JOURNAL OF EXPERIMENTAL ZOOLOGY 286:625–631 (2000)

Direct Influence of Melatonin on the Thyroid and Comparison With Prolactin

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ABSTRACT Melatonin administered in vivo had previously been shown to inhibit thyroid cell proliferation and subsequent in vitro thyroxine (T_4) secretion in anuran tadpoles. Melatonin in vitro also directly reduced the sensitivity of the thyroid to thyrotropin (TSH). The present work sought to determine whether melatonin directly affected baseline, unstimulated T_4 secretion, and to compare its effect with that of prolactin (PRL). Thyroids from larval *Rana catesbeiana* or adult *Rana pipiens* were incubated in control or melatonin (0.01 to 100 μg/ml) media. Melatonin directly inhibited T_4 secretion by thyroids from both tadpoles and frogs at all concentrations of melatonin used and at both prometamorphic and climax tadpole stages. PRL, used in vitro at 10 μg/ml, did not influence the response of the thyroid to TSH (0.2 μg/ml) in young tadpoles, or the baseline secretion of T_4 by thyroids at any stage of larval life except climax, when T_4 secretion was significantly decreased by the third day of culture. Thus although both melatonin and PRL have been shown to antagonize the action of T_4 in vitro, and to decrease metamorphic rate, melatonin is a much more effective thyroid gland inhibitor than PRL. *J. Exp. Zool. 286:625–631, 2000.*

Melatonin may be involved in the hormonal control of amphibian metamorphosis, a developmental event initiated by a rise in the thyroid hormones (TH). Numerous influences of melatonin on the rate of growth and development of tadpoles have been noted (Delgado et al., '84, '87; Gutierrez et al., '84; Edwards and Pivorun, '91), the majority of which were inhibitory. Melatonin decreased premetamorphic tadpole thyroid cell proliferation (Wright et al., '96) and inhibited the effect of T_4 at the peripheral level (Wright et al., '91).

Whether melatonin antagonizes or promotes metamorphosis depends, at least partly, on the melatonin concentration, the light/dark (LD) cycle, and the time of administration (Edwards and Pivorun, '91; Wright et al., '91). Melatonin may fit the profile of a thyroid hormone modulator like PRL, which antagonizes the peripheral effect of T_4 (Kikuyama et al., '93) but which can synergize with T_4 to promote hindlimb growth and development as the TH levels rise toward climax (Wright, et al., '94); or corticosteroids, which have different effects, at least on the hindlimb, depending on the TH concentration (Wright et al., '94). However, PRL (Clemons and Nicoll, '77; Yamamoto and Kikuyama, '82) and corticosteroid (Krug et al., '83; Kikuyama et al., '86) levels rise in the plasma at climax. In contrast, plasma melatonin decreases at climax of spontaneous metamorphosis (Wright and Racine, '97) and the decrease in the blood is induced prematurely by exogenous T_4 (Wright and Alves, '97). Concomitant administration of T_4 and melatonin increases the melatonin concentration of peripheral organs such as tail and limbs, but not of neural or endocrine organs (Wright et al., '97a). As T_4 rises in the plasma, increased melatonin in target organs might slow the rate of tissue change while the lowered plasma level removes thyroid inhibition. Since the secretion and rhythmicity of melatonin is related to an external variable, the light/dark cycle, this hormone may be involved in environmental control of metamorphic rate.

Previous work in our laboratory showed that melatonin injected prior to culture of tadpole thyroids altered the subsequent secretion pattern of both T_4 and triiodothyronine (T_3), and changed the T_3:T_4 ratio (Wright et al., '96). Melatonin administered in vitro directly inhibited the response of the thyroid to TSH (Wright et al., '97b). The present experi-

Grant sponsor: National Science Foundation; Grant number: IBN-9222504; Grant sponsor: National Institutes of Health; Grant number: DK46535.
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Received 30 June 1998; Accepted 21 September 1999

ments were performed to ascertain if melatonin affected baseline or unstimulated T_4 secretion in vitro in the absence of TSH. A number of different concentrations of melatonin were used and glands were tested from animals at different stages and in the adult frog. If melatonin were involved in the hormonal control of metamorphosis, it might have a different effect on the thyroid at different stages. The pituitary hormone PRL modulates the action of T_4 at the peripheral level (Kikuyama et al., '93), but its effect on the thyroid is less clear. Therefore, we also studied the influence of PRL on the thyroid at different stages of larval development.

