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PURINE METABOLISM IN THE CHICK EMBRYO: INFLUENCE OF 2-SUBSTITUTED THIADIAZOLES*

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Abstract—2-Ethylamino-1,3,4-thiadiazole, a compound that inhibits the growth of certain transplanted animal tumors is capable of increasing the synthesis of purines *de novo* in the developing chick embryo. The effect of 2-ethylamino-1,3,4-thiadiazole can be prevented by nicotinamide and by the nicotinamide antagonists, 3-acetylpyridine and 6-aminonicotinamide. The effect of 2-ethylamino-1,3,4-thiadiazole can be enhanced by 3,3-dimethyl-1-phenyltriazene, a compound that is otherwise similar to 6-aminonicotinamide in its effects on the developing chick embryo.

It is suggested that different tissues or biochemical systems have varying requirements for nicotinamide or are less discriminating in acceptance of substitutes.

IT HAS been shown previously that compounds which are known to influence purine biosynthesis in several biologic systems are capable of influencing the accumulation of uric acid in the developing chick embryo.[1–3]

2-Ethylamino-1,3,4-thiadiazole (EA-TDA) and several related compounds were found by Oleson *et al.*[4] and Burchenal and Dagg[5] to inhibit the growth of certain transplanted animal tumors. The antitumor effects of these compounds could be prevented by nicotinamide. Trials of the compounds in human subjects with advanced neoplastic diseases have produced no regression of tumors. However, it has been found that the 2-substituted thiadiazoles do produce an increase in the synthesis of uric acid *de novo* (a 'uricogenic' effect) in man and this, too, can be prevented by nicotinamide or nicotinic acid.[6] The present studies of these compounds in the developing chick embryo were undertaken in order to clarify their effects on uric acid synthesis and to attempt to define their relation to nicotinamide metabolism.

MATERIALS AND METHODS

Fertilized White Leghorn eggs of known days of incubation were used. At least fifteen eggs were used in each experimental group and a separate control group of equal size was used for each experiment. On the eighth day of incubation the appropriate compound, dissolved in normal saline, was injected into the yolk sac by tuberculin syringe and hypodermic needle. The aperture was sealed with paraffin. Eggs were candled daily to determine viability. At the time of sacrifice (usually 14 days in these experiments), the shells were cracked and the entire contents carefully transferred to Petri dishes. The embryos were freed from their membranes, weighed,

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and inspected for abnormalities. The embryos with the remainder of the content of the egg were then homogenized in a Waring blender in groups of three to five, transferred to volumetric flasks and diluted to volume. Small amounts of caprylic alcohol were used to prevent foaming. Aliquots of each pool were frozen until analyzed. It has been established that storage in the frozen state for up to three months does not influence the uric acid content.

The concentration of uric acid was determined colorimetrically by a modification of the method of Archibald[7] employing the enzyme, uricase.* This method has been described in detail in a previous publication.[8]

The structural formulae of the compounds used are shown in Fig. 1.†

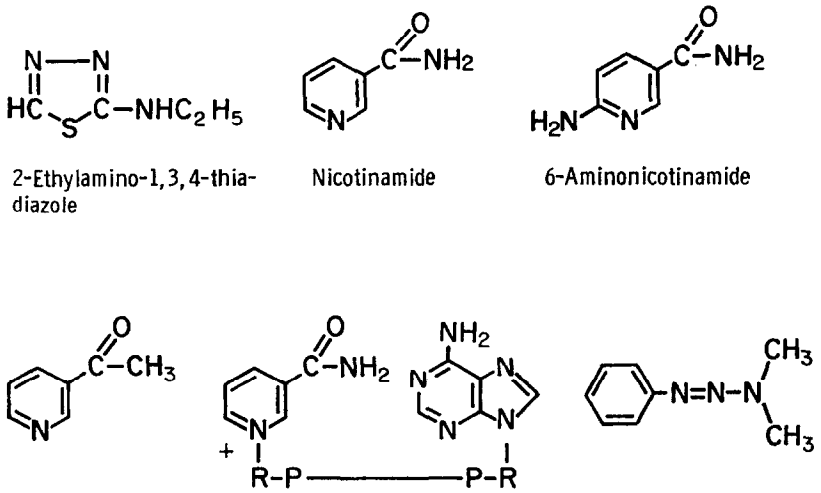

FIG. 1. Structural formulae of some compounds related to the effects of the 2-substituted thiadiazoles.

