cells forming differentiated embryo tissue. The long single-stranded regions might also be related to transcription of specific genes; the formation of gaps may be part of the mechanism by which histone genes are regulated during the mitotic cycle and early development.

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Regulation of Cutaneous Previtamin D_3 Photosynthesis in Man: Skin Pigment Is Not an Essential Regulator

Abstract. *When human skin was exposed to simulated solar ultraviolet radiation, epidermal 7-dehydrocholesterol was converted to previtamin D_3. During prolonged exposure to simulated solar ultraviolet radiation, the synthesis of previtamin D_3 reached a plateau at about 10 to 15 percent of the original 7-dehydrocholesterol content, and previtamin D_3 was photoisomerized to two biologically inert isomers, lumisterol$_3$ and tachysterol$_3$. Increases either in skin melanin concentration or in latitude necessitated increases in the exposure time to simulated solar ultraviolet radiation required to maximize the formation, but not the total content, of previtamin D_3. In order of importance, the significant determinants limiting the cutaneous production of previtamin D_3 are (i) photochemical regulation, (ii) pigmentation, and (iii) latitude.*

Although the photochemical pathway by which 7-dehydrocholesterol (7-DHC) in the skin is converted to previtamin D_3 (preD_3) by exposure to sunlight is established, little is known about the factors that directly influence the cutaneous photosynthesis of preD_3 (*1–4*). We exposed hypopigmented and hyperpigmented human skin to simulated solar ultraviolet radiation for various times and determined the photoproducts of 7-DHC in order to (i) study the sequential photochemical events quantitatively; (ii) examine regulatory processess, if any; (iii) investigate the role of melanin in the production of preD_3 in the skin; and (iv) determine the effect of latitude on preD_3 formation in the skin. In these experiments, the formation of preD_3 in hypopigmented skin reached a plateau after a short (15 minute) exposure to simulated noon equatorial ultraviolet radiation; further irradiation increased only the biologically inactive photoisomers, lumisterol$_3$ and tachysterol$_3$. As the melanin concentrations in skin increased, the time of exposure necessary to maximize the formation, but not the total content, of preD_3 also increased.

Surgically obtained hypopigmented (type III) and hyperpigmented (types V and VI) human skin specimens (*5*), obtained from different areas of the body with subcutaneous fat removed, were cut into samples (6.25 cm^2) and immersed in a water bath (60°C) for 30 seconds according to the method of Blank *et al.* (*6*). The skin was blotted dry, and the stratum-corneum side was exposed for various times to simulated solar ultraviolet radiation. The radiation source was a 2.5 kW xenon arc lamp (Schoeffel) coupled with a dichroic mirror and appropriate filters. Conditions were adjusted to approximate the ultraviolet radiation that reaches Earth in June at sea level at either the equator (0° latitude) or in Boston (42°20′N) at noon. Immediately after irradiation, the skin was separated into a top layer (stratum corneum, granulosum, and stratum spinosum) and a bottom layer (stratum basale and dermis). The basal cells (stratum basale) were mechanically scraped from the dermis. Control skin samples maintained in an ultraviolet-free environment for the same periods of time were similarly separated. The skin layers were confirmed histologically. The separated layers of skin were extracted with 8 percent ethyl acetate in *n*-hexane for 24 hours at −20°C. The extracts were centrifuged, and the supernatant was collected, taken to dryness under N_2, and weighed. A portion of each sample was then chromatographed in duplicate to determine the concentrations of 7-DHC, preD_3, lumisterol$_3$, and tachysterol$_3$ (Fig. 1, A to D).

The chromatography of the lipid extracts from skin was performed in ethyl acetate (8 percent) in *n*-hexane on a high-performance liquid chromatograph (HPLC) equipped with a radial compression module containing a Radial-Pak-B column (10 by 0.8 cm) coupled with an ultraviolet absorption detector at 254 nm (Waters Associates, model 440) and a printer-plotter Data Module Integrator (Waters Associates). This chromatographic system permits complete resolution to baseline of 7-DHC, preD_3, lumisterol$_3$, and tachysterol$_3$ (*2*). Quantitation of the concentration of 7-DHC, preD_3, lumisterol$_3$, and tachysterol$_3$ in skin lipid extracts was based on standard curves that were generated by plotting the integrated area under the peak as a function of known concentration of pure compound.

