IS AVIAN PANCREATIC POLYPEPTIDE (APP) TROPHIC FOR TISSUES OF THE EMBRYONIC CHICK GUT?

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

David Arlen Laurentz

December 1975

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ABSTRACT

Avian pancreatic polypeptide (APP) has been shown to be a gastric (proventricular) secretagogue in adult chickens. In mammals, gastrin (in the form of Pentagastrin) is a trophic hormone for tissues of the gut in addition to being the hormone regulating gastric secretion. The purpose of this study was to determine, using ablation of the APP secretory source and precocious administration of APP to developing embryos, if APP is also trophic for gut tissues in addition to stimulating gastric secretion.

The results of this study indicate that in the absence of APP there is a "wasting" response in the adult proventriculus mucosa. Injection of APP into two week old embryos caused an increase in proventricular total protein within two days, similar to the effect seen with Pentagastrin injected at this time. Within one hour of APP injection, radiolabeled amino acid incorporation into protein *in vitro* by the proventriculus was stimulated, and at higher doses the same effect seen in the liver.

The evidence presented suggests that APP exerts a trophic action on the embryonic proventriculus (secretory stomach), and possibly on the liver at higher doses.

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1.

INTRODUCTION

INTRODUCTION

Avian pancreatic polypeptide (APP) is a candidate hormone of the gut, the origin of which may eventually be ascribed to the A_1-cell, or one very similar to the A_1-type, of the avian endocrine pancreas. Since the original description of the A_1-cell in the avian pancreas (an argyrophilic metachromatic cell type referred to as the D-cell in mammals) its existence as an unique cell type has been a subject of controversy (Like, 1967). The contradictory evidence concerning the A_1 or D-cell has been reviewed by Fujita (1968) who concluded that it is an unique cell type functioning as a third endocrine element of the pancreatic islet. Thus established, interest has gathered in determining the nature of the secretory product of this third endocrine element.

In mammals, gastric hypersecretion caused by pancreatic adenomas prompted the use of indirect immunofluorescent (Lomsky, Langr and Vortel, 1969), direct immunofluorescent and radioimmunoassay techniques (Greider and McGuigan, 1971) which identified gastrin in the secretory granules of the D-cells in normal pancreata from man, pig, panther, rabbit, albino rat, and guinea-pig. The identity of the secretion of the D or A_1-cell in Aves is less well established.

Physiological amounts (0.15 µg/ml) of gastrin as well as water extracts of pigeon A_1-cells have been shown to inhibit glucose-stimulated insulin release from microdissected mouse B-islets (Lernmark, Hellman and Coore, 1969: Hellman and Lernmark, 1969). Gastrin could not be detected in the esophagus, crop, proventriculus, gizzard, or duodenum of chickens (Ruoff and Sewing, 1970), indicating that the A_1-cell hormone, if gastrin, could be the only source of gut secretagogic activity in Aves.

APP was originally reported as a polypeptide contaminant present during insulin purification from the chicken pancreas, and present in a twofold greater (protein) concentration than insulin in the same pancreas (Kimmel, Pollock and Hazelwood, 1968). Subsequent isolation and purification showed the peptide to be a straight chain of 36 amino acid residues, MW 4200, and a suggested amino acid sequence as given in Fig. 1 (Kimmel and Pollock, 1975). APP radioimmunoassay activity has been found in eight avian species (chicken, duck, goose, pigeon, guinea-fowl, great horned owl, red tailed hawk, and roseate spoonbill) and in turtles, but not in amphibia or snakes (Langslow, Kimmel and Pollock, 1973). No cross-immunoreactivity with anti- APP serum has been found in man, cow, pig, dog, or rabbit despite the isolation of a similar 36 amino acid peptide from bovine, ovine, porcine and

Figure 1. The amino acid sequence of APP (taken from Kimmel and Pollock, 1975)

Points marked T and C represent the bonds which are broken by trypsin and chymotrypsin, respectively. A polypeptide has subsequently been isolated from bovine pancreas (Lin and Chance, 1972) which is identical to APP in 16 of its 36 residues.

AMINO ACID SEQUENCE OF APP

Gly • Pro • Ser • Gln • Pro • Thr • Tyr • Pro • Gly •

Asp • Asp • Ala • Pro • Val • Glu • Asp • Leu • Ile •

Arg • Phe • Tyr • Asp • Asn • Leu • Gln • Gln • Tyr •

Leu • Asn • Val • Val • Thr • Arg • His • Arg • Tyr — NH_2

T C C

T T

human pancreata. The bovine pancreatic polypeptide (BPP) is homologous to APP in 16 out of the 36 amino acid positions. Neither APP nor its mammalian counterparts shows any structural homology to the 17 amino acid gastrin, however. Despite this lack of homology with gastrin, the numerous lines of evidence (above) suggesting that the A_1-cell would secrete gastrin led to investigation of a possible secretagogic effect of APP in Aves.