For analysis of nicotinamide-adenine dinucleotide (NAD), the embryos were inspected for abnormalities, weighed, and the embryos alone then frozen singly on dry ice. They were kept frozen until the analyses were performed, when they were placed in a Waring blender and homogenized with trichloroacetic acid. The supernatant was neutralized and analysed for NAD with yeast alcohol dehydrogenase, by the method of Racker.[9]

For determination of the incorporation of ^{14}C-labeled sodium formate into purines, EA-TDA was injected into eggs on day 8 of incubation, and 10 μc of ^{14}C-sodium formate (7·5 mc/mmole), was injected into treated and untreated eggs on day 12.

* Uricase was purchased from the Worthington Biochemical Corp., Freehold, N.J. (Worthington Uricase) or from Henley & Co., New York, N.Y. (Uricase Leo, Stero Kems Co., Copenhagen, Denmark).

† 2-Ethylamino-1,3,4-thiadiazole was obtained as a dry powder from Eastman Kodak Company, Rochester, N.Y.; nicotinamide as a dry powder from Nutritional Biochemicals Corp., Cleveland, Ohio; 6-aminonicotinamide from Frank Horner, Ltd., Toronto, Canada; 3-acetylpyridine from Aldrich Chemical Co., Milwaukee, Wis.; 3,3-dimethyl-1-phenyl triazine from Merck, Sharpe & Dohme, West Point, Pa.

The eggs were opened at various intervals after the injection of ^{14}C-formate and extracted with 4 vol. boiling ethanol. The extract was lyophilized and redissolved in 1 ml of water. A 40-μl aliquot was used for 2-dimensional descending chromatography with phenol–water in the first dimension and butanol–proprionic acid–water in the second. Radioautograms were prepared by exposing Ansco X-ray film to the air-dried chromatograms for 2–8 weeks. The radioactive spots were eluted from the chromatogram and identified spectrophotometrically and by Rf in comparison with known standards and the specific activities determined.*

RESULTS

The teratogenic effects of the various compounds employed in these studies have been reported in previous communications from these and other laboratories.[10, 11] These effects fell into three classes, which are shown diagrammatically in Fig. 2.

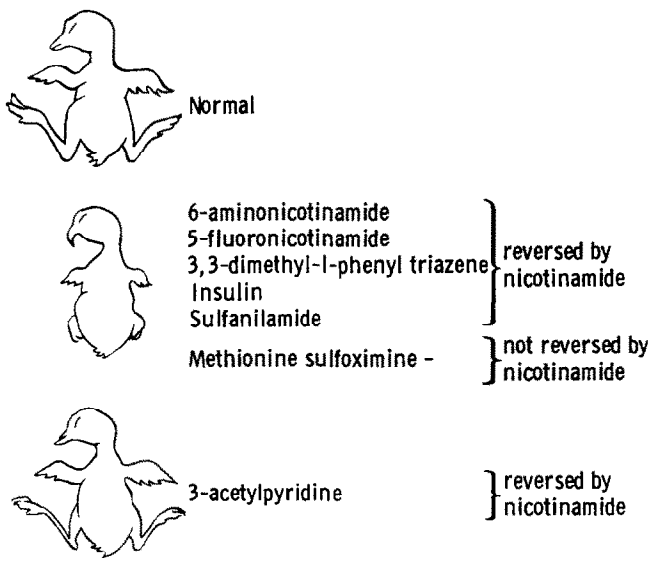

FIG. 2. Top: normal 14-day embryo. Next is an abnormal embryo with parrot beak and micromelia. These deformities are produced by some of the nicotinamide analogs as well as by some chemically unrelated substances, and the effects can be prevented by nicotinamide. The same abnormality is produced by methionine sulfoximine but the effects of that compound are not prevented by nicotinamide. 3-Acetylpyridine, which is also an analog of nicotinamide, produces a different type of defect with shortening of the upper beak and marked thinning of the legs due to inhibition of muscle development. 2-Ethylammo-1,3,4-thiadiazole (EA-TDA) produces no consistent abnormality.

