

# Physiological Responses of Larval and Postmetamorphic *Rana pipiens* to Growth Hormone and Prolactin

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**ABSTRACT** Bovine prolactin stimulates growth of tadpoles, whereas bovine growth hormone stimulates growth of postmetamorphic frogs. The biochemical composition of liver, muscle, and fat body were examined to determine whether there were any changes in carbohydrate, protein, fat, or nucleic acids which might correlate significantly with the growth effects of the hormones in the two stages of development. In the frog, the major effect of growth hormone was to depress carbohydrate and lipid stores, with little or no effect on protein and nucleic acids in most experiments. Prolactin had similar but smaller effects on carbohydrate, and no effect on fat body lipid in the frog. In tadpoles prolactin suppressed muscle glycogen, but otherwise did not affect tissue composition. Growth hormone had no effects in the tadpole. Ways in which the interrelated developmental and metabolic effects of growth hormone and prolactin could increase the adaptive significance of distinct larval and postmetamorphic growth-regulating hormones in the amphibians are discussed.

Amphibians exhibit a unique mechanism for regulation of growth in that prolactin stimulates growth in larval stages of development, whereas growth hormone stimulates growth of the post-metamorphic animals (Brown and Frye, '69b). Since the first experiment suggesting that prolactin causes growth in tadpoles (Etkin and Lehrer, '60), the growth effect of prolactin in larval amphibians has been substantiated in several species, including *Rana catesbeiana* (Berman et al., '64; Etkin and Gona, '67a,b), *Alytes obstetricians* (Remy and Bounhiol, '65, '66), and *Rana pipiens* (Etkin and Gona, '67b; Brown and Frye, '69a). In these studies injections of purified prolactin preparations caused an increase in length, wet weight, and dry weight, while growth hormone under the same conditions had little or no effect.<sup>3</sup>

Although the effect of prolactin on larval growth has been well documented, the mechanisms by which this growth is achieved is not entirely clear. Recent studies indicate that prolactin stimulates growth in part by acting as an antithyroid agent. Prolactin has been shown to act as an antithyroid agent in two ways: by blocking the output of thyroxin from

the thyroid gland (Gona, '67, '68), and by inhibiting the action of thyroxin in the tissues (Bern et al., '67; Derby and Etkin, '68). Since several other antithyroid compounds stimulate growth in tadpoles (Steinmetz, '54; Brown and Frye, '69a), it is possible that the growth promoting effect of prolactin is a consequence of its antithyroid action. However, Brown and Frye ('69a) showed that prolactin treatment of thyroidectomized or thiouracil treated animals caused a further stimulation of growth beyond that of control values. This indicates that the hormone has an additional growth effect which is independent of its antithyroid activity.

Fewer growth studies have been done with postmetamorphic amphibians than with the larval stages, but the reports which have been published to date indicate that, in contrast to the larval situation, growth hormone is the primary

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<sup>3</sup> In one exceptional case (Enmar et al., '68) both prolactin and growth hormone were effective in increasing length and weight of *Rana temporaria* larvae, the latter being the more effective of the two hormones.

growth hormone of the postmetamorphic frog. Growth hormone was shown to increase weight and length in young *Rana pipiens* (Brown and Frye, '69b) and in the toads *Bufo boreas* and *Bufo marinus* (Zipser et al., '69). Prolactin appears to have very little or no effect on either *Rana* or *Bufo* postmetamorphic animals.

In mammals, the growth promoting effects of growth hormone are accompanied by extensive and interrelated effects upon the metabolism of carbohydrate, fat, and protein. Specifically, growth hormone promotes nitrogen retention and protein synthesis (Lee and Ayres, '36); is glycostatic as reflected by maintenance of glycogen levels of muscle and liver in fasting hypophysectomized and normal rats (Russell and Bloom, '56; Altszuler et al., '68); and is lipolytic in that it increases free fatty acid concentration in plasma, fatty acid turnover, and oxidation to carbon dioxide (Winkler et al., '64).

Although mammalian growth hormone and prolactin are known to be growth promoting in amphibian postmetamorphic and larval stages, as described above, there has been very little study of the related metabolic effects. The purpose of this investigation was to examine the biochemical basis of the growth promoting effects of GH and prolactin, and to determine what effects they might have upon overall composition of carbohydrate, fat, and protein compared to the effects in mammals. Specifically, this paper reports the effects of prolactin and growth hormone upon the biochemical composition of various tissues after long term treatment. The principal tissues analyzed were liver and skeletal muscle, and the substances measured include water, carbohydrate, fat, protein, and nucleic acids. The purpose of the biochemical analyses was to compare the effects of prolactin and growth hormone in amphibians, and in addition to compare the effects of these hormones in amphibians with their action in mammals.

A subsequent paper will describe the effects of prolactin and growth hormone upon the *in vivo* incorporation of radioactive amino acid into the protein fraction of various tadpole and frog tissues after prolactin and growth hormone injection.

## MATERIALS AND METHODS

### Animals

Frogs of the species *Rana pipiens* were used in all experiments. Postmetamorphic frogs were obtained from either Lemberger Company, Oshkosh, Wisconsin or collected locally. The frogs used were of medium length (6–7 cm) and at the start of most experiments weighed between 15 and 30 grams. Prior to use they were fed crickets ad lib. two to three times/week. They were fed up to the beginning of the experiment and then fasted four to five days prior to the sacrifice of the animals. During experiments frogs were placed in individual plastic refrigerator dishes lined with wet paper toweling and kept in an incubator at 21–23°C on a 12-hour photoperiod.

Tadpoles were obtained by ovulation and artificial insemination by the method of Wright and Flathers ('61). The tadpoles were raised in aerated tap water in enamel pans and fed boiled spinach. During experiments tadpoles were maintained individually in four-inch finger-bowls and kept at 21–23°C on a 12 hour photoperiod. Tadpoles were selected for experimental use at Taylor-Kollros stages X–XIII (Taylor and Kollros, '46).

### General procedures

Hypophysectomy of frogs was performed through a hole drilled in the sphenoid bone. They were allowed to recuperate from the surgery for four to seven days before use in an experiment. Hypophysectomized frogs were kept in 0.3–0.5% amphibian Ringers.

Hormones were obtained from the National Institutes of Health as crystalline bovine growth hormone (NIH-GH-B12: 0.97 USP units/mg, and NIH-GH-B14: 1.04 USP units/mg) and bovine prolactin (NIH-P-32: 19.9 IU/mg). They were dissolved in 0.7% NaCl, pH 9.3–9.6. Injections were made intraperitoneally through the ventral abdominal body wall in frogs and through the tail muscle into the abdomen in tadpoles.

Growth was determined by length and weight increment. Frog length was taken as total snout-urostyle length, or as length of the tibia. Tadpole length was taken as total body plus tail length. Weights were

taken after blotting animals uniformly on a dry cotton towel.

#### *Chemical procedures*

*Tissue fractionation.* The method of Shibko et al. ('67) was used for fractionation of tissues to be analyzed for chemical composition, and modified to fit the amount of tissue and design of each experiment. In general, the method proved satisfactory except that significant amounts of protein were extracted into the lipid fraction. The lost protein was corrected for by chemically measuring the amount of protein in the lipid fraction and adding it to the protein fraction.