APP, when compared to the synthetic pentapeptide, Pentagastrin, was found to cause a proventricular secretory volume increase 100 times greater than that caused by the gastrin analog when injected into chickens (Hazelwood, Turner, Kimmel and Pollock, 1973). Acid, pepsin and total protein secretion were also significantly elevated. In the same laboratory, higher doses of APP were discovered to stimulate hepatic glycogenolysis and plasma hypoglycerolemia without a plasma glycemic response, suggesting a stimulation of hepatic lipogenesis. Because of the secretagogic activity of APP and the apparent absence of gastrin in birds, the possibility that APP served as an avian "gastrin" arose and further comparisons of the two were indicated.

In recent years there has accumulated clinical evidence that gastrin increases the growth of mucosa in the upper gastrointestinal tract (Crean, 1967), while the absence of gastrin

leads to gastric mucosal atrophy (MacDonald and Rubin, 1967). Subsequent investigations in several laboratories showed that Pentagastrin indeed acts as a pleiotypic effector in mucosal tissues of the stomach and small intestine, as do growth hormone and other trophic hormones in their target tissues (Tomkins, 1972), in that it increases amino acid incorporation (Johnson, Aures and Yuen, 1969), increases precursor incorporation into RNA (Chandler and Johnson, 1972) and stimulates DNA synthesis (Johnson and Guthrie, 1974). In addition Pentagastrin has been shown to increase fundic parietal cell density and total count, although the specific parietal cell response to Pentagastrin is qualitatively different from that presumably caused by gastrin observed after duodenal obstruction (Crean, Marshall and Rumsey, 1964). This now well established trophic effect of Pentagastrin on the mucosa of gastrointestinal tissues in mammals serves as the basis for the further investigation of similarities between gastrin and APP in birds proposed herein: to determine if Pentagastrin and/or APP possess a trophic as well as a secretagogic activity in Aves.

Classically, two approaches are used to determine the effect of an hormone: the hormone is replaced in an animal from which the source of secretion has been removed, or the hormone can be injected precociously into developing animals. Due to considerations of the quantity of APP available, the

latter approach was used in this study. A selection of the parameters which had been applied in previously mentioned investigations with gastrin would serve as indications of trophic activity of APP; gastrointestinal wet weight, organ length, weight, total protein, protein to DNA ratio, RNA to DNA ratio, and protein precursor uptake and incorporation. Increases in these parameters are considered to be indicative of a trophic response.

II.

STATEMENT OF THE PROBLEM

STATEMENT OF THE PROBLEM

The purpose of this study was to determine if avian pancreatic polypeptide (APP) has a trophic effect on gut tissues of the embryonic chick. To establish this system, Pentagastrin-injected eggs were compared with saline-injected controls with respect to gastrointestinal tract fresh weight, proventricular length, organ fresh weight, total protein, protein/DNA and RNA/DNA ratios. The dosage levels of Pentagastrin determined to be most effective were repeated substituting APP, measuring the same parameters and in addition the rate of incorporation of radiolabeled amino acid into gut tissue protein. Increases in these parameters are considered to be an indication of a trophic response.

III.

MATERIALS AND METHODS

MATERIALS AND METHODS

Animals

Eggs of a single-comb white leghorn (SCWL) strain known as Babcock B-300 were obtained from a local hatchery (Albers Hatchery and Feed, Inc., LaGrange, Texas). Those eggs which were not incubated immediately on receipt were stored upright in an air-conditioned room for not more than five days.. Incubation was in a Superior Incubator Co. model 600, maintaining a dry bulb thermometer reading of 99 3/4° F. and a wet bulb reading of 86° F. (indicating 48% humidity). Eggs were turned twice daily.

Female adult SCWL chickens were used to study the effects of "total" pancreatectomy on proventricular and duodenal mucosa. Pancreatectomy, performed by J. R. Colca, consisted of removal of 99% of the pancreas leaving the duodenum and its blood supply intact, followed eight days later by removal of the remaining splenic lobe (Colca and Hazelwood, 1976). Animals were sacrificed four days after the second stage of the operation. Total depancreatized and sham-operated birds were maintained on standard chicken feed (Purina) with dried bovine pancreas added (10%) to the feed of the operated birds as a source of pancreatic enzymes.

Injection

Eggs to be injected were candled to locate (and mark) a site for injection which would enter the yolk sac (subsequent observations at the time of sacrifice demonstrated that all of the injections did so). Maintaining the egg in an upright position throughout the candling and injection procedures, the shell was ground down to, but not penetrating through, the shell membrane using an Emesco dental drill. All injections consisted of a 0.1 ml volume using a 27 gauge 5/8" needle. The hole was then sealed with a drop of hot paraffin and the eggs returned to the incubator until the time of sacrifice.

Removal of embryonic gut tissues

At the time of sacrifice, the shell surrounding the air cell was removed with a pair of forceps. The shell membrane was peeled back and the location of the injection noted with respect to the yolk sac. The embryo was then removed rapidly and decapitated. Tissues to be assayed were dissected out within 30 seconds of decapitation using fine forceps and placed either in cold isotonic saline or cold incubation medium depending on the analyses to be performed.

Analyses

Protein, RNA and DNA

Tissue homogenates were extracted and assayed for RNA and DNA using the phloroglucinol and diphenylamine reactions as described by Schneider (1945). Colorimetric readings for the nucleic acids were made on a Beckman Model 25 Spectrophotometer. The final pellet of the extraction procedure was dissolved in 10 ml of 1 N NaOH and 1 ml used for protein determination according to the method of Lowry et al. (1951). Colorimetric readings for protein were made on a Bausch and Lomb Spectronic-20.

