peroxide to account for possible interference from other pyrroles (5).

All the data presented are derived from a minimum of 15 embryos in order to minimize individual differences.

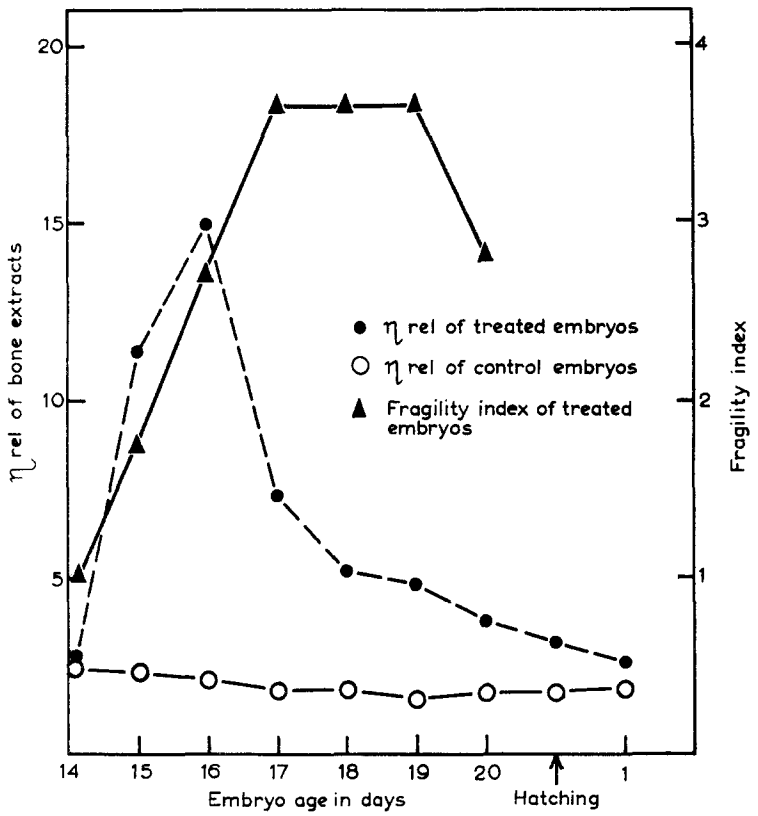
Skeletal Deformities.—In order to show that a compound is capable of producing skeletal deformities in the chick embryo it is necessary to inject the compound at 4 days of incubation instead of at 14 days. A minimum of 15 fertile embryos was therefore injected with the most suitable dose, 0.00253 mM, through a pinhole in the shell and the survivors at 14 days cleared and stained with alizarin in order to clearly demonstrate the skeleton (6).

Synthesis of Hydrazones of INAH.—Pyridoxal hydrazone of INAH was prepared by the mixture of solutions of pyridoxal hydrochloride, INAH, and sodium acetate and the resulting precipitate twice recrystallized from ethanol (m.p. 250°C.). The benzaldehyde and salicylaldehyde hydrazones of INAH were prepared by the addition of ethanolic solutions of the aldehydes to aqueous INAH and the resulting precipitates washed with water and ethanol (benzaldehyde hydrazone m.p. 193°C., salicylaldehyde hydrazone m.p. 248°C.). The streptomycin hydrazone of INAH used was "streptohydrazid"[3] (m.p. 230°C.). The melting points of the above compounds were determined using the Kofler micro-melting point apparatus, in order to establish identity with known compounds where possible, as well as the absence of INAH (m.p. 171°C.) and of pyridoxal (m.p. 165°C.).

[3] Streptohydrazid kindly supplied by the Pfizer Corporation, Harwich, England.

EXPERIMENTAL

INAH Effect.—Increase in the dosage of INAH resulted in increased extractibility of collagen, as measured by the relative viscosity and hydroxyproline content of the extracts (Text-fig. 1), reaching a maximum at 7.4 mg.