*Chemical assays.* Protein was determined by the biuret method of Wannenmacher et al. ('65) for concentrated solutions, or the method of Lowry ('51) for dilute samples.

The carbohydrate fraction was taken as the acid-soluble fraction after PCA or TCA treatment of the tissue homogenate. The phenol-sulfuric acid reaction of Ashwell ('66) was used to estimate the total carbohydrate of the sample.

RNA concentrations were determined by reading the samples in a Beckman spectrophotometer at 260  $m\mu$  referenced to a standard solution of hydrolyzed RNA. Diphenylamine reagent was used to determine DNA (Burton, '56). These results were usually consistent with those obtained by reading the DNA solution in a Beckman spectrophotometer at 268  $m\mu$  referenced to a standard of hydrolyzed salmon sperm DNA.

Frog liver lipid was determined gravimetrically after isolation according to the method of Shibko et al. ('67). Lipid content of tadpole liver was determined gravimetrically after homogenizing the tissue in chloroform:methanol (2:1) followed by two more extractions with the same solvent.

The per cent water in liver and muscle tissues was measured by placing the weighed tissue in a tared vessel and drying to constant weight at 60°C.

#### *Statistics*

Student's *t* test was used to analyze the data (Snedecor and Cochran, '69). A probability value (*P*) of 5% or less

was considered significant. Values in figures and tables are given as mean  $\pm$  standard error of the mean. The numbers in parentheses indicate the size of the groups.

#### *Experimental design*

The results reported in this paper are based on two experiments with frogs and four experiments with tadpoles. The plan of these experiments is summarized as follows:

*Frog experiment 1:* Hypophysectomized animals.

Hpx — hypophysectomized frogs (8 animals), injected with 0.7% NaCl per day for six weeks.

Hpx + GH — hypophysectomized frogs (7 animals), injected with 3.6  $\mu\text{g}$  growth hormone/gm body weight per day for six weeks.

Hpx + P — hypophysectomized frogs (6 animals), injected with 3.6  $\mu\text{g}$  prolactin/gm body weight, per day for six weeks.

Sham — sham operated injected with 0.7% NaCl per day for six weeks (7 animals).

*Frog Experiment 2:* Intact animals (9 animals/group).

C — 100  $\mu\text{l}$  0.7% NaCl per day for five weeks.

GH — 100  $\mu\text{g}$  growth hormone per day for five weeks.

P — 100  $\mu\text{g}$  prolactin per day for five weeks.

The animals used in Experiment 1 were collected in mid-winter (December) approximately two weeks prior to the beginning of the experiment. Frogs used in the second experiment were collected in spring at the time of emergence from hibernation.

*Tadpole Experiments 1, 2, and 3.*

C — 0.7% NaCl/day (15 animals).

GH — 5  $\mu\text{g}$  growth hormone/day (12 animals).

P — 5  $\mu\text{g}$  prolactin/day (16 animals).

The animals were weighed and measured at the beginning and end of the experiment. They were injected for either nine or ten days and killed approximately 24 hours after the last injection. Food was withdrawn at the time of the last injection in Experiments 1 and 3 but not Experiment 2.

*Tadpole Experiment 4.*

Same design as Experiment 1 except the growth hormone and prolactin groups were injected with 5  $\mu\text{g}$  hormone for three days, then 10  $\mu\text{g}$  for six days.

RESULTS

*Length and weight changes in tadpoles and frogs.* The results of this study confirm the previous reports (cited in the introduction) that prolactin promotes growth in tadpoles whereas growth hormone stimulates growth in postmetamorphic frogs (figs. 1, 2). Figures 1 and 2 represent growth data from Frog Experiment 2 and Tadpole Experiment 1 respectively.

*Frog liver.* In the first experiment (hypophysectomized animals) growth hormone injections decreased both absolute and relative liver weight (fig. 3). Mean liver weight of growth hormone-treated animals was little more than one-half that of the hypophysectomized controls ( $P < 0.001$ ) and only two-thirds as large as the prolactin-treated and sham control groups ( $P < 0.001$ ). Neither hypophysectomy nor prolactin treatment significantly increased liver weight above sham control values.

Reduction of liver size by growth hormone could be due to either a selective depletion of some liver components or a general depletion of all liver components. An examination of the amount of each biochemical component in the livers was undertaken to distinguish between the above two alternatives. The results of Ex-

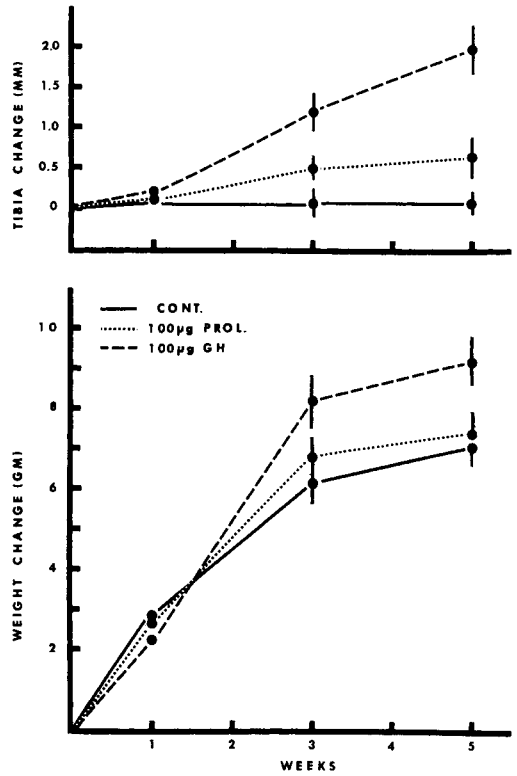


Fig. 1 Changes in tibia length and body weight in intact frogs during five weeks of treatment with growth hormone and prolactin (frog experiment 2).

periment 1 are shown in table 1. In terms of absolute amounts, the only two components affected by growth hormone were total carbohydrate and water. The hypophysectomized control had nearly seven times as much carbohydrate as the growth

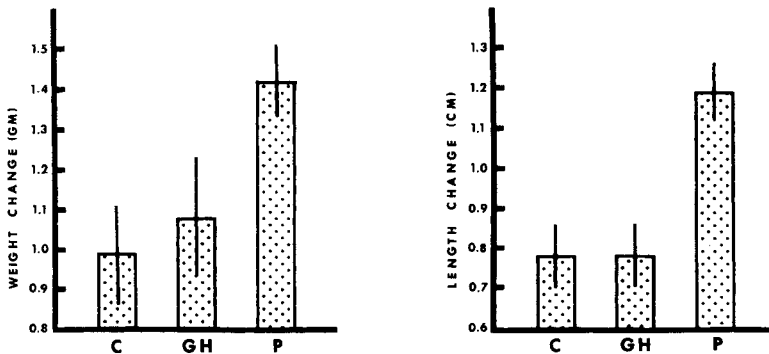


Fig. 2 Changes in total length and weight of tadpoles after nine days of treatment with growth hormone and prolactin (tadpole experiment 1). C, control; GH, 5  $\mu\text{g}$  growth hormone/day; P, 5  $\mu\text{g}$  prolactin/day.

hormone-treated animals. Even though livers of prolactin-treated and sham control animals had over four times as much carbohydrate as the growth hormone group, they had significantly less carbohydrate than the hypophysectomized control group ( $P < 0.05$ ). As would be expected, the total amount of water in the liver corresponded closely to the absolute liver size. Therefore, the absolute water content of the growth hormone group was also significantly below the hypophysectomized control group ( $P < 0.001$ ). The absolute amounts of protein, RNA, DNA, and lipid are not significantly

different in any of the four groups. Thus, the reduced levels of carbohydrate and water in the livers of growth hormone-treated animals account for the difference in absolute weight between this and the other groups. The same is true of any two groups: the difference in absolute liver weights can be accounted for entirely by the different amounts of carbohydrate and water.

