perienced the same temperature. (ii) Temperatures late in development have no influence on sex determination (on the basis of our temperature-shift experiments), and gonadal differentiation after hatching progresses in the direction expected from incubation temperature. These observations indicate that one of two clear-cut developmental pathways is undertaken in the embryo, and that the pathway is adhered to at least shortly after hatching. In map turtles raised 7 years from hatching, secondary sexual characteristics have remained constant from their inception at age 2 years (*11*). Histological studies on other turtles (*12*) showed no incidence of sex reversal or dysfunction in individuals of various ages. Therefore, we think it unlikely that infertility or changes in sex phenotype are common occurrences in these turtles, but this remains to be demonstrated conclusively.

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7. Here we consider *G. ouachitensis* as a valid species (R. C. Vogt, in preparation), although it is recognized by some as a subspecies of *G. pseudogeographica*. Our conclusions do not change if data for these two taxa are lumped.
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10. Diagnosis of sex was based on Yntema's criteria (*3*). Hatchlings and embryos near hatching show either a well-developed Mullerian duct and a long gonad (ovary) with a thick cortex and degenerate medulla, or (males) show a degenerate Mullerian duct, rudimentary cortex, with a well-developed medulla. Ovaries develop slightly enlarged follicles within 2 months after hatching. If the sex was not clear when viewed under a dissecting microscope, the gonad was squashed in aceto-orcein stain and viewed under a light microscope to facilitate distinguishing the cortex and medulla. Our observations on sex differentiation agree with those of (*3*) and P. I. Risley [*Z. Zellforsch. Mikrosk. Anat.* **18**, 493 (1933)].
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13. Eggs buried in the shade developed more slowly than those in the sun and were brought to the laboratory prior to hatching, but only after they had surpassed the developmental stages in which sex determination is sensitive to temperature. Many embryos were near hatching, some being dissected and sexed immediately; remaining eggs were divided between 25° and 30.5°C for the duration of incubation, but only males resulted.
14. John M. Legler initially brought our attention to sex determination in turtles. We thank C. Denniston, J. F. Crow, W. Engels, C. Preston, E. Wijsman, W. Reeder, J. Opitz, and R. Lande for help with the project design; M. Curie-Cohen, J. VandeBerg, R. Berg, P. Randolph, A. Muth, N. Rudolph, J. M. Legler, and the above for discussion; W. Kugler, C. Hughes, and A. Bull for technical assistance. W. Reeder and W. Porter provided materials for the field work; the University of Wisconsin Biotron and Genetics Laboratory provided some of the incubators. Supported by a University of Wisconsin Biomedical Research Grant; and an NIH postdoctoral fellowship (J. B.). This is paper 2380 from the University of Wisconsin Laboratory of Genetics.

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Target Cells for 1,25-Dihydroxyvitamin D_3 in Intestinal Tract, Stomach, Kidney, Skin, Pituitary, and Parathyroid

Abstract. *After mature rats that had been fed on a vitamin D_3–deficient diet were injected with tritium-labeled 1,25-dihydroxyvitamin D_3, radioactivity became concentrated in nuclei of luminal and cryptal epithelium of the duodenum, jejunum, ileum, and colon; in nuclei of the epithelium of kidney distal tubules including the macula densa, and in podocytes of glomeruli; in nuclei of the epidermis including outer hair-shafts and sebaceous glands; and in nuclei of certain cells of the stomach, anterior and posterior pituitary, and parathyroid. These results reveal cell types that contain receptors for 1,25-dihydroxyvitamin D_3 or metabolites of this compound both in known or hypothesized target tissues and in tissues that were previously unknown to participate in vitamin D_3 metabolism.*

Target tissues for 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] have been demonstrated in intestine, kidney, and bone by means of radioassay and biochemical techniques (*1*). However, little information is available on the morphological identification of target cells for this hormone. In autoradiographic experiments with chick duodenum, radioactivity was found in the region of nuclei of the epithelium (*2*). In rat metaphyseal bone, 25-hydroxyvitamin D_3 was found "in epiphyseal hypertrophic cells, epiphyseal matrix, osteoid, osteoblasts, and osteocytes" (*3*) with an autoradiographic procedure that is not suitable for determining the distribution of steroids (*4*). Our dry-mount and thaw-mount autoradiographic techniques (*4*), which exclude liquid fixatives, embedding media, and dehydration fluids, have been used successfully for determining the cellular and subcellular distribution of steroid hormones (*5*) and have facilitated demonstrations of steroid hormone target cells in the brain, pituitary, thymus, heart, blood vessels, ovary, testis, and skin (*5, 6*). In the experiments described here we injected 1,25$(OH)_2D_3$ into rats and obtained autoradiographic evidence for nuclear concentration and retention of radioactivity in rat duodenum, jejunum, ileum, colon, stomach, kidney, skin, pituitary, and parathyroid.

