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# A Study of Cartilaginous Fusions, DNA Synthesis, and Protein Synthesis in Embryonic Rat Limbs After Injection of Aminothiadiazole

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*ABSTRACT* Sprague-Dawley rats were injected with 2-amino-1,3,4-thiadiazole (ATDA), 100 mg/kg ip, on one of days 10–14 of pregnancy (sperm day = day 0). A supplementary ip injection of nicotinamide (N), 100 mg/kg, was administered to some rats immediately after the ATDA injection on day 12. Embryonic deaths, reduction limb malformations, and depressed fetal weights were attributable to ATDA treatment, but N coadministration was protective. Fetuses from rats dosed with ATDA or ATDA+N on day 12 were recovered on day 18 or 19 and their skeletons stained with methylene blue. Abnormal cartilaginous continuities or "fusions" between the various cartilage skeletal elements were present in 99% of limbs in the ATDA-treated group. Extent of the fusions varied widely. In the forelimb, fusions occurred only in the carpus and paw, whereas in the hindlimb, there were many abnormal fusions of the tibia and fibula in addition to the fusions in the tarsus and paw. Cotreatment with N prevented fusions. Other rats treated on day 12 were sacrificed 4, 10, 24, 48, and 72 hours later. Embryonic limbs were cultured in vitro for two hours in a medium containing $^{3}$H-thymidine or $^{14}$C-leucine. Isotope incorporation was determined. Ten hours after treatment with ATDA, DNA synthesis was depressed to 1.2%–5.1% of control levels. By 72 hours, the rate of synthesis returned to normal. N coadministration was protective against the ATDA-induced depression of DNA synthesis at 10 hours. Some changes in the rate of protein synthesis after ATDA treatment were apparent.

The work reported here was carried out to obtain information about the mechanism of the teratogenic action of ATDA (2-amino-1,3,4-thiadiazole) and to provide a basis for further investigations of the abnormal development of limbs after treatment with this teratogen. We believe that our results and the questions raised by this work will be of interest to other investigators of normal and abnormal development. ATDA is one of a series of thiadiazoles synthesized in the search for cancer chemotherapeutic agents (Oleson et al., '55). ATDA was shown to inhibit growth of solid tumors and leukemias in mice (Oleson et al., '55; Ciotti et al., '60), but its toxicity in clinical trials precluded its continued clinical use (Krakoff and Balis, '59).

Several authors (Beaudoin, '73, '74, '76; Maren and Ellison, '72; Scott et al., '73) have shown that treatment of pregnant rats with ATDA results in fetuses with reduction limb defects and other abnormalities, but the alizarin staining used in those studies did not allow critical evaluation of the skeletal defects. Maren and Ellison ('72) reported a differential effect of ATDA on the incidence of malformations of the right versus the left embryonic limb. Cell death and necrosis occurs in abnormally developing limbs after ADTA treatment, but biochemical changes in the limbs have not been studied. Scott et al. ('73) have, however, reported a severe and prolonged depression of the rate of DNA synthesis in whole embryos after maternal rat ATDA treatment. Nicotinamide coadministered with ATDA prevents the teratogenic action of ATDA (Beaudoin, '73, '74, '76; Scott et al., '73) and it also prevents the depression of DNA synthesis in whole embryos (Scott et al., '73). This protec-

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tive action of nicotinamide may indicate that ATDA interferes with NAD and NADP related metabolism.

It is tempting to speculate that the ATDA induced depression of DNA synthesis in whole embryos noted by Scott et al. ('73) reflects a similar change in the abnormally developing rat limb bud after ATDA treatment. We feel, however, that inferences about biochemical changes in an embryonic organ, such as the limb bud, based on biochemical data for whole embryos should be tenuous because of the heterogeneous composition of embryos. Factors contributing to this heterogeneity are the many organs in an embryo, the different developmental stages of these organs at any one time, and metabolic differences at various stages of development (Hermann and Tootle, '64; Mackler et al., '71).

Johnson ('65) and Rutter et al. ('68) have indicated the necessity of the synthesis of specific proteins for cytodifferentiation and other aspects of development, and Landauer ('69) has pointed out that many teratogens may interfere with protein synthesis. We are aware of no studies relating to possible effects of ATDA on protein synthesis.

For reasons outlined above, this study was designed to provide information about DNA and protein synthesis within limb buds after ATDA treatment. It was also designed to study the limb skeletal defects resulting from such treatment. The data reported here, as well as light microscopic observations of such limbs, have been reported in preliminary form (Wotring, '75a, b; '76).

## MATERIALS AND METHODS

### *Housing, breeding, and injecting animals*

Sprague-Dawley rats obtained from Spartan Research Animals (Haslett, Michigan) were maintained on Teklad Mouse and Rat Diet (Teklad Mills, Winfield, Iowa) plus water ad libitum. Day 0 of pregnancy was considered to begin between 10 AM and 11 AM on the morning that sperm were found in the vagina. Between 10 AM and 11 AM on one of days 10–14 of pregnancy, each animal received a single ip injection, 100 mg/kg body weight, of a 2% aqeous solution of 2-amino-1,3,4-thiadiazole HCl (ATDA) (Eastman Kodak, Rochester, New York) or distilled water (controls). A supplementary ip injection of a 2% aqueous nicotinamide (N) solution (Sigma, St. Louis, Missouri), 100/mg/kg, was given to some of the rats that received an ATDA injection on day 12. The N injection was given immediately after the ATDA injection.

### *Teratogenic testing*

Pregnant females dosed with ATDA or water on days 10, 11, 12, 13, or 14 of pregnancy, or with ATDA + N on day 12, were sacrificed between 10 AM and 11 AM on day 20 of pregnancy. The number of pregnant animals in each treatment group is indicated in Table 1. Dead or resorbed embryos were recorded as embryonic deaths. Live fetuses were weighed and their limbs were examined for the presence or absence of external malformations.

To study the effect of drug treatment on the limb skeleton, dams injected with ATDA, ATDA + N, or water on day 12 of pregnancy were sacrificed on days 18 and 19. Fetuses alive at delivery were fixed in 10% formalin and the cartilaginous portion of the skeletons was stained with methylene blue chloride (Gruneberg, '53). The limbs of 91, 100, and 49 fetuses from 9 control litters, 9 ATDA treated litters, and 5 ATDA + N treated litters, respectively, were examined with the aid of a dissecting microscope. Tables (Mainland et al., '56) based on the Chi-square four-fold contingency test were used to compare incidences of findings in left versus right limbs and in fore- versus hindlimbs.

### *DNA and protein synthesis*

Females were sacrificed by decapitation 4, 10, 24, 48, and 72 hours after injection of ATDA or distilled water on day 12, and 10 hours after injection of ATDA + N on day 12. Embryonic fore- and hindlimbs were excised at the lateral body wall. Fore- and hindlimbs were placed on grids in separate preequilibrated organ culture dishes containing either $^{3}$H-thymidine or $^{14}$C-leucine. The number of limb buds per dish ranged from 3 to 7. These dishes were transferred to the organ culture chamber at 37°C for two hours of continuous gassing with a mixture of 49% $O_2$, 4.7% $CO_2$, and 46.3% $N_2$.

Stock medium containing $^{3}$H-thymidine was prepared by adding thymidine-methyl-$^{3}$H solution (Nuclear Dynamics, El Monte, California) to Waymouth MB 752/1 medium (Grand Island Biological, Grand Island, New York) so that the resulting radioactivity was 2 μc/ml. Stock medium containing $^{14}$C-leucine was prepared by adding L-leucine-UL-$^{14}$C (Cal Atomic, Los Angeles, California) to Waymouth medium 752/1 that had been prepared by the manufacturer to contain no leucine. The radioactivity of this stock medium was 0.5 μc/ml.