(1) No abnormalities were produced by EA-TDA. (2) Micromelia and parrot beak were produced by 6-amino-nicotinamide, 5-fluoronicotinamide, and a group of unrelated compounds, all of which can be reversed by nicotinamide and also by methionine sulfoximine, which is not reversed by nicotinamide. (3) Thin legs and short upper beak were produced by 3-acetylpyridine and reversed by nicotinamide.

* The author is indebted to Dr. Glynn Wheeler, Southern Research Institute, for performing and interpreting the chromatograms and radioautograms using the methods described by Tomisek *et al.* A. J. TOMISEK, H. J. KELLY and H. E. SKIPPER. Chromatographic Studies of Purine Metabolism. I. The Effect of Azaserine on Purine Biosynthesis in *E. coli* Using Various C^{14}-labeled Precursors. *Arch. Biochem.* **64**, 437 (1956).

In the chick embryo, as in man, EA-TDA caused a definite increase in the production of uric acid at doses from 0·5 mg/egg to 10 mg/egg. This was not associated with any teratogenic effects, and there was no inhibition of growth of the surviving embryos. At higher doses the mortality was too high for evaluation of the uricogenic effect. As in man, the analogs substituted in the 2-position only (2-amino-1,3,4-thiadiazole and 2-acetylamino-1,3,4-thiadiazole) are active in causing an increase in uric acid production, and those substituted in both the 2 and 5 positions (2,5-diamino-1,3,4-thiadiazole and 2-acetylamino-1,3,4-thiadiazole, 5-sulfonamide (Diamox)) are inactive (Table 1).

The relation of EA-TDA to nicotinamide was examined in a series of experiments. Fig. 3 shows that nicotinamide alone produces no change in uric acid content but,

TABLE 1. INFLUENCE OF 2-SUBSTITUTED THIADIAZOLES ON URIC ACID PRODUCTION*

Compound	Dose,† mg/egg	Mortality	Uric acid, mg/egg	Weight, g/embryo
2-Ethylamino-1,3,4-thiadiazole	10	3/25	22·7 ± 2·7	7·6 ± 0·6
Saline control		0/15	11·4 ± 1·2	8·7 ± 0·7
2-Amino-1,3,4-thiadiazole	1	2/12	33·0 ± 1·8	6·6 ± 1·4
Saline control		1/10	13·1 ± 1·8	7·8 ± 1·0
2-Acetylamino-1,3,4-thiadiazole	1	4/12	26·2 ± 1·3	8·5 ± 0·6
Saline control		3/12	12·5 ± 1·3	8·9 ± 0·7
2,5-Diamino-1,3,4-thiadiazole	20	2/15	11·9 ± 0·8	6·3 ± 0·9
Saline control		1/10	13·1 ± 1·8	7·8 ± 1·0
2-Acetylamino-1,3,4-thiadiazole-5-sulfonamide	5	1/7	10·1 ± 2·2	7·0 ± 1·2
Carboxymethyl cellulose control		1/9	11·6 ± 0·2	8·7 ± 0·6

* Values given are ± S.D. Compound(s) injected on 8th day. Eggs opened on 14th day. The controls were simultaneously injected with the vehicle alone.

† The dose reported here is that which produced mortality not significantly greater than that of the vehicle-injected control.