TEXT-FIG. 2. Effect of 7.4 mg. of INAH injected at 14 days on subsequent fragility of embryo and extractability of collagen. Fragility Index used here was:

$$\frac{\text{Load required to rupture control embryos}}{\text{Load required to rupture treated embryos}}$$

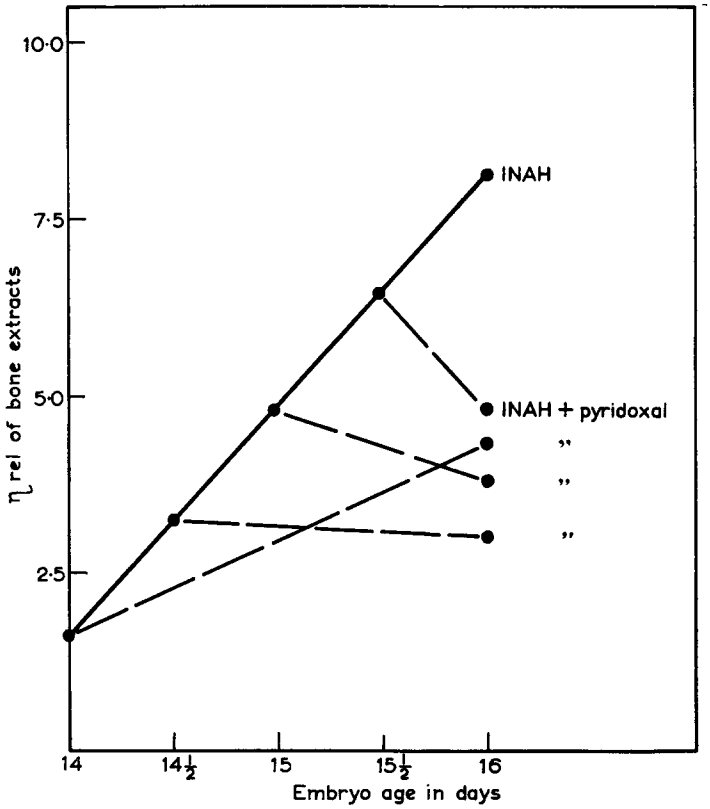
within 20 seconds.

of INAH (0.054 mM). With the exception of the normal extracts, all these extracts gelled at 37°C., going back into solution on subsequent cooling, thus behaving in this respect like normal collagen (7, 8) and like the extracts from BAPN-treated embryos.

Analyses of the collagen content of the residues after extraction (Table I) indicated that the failure of the higher doses of INAH to yield further extractable collagen was not due to its absence from the bones.

The effect of a single dose of 7.4 mg. of INAH as measured by the extract viscosity reached a peak 2 days after injection, slowly subsiding to almost normal at hatching time; changes in embryo fragility followed a parallel course although with a slight lag in time (Text-fig. 2).

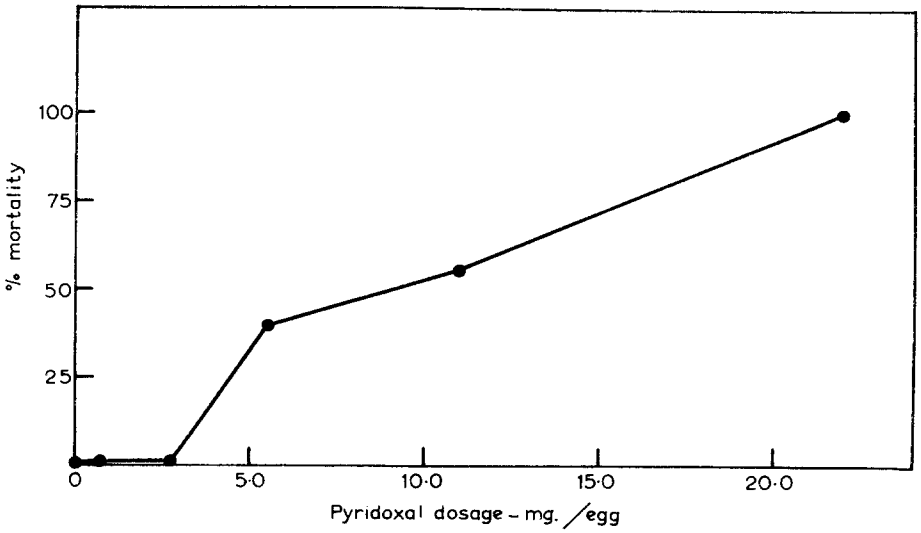
Reversal of INAH Effect with Pyridoxal.—The normal metabolism of vitamin



TEXT-FIG. 3. Effect of time of injection of 11.0 mg. of pyridoxal on extractability of collagen from embryos treated with 7.4 mg. of INAH at 14 days.