Amino acid incorporation

Proventriculus, gizzard and liver tissues were removed as above, blotted dry and weighed on a semi-micro torsion balance. The tissues were then homogenized by hand using a glass homogenizer in 0.9 ml of cold incubation medium consisting of 7.45 g/l KCl, 2.34 g/l NaCl, 2.42 g/l Tris pH 7.9, 0.42 ml/l mercaptoethanol, 2.03 g/l $MgCl_2 \cdot 6H_2O$, and 85.60 g/l sucrose. The homogenate was then poured into conical bottom polypropylene centrifuge tubes for incubation. Immediately before initiation of the incubation period, 0.1 ml of a stock solution containing 25 mM ATP, 1.0 mM GTP, and 0.5 μCi of uniformly labeled L-leucine-^{14}C (SA=240 mCi/mmole) was added to each tube. Incubation was carried out for 15 minutes in a Dubnoff metabolic shaking incubator at 42^{o} C. and

120 cycles/minute. After 15 minutes the reaction was stopped with 2 ml of 5% TCA containing 14 mM unlabeled L-leucine. To insure complete precipitation, the samples were left in the refrigerator overnight. Using centrifugation, the precipitate was washed twice with 5% TCA + 14 mM leucine, once in 5% TCA at 90°C. for 15 minutes to remove nucleic acids, and twice in ETOH:ether (3:1 vol/vol) to remove lipids. The pellet was then dried, digested in 1 ml Soluene (Beckman tissue solubilizer)/100 mg tissue, and transferred quantitatively to counting vials using a toluene based scintillation cocktail containing 4 g/l PPO and 50 mg/l POPOP. The sample was counted for 20 minutes (at 2% error) in a Beckman Model LS-150 liquid scintillation counting system. Results were expressed in disintegrations per minute per milligram of tissue as a percent of saline injected controls, assuming 95% isotope recovery.

EXPERIMENTAL PROTOCOL

A. Effects of total pancreatectomy on proventricular and duodenal mucosa

Preliminary evidence of a trophic effect by APP was sought by determining if a deficiency response could be elicited when the source of APP was removed from adult birds. Totally depancreatized and sham-operated birds were killed by

Nembutal (sodium pentobarbital) injection. The proventriculus and duodenal loop were excised, split longitudinally, and washed in ice-cold isotonic saline. A sample of mucosa (50 mg) was scraped off using a tared glass microscope slide, and the fresh weight was determined within 2 minutes by difference after reweighing on a Mettler analytic balance. The tissue was then homogenized using a motor driven pestle in 3 ml cold 5% TCA for protein, RNA and DNA analyses as described. No attempt was made to determine actual circulating levels of APP in this experiment: APP circulating levels are known to decrease approximately 50% (6-8 ng/ml to 2-3 ng/ml) as a result of pancreatectomy (Kimmel and Pollock, 1975).

B. Injection of Pentagastrin for determination of an effective dose

Pentagastrin (Peptavlon, Ayerst) was solubilized in 0.3 ml of 0.1 N NH_4OH. After addition of 20 ml of water, enough 0.1 N NH_4OH was added to keep the pH between 9.4 and 9.7 . This solution was stirred until clear, and 0.34 g NaCl was added and dissolved. The pH was adjusted to 7.5 with 0.1 N HCl and water added to reach a final volume of 40 ml. This solution was sterilized by filtering through a millipore filter with a pore size of 0.45 u, and diluted with isotonic saline to 0.40 and 0.04 µg/ml. Eggs at 8, 12 and 14 days of

development were given single 0.1 ml injections of either isotonic saline or one of the two Pentagastrin solutions (0.04 or 0.004 µg/egg). Sacrifice was in each case at day 21, and the wet weight of the intact gastrointestinal tract from the superior margin of the crop to the posterior end of the colon determined after blotting. In addition, the length of the proventriculus was determined using the calibrated ocular lense of an Olympus binocular microscope.

C. Response of organ wet weight, protein, DNA and RNA to Pentagastrin and to APP

For demonstration of a dose response, single injections of 0.04, 0.08 and 0.12 µg/egg of either Pentagastrin (prepared as above) or APP suspended in isotonic saline were compared to saline-injected controls. Day 12 was chosen as the day of injection because the embryo size was not indicated to be a determining factor for the magnitude of response; however, this is the period at which A_1-cells begin secretory activity. At sacrifice 48 hours later, proventriculus, gizzard and liver were excised and placed in ice-cold isotonic saline. After blotting dry they were weighed, then homogenized in 3 ml cold 5% TCA for nucleic acid and protein determination. These results are expressed as mg total organ protein, protein/DNA and RNA/DNA ratios.

D. Response of organ protein, DNA and ^{14}C-leucine incorporation 1 hour after APP injection

Embryos injected with either saline , 0.04, 0.08, or 0.12 µg APP/egg were sacrificed on day 14 one hour post-injection. Proventriculus, gizzard and liver tissues were excised, weighed after blotting, and homogenized in 3 ml of ice-cold 5% TCA for protein and DNA determination. In a separate experiment, tissues from identically treated embryos were homogenized (after weighing) in 0.9 ml of ice-cold incubation medium for determination of ^{14}C-leucine incorporation into TCA precipitable protein as described. Results from this experiment are expressed as disintegrations per minute per milligram of tissue as calculated from a counting efficiency standard curve (see Appendix).