After incubation, all limb buds from each dish were quickly rinsed in distilled water at 0°C. Limb buds were then homogenized in tissue grinders containing distilled water at 0°C. Parallel aliquots of homogenate were used for

determination of radioisotope incorporation, protein content, and DNA content. Four determinations of each parameter, for each treatment group and time interval were made. Protein was determined by the Lowry method (Lowry et al., '51) using bovine serum albumin standards (Sigma). DNA was determined by the ethidium bromide fluorescence technique (LePecq and Paoletti, '66; Prasad et al., '72) using calf thymus DNA standards (Sigma). Fluorescence measurements were made in a Turner Model 111 fluorometer using a 7–37 filter (Corning Glass Works, Corning, New York) and a Kodak Wratten 2-A filter to transmit, respectively, the 365 mμ excitation and 590 mμ emission wavelengths of the ethidium bromide-DNA complex.

Methodology for determining incorporation of radioactivity was adapted from that reported by Scott et al. ('71) and by Doyle et al. ('74). Equal volumes of homogenate and 0.6 N PCA at 0°C were combined and after one hour, the resulting precipitate was collected by vacuum filtration on 2.4 cm GF/C Whatman glass fiber filters. The filters were rinsed and dried by successively passing 0.3 N PCA, 100% ethanol, and air through them. Dry filters were then transferred to vials. Soluene (Packard Instrument Company, Downers Grove, Illinois) was added to those vials containing precipitate from the limb buds cultured in $^{3}$H-thymidine medium and digestion was carried out for 12 hours. No soluene was added to those vials containing precipitate from the limb buds cultured in $^{14}$C-leucine medium.

Omnifluor (New England Nuclear, Boston, Massachusetts) -toluene cocktail was added to all vials and counting was carried out in an Isocap 300 liquid scintillation counter (Searle Analytic, Des Plaines, Illinois) using programs designed to obtain sample channels ratio data. Incorporation data was expressed as dpm/limb bud, dpm/μg protein, dpm/μg DNA and was evaluated using the Student t-test. P value of less than 0.05 was considered indicative of a statistically significant difference between means for the control and experimental treatment groups.

The synthesis of DNA and protein was considered to be reflected by the amount of radioisotope in the perchloric acid precipitate of the limb bud homogenates.

## RESULTS

### *Teratogenic testing*

The findings at sacrifice on day 20 of pregnancy are summarized in Table 1. Dosing with ATDA on days 10, 11, 12, 13, or 14 resulted in 100%, 96.6%, 12.9%, 13.6%, and 20% embryonic death, respectively. The incidences of embryonic death after dosing on days 12 or 13 are near the 9.1% and 6.9% embryonic death rates in day 12 and day 13 controls. No malformed limbs were seen in the few survivors of day 11 dosing and only 2% of hindlimbs became malformed after day 14 dosing, but 100% of fore- and hindlimbs became malformed after day 12 dosing. ATDA treatment on day 13 resulted in 100% of hindlimbs being malformed, but only 65% of forelimbs were so affected. Most of the limb malformations were ectrodactyly and/or syndactyly. Hindlimbs lacking a normal knee flexure and oriented toward the tail of the fetus were said to be "clubbed." Dosing with ATDA on days 12, 13, or 14 resulted in approximately a ⅓ reduction in fetal weight as compared to control values. Nicotinamide coadministered with ATDA on day 12 protected against limb malformations, decrease in fetal weight, and increase in incidence of embryonic death.

The results of the study of methylene blue stained limb skeletons are summarized in Table 2. Figures 5–10 illustrate the abnormalities observed. It was found that 99.5% and 98% of fore- and hindlimbs, respectively, in the ATDA-treated group had abnormal cartilaginous continuities between the various cartilage elements. No continuity between these elements occurred in control fetuses or in fetuses from ATDA-plus-nicotinamide treated litters. These cartilaginous connections are referred to as "fusions," although this term is not intended to connote any indication of their developmental etiology which is discussed below. The extent of the cartilaginous fusions varied from a small strand of cartilage connecting two separate elements to an extensive connection between elements. Often a single abnormally large metacarpal, metatarsal, or phalanx of essentially normal shape was present in place of two or three separate or partially fused ones. Observations of this type were not recorded as fusions. In the forelimb, carpal to carpal and metacarpal to metacarpal fusions were especially prevalent compared with other types of fusions. No fusion or other abnormal morphology proximal to the carpus was noted (Figs. 5, 6a–e). In the hindlimb, the most prevalent types of fusions were tibia to fibula and tarsal to tarsal (Figs. 7–9). Tibia-fibula fusion occurred between the proximal ends of the tibia and fibula and was unlike the normal tibia-fibula fusion that occurs between the distal portions of the shafts of the tibia and fibula at a later stage in development. In some cases, (Fig. 10b), hindlimbs were clubbed as described above for day 20 fetuses.

*TABLE 1. Embryonic death, limb malformations, and fetal weights following treatment of rats on days 10-14 of pregnancy with ATDA, ATDA + N, or water*[1]

| Day of injection | Injection | Number of Rats injected | Number of Implantation sites | % Embryonic death | % Forelimbs malformed | % Hindlimbs malformed | Mean fetal wgt. (g) ± SE |
|---|---|---|---|---|---|---|---|
| 10 | Control | 3 | 36 | 5.6 | 0 | 0 | 3.7 ± 0.00 |
| | ATDA | 4 | 50 | 100.0 | – | – | – |
| 11 | Control | 3 | 26 | 2.8 | 0 | 0 | 3.9 ± 0.21 |
| | ATDA | 5 | 59 | 96.6 | 0 | 0 | 1.4[2] |
| 12 | Control | 3 | 22 | 9.1 | 0 | 0 | 3.9 ± 0.06 |
| | ATDA | 7[3] | 93 | 12.9 | 100 | 100 | 2.2 ± 0.03 |
| | ATDA + N | 5 | 46 | 8.7 | 0 | 0 | 3.7 ± 0.02 |
| 13 | Control | 3 | 29 | 6.9 | 0 | 0 | 3.8 ± 0.03 |
| | ATDA | 5 | 59 | 13.6 | 65 | 100 | 2.6 ± 0.02 |
| 14 | Control | 3 | 30 | 10.0 | 0 | 0 | 4.1 ± 0.02 |
| | ATDA | 6 | 65 | 20.0 | 0 | 2 | 2.6 ± 0.16 |

[1]Rats were sacrificed on day 20 of pregnancy following an ip injection(s) on one of days 10–14 of pregnancy. Injections were water (controls), 100 mg/kg 2-amino-1,3,4-thiadiazole hydrochloride (ATDA) or ATDA plus an equal amount of nicotinamide (N). Results of external examinations of limbs.

[2]No SE computed. Live fetuses in only one litter.

[3]Two rats injected with ATDA on day 12 died before day 20 of pregnancy and were not included in this table.

No statistically significant differences in the incidence of various types of fusions were noted when right and left forelimbs or right and left hindlimbs were compared. There were no fusions of long limb bones in forelimbs, but many of the analogous fusions were present in hindlimbs. The incidence of carpal to carpal fusions was not significantly different from the incidence of tarsal to tarsal fusions, but significantly more of all other types of paw fusions occurred in forepaws than in hindpaws.