When the data from this experiment are expressed as relative values (mg of substance/gm liver tissue; this calculation can be made from data in table 1) the relative concentrations of protein,

TABLE 1

*Absolute composition of frog liver after hypophysectomy and hormone replacement*

	Water mg/liver	Carbohydrate mg/liver	Protein mg/liver	RNA mg/liver	DNA mg/liver	Lipid mg/liver	Recovery mg recovered ave. liver wt.
HYPX + GH (7)	547 ± 42 <sup>2</sup>	35 ± 8.7 <sup>2</sup>	95.4 ± 8	4.43 ± 0.35	3.81 ± 0.26	33 ± 11	0.717 0.749
HYPX + Prolactin (6)	823 ± 74	141 ± 28 <sup>1</sup>	98.8 ± 9	4.97 ± 0.28	4.21 ± 0.33	26 ± 2	1.09 1.12
HYPX + Saline (8)	1021 ± 85	239 ± 30	104.5 ± 8	4.81 ± 0.32	4.20 ± 0.38	26 ± 3	1.40 1.42
Sham operated (7)	794 ± 65	157 ± 18 <sup>1</sup>	96.4 ± 6	4.30 ± 0.28	3.99 ± 0.32	20 ± 7	1.07 1.13

<sup>1</sup>  $p < 0.05$ .

<sup>2</sup>  $p < 0.001$ .

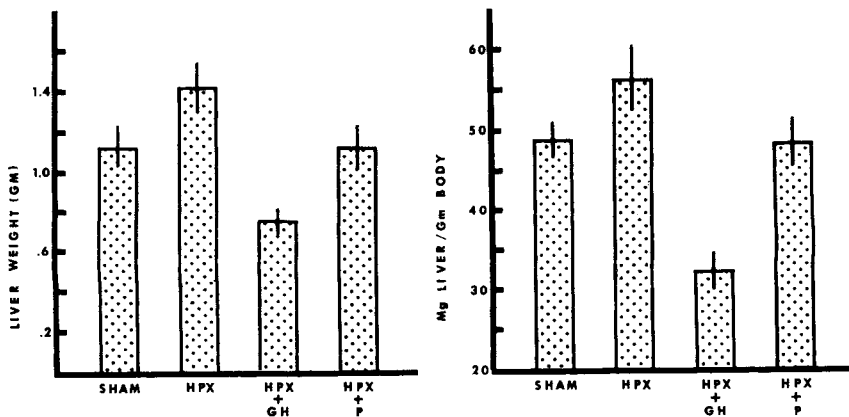


Fig. 3 Absolute and relative liver weights of hypophysectomized and sham-operated frogs after six weeks of treatment with growth hormone and prolactin (frog experiment 1).

RNA, DNA, and lipid in the livers of growth hormone-treated animals were highly elevated above the values seen in the other three experimental groups, whereas carbohydrate was lowered. However, since changes in carbohydrate and water account almost exactly for hormone effects on total liver size, we conclude that the hormonal effects are more accurately represented in terms of absolute changes and therefore that there was no real effect of growth hormone on protein, RNA, DNA, and lipid.

In Experiment 2 (intact animals) the mean liver size of the growth hormone-treated animals was again significantly below prolactin-treated ( $P < 0.05$ ) and control ( $P < 0.001$ ) values (table 2). Prolactin treatment also reduced absolute and relative liver size below control values ( $P < 0.001$ ). As was the case in Experiment 1, the absolute values for carbohydrate and water in Experiment 2 were the most drastically altered categories and accounted for most of the difference in liver weights between different groups (table 2, fig. 4). In addition, growth hormone treatment of intact animals decreased total protein content below the controls ( $P < 0.025$ ). Prolactin treatment produced results intermediate between the growth hormone and control groups. The total amount of DNA/liver was elevated by growth hormone injections ( $P < 0.05$ ). This differed from the results obtained with hypophysecto-

mized animals in Experiment 1 and therefore requires further verification.

*Frog gastrocnemius muscle.* In addition to liver, frog gastrocnemius muscle was analyzed after hormone treatment. The data from Frog Experiments 1 and 2 are summarized in tables 3 and 4. The results obtained with muscle are presented as relative values (mg chemical/

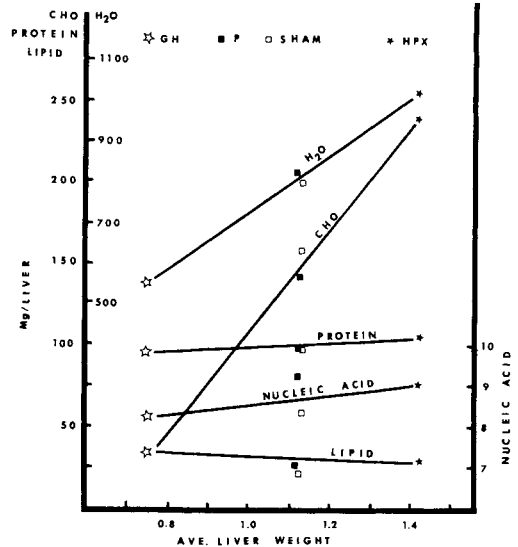


Fig. 4 Composition of frog liver after hypophysectomy and hormone replacement (frog experiment 1). CHO, carbohydrate; GH, growth hormone treated; P, prolactin treated; SHAM, sham hypophysectomized controls; HPX, hypophysectomized without hormone treatment.

TABLE 2

*Absolute composition of intact frog liver after hormone treatment*

	Water mg/liver	Carbohydrate mg/liver	Protein mg/liver	RNA mg/liver	DNA mg/liver	Recovery mg recovered ave. liver wt.
GH	769 ± 43 <sup>3</sup>	98 ± 12 <sup>3</sup>	134 ± 6 <sup>2</sup>	5.47 ± 0.35	5.14 ± 0.29 <sup>1</sup>	1.01
(9)						1.12
P	839 ± 49 <sup>3</sup>	128 ± 15 <sup>3</sup>	146 ± 9	5.94 ± 0.44	4.95 ± 0.44	1.12
(9)						1.28
C	1207 ± 61	298 ± 24	158 ± 8	5.78 ± 0.26	4.16 ± 0.31	1.67
(9)						1.82

<sup>1</sup>  $p < 0.05$ .  
<sup>2</sup>  $p < 0.025$ .  
<sup>3</sup>  $p < 0.001$ .

gm muscle). Data for absolute quantities of substances in muscle show the same result. In Experiments 1 and 2 there was no difference in total or relative (mg/gm) muscle weight among any of the groups (tables 3, 4). However, this probably reflects the wide range of terminal body sizes rather than the failure of growth hormone to stimulate muscle growth. If initial and terminal muscle weights were compared, a growth hormone stimulation probably would be observed.