Statistical Analyses

Statistical analysis of all experiments was accomplished using the Student's two-tailed t-test for difference between two means. Probability values of $p<0.05$ were considered to be significant.

IV.

RESULTS

RESULTS

A. Effects of total pancreatectomy on proventricular and duodenal mucosa

The changes in the proventricular and duodenal mucosa in response to pancreatectomy, or removal of the only known source of APP, are shown in Table 1. The wet weights of the entire proventriculus and of the duodenal loop cut immediately distal from the pylorus to the ascending portion adjacent to the pylorus, were determined before scraping of the mucosa. There was no significant change in those weights. The proventricular mucosa showed significant changes in all of the additional parameters that were measured, however. The percent protein (milligrams of protein per 100 milligrams of wet weight) of the proventricular mucosa from depancreatized animals was 60% of the value found in the sham-operated animals, significant at the 1 percent level. The milligrams of protein per milligram of DNA was 76.9% of the value in control birds, significant at the level of 2 percent. With respect to the RNA/DNA ratio, a reflection of the relative rates of transcription, the pancreatectomized and presumably APP-depleted animals showed a small but nevertheless marginally significant ($p=0.05$) increase over the sham-operated animals. Comparing the RNA/protein ratio,

Table 1. Response of adult chicken proventricular and duodenal mucosa to pancreatectomy

These data are taken from initial experimentation done with adult birds, from which the proventriculus and duodenum were removed four days after the second stage of a two part operation (removal of 99% of the pancreas followed eight days later by removal of the splenic lobe) for comparison of mucosa condition with sham-operated controls. Values represent means plus or minus standard error of the mean. Number of observations is indicated by parentheses ( ).

RESPONSE OF ADULT CHICKEN PROVENTRICULAR AND DUODENAL MUCOSA TO PANCREATECTOMY

		SHAM OPERATED (3)	PANCREATEC-TOMIZED (4)	% CHANGE	P-VALUE
		-WHOLE ORGAN-			
WET WEIGHT, GM	Proventr.	4.67 ±0.34	5.33 ±0.42	14.1	NS
	Duodenum	7.25 ±0.49	7.04 ±0.62	- 2.9	NS
		-MUCOSAL TISSUE ONLY-			
% PROTEIN	Proventr.	7.58 ±0.49	4.60 ±0.36	-39.3	<0.01
	Duodenum	7.90 ±0.83	7.74 ±0.41	- 2.0	NS
MG PROTEIN/MG DNA	Proventr.	48.01 ±1.78	36.94 ±2.51	-23.1	<0.02
	Duodenum	41.77 ±6.75	44.30 ±10.60	- 5.2	NS
MG RNA/MG DNA	Proventr.	3.41 ±0.27	4.53 ±0.38	32.8	= 0.05
	Duodenum	3.24 ±0.79	2.94 ±0.76	- 9.3	NS
MG RNA/MG PROTEIN (X 100)	Proventr.	7.12 ±0.56	12.23 ±0.44	71.8	<0.001
	Duodenum	7.54 ±1.33	6.61 ±0.86	-12.3	NS

( ) = Number of Observations

inversely indicating the rate of translation occuring versus the levels of RNA present, a large increase (78.8%) was observed in the pancreatectomized birds over the sham-operated ones, significant at the level of $p < .001$. By contrast, the biochemical properties of the duodenal mucosa were unchanged by pancreatectomy. It should be noted, however, that the values obtained for this tissue are very similar to the values for the proventricular mucosa in the sham-operated birds, with the indication that these tissues are similar in composition (if not in sensitivity to APP).