### *DNA synthesis*

Thymidine incorporation data for control and ATDA-treated limbs are shown as means in Table 3 and are expressed in graphical form in Figures 1 and 2. Incorporation by forelimb buds four hours after ATDA treatment (expressed per limb bud, per μg DNA, and per μg protein) was depressed to approximately half that occurring in control forelimb buds. Thymidine incorporation by hindlimb buds at four hours after ATDA administration was not significantly different from controls. Ten hours after ATDA administration, thymidine incorporation (expressed per limb bud, per μg DNA, and per μg protein) was markedly depressed in both fore- and hindlimb buds. At this time, incorporation rates for ATDA-treated forelimb buds were only 2.7% to 5.1% of the corresponding control values, and incorporation rates for ATDA-treated hindlimb buds were only 1.2% to 2.3% of the corresponding control values.

Recovery of the ability to incorporate thymidine was evident at 24 hours post ATDA treatment. At this time, thymidine incorporation (expressed per limb bud, per μg DNA, and per μg protein) by treated fore- and hindlimb buds ranged from 18% to 40% of control values. Thymidine incorporation by ATDA limbs continued to increase up to 72 hours post-treatment, at which time dpm per forelimb were only 55% of the corresponding control value. This was the only significant difference between experimental and control values at 72 hours.

Incorporation of thymidine by forelimb buds 10 hours after administration of nicotinamide-plus-ATDA was not significantly different from incorporation by 10 hour control forelimb buds. Thymidine incorporation by hindlimb buds 10 hours after administration of nicotinamide-plus-ATDA was 61%, 67%, and 60% of the corresponding control values for dpm/μg DNA, dpm/μg protein, and dpm/limb bud, respectively.

*Protein synthesis*

Leucine incorporation data for control and ATDA-treated limbs are shown as means in Table 4 and are expressed in graphical form in Figures 3 and 4. Leucine incorporation per μg DNA was not significantly different in control and ATDA-treated fore- and hindlimb buds at any of the intervals studied. When leucine incorporation was expressed per μg protein, 72 hour ATDA-treated fore- and hindlimbs both had significantly more incorporation than did 72 hour control fore- and hindlimbs. Leucine incorporation, expressed per limb bud, appeared to be generally decreased in ATDA-treated fore- and hindlimbs, but a decrease was statistically significant in forelimbs only at 24 and 72 hours and in hindlimbs only at 72 hours.

## DISCUSSION

The Sprague-Dawley rat was used in the present study, but other accounts of ATDA teratogenicity in rats have been based on studies with the Wistar strain (Maren and Ellison, '72; Beaudoin, '73; Scott et al., '73). We do not ascribe any differences between the results of the present study and the results of the studies using the Wistar rat to strain differences in the response of rats to this teratogen.

No differences in susceptibility of right and left embryonic rat limbs to ATDA teratogenicity were noted in this study or by Beaudoin ('73) and Scott et al. ('73), whereas Maren and Ellison ('72) did report a preferential effect on the right forelimb. This difference in results may be due to variations (dosage, route, and timing) in the dosing protocol used by Maren and Ellison, as compared to the dosing protocols used in the present study, by Beaudoin ('73), and by Scott et al. ('73).

The reduction type limb malformations reported by Maren and Ellison ('72), Beaudoin ('73), and Scott et al. ('73) included syndactyly, ectrodactyly, brachydactyly, and hemimelia. These authors did not report, however, any limb skeletal abnormalities, except retarded ossification, in their studies of alizarin stained skeletons. Beaudoin did point out the impossibility of evaluating the appendicular skeleton in his study because the cartilage was not stained. Abnormal cartilaginous connections between otherwise individual skeletal elements are referred to as "fusions," based on similar usage of the term by previous authors (Warkany et al., '43; Warkany, '69; O'Rahilly, '51, '53; Kelikian, '74). The term "fusion" implies that two initially separate unconnected elements have become connected. That other factors may be involved in the embryogenesis of ATDA-induced skeletal fusions is suggested by light microscopic observations of Wotring ('79, unpublished observations). O'Rahilly ('51, p. 160) has summarized the confusion about this type of finding by indicating that "Various ill-defined terms have been suggested as an alternative to 'fusion,' e.g., coalition, coalescence, synchondrosis, synarthrosis, syndesmosis, etc."

Significantly different incidences of analogous types of fusions in fore- and hindlimbs were noted in this study. These differences may be a reflection of the different developmental stages of the limbs at the time of ATDA treatment, and of the reparative processes subsequent to treatment (Scott et al, '73; Wotring, unpublished observations). The rat forelimb at day 12 of pregnancy is relatively more developed than the hindlimb, and this could explain the fact that fusions occurred in the proximal part of the hindlimb, but not in the proximal part of the forelimb. Fewer fusions in the hindpaws and tarsus than in forepaws and carpus could be due to the less differentiated state of the distal part of the hindlimb bud at the time of ATDA treatment, allowing recovery and repair subsequent to the insult.

Beaudoin ('73) and Scott et al. ('73) have shown that nicotinamide administered concomitantly with ATDA prevents the malformations caused by ATDA administered alone. The results of the present study fully support this finding. We also observed that nicotinamide coadministered with ATDA prevents the depression of DNA synthesis in forelimbs at 10 hours caused by ATDA alone, and partial protection was afforded the hindlimbs. We are uncertain that this represents a real difference between fore- and hindlimbs, but further experimentation will be necessary to resolve this matter. Scott et al. ('73) did not report looking for effects on DNA synthesis when nicotinamide was coadministered with ATDA, but they did report alleviation of the depression of DNA synthesis in whole embryos when nicotinamide administration was delayed 4, 8, and 12 hours.

In this study of limb buds, and in the Scott et al. ('73) study of whole embryos, ATDA administration resulted in a severe depression of DNA synthesis with subsequent recovery to essentially normal levels of synthesis. The

*TABLE 2. Incidence of analogous cartilaginous fusions in methylene blue stained fore- and hindlimbs of rat fetuses after an ip injection of ATDA into rats on day 12 of pregnancy*

| | | Types of Fusions[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total number limbs examined | Humerus ulna / femur tibia | Ulna radius / tibia fibula | Ulna carpal / fibula tarsal | Carpal carpal / tarsal tarsal | Carpal metacarpal / tarsal metatarsal | Metacarpal metacarpal / metatarsal metatarsal | Metacarpal phalanx / metatarsal phalanx | Phalanx[2] phalanx / phalanx[3] phalanx | Total number limbs with fusions |
| Left & right forelimbs | 200 | 0 | 0 | 44 | 190 | 59 | 170 | 29 | 60 | 199 |
| Left & right hindlimbs | 200 | 19 | 188 | 2 | 195 | 25 | 110 | 1 | 8 | 196 |
| Statistical[4] significance | – | ** | ** | ** | – | ** | ** | ** | ** | – |

[1]Analogous types of fusions in fore- and hindlimbs have been placed in the same column.
[2]Forepaw phalanx-phalanx fusion.
[3]Hindpaw phalanx-phalanx fusion.
[4]Tables (Mainland et al., '56) based on the Chi-square four-fold contingency test were used to compare incidences of the fingings in forelimbs versus hindlimbs. **Indicates statistically significant differences at the 1% level; - indicates no statistically significant difference at the 5% level.

most severe depression of DNA synthesis was noted in limb buds and whole embryos (Scott et al., '73) at 10 and 17 hours, respectively. At 10 hours post-ATDA treatment, all values for thymidine incorporation by limb buds were between 1.2% and 5.1% of control values. The maximal depression noted by Scott et al. ('73) was approximately 12% of control levels. These differences in severity and timing of maximal depression of DNA synthesis may be a result of a greater proportion of cells in the limb bud, as compared to the whole embryo, which are severely affected by cytotoxic properties of ATDA. Results of the present study of DNA synthesis confirm the effects of ATDA on DNA synthesis noted by Scott et al. and specifically identify these effects in an abnormally developing organ. Further localization of this depression of synthesis would be desirable and could presumably be studied by radioautography of limb buds exposed to radioactive thymidine. Such data might indicate whether comparable changes in DNA synthesis occurs in various limb bud components such as ectoderm, noncondensed mesenchyme, condensed mesenchyme, endothelium, etc. Such data should be helpful in further elucidating the mechanism of action of ATDA.