B6 in the higher animals has been shown to be inhibited by INAH; it was evident that the injection of an equimolar amount of pyridoxal hydrochloride into an embryo previously treated with INAH diminished the amount of collagen extractable from the bones, irrespective of the time of pyridoxal injection (Text-fig. 3). This reversal effect was more clearly demonstrated when INAH was injected at 14 days, pyridoxal at 15 days, and the bones harvested at 16 days, since the final extract viscosity, although never quite reaching normal, was much lower than that at the time of pyridoxal injection. Pyridoxal itself was found to be quite toxic to the embryo (Text-fig. 4) producing loss of

weight and a very slight diminution in the relative viscosity of bone extracts from that of the normal (Table II). When given with INAH the toxicity of pyridoxal was diminished (Table III) and whereas it still caused loss of weight it nevertheless produced increase in the embryo tensile strength with diminu-



TEXT-FIG. 4. Effect of pyridoxal dosage on mortality rate of 16 day embryos injected 2 days earlier.

TABLE II

Effect of Pyridoxal on Weight and Relative Viscosity of Bone Extracts in 16 Day Embryos Treated 2 Days Earlier

Pyridoxal dosage	Average embryo weight*	η rel
mg./egg	*gm.*	
0	16.4	1.8
0.70	16.8	1.9
2.75	15.3	1.7
11.0	15.1	1.5
22.0	Died	—

* A minimum of 15 eggs was used in each group.

tion in the amount of extractable collagen (Table IV) resulting in functional improvement with respect at least, to collagen.

Comparison of INAH Effect with That of BAPN.—Treatment of 14 day embryos with various doses of BAPN resulted in a threefold increase in the yield of extractable collagen as compared with the effect of an equimolar dose of INAH; the general shape of the curve however, was similar (Text-fig. 5).

The effect of 0.054 mM of BAPN on the embryo fragility and collagen extractability was also similar to, though larger in magnitude than, that of 0.054 mM of INAH (Text-fig. 6).

TABLE III

Effect of Pyridoxal on Mortality Rate of 16 Day Embryos Treated 2 Days Earlier with INAH

Experiment No.*	INAH dose	Mortality with INAH alone	Mortality with INAH and 11.0 mg. pyridoxal/egg
	mg./egg	*per cent*	*per cent*
1	3.7	11	45
2	7.4	0	12
3	7.4	0	30
4	7.4	0	40
5	7.4	10	10
6	7.4	0	10
7	7.4	10	10
8	7.4	0	10
9	14.8	11	11

* A minimum of 15 eggs was used in each experiment.

TABLE IV

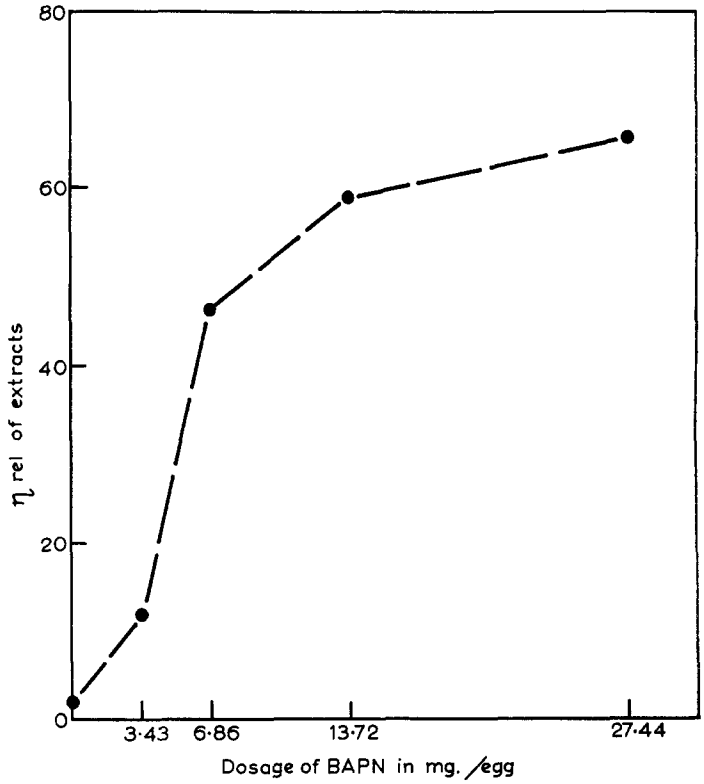
Effect of 11.0 Mg. of Pyridoxal on Embryos Treated with 7.4 Mg. of INAH at 14 Days and Examined at 16 Days