MATERIALS AND METHODS

Animals and pre-experiment treatment

Mature male and female *Rana pipiens* frogs were obtained shortly before use from Connecticut Valley Biological Supply (Southampton, MA) and kept in approximately 1-cm 10% Holtfreter's solution at 22°C on an 18L:6D cycle. They were fed *Tenebrio* larvae and the solution was changed every other day. *Rana catesbeiana* (bullfrog) tadpoles were obtained from Charles D. Sullivan Co. (Nashville, TN), and were usually used a short time after arrival. They were maintained at 22°C in 10% Holtfreter's solution or spring water changed three times a week, and fed washed, canned spinach once a day. Staging was according to the method of Taylor and Kollros ('46).

The animals in the melatonin experiments were kept on an 18L:6D cycle prior to thyroid removal for organ culture because previous work has shown that melatonin inhibits metamorphic progress under these conditions (Wright et al., '91). In the PRL experiments, a 12L:12D cycle was used since that is the standard LD cycle we use in most experiments. These preculture differences in the LD cycle should not invalidate comparisons between melatonin and PRL experiments. There is evidence from previous work, for example, that the preculture photoperiodic regimen has no effect on the nature of the thyroid response to TSH. Thyroids from tadpoles which had been kept on 18L:6D showed a stimulatory response to TSH (Wright et al., '97b) similar to that of thyroids from tadpoles kept on 12L:12D prior to thyroid culture in experiment P1 of the present work.

During the last 3 days, tadpoles were not fed and were kept in a suspension of 0.85% sulfadiazine (Sigma) in 10% Holtfreter's solution in order to sterilize the skin before obtaining tissues for culture. Frogs were treated with sulfadiazine in Holtfreter's solution for 2 days. All experiments were done in the summer except the frog melatonin experiment (exp. M4) which was done in the spring, and one PRL experiment (exp. P1) which used winter tadpoles. T_4 secretion and responses to hormones in vitro are not significantly affected by season in any way that would invalidate comparisons among the present experiments. The levels of baseline T_4 secreted in vitro by thyroids from summer and winter tadpoles, and cold-treated summer ones, were not significantly different (Wright et al., '99). Although the thyroids of summer tadpoles were more stimulated than those of winter, or cold-treated summer ones, the thyroids of all groups secreted significantly more T_4 upon addition of TSH to the culture media than the baseline level achieved without TSH stimulation (Wright et al., '99).

Thyroid culture

All procedures described were done with sterile instruments and solutions in a tissue culture hood previously sterilized by ultraviolet light. To obtain thyroids for culture, each animal was removed individually from the sulfadiazine treatment and pithed with a dissecting needle. The cranium and upper jaw were removed and placed ventral side up and the paired thyroids were dissected out and removed together with a small piece of the underlying cartilage. All muscle and other tissue was removed from the surface of the thyroid so that the follicles were exposed to the culture media.

After removal, the thyroids were twice rinsed briefly in cold sterile Holtfreter's solution with 300 units/ml penicillin and 300 μg/ml streptomycin, followed by two 3-hr rinses in the incubation media. Thyroid pairs were cultured in individual wells of 24-well plates in 0.25 ml (tadpoles) or 0.3 ml (frogs) of two-thirds strength L-15 medium with 0.1% bovine serum albumin, 50 μg/ml antibiotic/antimycotic solution and 150 μg/ml gentamycin. All culture reagents were obtained from GIBCO (Grand Island, NY). The cultures were incubated in the dark at 24°C and media collected and replaced at 8-hr intervals. Collected media were stored frozen at –20°C for RIA. Thyroids were fixed, sectioned, and affixed to slides, and the histology checked for viability in culture. No signs of necrosis were noted.

Experimental protocols

Melatonin experiments

The details following refer to most experiments and any exceptions are noted in the specific protocols below. Experiments started at 1600 hr, so

that tissue preparation and the two 3-hr rinses were over in time for the start of culture at 2330 hr, since melatonin had a greater effect on metamorphic rate when given late in the photophase of an 18L:6D cycle (Wright et al., '91). Thyroid media were collected and replaced at 8-hr intervals, at 0730, 1530, and again at 2330 hr. There were one pair of thyroids per well and a total of six samples in each group, control or experimental.