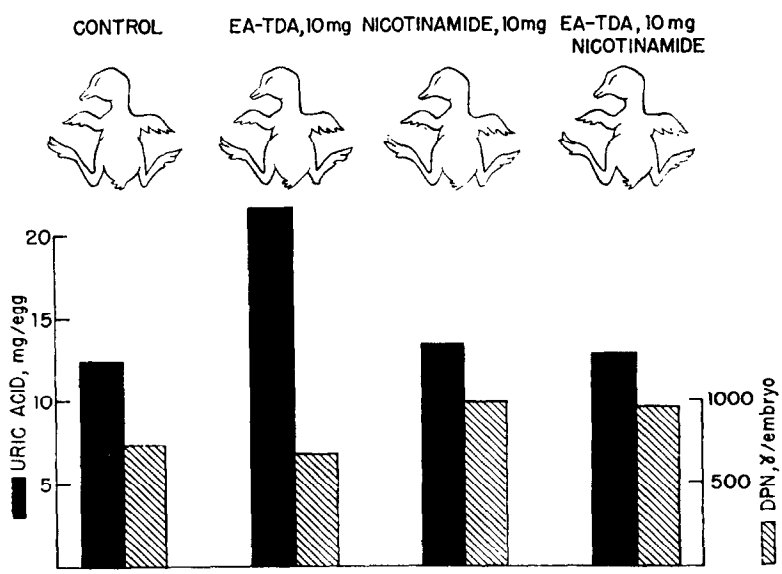

FIG. 3. Effects of 2-ethylamino-1,3,4-thiadiazole and nicotinamide on uric acid and NAD (DPN) content of 14-day chick embryos. Compound(s) injected on 8th day of incubation.

when given in combination with EA-TDA, it inhibits the effect of EA-TDA on uric acid production. EA-TDA did not influence the NAD content of the embryo. However, nicotinamide, which is one component of the NAD molecule, produced a moderate increase in NAD, and this was not influenced by EA-TDA.

When a smaller dose of nicotinamide (1 mg/egg) was used, the EA-TDA effect was also inhibited. At this dose nicotinamide produced no effect on NAD content. DL-Tryptophan, a nicotinamide precursor, also inhibited the EA-TDA effect at a dose (5 mg/egg) which did not influence NAD content. NAD itself could inhibit the EA-TDA effect only at a large dose (10 mg/egg). At all doses of NAD up to 10 mg/egg there was no significant increase in the NAD content of the embryos, suggesting that the administered NAD was broken down and merely contributed enough nicotinamide (approximately 2·5 mg when 10 mg NAD was used) to block the EA-TDA effect.

A series of experiments was done in which the incorporation of ^{14}C-labeled sodium formate into the alcohol-soluble purines of the developing chick embryo was determined with and without EA-TDA. The most marked differences between the treated and untreated eggs were seen in the specimens sacrificed 4 hr after injection of ^{14}C. Those values are shown in Table 2. It is apparent that a two- to four-fold increase

TABLE 2. INFLUENCE OF 2-ETHYLAMINO-1,3,4-THIADIAZOLE* ON INCORPORATION OF ^{14}C-FORMATE INTO PURINES AND AMINO ACIDS IN THE DEVELOPING CHICK EMBRYO

Compound	Control, cps	Treated, cps
Hypoxanthine	<2·0	6·34
Inosine	2·99	8·11
Xanthine		<2·0
Uric acid	2·53	8·02
Allantoin		3·27
Serine	<2·0	4·83
Total	9·52	32·57

* EA-TDA, 10 mg/egg injected on 8th day; ^{14}C-sodium formate, 10 μc/egg, injected on 12th day and egg opened 4 hr later.

occurred in the incorporation of ^{14}C into all the identifiable alcohol-soluble purines as well as into allantoin and serine. This indicates that EA-TDA, in this experiment, produced an increase in purine biosynthesis *de novo*.

In view of the demonstrated ability of EA-TDA to increase purine biosynthesis, a series of experiments was done in which EA-TDA was injected into the egg in combination with other agents known to be inhibitors of purine synthesis *de novo*. These compounds were the folic acid antagonist, 4-amino-N^{10}-methylpteroylglutamic acid (methotrexate, MTX) and the glutamine antagonists, 0-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (DON). The studies, summarized in Table 3, show that these compounds are capable of inhibiting the augmented uric acid production caused by EA-TDA, although DON fails to inhibit normal uric acid production in the chick embryo and azaserine inhibits it only slightly.[1, 2]

6-Mercaptopurine (6 MP), an inhibitor of purine nucleotide biosynthesis and interconversion, failed to affect the uricogenic activity of EA-TDA in the chick embryo.