Six 28-day-old male Sprague-Dawley rats were placed on a vitamin D_3–deficient diet for 35 days; the diet was supplemented with vitamins A, E, and K (*7*). All animals were injected intravenously with 1,25$(OH)_2$-[26,27-^{3}H]D_3 dissolved in 75 percent ethanol with isotonic saline. Two animals received 0.19 μg of the labeled compound (specific activity 90 Ci/mmole) per 100 g of body weight; and four animals received 0.16 μg/100 g (specific activity 160 Ci/mmole). The animals of the former group were killed 1 or 3 hours and those of the latter group 2 hours after the injection. Two of the rats that received the high dose were each injected subcutaneously with 1.0 μg of unlabeled 1,25$(OH)_2D_3$ 15 minutes before they were injected with the labeled compound. In addition we used one pregnant rat (*8*) that received the vitamin D_3–deficient diet from the start of pregnancy. On day 18 of pregnancy, the animal was injected intravenously with the labeled compound (0.18 μg/100 g of body weight; specific activity 160 Ci/mmole) dissolved in 70 percent ethanol (with isotonic saline) and killed 4 hours afterward. Tissues were excised, mounted, and frozen onto tissue holders, sectioned at 4 μm, and thaw-mounted on photographic emulsion (Kodak NTB3) coated slides. The autoradiograms were exposed for 3 to 11 months, then photographically processed and stained with methylene blue and basic fuchsin. The thaw-mount autoradiographic technique and the preparation of controls against chemography have been discussed (*4*). Cells are considered labeled when the

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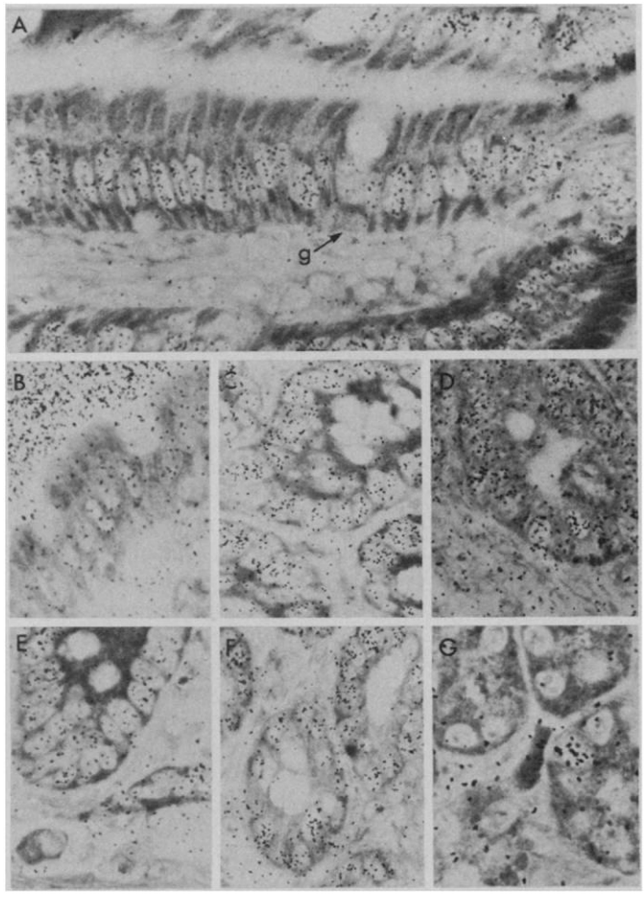
number of silver grains over the nucleus exceeds several times the silver grain density in adjacent tissue compartments.

At all dose levels and time intervals after the injection of $1,25(OH)_2$-[26,27-$^3H]D_3$, radioactivity was found to be highly concentrated in nuclei of epithelial cells of villi and crypts in the duodenum (Fig. 1, A and C), jejunum (Fig. ID), and ileum (Fig. 1E), and of epithelial cells of the luminal surface and crypts in the colon (Fig. 1F). Prior injection of the animal with unlabeled $1,25(OH)_2D_3$ diminished or abolished the nuclear uptake of radioactivity (Fig. 1B). The nuclear concentration of radioactivity was not confined to the duodenum, but included other segments of the small and large intestine, although the nuclear uptake appeared to be in general somewhat stronger in the small intestine. These results suggest that all segments of the intestinal tract participate to a varying degree in vitamin D_3–dependent calcium and phosphate absorption, and they support the concept of a genomically mediated synthesis of related specific intestinal proteins, such as calcium-binding protein, alkaline phosphatase, adenosine triphosphatase, and phytase (*9*). Goblet cell nuclei remain unlabeled (Fig. 1A), indicating that $1,25\text{-}(OH)_2D_3$ has no direct effect on mucus production. In various regions of the lumen of the intestine, high amounts of radioactivity can be seen, both in control animals and animals that received injections of unlabeled vitamin D_3 before the labeled compound (Fig. 1B). This probably represents biliary metabolites. The sharp separation between high lumenal and low epithelial radioactivity suggests the presence of an intralumenal binding protein or a barrier mechanism, or both. In the stomach, in contrast to the intestine, epithelial cells were generally unlabeled, except for dispersed individual cells in basal portions of gastric glands (Fig. 1G) in locations similar to those in which chromaffin cells are found.