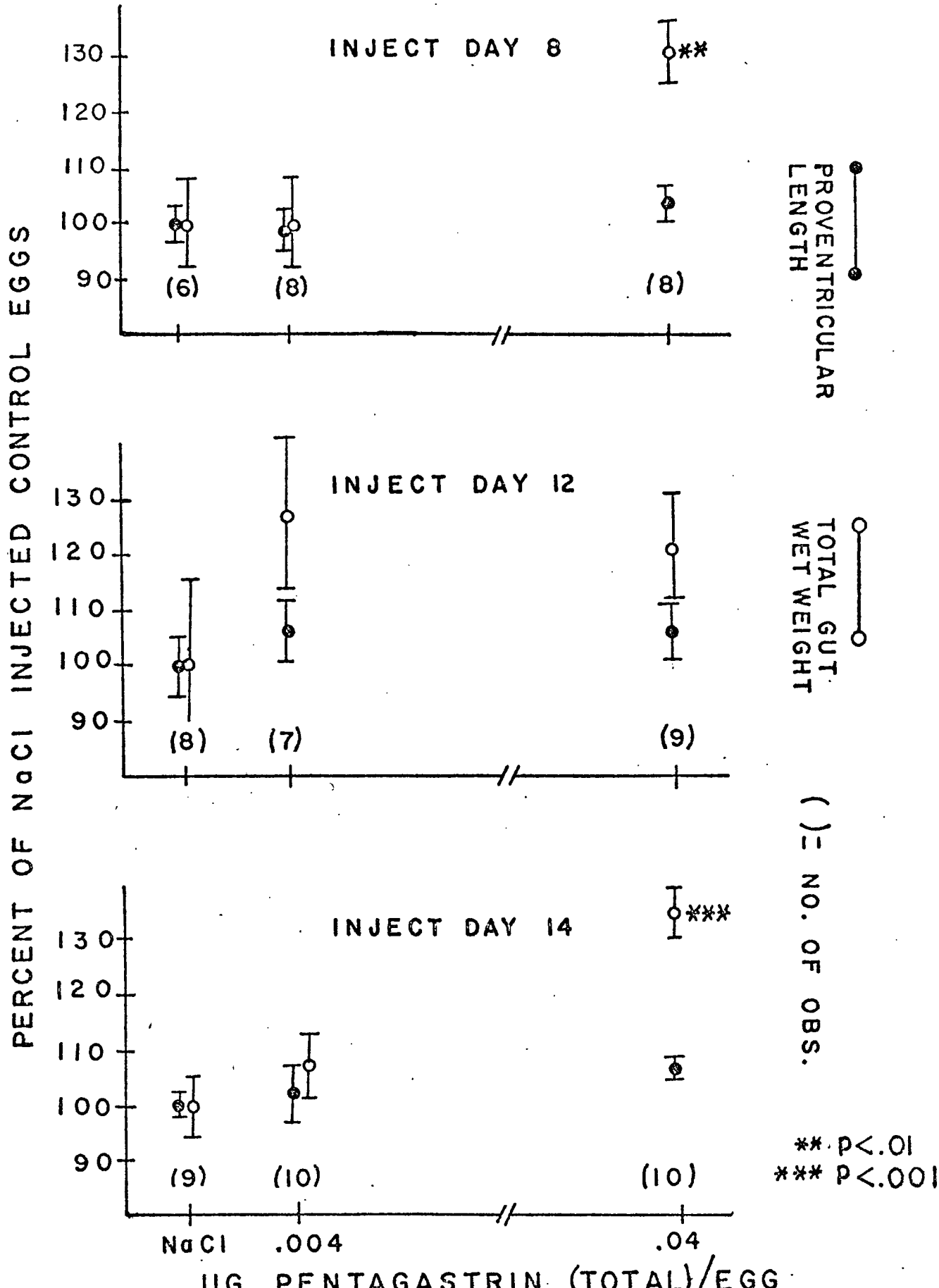
B. Injection of Pentagastrin for determination of an effective dose

In order to compare the trophic effects of APP with Pentagastrin, it was first necessary to establish the effectiveness of Pentagastrin in this system. The three graphs of Figure 2 present the results from injection of a broad dosage range of Pentagastrin (10X) at times of development which bracket the period at which A_1-cells begin active secretion as evidenced by morphological data. The wet weight of the entire gastrointestinal tract in response to 0.04 µg Pentagastrin/egg, when measured at day 21, shows an increase that is quantitatively the same regardless of the time of injection. The increases for this dose of Pentagastrin when

Figure 2. Effect of Pentagastrin on chick embryo gut tissue

Embryos were injected through the shell into the yolk sac either on day 8, 12, or 14 with Pentagastrin. In each case sacrifice was on day 21. Closed circles represent proventricular length, open circles the fresh weight of the entire gastrointestinal tract. Vertical bars represent standard error of the mean, ( ) the number of observations for each point.

EFFECT OF PENTAGASTRIN ON CHICK EMBRYO GUT TISSUE: INJECTED ON DAYS 8, 12, & 14; SACRIFICE ON DAY 21



injected at days 8 and 14 are significant at the level of $p<0.01$ and $p<.001$, respectively. The increase in weight in response to this dose injected on day 12 is not significant, though the increase in response to the lower dose is greater at this time (though still not significant).

The length of the proventriculus was measured with the intention of indicating a more specific trophic action of Pentagastrin, but there was no significant change in this parameter at any dose or time of injection. This lack of response, in the face of a general gastrointestinal weight increase, led to the substitution of organ wet weight for organ length in subsequent experiments.