Experiment M1. This experiment was performed to test the effect of a low concentration of melatonin (0.01 μg/ml) on thyroids from late prometamorphic tadpoles (stages XVII–XVIII).

Experiment M2. This experiment evaluated the effect of higher concentrations of melatonin (10 and 100 μg/ml) on thyroids from late prometamorphic tadpoles (stages XVI–XVII).

Experiment M3. This experiment was done to determine the effect of a low (0.01 μg/ml) and high (100 μg/ml) level of melatonin on secretion of thyroids from early climax tadpoles (stages XX–XXII). In this experiment there were two pairs of thyroids per well in order to get detectable T_4, since preliminary work indicated that baseline T_4 secretion was very low at climax.

Experiment M4. Mature, adult leopard frogs were utilized to study the effect of melatonin (100 μg/ml) on the adult anuran thyroid. There were equal numbers of male and female frogs in each group because tadpole experiments would be expected to contain both sexes. Media were collected at 0700, 1500, and 2300 hr, one-half hour earlier than in the tadpole experiments described previously.

PRL experiments

These experiments lasted for 3 days, since preliminary work had indicated that any effect of prolactin on the thyroid might take longer than in the melatonin work. Media were collected at the end of each day. The tissue preparation and culture methods were the same as described previously; the sample size was six per group, with one pair of thyroids per well, except as noted below. All experiments began early in the photophase at 0900 or 0930 hr. The PRL concentration in all experiments was 10 μg/ml. In one experiment where TSH was used, the TSH concentration was 0.2 μg/ml.

Experiment P1. Premetamorphic tadpoles (Stages IX–XI) were used. One group of thyroids were cultured with TSH, and the other with both TSH and PRL to see if PRL influenced TSH sensitivity of the thyroids.

Experiment P2. Thyroids from premetamorphic tadpoles (Stages X–XI) were cultured in control or PRL media to determine if PRL affected the thyroids of young tadpoles in the absence of TSH. There were two pairs of thyroids/well since baseline T_4 secretion would be expected to be very low at this early stage. N = 5 per group.

Experiment P3. The effect of PRL on thyroids from older, prometamorphic tadpoles (stages XV–XVIII) were tested in this experiment.

Experiment P4. In this experiment, thyroids from climax tadpoles (stages XX–XXI) were cultured in control or PRL media to see how PRL affected thyroids at the culmination of metamorphic changes.

Hormones

Hormones were administered in vitro. TSH (NIADDK-oTSH-12) was dissolved in 0.7% saline and used in the media at a concentration of 0.2 μg/ml. PRL (USDA-b-PRL-B-1) was dissolved in 0.7% saline at pH 8 and added to media to make a concentration of 10 μg/ml. Melatonin (Sigma) was dissolved in alcohol and diluted in distilled water to make a stock solution of 1 mg/ml. It was used in the culture media at a final concentration of from 0.01 to 100 μg/ml as specified in the different experiments. These concentrations of melatonin are comparable to the levels used in both mammalian and amphibian studies (see Wright et al., '97b). Moreover, probably only a small amount of the hormone penetrates the thyroids since it has been calculated that only about 15% of T_4 in the culture media actually gets into the tissue in culture (Robinson et al., '77).

Radioimmunoassay

T_4 secreted into the culture media was assayed using a canine total T_4 RIA kit (Diagnostic Products Corporation, Los Angeles, CA) employing antibody-coated tubes (as previously described in Wright et al., '95). Samples were assayed in duplicate. Tubes were counted in a Packard RIA-STAR gamma counter with a counting efficiency greater than 80%. The intra- and inter-assay coefficients of variation for T_4 were 5.6 and 7.9 %, respectively.

Statistical analysis

The data were evaluated using Student's *t*-test, or ANOVA followed by Duncan's multiple range test, to isolate differences among the means of the groups. Differences were considered to be significant if $P < 0.05$.