The depression of DNA synthesis in this study and in the study by Scott et al. ('73) can be interpreted as indicative of depressed cell proliferation and cytotoxicity. The ultimate cytotoxic effect, cell death, occurring in limb buds after ATDA treatment (Scott et al., '73; Wotring, '76, '79, unpublished observations) appears to be directly related to the development of anomalous limbs. Whether the depression of DNA synthesis is a cause or effect of cell death is unknown. Still unresolved is the problem of the initial molecular lesion of ATDA. Based on studies of the protective action of nicotinamide coadministration, Beaudoin ('74) has suggested that ATDA may interfere with either the synthesis or utilization of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Since NAD and NADP are necessary coenzymes for numerous dehydrogenases, interference with their synthesis or use would likely be reflected by altered enzymatic activity. NAD- and NADP-dependent dehydrogenases are of functional importance in many metabolic pathways, such as the phosphogluconate shunt which produces pentoses for nucleic acid synthesis, the glycocytic sequence, and tricarboxylic acid cycle which are important in the process of generating high energy molecules, and in the purine biosynthetic pathway.

*TABLE 3. $^3$H-thymidine incorporation by embryonic rat limb buds following treatment of dams with ATDA or water on day 12 of pregnancy*

| Hours post treatment | Treatment | Limb bud | Mean[1] dpm limb bud ± SD | Mean[1] dpm/μg DNA ± SD | Mean[1] dpm/μg protein ± SD |
|---|---|---|---|---|---|
| 4 | water | fore | 7,279 ± 1,072* | 2,119 ± 301* | 174 ± 28 |
| 4 | ATDA | fore | 3,587 ± 2,192 | 1,166 ± 682 | 95 ± 63 |
| 10 | water | fore | 8,519 ± 2,323** | 2,655 ± 1,508* | 156 ± 41** |
| 10 | ATDA | fore | 226 ± 85 | 112 ± 45 | 8 ± 1 |
| 24 | water | fore | 11,442 ± 4,348** | 2,643 ± 1,370* | 134 ± 47* |
| 24 | ATDA | fore | 2,006 ± 1,020 | 528 ± 277 | 43 ± 25 |
| 48 | water | fore | 16,807 ± 6,057 | 2,369 ± 445 | 161 ± 67 |
| 48 | ATDA | fore | 9,063 ± 3,272 | 1,591 ± 724 | 131 ± 59 |
| 72 | water | fore | 28,091 ± 7,254* | 1,794 ± 520 | 152 ± 33 |
| 72 | ATDA | fore | 15,524 ± 3,475 | 1,698 ± 262 | 162 ± 48 |
| 4 | water | hind | 4,460 ± 1,215 | 1,933 ± 492 | 178 ± 91 |
| 4 | ATDA | hind | 3,999 ± 2,479 | 1,798 ± 1,101 | 170 ± 133 |
| 10 | water | hind | 7,705 ± 2,030** | 2,453 ± 221** | 173 ± 23** |
| 10 | ATDA | hind | 89 ± 70 | 51 ± 22 | 4 ± 1 |
| 24 | water | hind | 10,539 ± 4,695* | 2,269 ± 716* | 134 ± 56* |
| 24 | ATDA | hind | 2,172 ± 1,059 | 715 ± 473 | 54 ± 33 |
| 48 | water | hind | 19,312 ± 4,887* | 2,282 ± 693* | 179 ± 77 |
| 48 | ATDA | hind | 8,518 ± 3,712 | 1,268 ± 357 | 114 ± 51 |
| 72 | water | hind | 26,374 ± 10,581 | 1,904 ± 850 | 135 ± 53 |
| 72 | ATDA | hind | 19,118 ± 3,839 | 1,423 ± 247 | 153 ± 42 |

[1]Means for the control (water) and ATDA treatment groups were compared by using the Student t-test. *Indicates $0.01 < P < 0.05$. **Indicates $P \leq 0.01$.

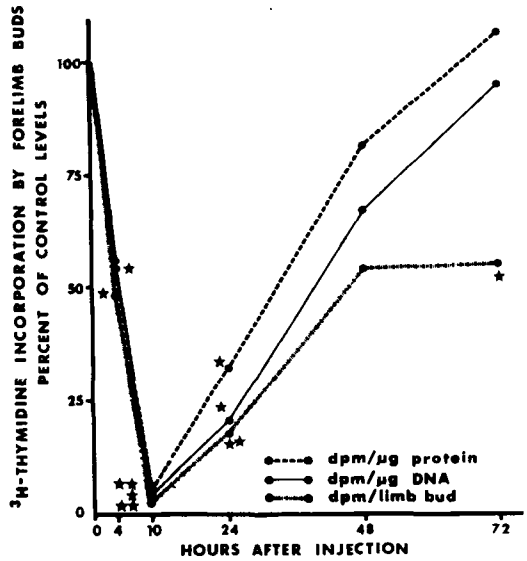



Fig. 1. In vitro $^3$H-thymidine incorporation, during a 2-hour culture period, by embryonic rat forelimbs after an ip injection of ATDA (100 mg/kg) into rats on day 12 of pregnancy. Each data point represents the average of 4 determinations from 4 separate litters. Each determination was made by using the homogenate of 3–8 limbs from 1 litter. The same number of control determinations were made. A single star indicates $0.01 < P < 0.05$ and the double star indicates $P \leq 0.01$.

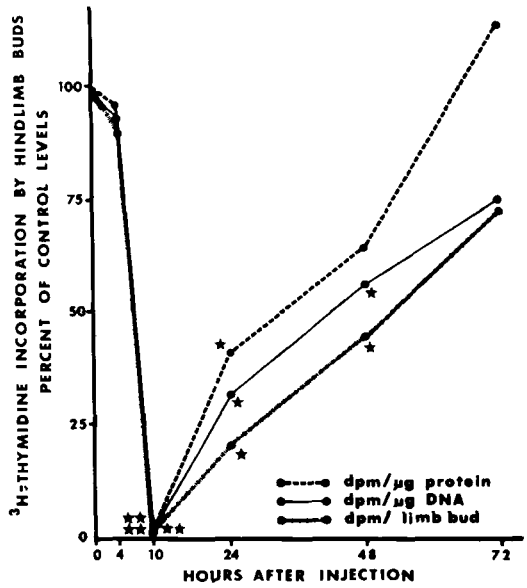



Fig. 2. In vitro $^3$H-thymidine incorporation during a 2-hour culture period, by embryonic rat hindlimbs after an ip injection of ATDA (100 mg/kg) into rats on day 12 of pregnancy. Each data point represents the average of 4 determinations from 4 separate litters. Each determination was made by using the homogenate of 3–8 limbs from 1 litter. The same number of control determinations were made. A single star indicates $0.01 < P < 0.05$ and a double star indicates $P \leq 0.01$.