Experiment No.*	Average embryo weight			Fragility index (Time to produce rupture / Average embryo weight)			η rel of bone extracts		
	Control	INAH	INAH + pyridoxal	Control	INAH	INAH + pyridoxal	Control	INAH	INAH + pyridoxal
	gm.	*gm.*	*gm.*						
1	17.3	17.4	16.0	7.0	0.3	5.4	1.8	13.1	3.6
2	16.4	16.8	15.8	7.0	0.4	3.0	1.8	14.7	7.0
3	16.4	16.8	15.2	7.0	0.4	3.3	1.8	14.7	5.4
4	17.0	17.1	16.5	8.0	0.3	5.2	1.9	21.2	6.5
5	17.4	17.6	16.5	—	—	—	1.8	16.4	5.0

* A minimum of 60 eggs was used in each experiment. In Experiments 1, 2, pyridoxal was injected at the same time as INAH; in Nos. 3, 4, and 5, pyridoxal was injected 24 hours after INAH.

Effect of Pyridoxal on BAPN-Treated Embryo.—Unlike INAH, BAPN enhanced the toxicity of pyridoxal (Table V), and whilst the average embryo weight was lowered, just as with INAH, there was however, no improvement in either the tissue tensile strength or in the relative viscosity of bone extracts (Table VI).

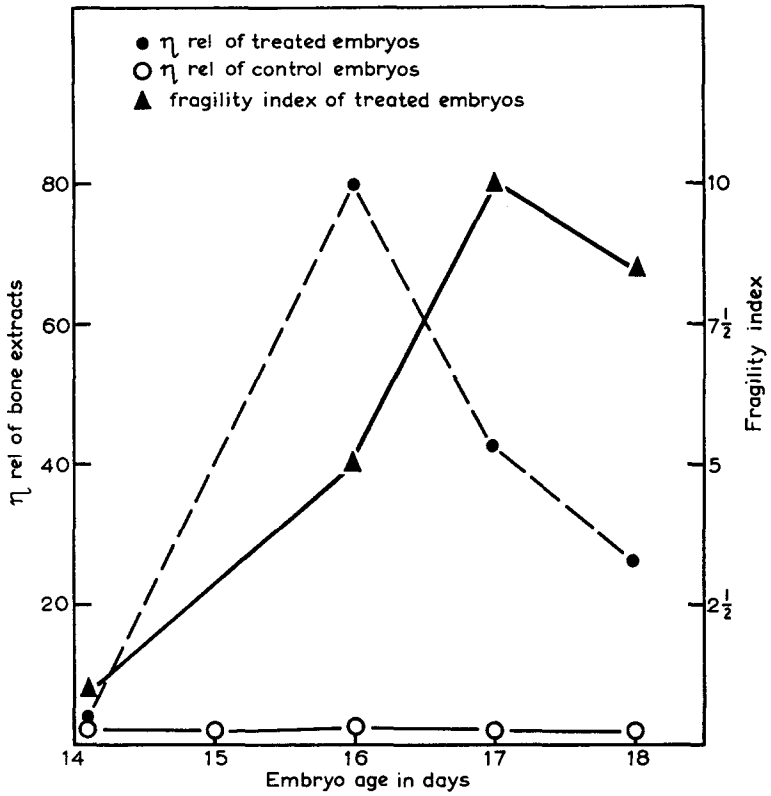
INAH-Pyridoxal Relationship.—The effect of varying either pyridoxal dosage (Text-fig. 7) or that of INAH (Table VII) suggested that interaction between the two compounds was based on a quantitative relationship; furthermore, injection of the two compounds either at two separate sites in the same egg, or at a single site after prior mixing *in vitro*, gave similar results (Table



TEXT-FIG. 5. Dosage effect of BAPN on relative viscosity of 1 M sodium chloride extracts of bones from 16 day embryos treated 2 days earlier.