In the liver (Fig. 2A), no nuclear labeling is seen. However, certain cells concentrated radioactivity in their cytoplasm. The chemical nature of this radioactivity needs to be clarified. These cells have smaller nuclei than hepatocytes and are located at sinusoids. The relative sparseness of these labeled cells and their position and stellate shape suggest that these are lipocytes, a cell type believed to store vitamin A (*10*) and previously demonstrated to concentrate radioactivity after the administration of ^3H-labeled progesterone (*6*). In the kidney (Fig. 2B) radioactivity occurred in the nuclei of epithelial cells of the distal tubules, including the macula densa, and in certain cells of the glomeruli, probably podocytes. Mesangial cells appeared to be unlabeled. Weak nuclear labeling was seen in collecting duct cells and, more sparsely, in cells of the proximal tubules.



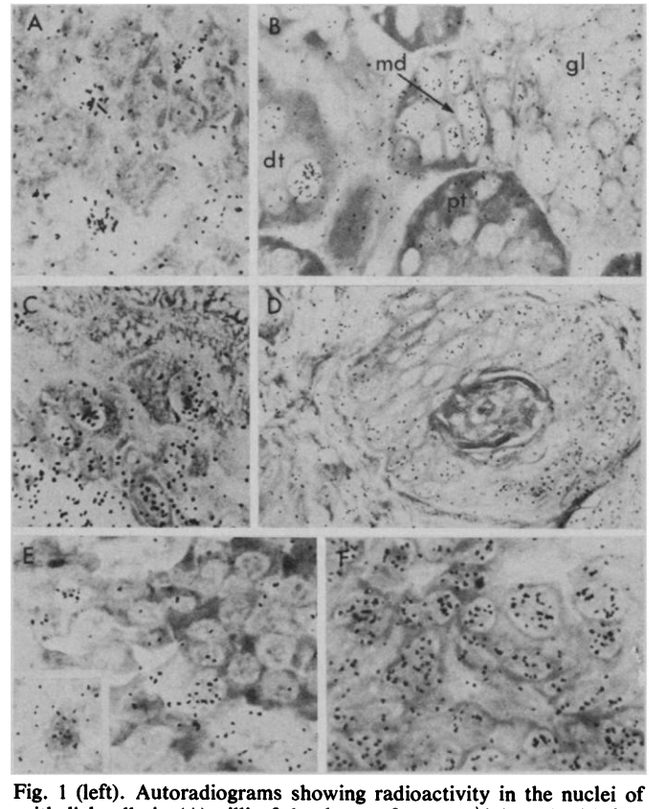


Fig. 1 (left). Autoradiograms showing radioactivity in the nuclei of epithelial cells in (A) villi of duodenum from rats injected with ^3H-labeled $1,25(OH)_2D_3$; (B) duodenal villi from rats injected first with the unlabeled and then with the labeled vitamin D_3; (C) crypts of duodenum; (D) jejunum; (E) ileum; (F) colon; and (G) endocrine cells of gastric glands. In (A) the goblet cells (*g*) are unlabeled; the arrow points at the unlabeled nucleus of a goblet cell that is located at the base. The tissues were prepared 1 hour (A, C, and E) or 2 hours (B, D, F, and G) after the labeled compound was injected; the autoradiograms were exposed for 11 months (A, C, and E) or 8 months (B, D, F, and G). Magnification ×450. Fig. 2. Autoradiograms showing the concentration of radioactivity (A) in the cytoplasm of certain liver cells, probably lipocytes or Kupffer cells; (B) in nuclei of epithelial cells of kidney distal tubules (*dt*), including the macula densa (*md*) adjacent to a glomerulus (*gl*), with the cells of the proximal tubules (*pt*) being unlabeled or very weakly labeled; (C) in nuclei of epithelial cells of epidermis and (D) outer hairshaft; (E) in nuclei of specific cells in the anterior pituitary and (E, inset) of pituicytes in the posterior pituitary; and (F) in nuclei of cells in the parathyroid. The tissues were prepared 1 hour (D), 2 hours (A, B, and C), 3 hours (E), or 4 hours (F) after the animals were injected with ^3H-labeled $1,25(OH)_2D_3$. The autoradiograms were exposed for 11 months (D and E), 7 months (A, B, and C), or 3 months (F). Magnification ×450 (A, B, and D) or ×600 (C, E, and F).