RESULTS

At prometamorphosis, 0.01 μg/ml melatonin significantly lowered Tμ secretion only at 0730 hr (Fig. 1A). As in all the melatonin experiments, the total of the 3 intervals was calculated to give the 24-hr secretion, and additional statistics performed. In this case, there was no significant difference between the control and melatonin groups. Higher concentrations of melatonin (10 and 100 μg/ml) significantly inhibited the secretion of T_4 at the 0730- and 1530 hr- collection intervals (Fig. 1B), but not at 2330 hr, and the total secretion (inset to Fig. 1B) was also significantly lower. In the climax experiment (Fig. 1C), melatonin significantly lowered T_4 secretion at 0730 and 1530 hr, and again the total of all intervals (inset to Fig. 1C) showed nearly equal inhibition of T_4 secretion by both the low and high concentration of melatonin.

Adult frog thyroids were significantly inhibited by 100 μg/ml melatonin (Fig. 2) at all the collection intervals and in the total 24-hr secretion. Thus, melatonin had a direct inhibitory effect on the thyroid baseline T_4 secretion in vitro at all stages in the life cycle from mid-metamorphosis to the adult.

PRL (Table 1) did not influence the response of the thyroid to TSH, or unstimulated, baseline secretion of T_4, at early stages (pre- and prometamorphosis) but significantly lowered T_4 secretion on day 3 of culture in climax tadpoles (Fig. 3); however, the mean daily secretion (inset to Fig. 3) of the PRL group was not significantly different from the control.

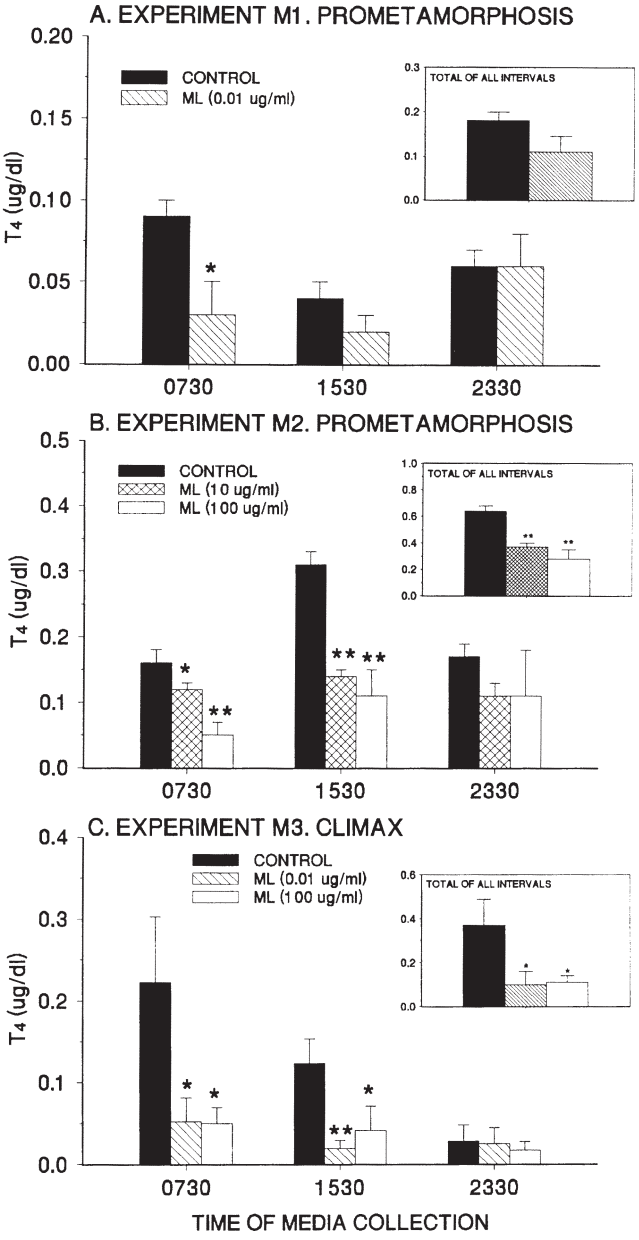

Fig. 1. Effect of various concentrations of melatonin on T_4 secretion by thyroids from prometamorphic (**A, B**) and climax (**C**) bullfrog tadpoles. Bars represent the means of samples of media collected at the designated intervals after 8 hr of culture (n = 6). Insets indicate the total T_4 collected in the three intervals of one day. Stars indicate significant differences between an experimental group and the control at the same interval (*$P < 0.05$; **$P < 0.01$). ML, melatonin.