As standards for the chromatography we used [3α-^{3}H]7-DHC, [3α-^{3}H]-preD_3, [3α-^{3}H]tachysterol$_3$, and [3α-^{3}H]-lumisterol$_3$; each compound had a specific activity of 4.8 Ci/mmole and was synthesized as previously described (*2*). Five milligrams of 7-DHC were exposed to ultraviolet radiation (*2*) to generate sufficient quantities of preD_3, lumisterol$_3$, tachysterol$_3$, and D_3 for the vitamin-D binding-protein (DBP) assay and for the standard concentration curves. Identification of the skin lipid peaks shown in Fig. 1 was based on cochromatography studies with radioactive tracers (*2*). In addition, 7-DHC was structurally characterized (*3*), and lumisterol$_3$ was isolated in pure form from surgically obtained human thigh epidermis that was exposed to 3 hours of simulated equatorial solar ultraviolet radiation. Determination of the structure of lumisterol

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was based on its ultraviolet absorption spectrum (λ_{max} at 280,271 nm), which is characteristic for this 5,7-diene isomer, and its mass spectrum with M^+ at 384.

The affinity of the DBP for 7-DHC, lumisterol$_3$, tachysterol$_3$, preD$_3$, and D$_3$ was determined (*7*) after each of the compounds was purified to homogeneity on the HPLC. There was no detectable displacement of labeled 25-hydroxyvitamin D$_3$ (25-OH-D$_3$) when we added, per tube, up to 1000 ng of 7-DHC, lumisterol$_3$, or preD$_3$ (Fig. 2). Fifty percent of labeled 25-OH-D$_3$ was displaced from the DBP when it was incubated with either 500 ng of tachysterol$_3$ or 30 ng of D$_3$ per tube.

Male weanling Holtzman rats (Madison, Wis.) were fed a vitamin-D-deficient diet adequate in calcium and phosphorus for 4 weeks, and then they were given intravenous doses of one of the following compounds (each dissolved in 50 μl of 95 percent ethanol): 0.25 μg of D$_3$, 10 μg of lumisterol$_3$, and either 1 μg or 10 μg of tachysterol$_3$. Fasted animals (six per group) were killed 24 hours later for determination of calcium transport activity in the intestine by the everted-gut-sac technique (*8*). Animals given D$_3$ (0.25 μg) showed increased intestinal calcium transport ($S/M = 3.5 \pm 0.2$) (*8*) compared to the control group ($S/M = 2.0 \pm 0.2$). The lumisterol$_3$ and 1.0 μg of tachysterol$_3$ failed to stimulate intestinal calcium transport ($S/M = 1.8 \pm 0.2$ and 2.0 ± 0.2, respectively), whereas 10 μg of tachysterol$_3$ produced a small but insignificant increase ($S/M = 2.3 \pm 0.3$).

In Fig. 1, B to D, we show representative chromatographic profiles of lipid extracts of the stratum basale separated from surgically obtained type III human skin samples that were exposed for various times to simulated equatorial solar ultraviolet radiation. After 15 minutes of exposure, the principal photoproduct in the stratum basale was preD$_3$ (Fig. 1B). In skin samples exposed for longer periods there were two additional photoproducts, lumisterol$_3$ and tachysterol$_3$ (Fig. 1, C and D), but, as shown in Fig. 1, C to E, tachysterol$_3$ formation reached a maximum at approximately 5 percent of the original 7-DHC concentration by 1 hour, whereas lumisterol$_3$ formation steadily increased to 50 percent by 8 hours. Correlated with the increase in photoproduct concentration was the decline of 7-DHC to 30 percent of its original concentration by 8 hours (Fig. 1E).

In Table 1 we compare the exposure time necessary to maximize preD$_3$ formation in type III and types V and VI skin. As pigmentation increases from type III to types V and VI, the exposure time necessary to maximize preD$_3$ formation increases from 30 minutes to 1 hour and 3 hours, respectively. However, regardless of skin type, preD$_3$ reaches a maximum and plateaus at about 15 percent of the original 7-DHC concentration and further exposure to ultraviolet radiation causes increases only in lumisterol$_3$. The exposure times necessary to maximize preD$_3$ formation in type III skin when it is exposed to simulated solar ultraviolet radiation at the equator or in Boston are shown in Table 1 and indicate the effect of latitude on preD$_3$ formation. The decrease in ultraviolet radiation (290 to 320 nm) from the equator to Boston (due to an increase in the zenith angle of the sun) increased the exposure time required to maximize preD$_3$ formation.