Hypophysectomy reduced the amount of muscle carbohydrate ( $P < 0.025$ ) and DNA ( $P < 0.025$ ), but not RNA ( $P < 0.08$ ) compared to the sham operated group. Neither growth hormone nor prolactin had any effect on muscle protein concentration. Growth hormone injected into hypophysectomized frogs elevated the levels of RNA and DNA above the sham control group while prolactin maintained these compounds at about the level of the intact animal. Growth hormone reduced muscle carbohydrate below the already reduced hypophysectomized control values ( $P < 0.05$ ). Prolactin treatment did not lower carbohydrate significantly below the hypophysectomized control.

The experiment with intact frogs (table 4) yielded similar results except that growth hormone did not increase DNA concentrations significantly above controls. In Experiment 2 muscle water was measured and was found to be increased by treatment with growth hormone ( $P < 0.001$ ) while prolactin had no effect on this parameter.

*Tadpole liver.* The effect of hormone injections on tadpole liver in Tadpole Experiment 1 are shown in table 5. The relative liver weight was significantly reduced by prolactin treatment ( $P < 0.001$ ). However, prolactin did not reduce the absolute liver size. Growth hormone had no effect on either relative or absolute liver size. Comparison of the hormone-treated with the control groups reveals no significant differences in the amounts of any major biochemical components. Carbohydrate, lipid, protein, RNA, and water concentrations were not different in any of the three groups. The smaller absolute size of the livers of prolactin-treated animals appears to reflect a failure of the liver to grow in proportion to body growth, and not to a specific depletion of any component of the liver. Essential similar results were obtained in the repeat experiments. One exception was observed in Experiment 4, in which liver RNA concentration of the growth hormone and the prolactin-treated groups was reduced below control values.

*Tadpole tail muscle.* Because carbohydrate and RNA levels were most responsive to hormone treatment in frog muscle, these parameters were measured in tadpole muscle. The results from the two tadpole experiments are summarized in table 6. Contrary to the results obtained with frogs, prolactin rather than growth hormone lowered carbohydrate. Prolactin reduced carbohydrate to nearly half the control level in Experiment 2 ( $P < 0.001$ ). In Tadpole Experiment 4 prolactin-induced

TABLE 3

*Chemical composition of frog gastrocnemius muscle after hypophysectomy and hormone replacement*

	Carbohydrate mg/gm	Protein mg/gm	RNA $\mu\text{g/gm}$	DNA $\mu\text{g/gm}$	mg Muscle/ gm body	Total muscle wt.
HYPX + GH (7)	6.04 $\pm$ 0.58 <sup>1</sup>	116 $\pm$ 3	864 $\pm$ 52 <sup>2</sup>	749 $\pm$ 60 <sup>1</sup>	19.8 $\pm$ 1.0	472 $\pm$ 43
HYPX + prolactin (6)	6.63 $\pm$ 0.83	120 $\pm$ 2	797 $\pm$ 60	704 $\pm$ 43	18.1 $\pm$ 0.7	424 $\pm$ 42
HYPX + saline (8)	8.12 $\pm$ 0.65	122 $\pm$ 3	716 $\pm$ 31	630 $\pm$ 20	18.0 $\pm$ 1.9	453 $\pm$ 23
Sham operated (7)	12.28 $\pm$ 1.6 <sup>2</sup>	116 $\pm$ 3	806 $\pm$ 27	710 $\pm$ 21 <sup>2</sup>	17.9 $\pm$ 0.6	414 $\pm$ 27

<sup>1</sup>  $p < 0.05$ .

<sup>2</sup>  $p < 0.025$ .

TABLE 4  
*Chemical composition of intact frog gastrocnemius muscle after hormone treatment*

	Water mg/gm	Carbohydrate mg/gm	Protein mg/gm	RNA $\mu$ g/gm	DNA $\mu$ g/gm	mg muscle/ gm body	Total muscle wt.
GH (9)	784.5 $\pm$ 0.8 <sup>2</sup>	11 $\pm$ 0.6 <sup>1</sup>	156 $\pm$ 1.2	675 $\pm$ 34 <sup>1</sup>	605 $\pm$ 31	49.6 $\pm$ 1.62	1674 $\pm$ 82
Prolactin (9)	779 $\pm$ 2.1	10.4 $\pm$ 1 <sup>1</sup>	157.7 $\pm$ 2.3	621 $\pm$ 20	541 $\pm$ 17	47.7 $\pm$ 0.86	1544 $\pm$ 56
Saline (9)	780 $\pm$ 1.3	14 $\pm$ 0.9	152.5 $\pm$ 1.4	564 $\pm$ 27	561 $\pm$ 19	45.9 $\pm$ 1.04	1461 $\pm$ 62

<sup>1</sup> p < 0.025.  
<sup>2</sup> p < 0.001.

TABLE 5  
*Relative composition of intact tadpole liver after hormone treatment*

	Water mg/gm	Carbohydrate mg/gm	Protein mg/gm	RNA mg/gm	Lipid mg/gm	Liver weight mg/gm body
GH (10)		13.7 $\pm$ 2.1	91.5 $\pm$ 2.2	8.60 $\pm$ 0.26	72.2 $\pm$ 6.4	24.0 $\pm$ 1.5
P (10)	812 $\pm$ 13	15.4 $\pm$ 1.2	88.4 $\pm$ 1.4	8.44 $\pm$ 0.30	67.1 $\pm$ 5	20.5 $\pm$ 0.77 <sup>1</sup>
C (10)	795 $\pm$ 8	13.9 $\pm$ 1.5	86.5 $\pm$ 1.5	8.55 $\pm$ 0.23	76.9 $\pm$ 5	27.5 $\pm$ 1.5

<sup>1</sup> p < 0.001.

TABLE 6  
*Comparison of carbohydrate and RNA levels in tadpole tail muscle after treatment with prolactin and growth hormone*

	Carbohydrate (mg/gm muscle)		RNA (mg/gm muscle)	
	Exp. 2	Exp. 3	Exp. 2	Exp. 3
Control	8.31 $\pm$ 0.61	3.09 $\pm$ 0.27	1.21 $\pm$ 0.06	1.13 $\pm$ 0.04
Prolactin	4.72 $\pm$ 0.47 <sup>2</sup>	2.45 $\pm$ 0.10 <sup>1</sup>	1.20 $\pm$ 0.09	1.11 $\pm$ 0.03
GH	6.54 $\pm$ 0.75	3.41 $\pm$ 0.28	1.31 $\pm$ 0.06	1.16 $\pm$ 0.04

<sup>1</sup> p < 0.05.  
<sup>2</sup> p < 0.001.

reduction of carbohydrate was not great but was significant ( $P < 0.05$ ). The difference between the results of the two experiments was probably due to the fact that in Experiment 4 the carbohydrate had been depleted by the previous 24-hour fast. The effect of growth hormone upon carbohydrate was not significant in either experiment. Neither hormone had any effect on the levels of RNA in tail muscle.

