of approximately ten times the usual specific radioactivity and of an additional paper chromatographic purification (*13*).

7. Skin was obtained from the abdominal wall of a surgical patient. Dermis, separated from epidermis by "stretching" [E. J. Van Scott, *J. Invest. Dermatol.* **18**, 377 (1952)], was frozen in liquid nitrogen, shattered to a powder, triturated with sand and buffer, and finally, sonically disrupted. Cystathionine synthase and protein were assayed in the whole homogenate and in the supernatant fluid. To determine DNA-P, fibroblasts or powdered tissue were washed twice with cold 5 percent trichloroacetic acid (TCA), then extracted with 5 percent TCA at 90°C for 15 minutes. DNA was determined by a published method [D. N. Croft and M. Lubran, *Biochem. J.* **95**, 612 (1965)]. Interference by acid mucopolysaccharides or by nonspecific turbidity [K. W. Giles and A. Myers, *Nature* **206**, 93 (1965)] was ruled out by demonstration of the expected ratio of absorbance at 600 nm to that at 550 nm [D. N. Croft and M. Lubran, *Biochem. J.* **95**, 612 (1965)] and a negligible correction for nonspecific turbidity. There was 43.7 μg of DNA-P per milligram of dermis. If a dry weight of 35 percent is assumed for dermis [S. Rothman, *Physiology and Biochemistry of the Skin* (Univ. of Chicago Press, Chicago, 1954), p. 495], this is equivalent to 125 μg of DNA-P per gram of dry dermis, in good agreement with the published value of 190 ± 100 μg [P. Santoianni and M. Ayala, *J. Invest. Dermatol.* **45**, 99 (1965)].
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3-Acetylpyridine: Effects in vitro Related to Teratogenic Activity in Chicken Embryos

Abstract. *Production of skeletal muscle hypoplasia by 3-acetylpyridine and its complete reversal by nicotinamide in developing chicken embryos have been confirmed. Cultures of developing embryonic chicken muscle show degenerative effects produced by 3-acetylpyridine; these effects are reversed by nicotinamide. Cartilage production in cultured chondrogenic cells is potentiated by 3-acetylpyridine; this potentiation is completely reversed by nicotinamide. It is suggested that nicotinamide- or pyridine-nucleotide–dependent reactions influence normal differentiation of limb mesoderm cells by inhibiting chondrogenic-cell and potentiating muscle-cell expression or proliferation.*

Landauer (*1*) demonstrated that 3-acetylpyridine is teratogenic when introduced into the yolk sac of 96-hour-old chicken embryos. The most striking aberration produced under this condition was skeletal muscle hypoplasia such that little or no leg musculature was apparent at hatching. Bone, skin derivatives (*1*), heart, and eye were normal (*2*). Adequate supplements of nicotinamide given at the 96-hour stage lowered the toxicity of co-injected 3-acetylpyridine and completely protected against its teratogenicity. Landauer suggested that 3-acetylpyridine interfered with synthesis or utilization of nicotinamide by the early chicken embryo, or both.

In order to perform a detailed biochemical analysis of the effects of nicotinamide-antagonized teratogens, it seems necessary to isolate certain developmental events from the complex biochemistry of the whole chicken embryo. Systems in vitro are available for this purpose, and we now report establishment of a correlation between the action of 3-acetylpyridine in vivo and in vitro. Limb cartilage-producing and muscle-producing cell cultures were employed for two reasons. (i) Since both of these cell types originate from the single-cell type of the mesoderm in limb development (*3*), a study of the action of nicotinamide-antagonized teratogens might reveal details of the control system that dictates into which of the two possible cell types mesodermal cells eventually develop, and (ii) nicotinamide-antagonized teratogens seem to affect either muscle or bone development, rarely both (*4*). Thus, the contrast between muscle- and cartilage-producing cells may serve in the evaluation of the site of action of these teratogens.

Suspensions of leg muscle myoblasts were plated at two density ranges and cultured in Konigsberg medium (*5*). Most experiments were performed with 0.5 to 2 × 10^6 cells in 3 ml of medium per 5-cm plastic petri dish (*6*). In the few cases where confluent cultures in which rapid formation of multinuclear cells were desired, 10 to 20 × 10^6 cells were plated. These single-cell suspensions consist, for the most part, of myoblasts with some contaminating fibroblasts. The myoblasts fuse to form myofibril-producing multinuclear cells. The culture medium was removed and replaced with fresh medium every 24 hours. From 0.045 to 4.5 mg of 3-acetylpyridine per 3 ml of culture medium was added either at the time of plating, or 24, 48, 72, or 96 hours after plating. The 3-acetylpyridine was renewed at each 24-hour medium change in one series (that is, continuous application) and was eliminated from the medium of other series after 24, 48, 72, 96, or 120 hours of exposure. Control myoblast cultures were compared to those supplemented with nicotinamide or 3-acetylpyridine or both. Two amounts of 3-acetylpyridine (0.23 or 2.3 mg per 3 ml of culture medium) and two amounts of nicotinamide (0.30 and 3.0 mg) were added to the culture medium either separately or in combination.

Cartilage-producing cultures of limb-bud mesoderm cells were grown in the presence of (i) 0.45 mg or 4.5 mg of 3-acetylpyridine or (ii) 0.60 or 6.0 mg of nicotinamide, or a combination of (i) and (ii). Suspensions of single cells were obtained from the mesoderm of stage 24 (*3*) limb buds (*7*). From 15 to 25 × 10^6 cells were plated on plastic petri dishes (5 cm) in Eagle's medium (*8*) with 7 percent horse serum (*9*), 3 percent fetal calf serum (*10*), and 5 percent chicken embryo extract. Cells at this density form multilayered cultures in which cartilage nodules may be seen in the living state under phase optics, as highly refractile arrays of cells or by their metachromatic staining with toluidine blue in fixed cultures (*11*).