*TABLE 4. $^{14}$C-leucine incorporation by embryonic rat limb buds following treatment of dams with ATDA or water on day 12 of pregnancy*

| Hours post treatment | Treatment | Limb bud | Mean[1] dpm/limb bud ± SD | Mean[1] dpm/μg DNA ± SD | Mean[1] dpm/μg protein ± SD |
|---|---|---|---|---|---|
| 4 | water | fore | 1,927 ± 509 | 618 ± 232 | 46 ± 15 |
| 4 | ATDA | fore | 1,787 ± 885 | 605 ± 246 | 48 ± 23 |
| 10 | water | fore | 1,813 ± 490 | 532 ± 138 | 34 ± 6 |
| 10 | ATDA | fore | 1,281 ± 693 | 528 ± 223 | 37 ± 6 |
| 24 | water | fore | 3,517 ± 303** | 756 ± 195 | 39 ± 4 |
| 24 | ATDA | fore | 2,089 ± 619 | 663 ± 129 | 46 ± 12 |
| 48 | water | fore | 3,639 ± 804 | 503 ± 145 | 33 ± 10 |
| 48 | ATDA | fore | 2,467 ± 733 | 463 ± 148 | 37 ± 16 |
| 72 | water | fore | 4,774 ± 854* | 341 ± 80 | 25 ± 7** |
| 72 | ATDA | fore | 3,552 ± 325 | 421 ± 94 | 38 ± 3 |
| 4 | water | hind | 1,442 ± 343 | 541 ± 122 | 58 ± 27 |
| 4 | ATDA | hind | 1,113 ± 90 | 545 ± 118 | 49 ± 13 |
| 10 | water | hind | 1,535 ± 394 | 464 ± 27 | 36 ± 4 |
| 10 | ATDA | hind | 892 ± 672 | 450 ± 185 | 37 ± 10 |
| 24 | water | hind | 2,562 ± 780 | 507 ± 149 | 34 ± 8 |
| 24 | ATDA | hind | 1,772 ± 601 | 502 ± 115 | 39 ± 9 |
| 48 | water | hind | 4,148 ± 1,168 | 388 ± 60 | 38 ± 19 |
| 48 | ATDA | hind | 2,740 ± 346 | 475 ± 138 | 35 ± 3 |
| 72 | water | hind | 5,478 ± 626* | 413 ± 111 | 29 ± 5* |
| 72 | ATDA | hind | 4,148 ± 603 | 376 ± 138 | 38 ± 2 |

[1]Means for the control (water) and ATDA treatment groups were compared by using the Student t-test.
*Indicates $0.01 < P < 0.05$.
**Indicates $P \leq 0.01$.

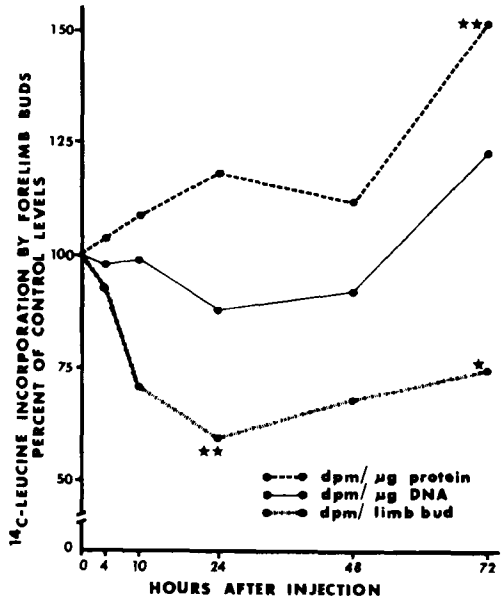



Fig. 3. In vitro $^{14}$C-leucine incorporation during a 2-hour culture period, by embryonic rat forelimbs after an ip injection of ATDA (100 mg/kg) into rats on day 12 of pregnancy. Each data point represents the average of 4 determinations from 4 separate litters. Each determination was made by using the homogenate of 3-8 limbs from 1 litter. The same number of control determinations were made. A single star indicates $0.01 < P < 0.05$ and a double star indicates $P < 0.01$.

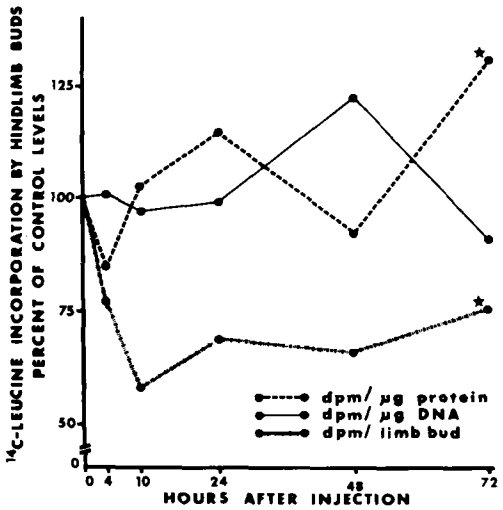


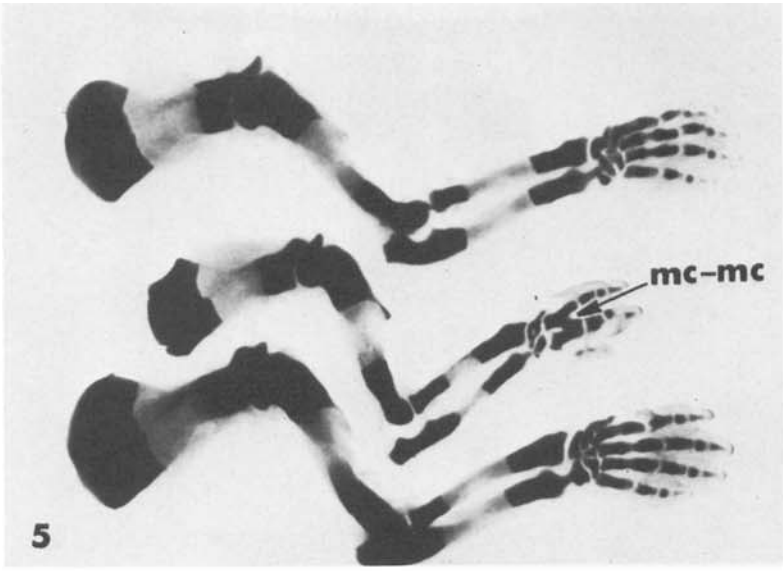

Fig. 4. In vitro $^{14}$C-leucine incorporation during a 2-hour culture period, by embryonic rat hindlimbs after an ip injection of ATDA (100 mg/kg) into rats on day 12 of pregnancy. Each data point represents the average of 4 determinations from 4 separate litters. Each determination was made by using the homogenate of 3-8 limbs from 1 litter. The same number of control determinations were made. A single star indicates $0.01 < P < 0.05$ and a double star indicates $P \leq 0.01$.

*Abbreviations*

c-c, carpal to carpal fusion
fib, fibula
mc-mc, metacarpal to metacarpal fusion
mc-p, metacarpal to phalanx fusion
mt-mt, metatarsal to metatarsal fusion
p-p, phalanx to phalanx fusion
r, radius
t, tarsal
tib, tibia
t-mt, tarsal to metatarsal fusion
t-t, tarsal to tarsal fusion
tib-fib, tibia to fibula fusion
u-ulna

Fig. 5. Right forelimbs of 19-day fetuses. The upper limb is that of a fetus from a dam that received a water injection on day 12 of pregnancy and the lower limb is that of a fetus from a dam that received ATDA plus nicotinamide on day 12; these limbs are essentially indistinguishable and cartilaginous fusions are not present in either limb. The limb in the center is that of a fetus from a dam that received an ATDA injection on day 12; note the metacarpal to metacarpal fusion and presence of only three digits. The ATDA-treated limb is small but otherwise normal proximal to the carpus. ×5.5.