*Fat body size.* Growth hormone is known to decrease lipid stores in some animals. Fat bodies of growth hormone and prolactin-treated animals were weighed in order to compare the effects of these two hormones upon amphibians and higher animals and in pre- and post-

metamorphic animals. In Frog Experiment 2, but not Frog Experiment 1, growth hormone significantly ( $P < 0.05$ ) reduced the ratio of fat body weight (figs. 5a,b). Prolactin had no effect on fat body size in either experiment. The absolute fat body sizes in these two experiments generally paralleled the relative fat body values.

Fat bodies from Tadpole Experiment 4 were also measured (fig. 6). Neither prolactin nor growth hormone treatment significantly altered the relative (mg/gm body) or absolute fat body weight. These experiments indicate that growth hormone tends to decrease lipid stores in frogs but not in tadpoles. Prolactin has



no significant effect on fat stores in either frog or tadpole.

*Blood glucose.* Because growth hormone has been shown to increase blood glucose levels in animals, pre- and post-metamorphic *Rana pipiens* were tested after growth hormone and prolactin injections. Animals tested were from Frog Experiment 2 and Tadpole Experiments 2 and 4. Growth hormone injected into intact frogs increased blood sugar levels by nearly 10 mg% over controls ( $P < 0.005$ ). The prolactin group mean was somewhat higher than controls but not significantly so (fig. 7a). Neither growth hormone nor prolactin have any effect on tadpole blood glucose in these two experiments (fig. 7b). The normal (control) level

of blood glucose in tadpoles is only about half that of frogs.

DISCUSSION

I. General body growth

Until recently, little information was available concerning growth regulation by pituitary hormones in lower vertebrates. However, recent work (cited in the Introduction) has established a role of the pituitary in growth regulation in amphibians, and has brought to light the interesting observation that larval growth is regulated by prolactin while postmetamorphic frogs grow primarily in response to growth hormone. The results of this

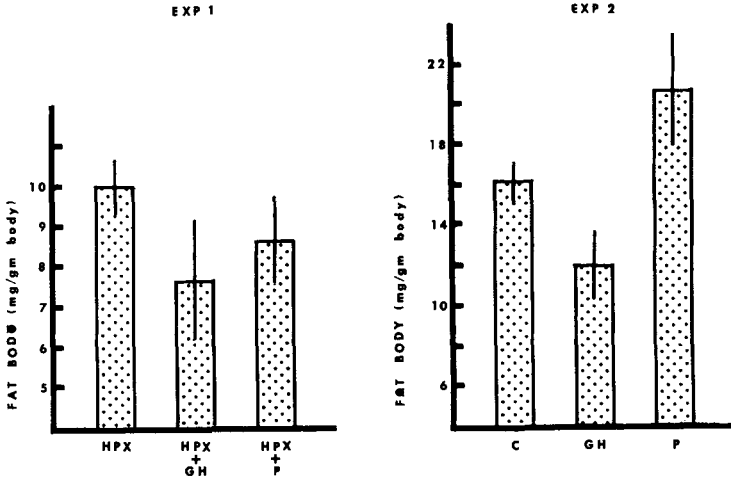


Fig. 5 Fat body weights of intact and hypophysectomized frogs treated with growth hormone and prolactin (frog experiments 1, 2).

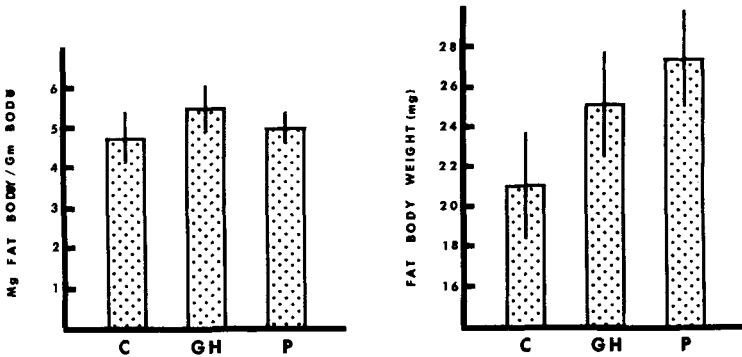


Fig. 6 Fat body weights of tadpoles after treatment with growth hormone and prolactin (tadpole experiment 4).

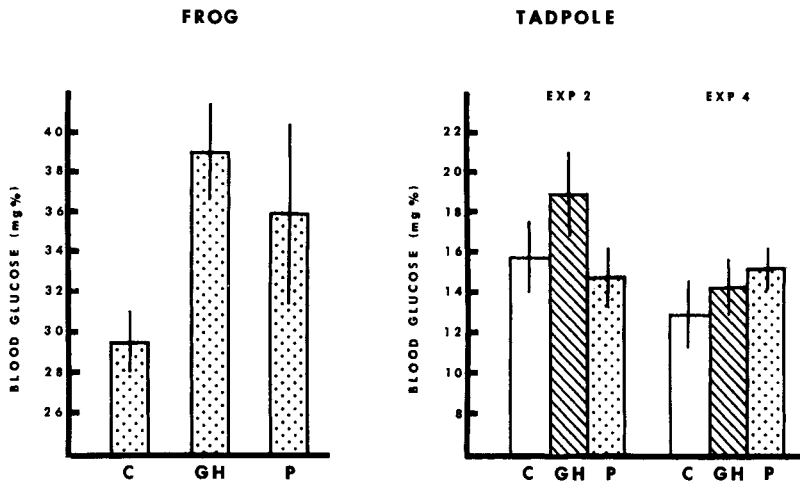


Fig. 7 Blood glucose levels of intact frogs (experiment 2) and tadpoles (experiments 2, 4) after treatment with growth hormone and prolactin.

study confirm the previous reports demonstrating growth effects of growth hormone and prolactin in frogs and tadpoles respectively. Interpretation of these results must be qualified since all work to date has been done with mammalian hormones. We presume that homologous molecules, similar to prolactin and growth hormone are the physiological growth regulating hormones of the pre- and post-metamorphic stages of amphibian development (Brown and Frye, '69a,b).

## II. Chemicals composition

In mammals, the physiological role of growth hormone is viewed to be one of regulating a balance in the metabolism of protein, carbohydrate, and fat. Regulation of the exact proportionality of this balance is not well understood, but appears to depend upon the nutritional balance of the animal, the hormonal background against which growth hormone acts, and the developmental state of the animal. For example, on a limited diet, too small to support growth, net growth can be induced by growth hormone, which promotes the utilization of lipid stores and thus spares ingested protein for growth (Lee and Ayres, '36). Similarly, during starvation, growth hormone spares glucose and amino acids at the expense of body fat. Only during advanced fasting

or starvation are these effects of growth hormone counteracted, presumably by suppression of growth hormone secretion and accelerated secretion of glucocorticoids which promote protein degradation. A major objective of this study has been to determine whether growth stimulation by growth hormone and prolactin is accompanied by a similar pattern of metabolic effects conducive to protein anabolism and carbohydrate conservation. On the basis of such information we hoped to see a relationship between the growth effects and the metabolic effects of these hormones which might be related to differences in the biology of the larval and postmetamorphic stages of development.