DISCUSSION

The results showed that concentrations of melatonin from 0.01 to 100 μg/ml directly inhibited baseline secretion of T_4 by thyroids from mid-metamorphic tadpoles to the adult. These findings extend previous work which showed that injected melatonin decreased thyroid cell proliferation in young tadpoles in vivo and inhibited subsequent T_4 secretion in vitro by thyroids from prometamorphic tadpoles (Wright et al., '96), and confirm melatonin's antagonism of thyroid secretion. Melatonin also depressed the response of the thyroid to TSH (Wright et al., 97b) but only with any consistency when a high concentration (100 μg/ml) of melatonin was used. The present findings show that melatonin does not only inhibit thyroid sensitivity to TSH, but also its secretion of T_4 in nonstimulatory conditions, and that this latter effect does not require as high a concentration of melatonin.

TSH was able to overcome melatonin inhibition of the thyroid except at high melatonin concentrations (Wright et al., '97b). Although TSH has not been measured in the plasma of tadpoles,

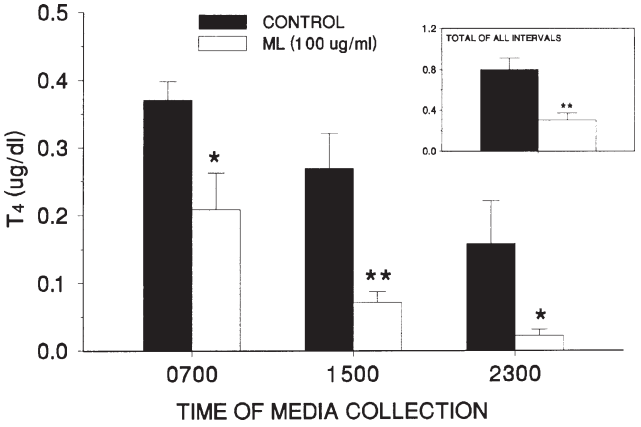

Fig. 2. Experiment M4. Adult frog. Effect of melatonin on T_4 secretion by thyroids from adult *Rana pipiens* frogs. Bars represent the mean of T_4 in samples of media collected at the designated intervals after 8 hr of culture (n = 6). The inset indicates the total daily secretion. Stars indicate significant differences between melatonin group and control (*$P < 0.05$; **$P < 0.01$). ML, melatonin.

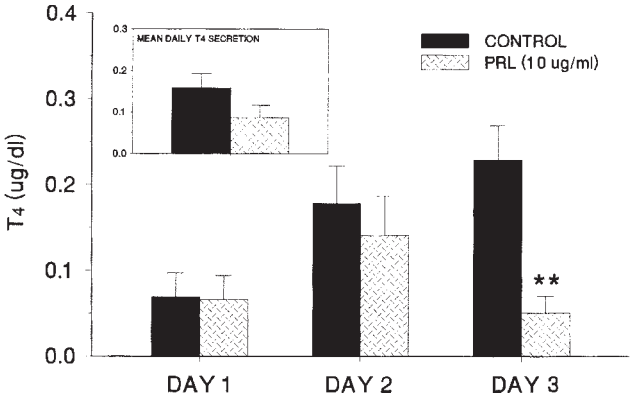

Fig. 3. Experiment P4. Climax. Effect of PRL on T_4 secretion in climax bullfrog tadpoles. Bars represent the means of samples of media collected at the end of each day (n = 6). The inset indicates the mean daily secretion, for comparison to the daily total of T_4 secretion in the insets to the melatonin graphs (Figs. 1 and 2). Stars indicate a significant difference between control and PRL ($P < 0.01$). ML, melatonin; PRL, prolactin.

TSH-containing cells have been localized in the pituitary. Immunocytochemical studies of the pituitary indicate that the content of TSH rises during metamorphosis, with a period of hormone release at climax (Miranda et al., '96). Melatonin's antagonism of baseline T_4 secretion might be important early in larval life, when TSH concentrations in the plasma are presumably low. Toward the end of metamorphosis, when thyroid activity increases due to TSH stimulation, melatonin may be less effective in inhibition of the thyroid. Furthermore, recent work in our lab showed that plasma melatonin levels fell at the metamorphic climax (Wright and Racine, '97) and that this decline appeared to be induced by the rise in T_4 (Wright and Alves, '97; unpublished data).