VIII). Pyridoxine had no effect on the relative viscosity of extracts from INAH-treated embryos, suggesting that the aldehyde group of pyridoxal was essential for reversal to take place. In order to test this, other aldehydes were injected into INAH-treated embryos. It was seen (Text-fig. 8) that whereas streptomycin had no effect, and salicylaldehyde and benzaldehyde a small effect, that of pyridoxal was the greatest; none of the aldehydes injected alone possessed lathyrogenic activity. Moreover, with the exception of streptomycin hydrazone of INAH, very little lathyrogenic activity was produced by the pyridoxal and benzaldehyde hydrazones of INAH, whilst the salicylaldehyde

hydrazone proved to be too toxic a compound. These results suggested a possible mode of action of pyridoxal, by the formation of a Schiff's base with INAH; the fact that both pyridoxal and benzaldehyde could inactivate INAH



TEXT-FIG. 6. Effect of 10.0 mg. BAPN injected at 14 days on the subsequent fragility of embryo and extractability of collagen. Fragility Index used here was:

$$\frac{\text{Load required to rupture control embryos}}{\text{Load required to rupture treated embryos}}$$

within 20 seconds.

by complexing with it *in vitro*, yet only pyridoxal effectively reversed the effect of INAH *in vivo*, pointed to the relatively specific action of pyridoxal.

Do Other Known Nicotinamide-Antagonists Possess Lathyrogenic Activity?—Since INAH has been shown in other systems to be an antinicotinamide agent (9, 10), the effect of 3-acetyl pyridine and 6-amino nicotinamide was tested to see whether they were lathyrogenic; they were inactive.

Are the Degradation Products of INAH Lathyrogenic?—INAH is metabolized

in the human and excreted in the urine as isonicotinic and isonicotinuric acids (11). These two compounds were tested for lathyrogenic activity but were found to be inactive.[4]

Comparison of Deformity-Producing Effects of INAH and BAPN.—When

TABLE V

Effect of Pyridoxal on Mortality Rate of 16 Day Embryos Treated 2 Days Earlier with BAPN

Experiment No.*	BAPN dose	Mortality with BAPN alone	Mortality with BAPN and 11.0 mg. pyridoxal/egg
	mg./egg	*per cent*	*per cent*
1	2.5	16	100
2	5.0	16	90
3	5.0	10	64
4	6.86	10	40
5	6.86	0	88
6	10.0	33	100
7	10.0	50	100

* In Experiments 1, 2, 6, and 7, pyridoxal was injected at 14 days; in Experiments 3, 4 and 5, at 15 days.

A minimum of 15 eggs was used in each experiment.

TABLE VI

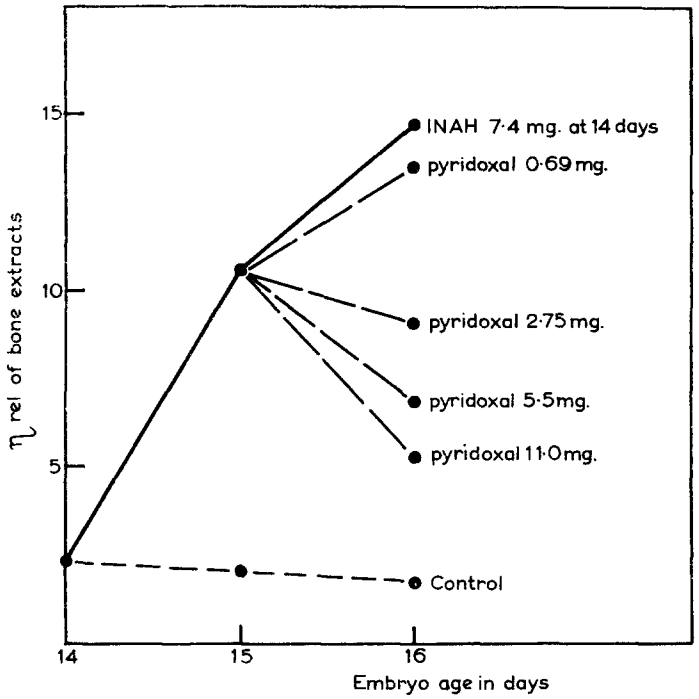
Effect of Pyridoxal (11.0 mg./egg) on Embryos Treated with BAPN at 14 Days and Examined at 16 Days