Following the observation of Oettgen *et al.*[12] that 6-aminonicotinamide (6-AN) prevented the antileukemic effect of 2-amino-1,3,4-thiadiazole in transplanted mouse leukemia, and in order to explore the interrelations of some of these nicotinamide-related substances, the effects of various combinations were studied in the chick embryo. 6-Aminonicotinamide is an analog of nicotinamide which has been shown

TABLE 3. EFFECT OF INHIBITORS OF PURINE SYNTHESIS ON THE URICOGENIC EFFECT OF EA-ETA

EA-TDA,* mg/embryo	Inhibitor*	Mortality	Uric acid, mg/embryo	Weight, g/embryo
0	0	4/10	11·8 ± 0·2	8·5 ± 0·4
10	0	4/15	27·6 ± 2·9	8·2 ± 0·8
10	MTX, 8 μg	59/65	8·9 ± 2·6	7·8 ± 1·1
0	0	1/12	10·6 ± 0·9	7·9 ± 0·6
10	0	6/12	26·2 ± 2·5	8·0 ± 0·6
10	AZS, 0·5 mg	5/25	10·0 ± 2·9	6·2 ± 1·0
0	0	1/12	10·6 ± 0·9	7·9 ± 0·6
10	0	6/12	26·1 ± 2·5	8·0 ± 0·6
10	DON, 0·1 mg	14/25	13·2 ± 2·7	4·1 ± 0·6

* Compound(s) injected on 8th day of incubation. Eggs opened on 14th day.

to be incorporated into the NAD molecule *in vitro* by exchange with the nicotinamide moiety.[13] It is toxic to the 8-day chick embryo at a dose of 0·07 mg, and its effects can be prevented by nicotinamide.[14] As shown in Fig. 4, 6-AN alone had no effect on uric acid production at a dose that caused characteristic developmental anomalies

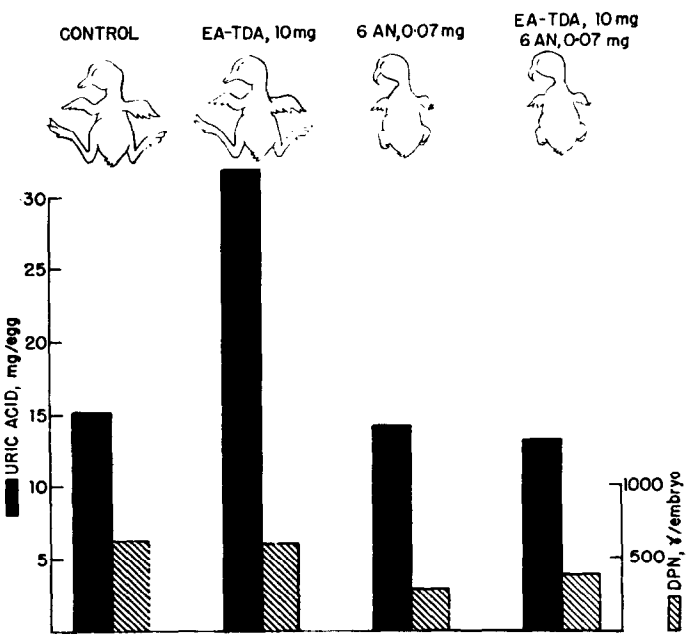

FIG. 4. Effects of 2-ethylamino-1,3,4-thiadiazole and 6-aminonicotinamide on uric acid and NAD (DPN) content of 14-day chick embryos. Compound(s) injected on 8th day of incubation.

and a decrease in NAD content. When used in combination with EA-TDA, it inhibited the uricogenic effect of EA-TDA. This inhibition occurred along with the decrease in NAD content and the teratogenic effects that were produced by 6-AN alone. It therefore appears that 6-AN, itself a potent nicotinamide antagonist, is able to substitute for nicotinamide in blocking the effects of EA-TDA. The dose of 6-AN required is only 0·07 mg, approximately 1/8 of the lowest dose of nicotinamide (approximately 0·5 mg) that is able to block the EA-TDA effect.