It has been clear for some time that melatonin inhibits the thyroid gland of mammals when administered in vivo (Haldar and Shavali, '92), where it has been reported to act as a goitrogen and produce elevated levels of TSH (Panda and Turner, '68). The mechanism of action of melatonin at the thyroid level is not yet clear. Melatonin can bind to membrane receptors, or enter the cell and exert direct intracellular effects, since it is a known free radical scavenger and antioxidant. It also has binding sites in the nuclei of varied types of cells where it may exert an effect on the genome (Reiter et al., '96). In mammals, it has been reported to affect the synthesis of tubulin and assembly of microtubules in cells of the hypothalamus, inhibit cyclic AMP and GMP accumulation in pituitary cells (Haldar and Shavali, '92), and inhibit iodide uptake into thyroid cells (Panda and Turner, '68). However, in amphibians, melatonin failed to affect TSH stimulation of iodide uptake into the thyroid of the adult tiger salamander (Norris and Platt, '73).

TABLE 1. In vitro effect of PRL on baseline T_4 secretion and response to TSH by cultured thyroids from tadpoles at different stages of metamorphosis[1]

Experiment	Groups	T_4 (μg/dl)
P1. Premetamorphosis	TSH	2.4 ± 0.3
	PRL/TSH	2.5 ± 0.3
P2. Premetamorphosis	Control	0.02 ± 0.01
	PRL	0.03 ± 0.01
P3. Prometamorphosis	Control	0.12 ± 0.02
	PRL	0.10 ± 0.01

[1]Values represent means ± S.E. of 3 days of secretion in vitro (n = 5–6). There were no significant differences.

Cultures of thyroids with PRL showed that this hormone had no influence on baseline T_4 release except at climax, when PRL inhibited T_4 secretion on the second and third days in vitro, with statistical significance on the third day. In premetamorphic tadpoles, PRL failed to affect the response of the thyroids to TSH. These findings are in contrast to melatonin, which reduced baseline T_4 secretion at all stages studied (present work) and which inhibited thyroid TSH sensitivity in vitro, most effectively at high melatonin concentrations (Wright et al., '97b). Thus, it is clear that melatonin has a much greater direct effect on the thyroid than PRL. PRL's main influence seems to

be at the tissue level, where it directly antagonized both tail regression and limb development in vitro (Tata et al., '91) by preventing TH-induced upregulation of thyroid hormone receptor mRNAs (Baker and Tata, '92). However, melatonin may also inhibit the action of T_4 in peripheral tissues, since it antagonized T_4-induced tail tip resorption in vitro (Wright et al., '91).

Earlier studies examining the effect of PRL on the thyroid had variable results. In adult red-spotted newts, PRL did not alter T_4 secretion, but TSH and PRL were more effective than TSH alone in stimulating in vitro T_4 secretion (Anderson and Dent, '82). On the other hand, very high doses of PRL (100 mg and above) for 7–10 days in vivo appeared to have a goitrogenic effect on the thyroid of bullfrog tadpoles (Gona, '67), and in hypophysectomized *Xenopus laevis* tadpoles, TSH given alone had a more stimulatory effect on the thyroid than when TSH and PRL were administered together (Regard and Mauchamp, '73). PRL did not affect TSH stimulation of iodide uptake in the adult tiger salamander (Norris and Platt, '73), whereas in hypophysectomized *Xenopus laevis*, PRL depressed TSH-stimulated iodide uptake in vitro (Regard and Mauchamp, '73). Some of the variable results cited previously may be explained by PRL concentrations or species differences, but it is also likely that they reflect stage differences or different thyroidal states. The present report is the only one, to our knowledge, that shows direct inhibition of TH secretion by PRL, at metamorphic climax. Perhaps the rise in PRL levels in the plasma at climax (Kikuyama et al., '93), coupled with PRL inhibition of the thyroid at that time, facilitates the conclusion of metamorphosis and the return of T_4 levels to normal.

ACKNOWLEDGMENTS

We are indebted to Christina Alves, Carol Racine, Agata Pikula, Robert Weir, and Beata Rzasa for assistance in some experiments. TSH and PRL were gifts of the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program.

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