Experiment*	Average embryo weight			Fragility index (Time to produce rupture / Average embryo weight)			η rel of bone extracts		
	Control	BAPN	BAPN + pyridoxal	Control	BAPN	BAPN + pyridoxal	Control	BAPN	BAPN + pyridoxal
	gm.	*gm.*	*gm.*						
5.0 mg. BAPN/egg	18.3	16.9	15.6	8.0	1.0	0.3	2.0	33.0	30.5
6.86 mg. BAPN/egg	17.7	17.6	15.9	8.0	1.6	1.6	1.7	31.3	38.0

* A minimum of 60 eggs was used in each experiment.

0.00253 mM of BAPN was injected into 4 day embryos and the survivors examined 6 days later, severe tibial bowing and deformity of the beak was observed (1). Associated with the deformities there was a slight, but for such an early embryo, significant increase in the relative viscosity of bone extracts, and a large increase in the embryo fragility; the INAH-treated embryos,

[4] The generous gift of Dr. D. Dearnaley, Biochemistry Department, University of Oxford.



TEXT-FIG. 7. Dosage effect of pyridoxal injected at 15 days, on extractability of collagen from embryos treated with 7.4 mg. of INAH at 14 days.

TABLE VII

Effect on Fragility and Relative Viscosity of Bone Extracts in 16 Day Embryos Treated 2 Days Earlier with Varying Doses on INAH and 11.0 Mg. of Pyridoxal

INAH dosage	Fragility Index*		η rel	
	INAH	INAH + pyridoxal	INAH	INAH + pyridoxal
mg./egg				
0	8.0	—	1.9	—
3.7	4.8	7.0	11.9	4.2
7.4	0.3	5.2	21.2	6.5
14.8	0.1	0.8	24.5	11.6

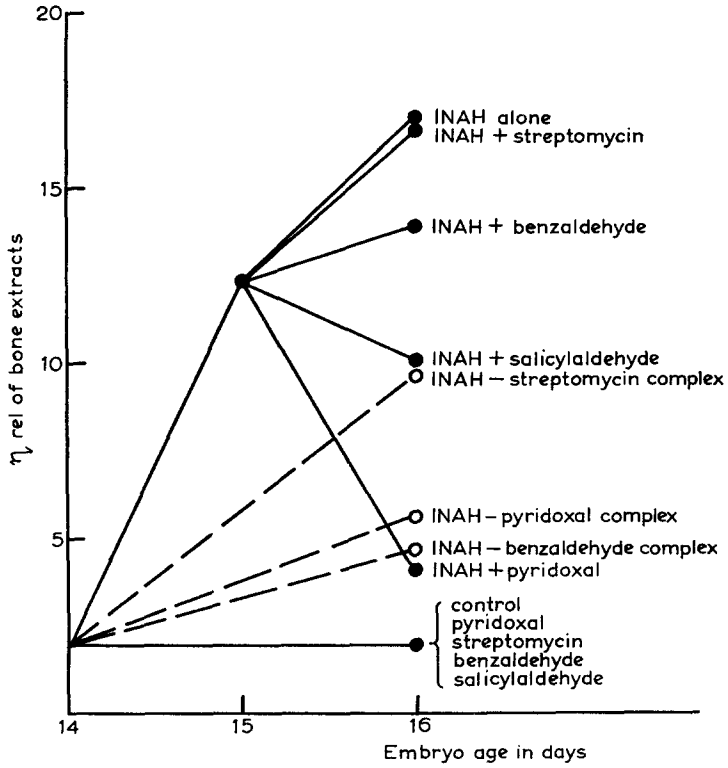
* Fragility index $= \frac{\text{Time for rupture}}{\text{Average embryo weight}'}$, using constant load of 140 gm.

whilst showing no sign of beak or tibial deformity even at 12 times the dosage, (Fig. 1), nevertheless still showed increased relative viscosity of bone extracts and embryo fragility (Table IX), suggesting that the two phenomena are possibly dissociated.