In the skin, nuclei of cells of the epidermis (Fig. 2C) were labeled, most strongly in the basal layers (Fig. 2, C and D), in the external root sheath of the hair (Fig. 2D), and in the basal layer of the sebaceous glands.

In the pituitary, radioactivity was observed in nuclei of certain cells (Fig. 2E) in the pars distalis (*11*). In addition, pituicytes in the infundibular process were weakly labeled as in animals injected with ^{3}H-labeled estradiol (*6*). In the parathyroid most of the nuclei of parenchymal cells were labeled (Fig. 2F). This provides evidence of a direct action by $1,25(OH)_2D_3$ on the parathyroid hormone system as hypothesized previously (*1*).

The localization of target cells in the pituitary, parathyroid, and stomach indicates that calcium homeostasis is regulated by central as well as peripheral endocrine mechanisms. Although nothing is known about the involvement of the pituitary, the cells in the stomach may be hormone (probably gastrin) producing cells, because gastrin has been shown to modulate the concentration of calcitonin in the blood and vice versa (*12*). The occurrence of radioactivity in presumed endocrine cells of the stomach suggests that $1,25(OH)_2D_3$ is a modulator in the interaction between gastrin and calcitonin.

Our results demonstrate the potential of the histological approach. Not only were we able to confirm and cytologically define biochemically characterized target tissues, but we were also able to add several new cell types to those already described. With the discovery of previously unidentifed target cells of $1,25(OH)_2D_3$ or metabolites of the compound, modifications must be made in the present models for calcium homeostatic mechanisms. Although we do not know what effects the hormone has on podocytes, cells of the macula densa, cells of the pars distalis, pituicytes, and endocrine cells of the stomach, extensive studies of other tissues, such as bone and thyroid, as well as of the cell types identified herein, should help to answer this question.

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A Glucose-Controlled Insulin-Delivery System: Semisynthetic Insulin Bound to Lectin

Abstract. *A stable, biologically active glycosylated insulin derivative that is complementary to the major combining site of concanavalin A has been synthesized. Hormone release is proportional to the quantity of glucose present. Glucose regulation of exogenous insulin delivery could have important applications in the therapy of diabetes mellitus.*

Although injectable insulin has been available for the treatment of diabetes mellitus for more than 50 years, the simple replacement of the hormone is not sufficient to prevent the pathological sequelae associated with this disease. The development of these sequelae is believed to reflect an inability to provide exogenous insulin proportional to the patients' varying concentrations of blood glucose (*1*). In fact, recent surveys in which hemoglobin A_{1c} measurements were used have revealed that a large percentage of diabetic outpatients have poor diabetic control (*2*). In recent years, several biological (*3, 4*) and bioengineering (*5*) approaches have been suggested for the development of a more physiological insulin delivery system and these are in various stages of exploration. In this report we propose a chemical approach to the problem. The principle is to synthesize insulin derivatives with attached oligosaccharides, which are complementary to the binding site of lectins. The release of bound insulin derivative would be a function of the derivative's binding constant to the lectins and of the glucose concentration.

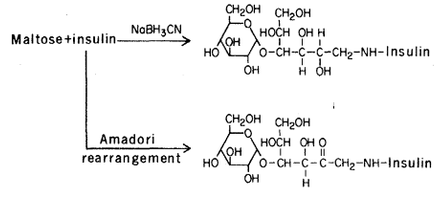

Fig. 1. Formation of insulin derivatives having terminal α-D-glucose residues. The Schiff base adduct between maltose and insulin undergoes an Amadori rearrangement to form the 1-deoxyfructosyl-α-1,4-glucopyranoside. In the presence of sodium cyanoborohydride, the imminium moiety is reduced to the corresponding glycitol.

Insulin derivatives complementary to the major combining site of the lectin concanavalin A were synthesized by using modifications of previously described methods for coupling aldehydes to primary amines (*6, 7*) (Fig. 1). Oligosaccharides, selected according to their differential affinities for binding to concanavalin A (*8*), were incubated with porcine insulin at molar ratios ranging from 12:1 to 650:1 for periods of 1 to 14 days at 37°C in 0.1*M* sodium phosphate buffer, *p*H 8.0. Incubations were carried out in the presence and in the absence of 0.25*M* sodium cyanoborohydride. Unreacted oligosaccharides were removed by gel filtration on Bio-Gel P-6, and unreacted insulin was separated by affinity chromatography on concanavalin A

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