*Liver.* In mammals, growth hormone causes increases in absolute liver weight, ratio of liver weight to body weight, total protein content (Li and Evans, '48), and liver glycogen (Russell and Bloom, '56; Altszuler et al., '68). The results of the present study on frogs are in marked contrast to these observations. Growth hormone brought about a decrease in liver size, carbohydrate, and in one experiment, total protein. Prolactin also reduced liver size and carbohydrate, though to a smaller extent than growth hormone, but had no effect on protein content. Zipser et al. ('69) found that both growth hormone and prolactin treatment of *Bufo boreas* and *Bufo marinus* reduced the to-

tal liver size, although prolactin was only about one-fifth as potent as growth hormone. These authors suggested that the effect of prolactin was probably due to growth hormone contamination of their prolactin preparations (which could also be true in our experiments). In *B. boreas* glycogen content was reduced to less than one-half the control value, which would probably account for the reduced liver size. In *B. marinus* they found that the reduction in liver size was due to loss of lipid.

In tadpoles prolactin had no effect upon either liver carbohydrate or absolute liver size. It is noteworthy that prolactin failed to elicit an increase in liver size in tadpoles under conditions when an overall increase in body size was stimulated. As noted earlier, prolactin tended to have the same effects as growth hormone on frog liver, although the magnitude was usually smaller. Reports in the literature concerning the effects of prolactin on mammalian liver size and glycogen content are conflicting (Elghamry et al., '66; Tutwiller, '70) and comparisons with amphibians will not be made at this time.

**Muscle.** Mammals are known to respond to growth hormone stimulation with increases in total muscle size paralleled by an increase in content of protein, water, and nucleic acid (Li et al., '49; Scow and Hagan, '65; Cheek et al., '65; Reid, '56; Di Stefano et al., '53). In addition growth hormone has been shown to have a glycostatic effect on muscle of fasting intact and hypophysectomized mammals (Russell and Bloom, '56). In the present study, growth hormone did not increase total muscle size or total protein content significantly, but this was probably due to the fact that initial and final muscle size could not be compared rather than to the lack of growth hormone effect. Growth hormone did increase the levels of nucleic acids and water in frog muscle, and in that sense a growth effect was observed. In addition, in another phase of this study to be reported separately, stimulation of incorporation of  $^{14}\text{C}$ -labeled amino acid into muscle protein was observed (Snyder, '70).

Frog and mammalian muscle carbohydrate respond differently to growth hormone stimulation. In frogs, hypophysec-

tomy reduced muscle glycogen, as it does in mammalian muscle, but unlike the mammalian response, growth hormone lowered the carbohydrate levels even below the level of hypophysectomized animals. Muscle carbohydrate of intact frogs was also reduced by growth hormone.

A striking difference was observed between the response of tadpole and frog muscle in that tadpole tail muscle did not respond to growth hormone in any of the parameters measured. Prolactin lowered carbohydrate levels in both frogs and tadpoles but stimulated growth only in tadpoles. Thus, there is clearly a difference in the hormone sensitivity of tadpole tail muscle and frog gastrocnemius muscle. Unfortunately, it was not possible to examine homologous muscles in the two developmental stages. Consequently it is not possible to determine whether the difference in response is inherent in the two types of muscle studied, or whether there is a change in the hormone sensitivity of skeletal muscle in general at metamorphosis.

**Lipid stores.** Injection of growth hormones into mammals has long been known to reduce carcass lipid stores (Lee and Ayres, '36; Li et al., '49; Li and Evans, '48). Liver lipid of hypophysectomized rats is also reduced by growth hormone injections (Li et al., '49). In this study, growth hormone treatment reduced fat body lipid but had no effect on liver lipid of frogs. Prolactin did not significantly change frog fat body or liver lipid levels. This result is similar to the observation of Zipser et al. ('69) that growth hormone reduces fat body weights in *Bufo boreas* and *Bufo marinus*. In *B. marinus*, but not in *B. boreas*, liver lipid was also reduced by growth hormone. Prolactin had no effect on toad lipid when given at the same dose as growth hormone. Thus, in frogs, toads, and mammals, there is a correlation between growth stimulation by growth hormone and reduction in total energy stores, but no such correlation exists between prolactin-induced growth and energy stores in the tadpole.

In mammals, the lipolytic effect of growth hormone has secondary effects on carbohydrate metabolism (Randle, '63). It is thought that growth hormone promotes the mobilization of free fatty acids

(FFA) from lipid stores which in muscle are oxidized preferentially over glucose. Tissue glycogen and blood glucose levels are partially spared during fasting, exercise, etc. This metabolic interrelationship between lipid and carbohydrate metabolism would appear not to occur in amphibians since growth hormone simultaneously reduces fat body lipid and carbohydrate stores. Although the mechanism by which growth hormone induces carbohydrate and lipid breakdown in frogs is not known, the results of this study suggest that it must be different from mammals.

### III. *Biological significance of the growth and metabolic effects of growth hormone and prolactin in amphibians*

In view of the adaptive significance which has been attached to the inter-related growth and metabolic effects of growth hormone in mammals, departures from this pattern, especially with respect to the glycogenolytic rather than glyco-static effect, are surprising and require explanation. At present there are very few known facts about amphibian carbohydrate metabolism from which to weave a consistent interpretation of the glycogenolytic, lipolytic, and protein anabolic effects of growth hormone. The pertinent points which are presently known, at least tentatively, which bear upon this point are:

a. The primary diet of frogs is insects of which the two largest components are protein and fat. As is characteristic of carnivores in general, carbohydrate is a relatively smaller proportion of the caloric intake than in herbivores.

b. Glycogen is an important metabolic substrate, perhaps the major one in certain tissues. This may be surmised from two observations: that glycogen levels of liver and skeletal muscle are unusually high compared to mammals (Farrer, thesis in preparation); and that frog skeletal muscle is "white" muscle which is very poorly vascularized, fatigues rapidly, and generates large amounts of lactic acid during exercise. It presumably depends, therefore, upon glycogen metabolism

through glycolysis and the Cori cycle as the major source of energy for contraction.

c. Frogs undergo prolonged fasting during winter hibernation, during which time they maintain high levels of liver and muscle glycogen (Farrer, thesis in preparation; Mizell, '65). Calculations based on the metabolic rate and the total fat and glucogen stores indicate that high levels of liver and muscle glycogen must be maintained by gluconeogenesis (Farrer, thesis in preparation).

d. Blood glucose values are low and variable. Typical values for postmetamorphic amphibians range from 15-40 mg% with values sometimes reported to be unmeasurably low (Wright, '59; Bartell, '69; Farrer, thesis in preparation).