Fig. 6a. Right forepaw of a 19-day control fetus. No cartilaginous fusion is present, although it appears to be in the carpus. The carpus, indicated by brackets, is shown in enlarged form in Figure 6b. × 12.4. b. Enlarged outline drawing of carpus in Figure 6a. The proximal ends of the five metacarpals are indicated by the arrows. Seven carpals are present. Partial superimposition of carpals by the distal end of the ulna and of one carpal by another explains what look like fusions in Figure 6a. × 25. c. Right forepaw of a 19-day fetus from a dam that received ATDA on day 12. One metacarpal and one phalanx are disproportionately large. Carpal to carpal fusion is present. × 12.4. d. Left forepaw of a 19-day fetus from a dam that received ATDA on day 12. Note the presence of only three digits and two metacarpals, carpal to carpal fusion, and metacarpal to phalanx fusion. × 12.4. e. Right forepaw of a 19-day fetus from a dam that received ATDA on day 12. Note the carpal to carpal fusion, phalanx to phalanx fusion, and metacarpal to metacarpal fusion (unlabeled arrow). The middle digit is disproportionately short. × 12.4.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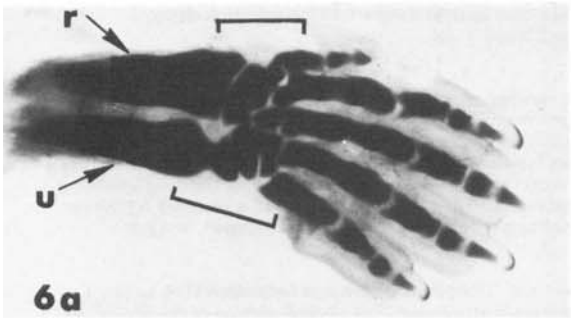
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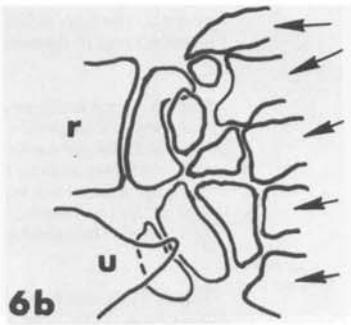
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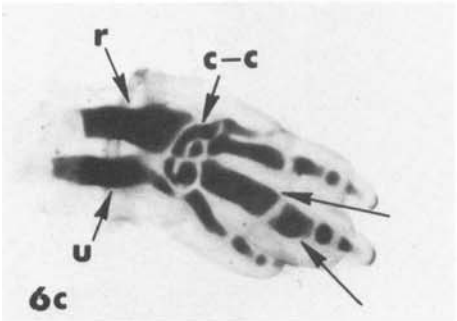
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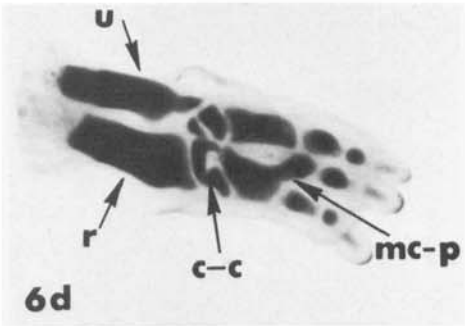
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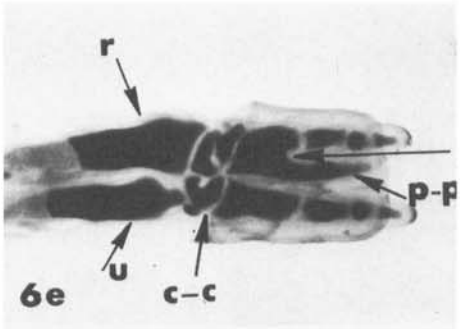
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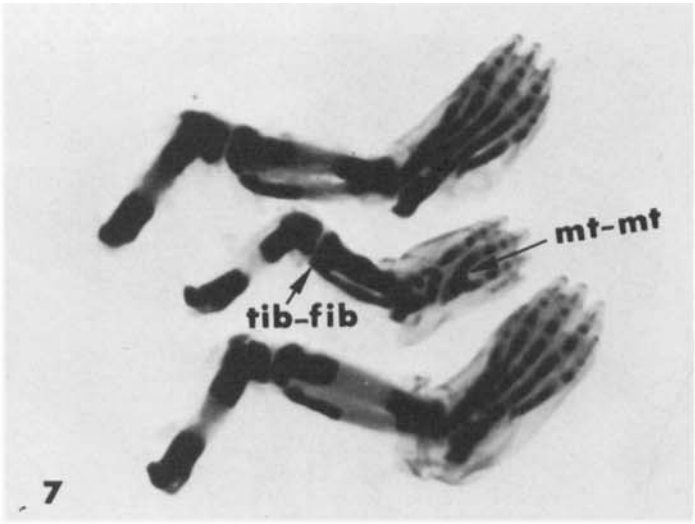
Fig. 7. Right hindlimbs of 19-day fetuses. The upper limb is that of a fetus from a dam that received a water injection on day 12 of pregnancy and the lower limb is that of a fetus from a dam that received ATDA plus nicotinamide on day 12; these limbs are essentially indistinguishable and cartilaginous fusions are not present in either limb. The limb in the center is that of a fetus from a dam that received an ATDA injection on day 12; note the metatarsal to metatarsal fusion and tibia to fibula fusion. × 5.5

Fig. 8a. Right hindpaw of a 19-day control fetus. No cartilaginous fusion is present, although it appears to be in the tarsus. That portion of the tarsus indicated by the brackets is shown in enlarged outline form in Figure 8b. × 12.4 b. Enlarged outline drawing of tarsus in Figure 8a. Partial superimposition of one metatarsal by another and of one metatarsal by a tarsal explains what look like fusions in Figure 8a. × 25. c. Right hindpaw of a 19-day fetus from a dam that received ATDA on day 12. Several types of fusions are present. Note the distal convergence of the two central digits. × 12.4. d. Right hindpaw of a 19-day fetus from a dam that received ATDA on day 12. Note the tarsal to tarsal fusion and metatarsal to metatarsal fusion (unlabeled arrow). × 12.4.