Hence the photochemical conversion of preD$_3$ to lumisterol$_3$ appears to limit preD$_3$ accumulation in human skin dur-

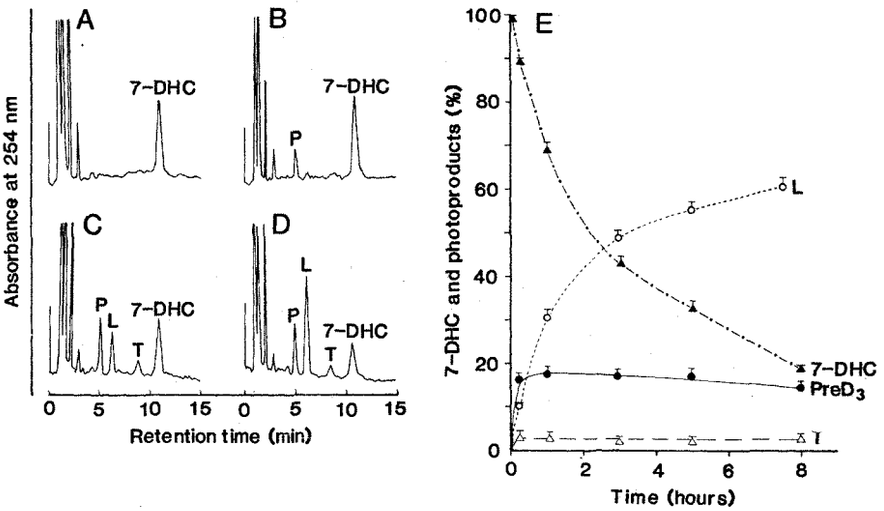

Fig. 1. High-performance liquid chromatographic profiles of a lipid extract from the basal cells of surgically obtained hypopigmented skin that was previously (A) shielded from or (B to D) exposed to equatorial simulated solar ultraviolet radiation that reaches Earth at sea level at noon for 10 minutes (B), 1 hour (C), or 3 hours (D). (E) An analysis of the photolysis of 7-dehydrocholesterol (*7-DHC*) in the basal-cell layer and the appearance of the photoproducts previtamin D$_3$ (*PreD$_3$*), lumisterol$_3$ (*L*), and tachysterol$_3$ (*T*) with increasing time of exposure to equatorial simulated solar ultraviolet radiation. Bars above data points show the standard error of the mean of three determinations.

Table 1. The effect of melanin concentration and latitude on the formation of previtamin D$_3$ and lumisterol$_3$ in human skin exposed to simulated solar ultraviolet radiation. Radiation was provided by a 2.5 kW xenon arc lamp appropriately filtered, passed through a quartz field lens and reflected by a maximum reflector of ultraviolet light onto the skin. Spectral irradiance agreed well with 1972 CIE (*15*) standard for solar simulation. Total ultraviolet irradiance (290 to 400 nm) was 5.88×10^{-6} W/m^2. The 290 to 320 nm irradiance was 5.170×10^{-7} W/m^2 for 0° latitude and 3.619×10^{-7} for 42°20′N latitude.

Skin type*	Simulated geographic location†	Time of exposure to maximize preD$_3$ formation‡ (hours)	Lumisterol$_3$ formation after 3 hours of exposure (%)
Type IIIa	Equator	0.50 to 0.75	40
Type V	Equator	0.75 to 1.50	20
Type VI	Equator	3.00 to 3.50	14
Type IIIb	Equator	0.25 to 0.50	50
Type IIIb	Boston	0.50 to 1.00	30

*Types IIIa, V, and VI were surgically obtained foreskin specimens from healthy males (26 to 36 years old); type IIIb was surgically obtained thigh skin from a 28-year-old male. †A WG-305-C Schott filter (Schott Optical Glass) was used to simulate noon sunlight at the equator at sea level. The radiation level was based on a CIE report (*15*) that 1 square centimeter of skin exposed to sunlight on a cloudless day for an 8-hour period will be irradiated with approximately 175 J of ultraviolet-A (320 to 400 nm) and 14 J of ultraviolet-B (290 to 320 nm). A WG-320-B Schott filter was used to simulate Boston (latitude 42°20′N) sunlight at noon in June at sea level at a zenith angle of 20°. The radiation level was based on the report (*15*) that 1 square centimeter of skin exposed to sunlight on a cloudless day for an 8-hour period will be irradiated with approximately 140 J of ultraviolet-A (320 to 400 nm) and 9 J of ultraviolet-B (290 to 320 nm). ‡Because of the high intensity of this light system, exposure times to simulated solar ultraviolet radiation were significantly decreased compared with natural conditions. For example, the amount of time it took to deliver the equivalent of 3 to 3½ hours of simulated natural equatorial solar ultraviolet radiation was approximately 10 minutes. Each value represents an average of three separate determinations. There was excellent agreement for each value, with less than 10 percent variation. All the experiments were performed within 6 hours of surgically obtaining the skin. Previous experience demonstrated that the viability and 7-dehydrocholesterol content of these skin samples does not significantly change for up to 72 hours when they are kept moist (with 0.9 percent saline) at 4°C.