Both 3-acetylpyridine and nicotinamide were made up in calcium- and magnesium-free Tyrode's solution and then passed through an HA Millipore filter. Portions (0.1 ml) were delivered to each experimental culture. The various controls to which 0.1 ml of calcium- and magnesium-free Tyrode's solution was added showed none of the effects observed when 3-acetylpyridine or nicotinamide was present. Cultures were observed daily with phase-contrast optics, and were fixed and stained at the end of

the experimental period. Myogenic cultures were fixed with Zenker's fixative and stained with Heidenhain's iron-hematoxylin. Cartilage-producing cultures were fixed with 10 percent Formalin and stained with 1 percent toluidine blue O.

In myoblast cultures 4.5 mg of 3-acetylpyridine interfered with cell proliferation and was lethal to cultures that received continuous exposure. The first evidence of the effect of 3-acetylpyridine was the appearance of from two to ten intracellular refractile vacuoles per cell. These vacuoles appear from 24 to 48 hours after initial exposure. If 3-acetylpyridine is eliminated from cultures in which about 50 percent of the cells have survived prolonged exposure to this agent, little if any proliferation is subsequently observed. However, these single cells affected by 3-acetylpyridine retain the capacity to fuse and form multinuclear cells. After 2 or 3 days in the absence of 3-acetylpyridine, over 90 percent of the surviving cells are multinuclear; this result is in agreement with that of Konigsberg (*5*) who showed that exposure to nitrogen mustard affects myoblast proliferation but not multinuclear cell formation. If 3-acetylpyridine is withdrawn before cellular destruction is noticeable, proliferation will take place after a lag of 2 to 3 days. After this lag, proliferation and subsequent multinuclear cell formation take place at almost normal rates.

In platings of myoblasts at high densities (10 to 20 $\times$ 10^6 cells), 3-acetylpyridine does not seem to destroy cells, but inhibits the rate of formation of multinuclear cells while the extent of such formation is not affected. This statement is based on comparisons at these cell densities of treated cultures with untreated controls; cell proliferation is minimum in both. However, multinuclear cell formation occurs much more rapidly in untreated cultures; the final number of multinuclear cells is eventually the same in both treated and control plates. Thus we conclude that 3-acetylpyridine exerts a marked inhibitory effect on the formation of multinuclear cells under conditions in which myoblasts are not destroyed.

Just as Landauer (*1*) observed in experiments in ovo, nicotinamide completely relieves the effects of 3-acetylpyridine when both are added to cultures of myoblast cells. This relief by nicotinamide is concentration-dependent, just as the effects of 3-acetylpyridine are concentration-dependent. Nicotinamide, in approximately equal amounts to 3-acetylpyridine, is necessary to overcome the effects of 3-acetylpyridine. This observation suggests that 3-acetylpyridine is competitively interfering with the action of nicotinamide in promoting muscle development.

Concentrations of 3-acetylpyridine which produce degenerative effects in myoblast cultures greatly stimulate the production of cartilage in chondrogenic cell cultures. In these cultures, 4.5 mg stimulates cartilage production by a factor of 10 to 100 in a period of 5 to 8 days; 0.45 mg stimulates this production by a factor of 2 to 5. Whether increased cartilage production represents increased cell proliferation or an increase in the number of cells which are secreting cartilaginous material is yet to be determined.

Nicotinamide counteracts the stimulation of cartilage production by 3-acetylpyridine when equivalent amounts are added simultaneously. In three control experiments, nicotinamide alone (4.5 mg per plate) showed some inhibition of cartilage formation. This is in marked contrast to the lack of effect of nicotinamide alone on myoblast cultures. The stimulation of cartilage formation by 3-acetylpyridine and the nicotinamide-sponsored inhibition of this stimulation as well as nicotinamide's inhibition of normal cartilage nodule formation indicates that nicotinamide inhibits the cartilage production process. Presumably 3-acetylpyridine effectively stimulates cartilage formation by competing with nicotinamide and thereby limiting the inhibitory effects of nicotinamide on cartilage nodule formation.

In general, 3-acetylpyridine seems to profoundly affect the proliferative activities of a variety of cells in culture (*12*). When proliferation is minimum, such as in the high-density chondrogenic cell cultures and myoblast cultures used here, 3-acetylpyridine exhibits a differential effect: stimulatory in the case of cartilage production and inhibitory in the case of myoblast differentiation. The extension of our observations to the effects of 3-acetylpyridine in ovo would suggest that, while general proliferation is inhibited by 3-acetylpyridine, as evidenced by the fact that treated embryos are smaller than the controls, the expression of cartilage production is favored over that of muscle development. Those mesodermal cells whose proliferative activity is not greatly affected by 3-acetylpyridine would be expected to produce cartilage more readily than muscle elements. Presumably, the effects of 3-acetylpyridine reflect a competitive stress that is put on the nicotinamide-dependent reactions or nicominamide-incorporating molecules such as nicotinamide-adenine dinucleotide or nicotinamide-adenine dinucleotide phosphate (NAD or NADP). This stress is observed as aberrant metabolic or developmental events, or both.

Thus, nicotinamide may participate in the control of differentiation of limb structure in that it is capable of sustaining normal muscle formation and development while at the same time inhibiting extensive cartilage formation. The teratogenic properties of 3-acetylpyridine may be an expression of the balance between nicotinamide potentiation and inhibition of the developmental events of mesodermal cells which are capable of forming either cartilage or muscle. These suggestions form the basis for further experimentation into the fate and mode of action of nicotinamide-linked teratogens.

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