Fig. 9. Tibia and fibula from the hindlimb of a 19-day fetus. The dam had been injected with ATDA on day 12. Note fusion of the tibia and fibula indicated by the unlabeled arrowhead. The ossified portion of the fibula (arrow) is not stained. Normal fusion of the tibia and fibula should take place later at the level indicated by the asterisk. × 12.4.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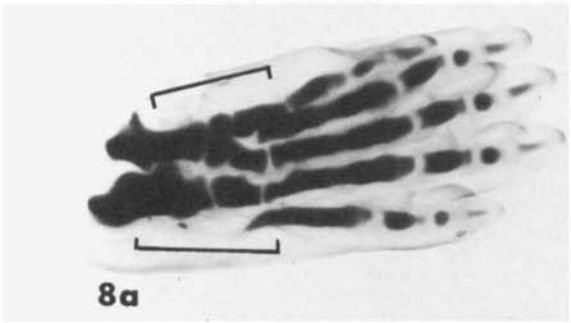
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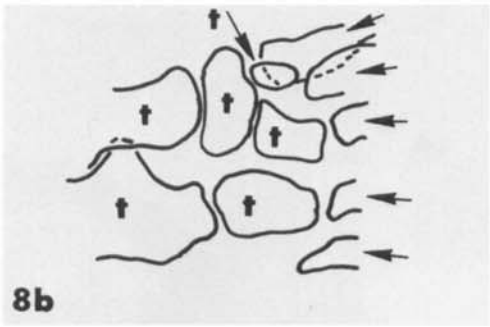
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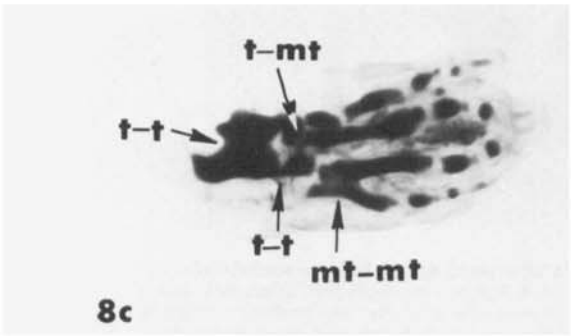
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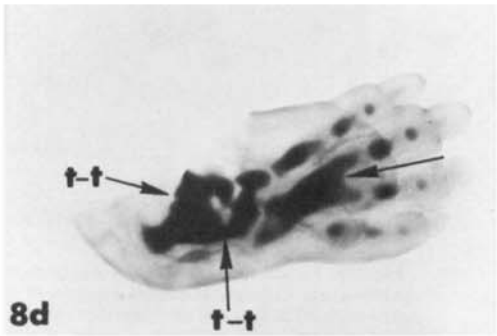
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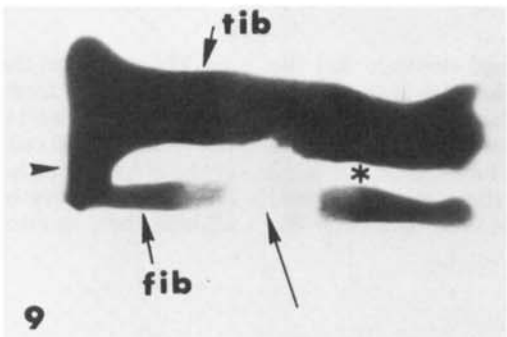
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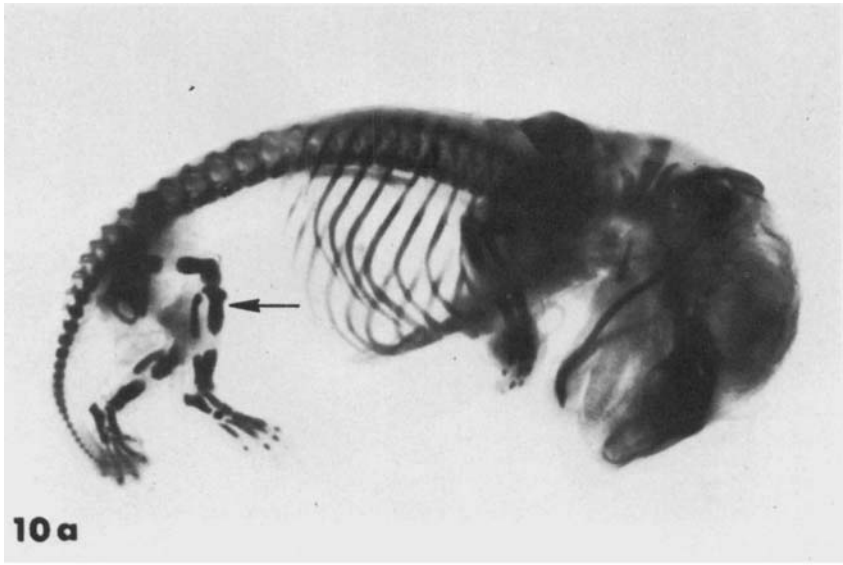


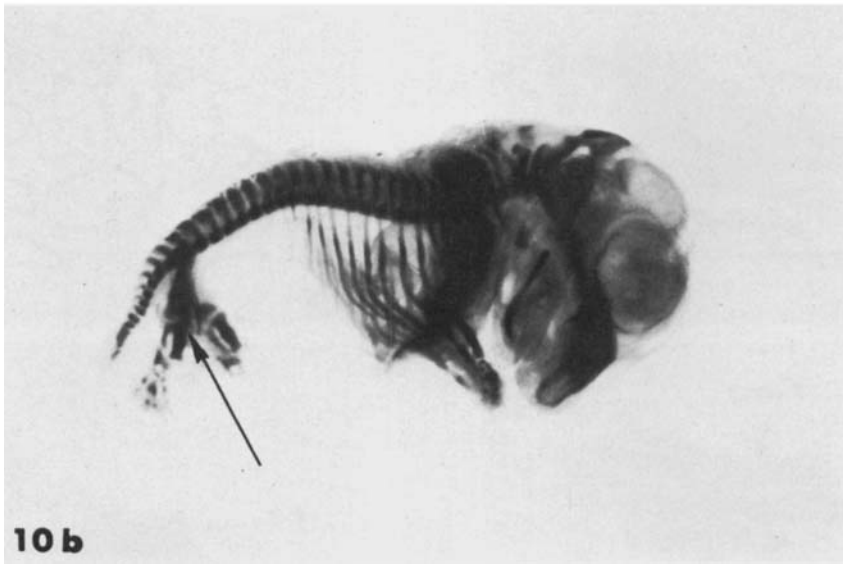


**Fig. 10a. Eighteen-day fetus from a dam that received a water injection on day 12. Note the normal posture of the hindlimbs. The right hindlimb is indicated by an arrow. × 4.4. b. Eighteen-day fetus from a dam that was injected with ATDA on day 12. Note that the hindlimbs are not flexed at the knee. The right hindlimb is oriented toward the tail and is said to be clubbed. Fusion of the femur, tibia, and fibula is indicated by an arrow. × 4.4**

Nelson et al. ('76) obtained evidence that the synthesis of guanine nucleotides is blocked in leukemia L1210 cells from ascitic fluid of mice treated with ATDA. Ritter et al. ('77) found that in rat embryos 4–12 hours after maternal treatment with ATDA, there were increased IMP levels and decreased GDP and GTP levels. The results of these two studies suggest a block, resulting from ATDA treatment, in the purine biosynthetic pathway. That this blockade in the L1210 cells may be due to inhibition of the NAD-dependent IMP dehydrogenase is indicated by data of Nelson et al. ('77) who showed that, in vitro, IMP dehydrogenase is

inhibited by an aminothiadiazole analog of NAD and also by the aminothiadiazole nucleotide. That the aminothiadiazole nucleotide may be the IMP dehydrogenase inhibitory molecule in vivo is indicated by its presence, and the absence of the aminothiadiazole analog of NAD in cells exposed to $^{14}$C-ATDA (Nelson et al., '77). An investigation of dehydrogenase activities and nucleotide pools in ATDA-treated embryonic rat limbs has not been carried out, but should be helpful in assessing the role of molecular changes, such as those noted above, in ATDA-induced abnormal limb development.

Incorporation of thymidine and leucine was expressed on the basis of DNA and protein content as well as per limb bud. The utilization of multiple normalization bases was considered to be advisable because it was thought that aspects of normal and abnormal limb bud development, such as limb bud size and the presence of extracellular proteins, might have had effects on the incorporation results which would vary depending on the normalization base used. Relatively consistent results for thymidine incorporation, however, were obtained regardless of the normalization base used. Less consistent results were obtained for the leucine incorporation data. Only when leucine incorporation – i.e., protein synthesis – was expressed per limb bud did a general depression from control levels seem apparent for ATDA-treated limb buds. Maximal depression for both fore- and hindlimbs was to approximately 60% of control levels and was noted at 10 and 24 hours in the hind- and forelimb buds, respectively. The depressed leucine incorporation at 10 hours in hindlimbs was not statistically significant. Whether the apparent decrease in protein synthesis is a cause or effect of the marked decrease in DNA synthesis is unknown, but it is assumed to be a result. This assumption is based on the lack of a statistically significant decrease until 24 hours, i.e., after the marked depression of DNA synthesis at 10 hours and on the less severe depression of protein synthesis than of DNA synthesis. The apparent general decrease in leucine incorporation, when expressed per limb bud, is thought to reflect the smaller size of ATDA limb buds as compared to control limb buds.