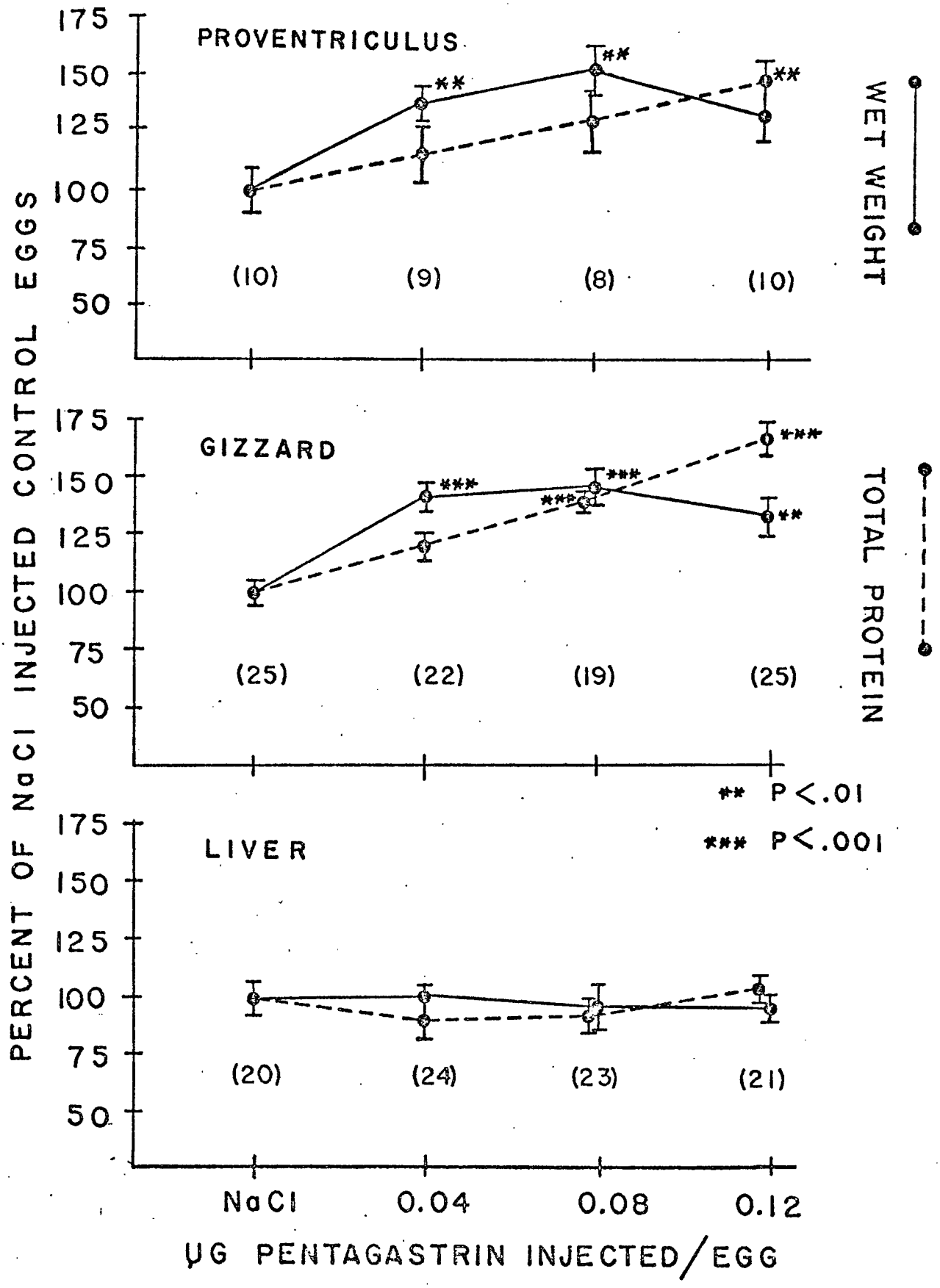
C. Response of organ wet weight, protein, DNA and RNA to Pentagastrin and to APP

In contrast with the 7 to 13 day post-injection sacrifice of the above procedure, sacrifice in this series was made 2 days post-injection. Figure 3 presents the response to Pentagastrin of the proventriculus, gizzard and liver with respect to wet weight and total organ protein. The proventricular wet weight increased with injection of 0.04 and 0.08 µg/egg, significant when compared to saline-injected controls at the level of 1 percent, while there is a similar increase in the gizzard ($p<.001$) at both doses. At 0.12 µg

Figure 3. Effect of Pentagastrin on embryo gut tissue wet weight and total protein

All embryos were injected on day 12 and sacrificed on day 14. Top graph represents response of the proventriculus, middle graph the response of the gizzard, and bottom the response of the liver. Solid lines are the organ fresh weight, and broken lines the organ total protein. Vertical lines represent standard error of the mean. The number of observations for each group is in parentheses ( ).

EFFECT OF PENTAGASTRIN ON EMBRYO GUT TISSUE WET WEIGHT & TOTAL PROTEIN: INJECTED ON DAY 12, SACRIFICE DAY 14



Pentagastrin/egg both the proventriculus and gizzard responses attenuate equally, but the gizzard elevation above control values remains significant. There was no change in liver wet weight in response to any dose of Pentagastrin.

Proventriculus and gizzard total protein increased linearly with increase in dose of Pentagastrin up to 148% ($p<0.01$) and 168% ($p<.001$) of saline controls, respectively. There was no change in liver total protein.

Figure 4 presents the response of protein to DNA and RNA to DNA ratios to the same Pentagastrin injections. The proventriculus did not change in these parameters. The gizzard RNA/DNA ratio increased 13% ($p<0.05$) with both 0.04 and 0.08 µg Pentagastrin/egg injections. There was a single significant increase in liver RNA/DNA with the 0.04 µg/egg injection. At no time did a protein/DNA ratio change significantly from the control value.