On the basis of these points we can theorize that the major mechanism for regulating carbohydrate content of the body in frogs is gluconeogenesis and that a growth hormone glyco-static mechanism as observed in mammals would be relatively unimportant. The relative unimportance of the glyco-static mechanism is suggested by the observation of high tissue levels of glycogen in both intact and hypophysectomized frogs, and by the fact that even at unmeasurably low levels of blood glucose adverse effects upon the central nervous system are not observed. In short, glucose *per se* may not be an important or essential substrate for nervous tissues as it is in mammals and, therefore, the importance of conserving glycogen and blood glucose during fasting may be slight. In fact, if one presumes that carbohydrate is always produced in abundance through gluconeogenesis, then the glycogenolytic effect of growth hormone seen in frogs may be viewed as a mechanism for diverting carbohydrate into energetic pathways, including growth, while conserving tissue protein. This idea is consistent with the observation that liver and muscle glycogen are lowest in the summer when growth and presumably growth hormone secretion is highest (Farrer, thesis in preparation). Nothing is known, of course, about seasonal patterns of growth hormone secretion in amphibians, but in terms of correlation between growth hormone and the inter-related metabolic effects described in this study, it would be expected to be highest

during summer when food is most abundant.

A mechanism must exist by which the protein anabolic effects of growth hormone and the protein catabolic effects of gluconeogenic hormones are kept from being antagonistic. In the frog, the balance between protein anabolism (under the influence of growth hormone) and protein catabolism (presumably under the control of glucocorticoids) could be regulated by seasonal variations in the relative activity of these two hormones. Although existing evidence does not allow for positive identification of the hormones responsible for gluconeogenesis, the glucocorticoids are implicated by the fact that they are gluconeogenic in mammals, and by the fact that adrenalectomy causes a depletion of frog muscle and liver glycogen (Gorbman, '64). Once growth hormone and the gluconeogenic hormones set the pattern of metabolism other hormones may operate to augment these patterns. Thus, in *Rana pipiens*, substrates have been shown to be metabolized primarily to glycogen in winter months and toward oxidation in summer. Insulin increased both summer and winter depending upon the season it was tested (Gourley et al., '69).

The findings of this study additionally suggest that growth hormone stimulates net protein catabolism in liver while stimulating overall growth and protein synthesis in the rest of the body. Growth hormone appears to combine the properties of promoting protein breakdown in the major gluconeogenic tissue, liver, while promoting growth in other tissues. Thus, carcass protein can be conserved under the influence of growth hormone while dietary protein catabolism is augmented, a condition which would appear to be necessary in an organism which uses large amounts of dietary protein as an energy substrate and as a source of amino acids for growth.

One of the most interesting aspects of amphibian growth concerns the reason why there should be different growth factors for the pre- and postmetamorphic stages of development. While there is no satisfactory explanation at this time, the difference in the two stages may be related to the effects of growth hormone

and prolactin on fat and carbohydrate stores. There are several ways in which tadpole energy metabolism differs from that of frog. First, tadpoles do not normally experience periods of starvation—food is always present in the digestive system. Secondly, anuran tadpoles are herbivores and consume relatively more carbohydrate and less protein than frogs. Thus, hormonal mechanisms for regulating carbohydrate homeostasis and gluconeogenesis may not be as important in tadpoles as in frogs. Third, lipid is the primary form of energy storage in tadpoles (cf. table 7 and figs. 23, 24). This is probably advantageous during the energetically demanding period of metamorphosis since fat is the most efficient way to store large amounts of energy in a small space. In view of this, it is adaptively advantageous that tadpole growth is regulated by prolactin which is conducive to lipid deposition rather than by growth hormone which causes fat breakdown.

In addition, at least part of the growth promoting effect of prolactin in tadpoles is caused by suppression of thyroid hormone secretion or antagonism of thyroid hormone action in target tissues (Gona, '67; Bern et al., '67). Selection of prolactin as the larval growth regulator may have been favored by the fact that this molecule combines the properties of blocking metamorphosis and stimulating growth—processes that are antagonistic during larval life.

One of the most important problems in biology involves the changing patterns of gene expression during development. The fact that amphibians appear to have different growth hormones at different stages of development implies that a developmental change in hormone sensitivity occurs at metamorphosis. This study documents this change by demonstrating an alteration in hormone sensitivity of muscle, liver, and fat body. The mechanism by which this changeover occurs is of considerable interest. One possibility is that different tissues in tadpoles and frogs are sensitive to prolactin and growth hormone respectively, and it is the relative proportion of these tissues that change at metamorphosis. Thus, the large tail of the tadpole might be sensitive to

prolactin while the skeletal muscle and liver of frog may respond to growth hormone. In this case, changing patterns of prolactin and growth hormone sensitivity at metamorphosis would not involve cellular differentiation *per se*, but would reflect a decline in numbers of prolactin-sensitive cells, and a rise in numbers of growth hormone sensitive cells comprising the hormone sensitive tissues.

Alternatively, at metamorphosis a change in hormone sensitivity may occur within the individual cells of prolactin and growth hormone responsive tissues. This would amount to a process of cellular differentiation in which a growth-hormone response mechanism would be synthesized and/or activated and the prolactin response mechanism inactivated at the time of metamorphosis. There are of course a large number of levels and ways in which such a change might be effected, ranging from changes in DNA activity to changes in membrane structure or protein conformation. If we assume that the same populations of cells which comprise the liver and the fat body of the tadpole, constitute these same structures in the frog, then the results of this study suggest that the mechanism of change in prolactin and growth hormone sensitivity is indeed one of cellular differentiation. The assumption that the bulk of the cells of the larval tissues do persist and form the liver and fat bodies of the frog has not, to our knowledge, been critically tested.

Finally, the change in hormone sensitivity at metamorphosis could reflect a change in sensitivity to, or secretion of, another hormone which modifies the action of growth hormone or prolactin. For instance, prolactin may produce part of its growth effect in tadpoles by inhibiting the thyroxin-induced breakdown of tadpole tissues [note the lack of stimulation of amino acid incorporation obtained in this study and the prolactin inhibition of thyroxin-induced tail breakdown observed by Blatt et al. ('69) and Derby and Etkin ('68)]. Although prolactin appears to have a growth effect independent of its antithyroid effect (Brown and Frye, '69), hormone interactions of this type cannot yet be discounted in explaining the dif-

ferences in the responses of tadpoles and frogs to prolactin and growth hormone.