The results of combination studies with 3-acetylpyridine (3-AP) and EA-TDA (Fig. 5) were similar to those with 6-AN and EA-TDA. Like 6-AN, 3-AP is extensively

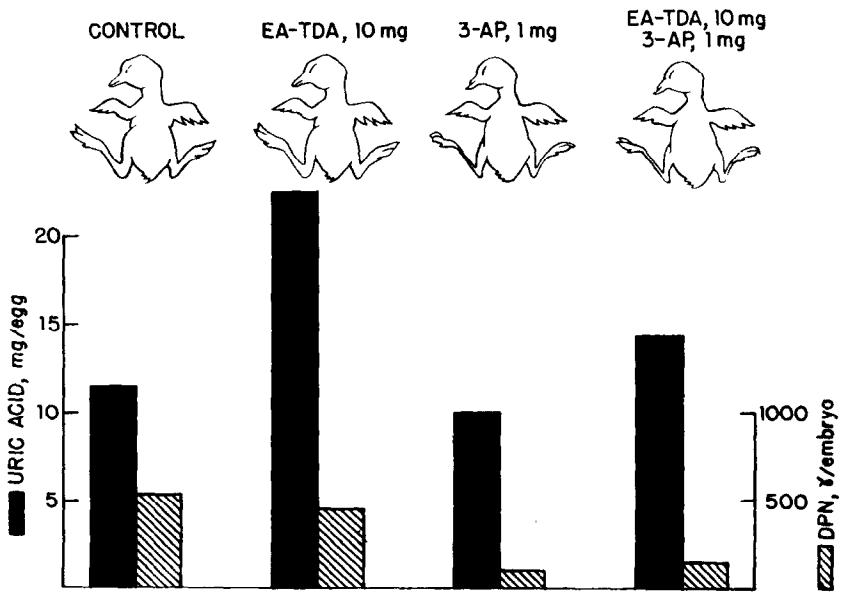

FIG. 5. Effects of 2-ethylamino-1,3,4-thiadiazole and 3-acetylpyridine on uric acid and NAD (DPN) content of 14-day chick embryos. Compound(s) injected on 8th day of incubation.

incorporated into the NAD molecule *in vitro*,[15] it is toxic to the developing chick embryo[11] (although the deformities it produces are different from those caused by 6-AN), and its effects in the chick embryo are prevented by nicotinamide. 3-AP alone did not influence uric acid production, but it was able to modify the effects of EA-TDA while producing its characteristic developmental abnormalities and a reduction in NAD content. It appears that this nicotinamide antagonist is also able to substitute for nicotinamide in blocking the effects of EA-TDA.

The ability to substitute for nicotinamide in blocking EA-TDA effects is not common to all nicotinamide analogs. Figure 6 shows the results of an experiment in which 5-fluoronicotinamide (5-FN) was given in combination with EA-TDA. Although 5-FN produced teratogenic effects in the chick embryo which were identical with those caused by 6-AN, it failed at toxic doses to inhibit the EA-TDA effect on uric acid production. 5-FN, in contrast to 6-AN, did not cause a decrease in NAD content of the embryo.

A group of structurally unrelated compounds, including insulin and sulfanilamide, produces teratogenic effects in the developing embryo which are similar to those caused by 6-AN (Fig. 2), and these too can be prevented by nicotinamide. These compounds were ineffective in blocking the effects of EA-TDA. The methionine analog, methionine sulfoximine (MSO), also produces parrot beak and micromelia in the developing embryo. The effects of this compound, however, are not prevented by nicotinamide, and MSO does not block the EA-TDA effects.