TABLE VIII

Comparison of in Vivo and in Vitro Effects of INAH-Pyridoxal Admixture, in 16 Day Embryos Treated 2 Days Earlier

INAH dosage	η rel of bone extracts		
	INAH alone	INAH and pyridoxal 11.0 mg.: injected at separate sites *in vivo*	INAH and pyridoxal 11.0 mg.: injected after mixing *in vitro*
mg./egg			
0	1.9	—	—
3.7	11.9	4.2	3.2
7.4	21.2	6.5	5.0
14.8	24.5	11.6	12.2



TEXT-FIG. 8. Comparison of (*a*) the effects of various aldehydes on INAH-treated embryos and (*b*) the effects of INAH with those of various aldehyde-INAH complexes.

DISCUSSION

Although both INAH and BAPN are lathyrogenic compounds, their modes of action appear to differ; BAPN produces skeletal deformities, but INAH does not; BAPN treatment results in a large amount of collagen becoming soluble, whereas INAH releases much less, and whilst pyridoxal has no effect on the former, it reverses the latter.

TABLE IX

Comparison of Effects of BAPN and INAH-Treated Embryos Injected at 4 Days with 0.00253 mM and Examined at 10 Days

Dosage*	Average weight of embryos	Fragility index (Time to produce rupture / Average embryo weight with constant load of 5 gm.)	η rel of bone extracts
mg.	*gm.*		
0	1.85	40+	1.9
BAPN 0.32 mg.	2.00	0.5	3.1
INAH 0.25 mg.	2.03	40+	2.3
INAH 0.50 mg.	2.20	40+	2.7
INAH 2.00 mg.	2.00	32	2.9
INAH 4.00 mg.	1.83	13	2.5

* A minimum of 15 eggs was used in each experiment.

In considering firstly the INAH effect, two obvious possibilities emerge; firstly INAH may act by combining to form a Schiff's base with the embryo's available pyridoxal and so produce a deficiency. There is fairly good evidence that pyridoxal will combine with specific groups of certain biologically active compounds whose activity is consequently lost; examples of this include the reversal in the rat of the toxic effect of *l*-penicillamine by pyridoxal with which it combines chemically (12, 13), the loss of biological activity of many acid hydrazides in the presence of vitamin B6 (14), and the reversal by pyridoxal of the *in vitro* inhibition of *Mycobacterium tuberculosis* induced by INAH (15). In the latter case, the aldehyde group was shown to be essential for reversal,

since neither pyridoxine nor pyridoxamine were effective. A second more remote possibility is that INAH may displace the nicotinamide portion of diphosphopyridine nucleotide (DPN) as demonstrated in another system by Zatman and his colleagues (9) who isolated and characterized the DPN-INAH analogue (10).

In considering the reversal of the INAH effect by pyridoxal, it is not yet apparent whether it acts simply by curing pyridoxal deficiency should it exist, or by combining with as yet unmetabolized INAH and so speeding the natural healing.

The small amount of lathyrogenic activity of the pyrodoxal hydrazone of INAH may have been due to the small amount of INAH, which, it has been suggested, is released by the *in vivo* hydrolysis of the complex (16).

No direct evidence has been presented here to show that the DPN system is involved; indeed, if it were, one might have expected antinicotinamide agents other than INAH to be lathyrogenic, but this was not found to be the case. However the indirect evidence appears sufficiently strong to warrant consideration of the possibility that the mobilization of collagen in fibrillar form is mediated through an enzyme system, particularly since knowledge concerning collagen-mobilizing systems is scanty (17).

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SUMMARY

When applied to the chorio-allantoic membrane of the chick embryo, isoniazid was shown to produce an increase in the fragility of the embryo and in the amount of collagen which was extractable from the bones with cold 1 M sodium chloride. The administration of pyridoxal reversed these phenomena almost completely.

The effect of isoniazid differed from that of β-aminopropionitrile in that the latter was of greater magnitude, and was not affected by pyridoxal; whereas β-aminopropionitrile caused skeletal deformities, isoniazid even at 12 times the concentration produced no deformities.

The aldehyde group of pyridoxal was shown to be necessary for its interaction with isoniazid.