Figure 5 presents the results of an identically performed experiment using APP injections in place of Pentagastrin injection. The wet weight of the proventriculus at 0.12 µg APP/egg rose to a value 35% over the saline control but this increase was not statistically significant. The total protein of this organ rose linearly in response to APP however, almost identical to the response to Pentagastrin. The final level was 152% of the control value,

Figure 4. Effect of Pentagastrin on embryo gut tissue protein/DNA and RNA/DNA

These embryos are the identical ones seen in Fig. 3, with the results of different assays. Solid lines represent the tissue protein/DNA ratio, broken lines represent the tissue RNA/DNA ratio. The top graph represents the proventriculus, the middle gizzard, and the lower the liver. Vertical lines represent the standard error of the mean, ( ) the number of observations for each point.

EFFECT OF PENTAGASTRIN ON EMBRYO GUT TISSUE PROTEIN/DNA & RNA/DNA: INJECTED ON DAY 12, SACRIFICE DAY 14

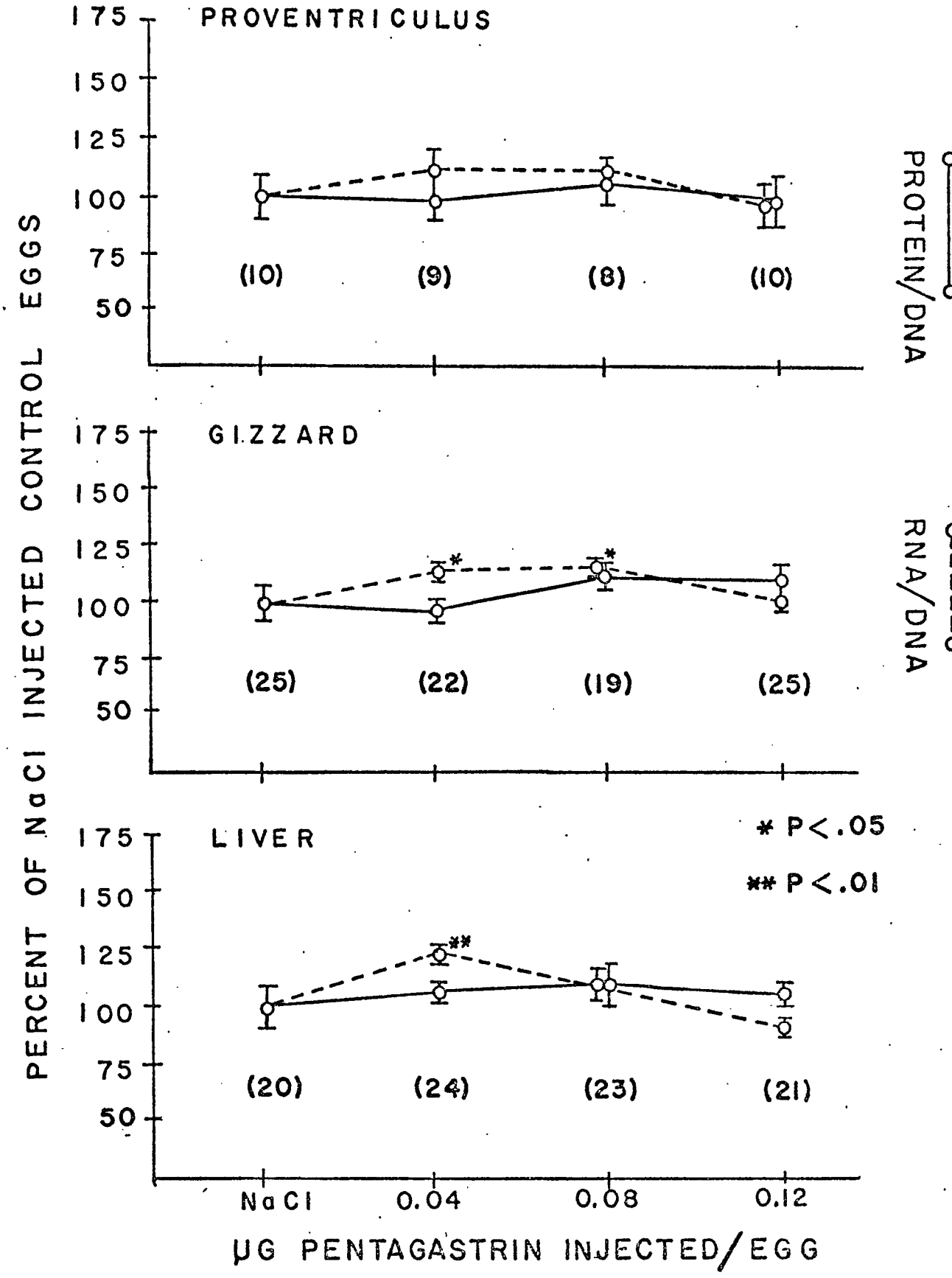
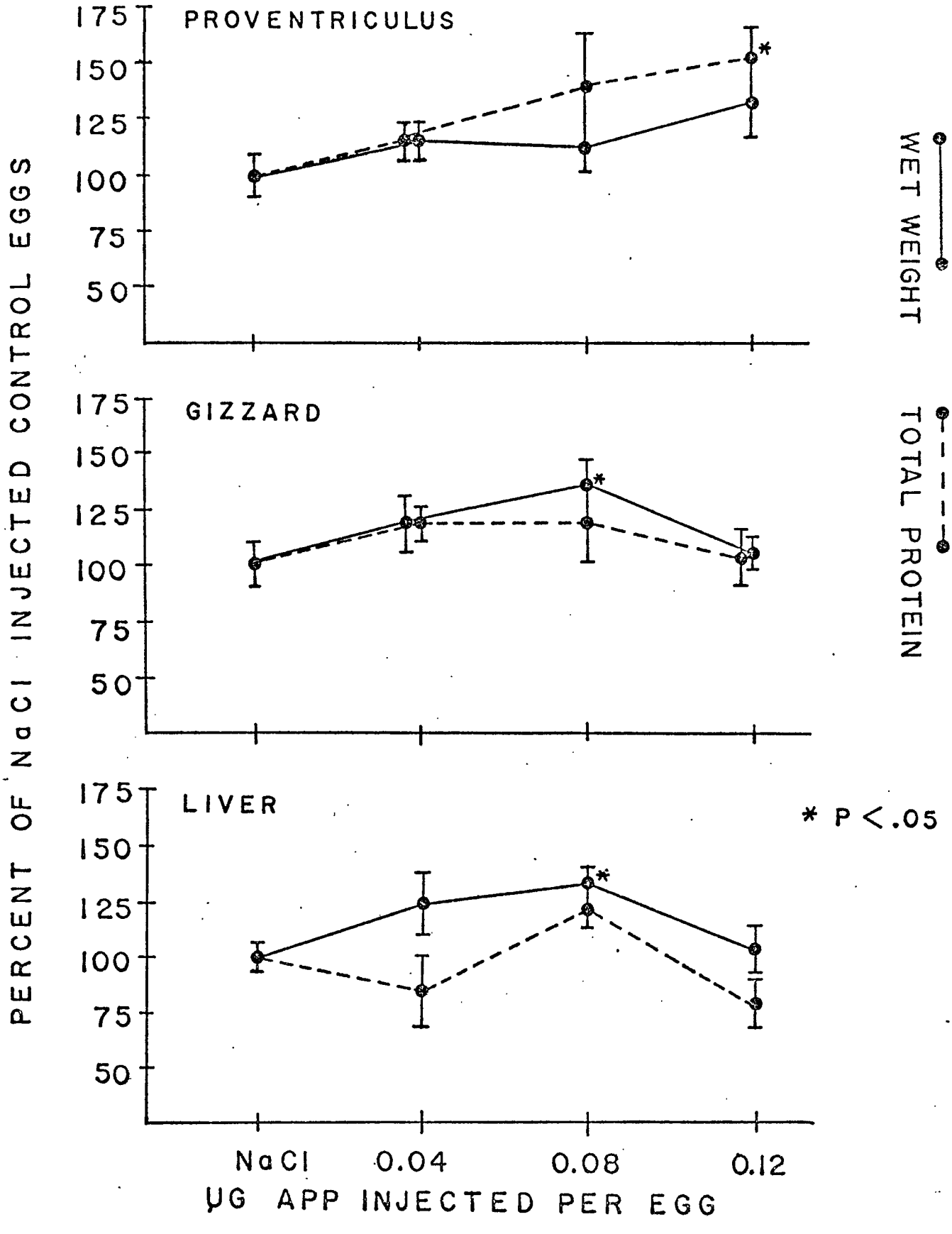