ing excessive exposure to the sun. Loomis (*9*) popularized the theory (*10*) that different skin colors in man are adaptations to geographical conditions. Loomis suggested that skin color regulated the transmission of solar ultraviolet so that vitamin radiation-D_3 photosynthesis would be relatively constant, and that natural selection favored black skin near the equator because it prevents toxicity due to excessive amounts of cutaneous vitamin-D_3 formation (*11*). Our data suggest that the photochemical conversion of preD_3 to lumisterol and tachysterol (*12*) is the major factor that prevents vitamin-D_3 intoxication after a single prolonged exposure to the sun. To our knowledge there are no documented cases of vitamin-D toxicity due to prolonged exposure to the sun (*13*). The experiments described here indicate that, if a hypopigmented person and heavily pigmented person are exposed to 3 hours of equatorial ultraviolet radiation (Fig. 1 and Table 1), the amount of preD_3 formed is essentially the same (that is, about 15 percent of the initial 7-DHC content in the epidermis). The major difference is that the photoproduction of lumisterol$_3$ is significantly greater in hypopigmented skin than in heavily pigmented skin.

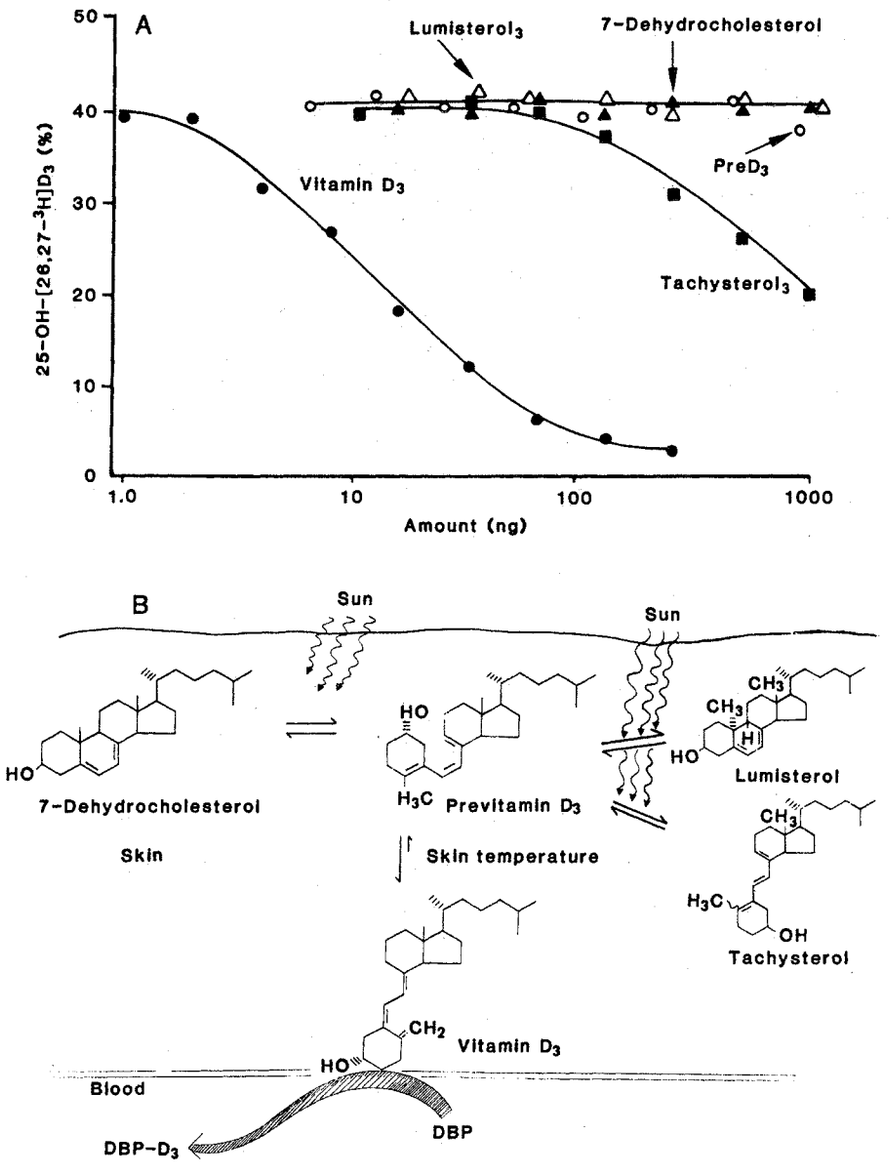

Fig. 2. (A) Displacement of ^{3}H-labeled 25-OH-D_3 from rat vitamin-D binding protein by vitamin D_3, previtamin D_3 (*PreD$_3$*), lumisterol$_3$, tachysterol$_3$, and 7-dehydrocholesterol. Each point represents an average of three determinations. (B) Schematic representation of the formation of previtamin D_3 in the skin during exposure to the sun and its thermal isomerization to vitamin D_3, which is specifically translocated by the vitamin-D binding protein (*DBP*) into the circulation. During continual exposure to the sun, previtamin D_3 also photoisomerizes to lumisterol$_3$ and tachysterol$_3$, which are biologically inert photoproducts; that is, they do not stimulate intestinal calcium absorption. Because the vitamin-D binding protein has no affinity for lumisterol$_3$ but has minimal affinity for tachysterol$_3$, the translocation of these photoisomers into the circulation is negligible, and these photoproducts are sloughed off during the natural turnover of the skin. Because these photoisomers are in a quasi-stationary state (*12*) as soon as previtamin D_3 stores are depleted (because of thermal isomerization to D_3), exposure of lumisterol and tachysterol to ultraviolet radiation will promote the photoisomerization of these isomers to previtamin D_3.