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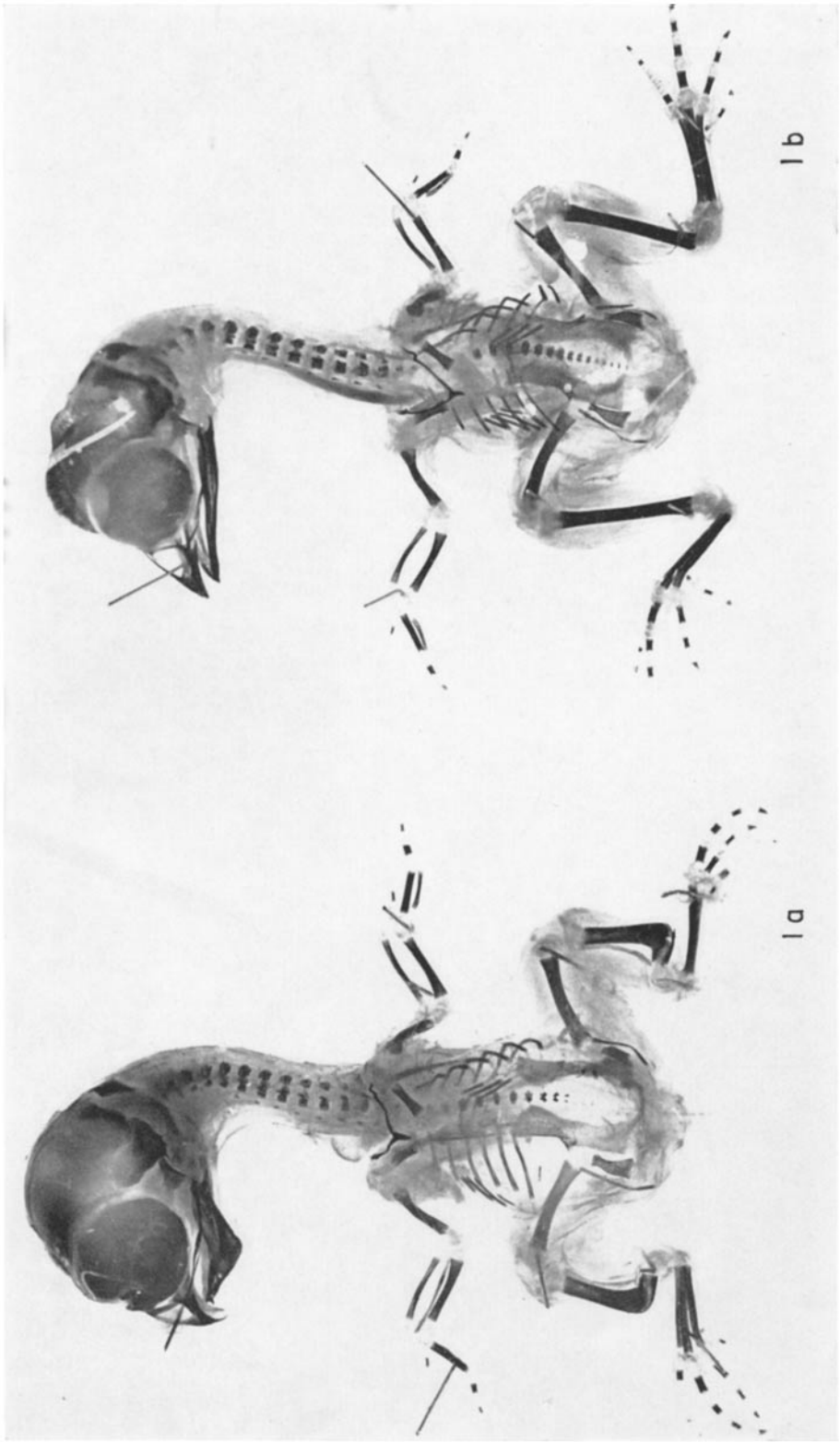
EXPLANATION OF PLATE 79

FIG. 1. Effect on skeleton of 0.00253 mM injected at 4 days and examined at 14 days.

1 a. BAPN-treated embryo showing bowed tibiae and deformed beak.

1 b. INAH-treated embryo showing absence of deformities.



(Levene: Isonicotinic acid hydrazide)