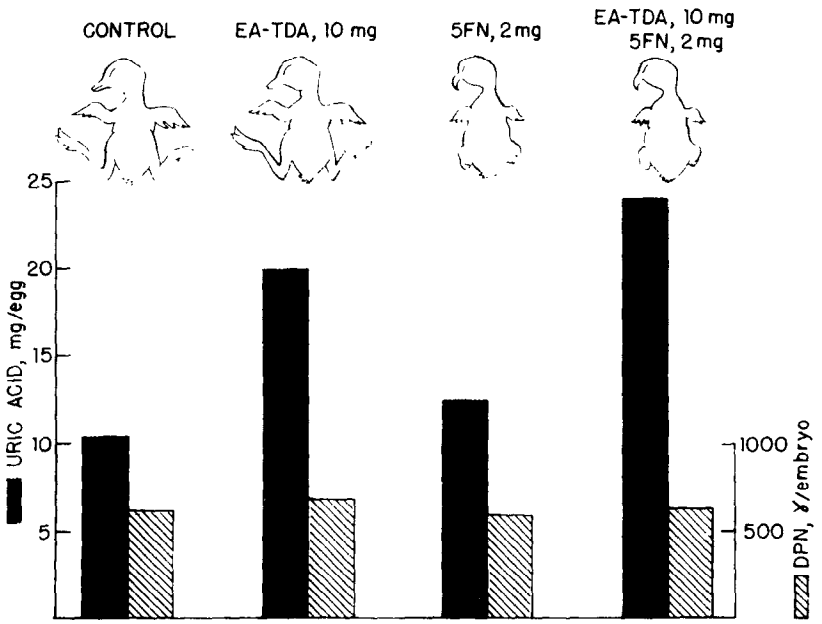

FIG. 6. Effects of 2-ethylamino-1,3,4-thiadiazole and 5-fluoronicotinamide on uric acid and NAD (DPN) content of 14-day chick embryos. Compound(s) injected on 8th day of incubation.

Another compound which is structurally unrelated to nicotinamide, 3,3-dimethyl-1-phenyltriazene (DMPT), has been found to have still another relationship to EA-TDA. This compound when used alone produces parrot beak and micromelia and, as is the case with other substances producing these deformities, its effects are blocked by nicotinamide. When DMPT was used in combination with EA-TDA, it was found to be extremely toxic. Combination studies (Fig. 7) were done with small doses of each compound. It is evident that mutual synergism exists between the two compounds. The administration of an ineffective dose of EA-TDA together with an ineffective dose of DMPT evoked both the uricogenic effect of EA-TDA and the teratogenic effect of DMPT.

DISCUSSION

The mechanism by which the 2-substituted thiadiazoles increase the production of uric acid is not known. Studies of these compounds in man, employing ^{14}C-labeled purine precursors, have clearly shown that they cause an increase in synthesis of uric

acid *de novo*.[6, 16] In the chick embryo, as in man, the evidence indicates that the increased uric acid production is due to increased *de novo* synthesis of purines. The weight of embryos treated with EA-TDA is normal, indicating that increased tissue breakdown is not responsible for the increase in uric acid.

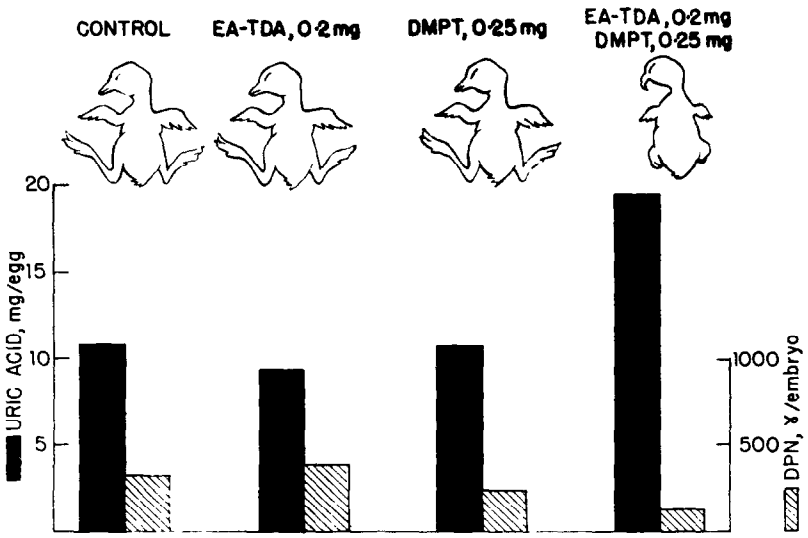

FIG. 7. Effects of 2-ethylamino-1,3,4-thiadiazole and 3,3-dimethyl-1-phenyltriazene on uric acid and NAD (DPN) content of 14-day chick embryos. Compound(s) injected on 8th day of incubation.

Although the point at which EA-TDA affects the biosynthetic pathway has not been determined, further evidence that it augments synthesis *de novo* is seen in the fact that the increased uric acid production is blocked by methotrexate, azaserine, and DON—compounds whose major sites of action have been localized. Methotrexate is known to inhibit the incorporation of single carbon fragments into the 2 and 8 positions of the purine ring. It has been shown in this laboratory that methotrexate markedly inhibits normal uric acid synthesis in the chick embryo[1, 2] as well as opposing the uricogenic effect of EA-TDA. Azaserine and DON are glutamine antagonists which prevent the incorporation of nitrogen into the 3 position of the purine ring, and although DON fails to inhibit normal uric acid production in the chick embryo and azaserine does so only weakly, these compounds too are capable of inhibiting the uricogenic effect of EA-TDA in the embryo.