#### LITERATURE CITED

- Altzuler, N., I. Rathgeb, B. Winkler and R. C. de Bodo 1968 The effects of growth hormone on carbohydrate and lipid metabolism in the dog. *Ann. N. Y. Acad. Sci.*, 148: 441-458.
- Ashwell, G. 1966 New colorimetric methods of sugar analysis. VII. The phenol-sulfuric acid reaction for carbohydrates. In: *Methods in Enzymology*. Vol. 8. E. F. Neufeld and V. Ginsburg, eds. Academic Press, New York, pp. 93-94.
- Bartell, M. 1969 The role of the pituitary in blood glucose regulation in larval and adult salamanders, *Ambystoma tigrinum*. Ph.D. Dissertation, Department of Zoology, University of Michigan, Ann Arbor, Michigan, 145 pp.
- Berman, R., R. C. Strohman, C. S. Nicoll and H. Bern 1964 Growth-promoting effects of mammalian prolactin and growth hormone in tadpoles of *Rana catesbeiana*. *J. Exp. Zool.*, 156: 353-360.
- Bern, H. A., C. S. Nicoll and R. C. Strohman 1967 Prolactin and tadpole growth. *Proc. Soc. Exptl. Biol. Med.*, 126: 518-520.
- Blatt, L. M., K. A. Slickers and K. H. Kim 1969 Effect of prolactin on thyroxin-induced metamorphosis. *Endocrinol.*, 85: 1213-1215.
- Brown, P., and B. E. Frye 1969a Effects of prolactin and growth hormone on growth and metamorphosis of tadpoles of the frog, *Rana pipiens*. *Gen. Comp. Endocrinol.*, 13: 126-138.
- 1969b Effects of hypophysectomy, prolactin, and growth hormone on growth of post-metamorphic frogs. *Gen. Comp. Endocrinol.*, 13: 139-145.
- Burton, K. 1956 A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.*, 62: 315-323.
- Cheek, D. B., G. K. Powell and R. E. Scott 1965 Growth of muscle cells (size and number) and liver DNA in rats and Snell Smith mice with insufficient pituitary, thyroid, or testicular function. *Bull. Johns Hopkins Hosp.*, 117: 306-321.
- Derby, A., and W. Etkin 1968 Thyroxine induced tail resorption *in vitro* as affected by anterior pituitary hormones. *J. Exp. Zool.*, 169: 1-8.
- Di Stefano, H. S., H. F. Diermeier and J. Tepperman 1953 Effects of growth hormone on nucleic acid and protein content of rat liver cells. *Endocrinol.*, 57: 158-167.
- Elghamry, M. I., A. Said and S. A. Elmougy 1966 The effect of lactogenic hormone on liver glucogen and blood glucose in ovariectomized mice. *Naturwissenschaften*, 53: 530.
- Enemar, A., B. Essvik and R. Klang 1968 Growth-promoting effects of ovine somatotrophin and prolactin in tadpoles of *Rana temporaria*. *Gen. Comp. Endocrinol.*, 11: 328-331.
- Etkin, W., and R. Lehrer 1960 Excess growth in tadpoles after transplantation of the adeno-hypophysis. *Endocrinol.*, 67: 457-466.

- Etkin, W., and A. G. Gona 1967a Antithyroid action of prolactin in the frog. *Life Sci.*, 6: 703-707.
- 1967b Antagonism between prolactin and thyroid hormone in amphibian development. *J. Exp. Zool.*, 165: 249-258.
- Gona, A. G. 1968 Radioiodine studies on prolactin action in tadpoles. *Gen. Comp. Endocrinol.*, 11: 278-283.
- 1967 Prolactin as a goitrogenic agent in amphibia. *Endocrinol.*, 81: 748-754.
- Gorbman, A. 1964 Endocrinology of the amphibia. In: *Physiology of the Amphibia*. J. A. Moore, ed. Academic Press, New York, pp. 371-425.
- Gourley, D. R. H., T. K. Suh and L. L. Brunton 1969 Seasonal differences and the effect of insulin on pyruvate uptake, oxidation and synthesis to glycogen by frog skeletal muscle. *Comp. Biochem. Physiol.*, 29: 509-524.
- Lee, M. O., and G. B. Ayres 1936 The composition of weight lost and the nitrogen partition of tissues in rats after hypophysectomy. *Endocrinol.*, 20: 489-495.
- Li, C. H., and H. M. Evans 1948 The biochemistry of pituitary growth hormone. *Recent Progr. Horm. Res.*, 3: 3-44.
- Li, C. H., M. E. Simpson and H. M. Evans 1949 The growth of hypophysectomized female rats following chronic treatment with pure pituitary growth hormone. *Growth*, 13: 171-174.
- 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.
- Mizell, S. 1965 Seasonal changes in energy reserves in the common frog, *Rana pipiens*. *J. Cell. and Comp. Physiol.*, 66: 251-258.
- Randle, P. J. 1963 Endocrine control of metabolism. *Ann. Rev. of Physiol.*, 25: 291-324.
- Reid, E. 1956 Hormonal effects on liver and kidney cytoplasm. *J. Endocrinol.*, 13: 319-329.
- Remy, C., and J. J. Bounhiol 1965 Croissance exageree des tetards de crapaud accoucher entires ou prives de leur hypophyses et subissant un traitement par la prolactine. *Comp. Rend. Soc. Biol.*, 159: 1532-1535.
- Remy, C., and J. J. Bounhiol 1966 Gigantisme experimental obtenu par intervention, chirurgicale et traitements hormonaux chez le tetard der crapaud accoucher. *Ann. Endocrinol.*, Paris, 27: Suppl., 377-382.
- Russell, J. A., and W. Bloom 1956 Hormonal control of glycogen in the heart and other tissues in rats. *Endocrinol.*, 58: 83-94.
- Scow, R. O., and S. N. Hagan 1965 Effect of testosterone propionate and growth hormone on growth and chemical composition of muscle and other tissues in hypophysectomized male rats. *Endocrinol.*, 77: 852-858.
- Shibko, S., P. Kiovistoinen, C. A. Tratnyek, A. R. Newhall and L. Friedman 1967 A method for sequential quantitative separation and determination of protein, RNA, DNA, lipid, and glycogen from a single rat liver homogenate or from a subcellular fraction. *Anal. Biochem.*, 19: 514-528.
- Snedecor, G. W., and W. G. Cochran 1967 *Statistical Methods*. Iowa State University Press, Ames, Iowa.
- Snyder, B. W. 1970 Physiological responses of larval and postmetamorphic *Rana pipiens* to growth hormone and prolactin. Ph.D. Dissertation, Department of Zoology, University of Michigan, Ann Arbor, Michigan, 95 pp.
- Steinmetz, C. 1954 Some effects of thyroxin and antithyroid compounds on tadpoles and their relation to hormonal control of growth. *Physiol. Zool.*, 27: 28-40.
- Taylor, A. C., and J. J. Kollros 1946 Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.*, 94: 7-23.
- Tutwiler, G. F. 1970 Physiochemical and biological characterization of a diabetogenic peptide from bovine adenohypophysis. Ph.D. Dissertation, Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan, 159 pp.
- Wannemacher, R. W., W. L. Banks and W. H. Wunner 1965 Use of a single tissue extract to determine cellular protein and nucleic acid concentrations and rate of amino acid incorporation. *Anal. Biochem.*, 11: 320-326.
- Winkler, B., R. Steele, N. Altszuler and R. C. De Bodo 1964 Effect of growth hormone on fatty acid metabolism. *Am. J. Physiol.*, 206: 174-178.
- Wright, P. A. 1959 Blood sugar studies in the bullfrog *Rana catesbeiana*. *Endocrinol.*, 64: 551-558.
- Wright, P. A., and A. R. Flathers 1961 Facilitation of pituitary induced ovulation by progesterone in early fall. *Proc. Soc. Exptl. Biol. Med.*, 106: 346-347.
- Zipser, R. D., P. Licht and H. Bern 1969 Comparative effects of mammalian prolactin and growth hormone on growth in the toads *Bufo boreas* and *Bufo marinus*. *Gen. Comp. Endocrinol.*, 13: 383-391.