At 72 hours after ATDA treatment, protein synthesis in both fore- and hindlimbs, when expressed per $\mu$g protein, was higher than in controls. The cause of this difference may be related to the fact that in controls at this time, a significant amount of extracellular cartilaginous matrix is evident, whereas less matrix is evident in 72-hour ATDA-treated limb buds (Wotring, unpublished observations). Limb bud extracellular cartilage matrix contains a significant amount of protein (Skalko and Cowden, '73; Linsenmayer et al., '73). Since extracellular protein is not involved in producing more protein, its presence may be expected to lower the rate of protein synthesis when synthesis is expressed on the basis of total protein content.

Utilization of the limb bud organ culture system in the work reported here had certain advantages over the in vivo incorporation system used by Scott et al. ('73). Using in vitro incorporation, the amount of radioisotopically labeled precursor available to each limb bud was precisely controlled and the amount of precursor available to a limb bud was assuredly unaffected by any possible ATDA-related changes in the placenta or dam.

ACKNOWLEDGMENTS

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LITERATURE CITED

Beaudoin, A.R. (1973) Teratogenic activity of 2-amino-1,3,4-thiadiazole hydrochloride in Wistar rats and the protection afforded by nicotinamide. Teratology, *7*:65-72.

Beaudoin, A.R. (1974) Thiadiazole-induced myelodysplasia in rats. Teratology, *9*:179-190.

Beaudoin, A.R. (1976) NAD precursors as antiteratogens against aminothiadiazole. Teratology, *13*:95-100.

Ciotti M.M., S.R. Humphreys, J.M. Vendetti, N.O. Kaplan, and A. Goldin (1960) The antileukemic action of two thiadiazole derivatives. Cancer Res., *20*:1195-1201.

Doyle, C.M., R. Zak, and D.A. Fischman (1974) The correlation of DNA synthesis and DNA polymerase activity in the developing chick heart. Dev. Biol., *37*:133-145.

Grüneberg, H. (1953) Genetical studies on the skeleton of the mouse. J. Genet., *51*:327-360.

Herrmann, H., and M.L. Tootle (1964) Specific and general aspects of the development of enzymes and metabolic pathways. Physiol. Rev., *44*:289-371.

Johnson, E.M. (1965) Electrophoretic analysis of abnormal development. Proc. Soc. Exp. Biol. Med., *118*:9-11.

Kelikian H. (1974) Congenital Deformities of the Hand and Forearm. W.B. Saunders, Philadelphia, pp. 732-745.

Krakoff, I., and M. Balis (1959) Studies of the uricogenic effect of 2-substituted thiadiazoles in man. J. Clin. Invest., *38*:907-915.

Landauer, W. (1969) Dynamic aspects of hereditary and induced limb malformations. In: Limb Development and Deformity. C.A. Swinyard, ed. Charles C. Thomas, Springfield, Illinois, pp. 120-135.

LePecq, J.-B., and C. Paoletti (1966) A new fluorometric method for RNA and DNA determination. Anal. Biochem., *17*:100-107.

Linsenmayer, T.F., B.P. Toole, and R.L. Trelsted (1973) Temporal and spatial transitions in collagen types during embryonic chick limb development. Dev. Biol., *35*: 232-239.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., *193*:265–275.

Mackler, B., R. Grace, and H.M. Duncan (1971) Studies of mitochondrial development during embryogenesis in the rat. Arch. Biochem. Biophys., *144*:603–610.

Mainland, D. L. Herrera, and M.I. Sutcliffe (1956) Tables for Use With Binomial Samples. Dept. Medical Statistics, New York University College of Medicine, New York.

Maren, T.H., and A.C. Ellison (1972) The teratological effect of certain thiadiazoles related to acetazolamide with a note on sulfanilamide and thiazide diuretics. Johns Hopkins Med. J., *130*:95–104.

Nelson, J.A., L.M. Rose, and L.L. Bennett (1976) Effects of 2-amino-1,3,4-thiadiazole on ribonucleotide pools of leukemia L1210 cells. Cancer Res., *36*:1375–1378.

Nelson, J.A., L.M. Rose, and L.L. Bennett (1977) Mechanism of action of 2-amino-1,3,4-thiadiazole (NSC 4728). Cancer Res., *37*:182–187.

Oleson, J., A. Sloboda, W. Troy, S. Holliday, M. Landes, R. Angier, J. Semb, K. Cyr, and J. Williams (1955) The carcinostatic activity of some 2-amino-1,3,4-thiadiazoles. J. Am. Chem. Soc., *77*:6713–6714.

O'Rahilly, R. (1951) Morphological patterns in limb deficiencies and duplications. Am. J. Anat., *89*:135–193.

O'Rahilly, R. (1953) A survey of carpal and tarsal anomalies. J. Bone Joint Surg. (Am.) *35*:626–642.

Prasad, A.S., E. DuMouchelle, D. Koniuch, and D. Oberleas (1972) A simple fluorometric method for the determination of RNA and DNA in tissues. J. Lab. Clin. Med., *80*:598–602.

Ritter, E.J., W.J. Scott, J.G. Wilson, and L. Bruce (1977) Mechanisms of teratogenic action of aminothiadiazole (ATD). Teratology, *15*:31A (Abstr.).

Rutter, W., J. Kemp, W. Bradshaw, W. Clark, R. Ronzio, and T. Sanders (1968) Regulation of specific protein synthesis in cytodifferentiation. J. Cell Physiol (Suppl. 1) *72*:1–18.

Scott, W.J., E.J. Ritter, and J.G. Wilson (1971) DNA synthesis inhibition and cell death associated with hydroxyurea teratogenesis in rat embryos. Dev. Biol., *26*:306–315.

Scott, W.J., E.J. Ritter, and J.G. Wilson (1973) DNA synthesis inhibition, cytotoxicity, and their relationship to teratogenesis following administration of a nicotinamide antagonist, aminothiadiazole, to pregnant rats. J. Embryol. Exp. Morphol., *30*:257–266.

Skalko, R.G., and R.R. Cowden (1973) Histochemical studies of cartilage development in the mouse limb-bud. Acta Anat., *85*:499–513.

Warkany, J. (1969) Experimental production of mammalian limb malformations. In: Limb Development and Deformity, Problems of Evaluation and Rehabilitation. C.A. Swinyard, ed. C.C. Thomas, Springfield, Illinois, pp. 140–160.

Warkany, J., R.C. Nelson, and E. Schraffenberger (1943) Congenital malformations induced in rats by maternal nutritional deficiency. J. Bone Joint Surg. (Am.), *25*: 261–270.

Wotring, G.E. (1975a) Tritiated thymidine incorporation, total DNA and total protein content of rat limb buds after a teratogenic dose of 2-amino-1,3,4-thiadiazole hydrochloride. Anat. Rec., *181*:513–514 (Abstr.)

Wotring, G.E. (1975b) Tritiated thymidine incorporation, total DNA and total protein content of rat limb buds after a teratogenic dose of 2-amino-1,3,4-thiadiazole hydrochloride (ATDA). Teratology, *11*:38a (Abstr.).

Wotring, G.E. (1976) A histological study of abnormal rat limb development resulting from maternal treatment with the teratogen 2-amino-1,3,4-thiadiazole hydrochloride. Anat. Rec., *184*:568 (Abstr.).