6-Mercaptopurine is a purine analog, the ribonucleotide of which is known to inhibit the conversion of inosinic acid to adenylic acid.[17] It has also been shown in an *in vitro* system to block an early step in purine synthesis, the conversion of phosphoribosyl pyrophosphate to phosphoribosyl amine.[18] Although 6 MP has been shown to block the uricogenic effect of EA-TDA in man,[19, 20] it has consistently failed to do so in the chick embryo. This parallels the situation that exists in regard to normal purine biosynthesis in the chick embryo, which is inhibited by methotrexate and to some extent by azaserine but not by 6 MP. It is not known what differences in purine biosynthesis between the chick embryo and man are responsible for the difference in response of the uricogenic effect of EA-TDA to 6 MP inhibition. However, the fact

that such differences exist suggests that avian and human purine biosynthesis cannot be considered identical and that qualitative or important quantitative differences may exist between them.

The final evidence that the increase in uric acid production is due to an increase in synthesis *de novo* is the marked and rapid accentuation of incorporation of ^{14}C-formate into alcohol-soluble purines and uric acid in EA-TDA-treated eggs as contrasted with untreated eggs.

It has been postulated that the mechanism of the EA-TDA effect is in blocking the incorporation of newly synthesized purine molecules into purine polynucleotides and/or nicotinamide-containing coenzymes. This could stimulate a compensatory exaggeration of purine biosynthesis *de novo*, resulting in the observed increase in uric acid accumulation. Such a metabolic block has not been demonstrated. Quantitative measurements of NAD content of embryos treated with EA-TDA have shown no abnormalities, although embryos treated with other compounds which are known to be nicotinamide antagonists (i.e. 6-AN and 3-AP) are low in NAD content. Ciotti *et al.*[21] have shown that EA-TDA can exchange sparingly with the nicotinamide moiety of NAD *in vitro* but not *in vivo*. Therefore, although EA-TDA may function by conversion to a NAD analog, this has not been proved.

Although it has been shown by Kaplan *et al.*[22] that 3-AP can be converted to nicotinamide *in vivo*, it seems unlikely that this is the mechanism by which 3-AP and 6-AN antagonize the EA-TDA effect, since they retain their teratogenic properties *while* antagonizing EA-TDA. Furthermore, the dose of 6-AN that antagonizes EA-TDA is so small that even if it were entirely converted to nicotinamide the dose would be far short of the amount of nicotinamide required to antagonize EA-TDA. Although Dietrich *et al.*[23] have demonstrated quantitative differences in the effect of 6-AN on various tissues in the mouse, what is seen here is a divergent and qualitatively different effect on two aspects of growth-anatomic development and purine synthesis.

An additional unexplained facet of the activities of EA-TDA is the mutual potentiation between EA-TDA and DMPT. The mechanism by which DMPT produces its teratogenic effect (reversible by nicotinamide), its effect in lowering NAD content, and its minor antitumor activity is unknown. This combination of effects leads to the hypothesis (not yet tested) that DMPT may function as a nicotinamide antagonist, substituting for nicotinamide in NAD synthesis.

The relationships between these substances appear very complex and cannot be simply summarized. It appears that there is a large group of compounds, structurally diverse and with a variety of biologic effects, which are all antagonized by nicotinamide and therefore might be considered, in a very general sense, 'nicotinamide antagonists'. One of these, 2-ethylamino-1,3,4-thiadiazole is also antagonized by other nicotinamide antagonists, among which are some (6-AN and 3-AP) which have been shown to function as antimetabolites to nicotinamide.

These facts suggest that some metabolic processes that require nicotinamide or nicotinamide-containing coenzymes are less discriminating than others in accepting a substitute. This may be because nicotinamide functions by different mechanisms in these different processes or because there are quantitative variations from one tissue to another in their requirement for nicotinamide. Therefore, a potent nicotinamide antagonist may produce a characteristic derangement while in the same

organism it is serving as a nicotinamide substitute in opposing the effect of a different type of antagonist.

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