Since melanin competes with 7-DHC for ultraviolet photons, increasing the melanin in human skin also increases the time of exposure to ultraviolet radiation that is needed to maximize preD_3 formation (Table 1). Our data show that skin melanin can limit preD_3 formation during limited exposure to solar ultraviolet radiation, and support experimentally the observation (*14*) that pigmented individuals living in the industrialized cities of the North are more prone to the development of osteomalacia or rickets.

Our data provide new insights into a basic mechanism controlling cutaneous photosynthesis of preD_3. When the skin is initially exposed to solar ultraviolet radiation the 7-DHC is converted to preD_3. Previtamin D_3 in the skin can undergo either thermal isomerization to D_3 or photoisomerization to lumisterol$_3$ and tachysterol$_3$ (Fig. 2). During prolonged exposure to the sun, the synthesis of preD_3 reaches a plateau at about 10 to 15 percent of the original 7-DHC concentration, and the preD_3 undergoes photoisomerization to two biologically inert products, lumisterol$_3$ and tachysterol$_3$. Tachysterol$_3$ formation also plateaus at about 5 percent, whereas lumisterol$_3$ formation continues to increase. Because the DBP has no affinity for lumisterol$_3$ and minimum affinity for tachysterol$_3$, the translocation of these photoisomers into the circulation is negligible, and thus these products are probably sloughed off during the natural turnover of the skin. Furthermore, because these photoisomers are in a quasi-stationary state with preD_3 (*12*), as soon as preD_3 stores are depleted (because of thermal isomerization to D_3), exposure of cutaneous lumisterol$_3$ and tachysterol$_3$ to solar ultraviolet radiation may promote the photoisomerization of these products to preD_3. In order of importance, the significant determinants limiting cutaneous preD_3 production seem to be (i) photochemical regulation, (ii) pigmentation, and (iii) latitude.

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2,4,5-Trichlorophenoxyacetic Acid Causes Behavioral Effects in Chickens at Environmentally Relevant Doses

Abstract. *Administration of the herbicide 2,4,5-trichlorophenoxyacetic acid to incubating chicken eggs alters behavior after hatching. Single doses, with no morphological effects, retard learning (lowest dose, 7 milligrams per kilogram of body weight) and increase general activity (27 milligrams per kilogram) and jumping (13 milligrams per kilogram). Day 15 of incubation is the most susceptible stage of development.*

As far as we know, there has been only one earlier study reporting teratological effects of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) on behavior (*1*). Administration of 2,4,5-T (100 mg/kg) to pregnant rats produced no morphological deformities in the offspring, but it did cause them to have learning deficits and to exhibit increased activity in the open field. We have shown that even lower doses can produce behavioral abnormalities in the chicken. A single dose of 2,4,5-T [Sigma; containing 0.03 part per million of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), according to the Australian Government Analytical Laboratory] was administered to the chicken egg on either day 8 or day 15 of incubation (*2*). A third group received 2,4,5-T by intraperitoneal injection on day 2 after hatching. The 2,4,5-T was suspended in a 0.5 percent solution of gum tragacanth made with sterilized, distilled water. Control animals (matched for each separate hatching) received the vehicle alone. Doses were calculated according to the weight of the chicken or egg at the time of injection and ranged from 7 to 53 mg/kg for eggs and 75 to 225 mg/kg for chicks after hatching.

The median lethal dose (LD_{50}) on day 2 after hatching was 200 mg/kg, and on day 15 of incubation it was 53 mg/kg. There was 70 percent hatching in eggs given 2,4,5-T on day 15 of incubation at doses up to 27 mg/kg. Some 5 to 10 percent of the hatched chicks, irrespective of dose, showed abnormal leg development; they either dragged one leg or held it off the ground. Approximately 5 percent of the treated chicks showed depigmentation of feathers and down. Both of these effects have been reported (*3*). No differences in body weight gain were observed in chicks receiving 2,4,5-T during incubation or at doses less than 150 mg/kg on day 2 after hatching.

The chicks without apparent morphological deformities (after doses shown in Fig. 1) were given behavioral tests in week 2 after hatching. The tester was unaware of the treatment that each chick had received. There were 8 to 20 chicks in each group tested. We measured the activity in an unfamiliar environment on day 7 of life by placing the chick in a gray-walled box (30 cm by 30 cm with 25-cm walls). Cumulative activity for a 3-minute interval ("general activity") was scored by an Animex activity meter, and the number of jumps made was counted by eye. We scored ambulation for some groups by dividing the floor of the box into quadrants and counting the number of times the chick crossed into a new quadrant. We tested visual discrimination learning on day 9 of life by using the "pebble floor" test (*4*), which consists of a search for grains of food scattered over a background of small pebbles adhering to the floor. Within a 60-peck trial, a measure of visual learning is generated by recording the number of incorrect responses (pecks at pebbles) within consecutive 20-peck intervals.

General activity, jumping, and visual learning rate were altered by 2,4,5-T treatment (Fig. 1). Since the same doses were administered on day 8 and day 15 of incubation, these data could be examined by a two-way analysis of variance (that is, dose, age, and their interaction). A one-way analysis of variance was used for the data from day 2 after hatching. General activity scores conformed to a parametric distribution, but jumping and learning scores required logarithmic transformation.

Jumping behavior proved to be the most sensitive parameter affected by 2,4,5-T. For those treated on days 8 and 15 of incubation, jumping showed both a significant dose effect ($F = 2.78$; d.f. = 3, 90; $P < .05$) and significant interaction between age and dose ($F = 3.33$; d.f. = 3, 90; $P < .05$). Comparisons of the groups given the two highest doses with their control groups (t-test) showed a significant increase in jumping in the groups treated with 13 mg/kg ($P < .05$) and 27 mg/kg ($P < .01$) on day 15 of incubation, but there were no significant differences in those treated on day 8 (see Fig. 1). There was also a significant dose effect for jumping in the groups treated on day 2 after hatching ($F = 4.06$; d.f. = 2, 20; $P < .05$), and a t-test of data for the control group and the group receiving a dose of 150 mg/kg revealed a significant increase in jumping after 2,4,5-T treatment ($P < .025$), this dose being tenfold higher than the lowest dose found to be effective on day 15 of incubation.

General activity, which includes measures of jumping, ambulation, and other movements, shows a similar tendency to increase after 2,4,5-T treatment on day 15 of incubation and day 2 after hatching (Fig. 1). Analyses of variance just failed to reveal significant effects, but t-tests of data for the control groups and those groups that had received the highest doses at each age revealed a significant elevation of general activity in the groups treated with 27 mg/kg on day 15

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