Figure 5. The effect of APP on tissue wet weight and total protein in the embryo gut

Embryos were injected on day 12 and sacrificed on day 14 (as were those seen in Figures 3 and 4), with APP injections substituted for Pentagastrin. Top graph represents the proventriculus, middle the gizzard, and lower the liver. Solid lines are the organ fresh weight and broken lines the total protein. Vertical lines represent the standard error of the mean and each point the mean of 5 observations.

EFFECT OF APP ON TISSUE WET WEIGHT & TOTAL PROTEIN IN EMBRYO GUT: INJECTED DAY 12, SACRIFICE DAY 14



with a $p<0.05$. The gizzard and, in contrast to the Pentagastrin series, the liver increased 35% ($p<0.05$) in wet weight at the 0.08 µg APP/egg dose. The total protein did not change significantly for either of these tissues. Figure 6 illustrates the response of the protein to DNA ratio and RNA to DNA ratio to APP injection. The proventriculus underwent a significant change in both of these parameters at the same higher APP dose (0.12 µg/egg) as did the total protein in this organ (Fig. 5), while the general shape of these curves are identical to that of the change in proventricular wet weight. Neither the gizzard nor the liver changed significantly with respect to these two ratios.

D. Response of organ protein, DNA, and ^{14}C-leucine incorporation 1 hour after APP injection

With the intention of measuring a more immediate and specific response to APP, the time of injection was changed to within one hour of sacrifice, using the same amounts of APP as were used previously. Figure 7 gives the *in vitro* radiolabeled amino acid incorporation into TCA precipitable protein by proventriculus, gizzard and liver one hour after APP injection, together with the protein/DNA ratio measured in a separate group of identically treated embryos. In the proventriculus, the protein/DNA ratio follows the same

Figure 6. Effect of APP on embryo gut protein/DNA and RNA/DNA ratios

These data are the results of different assays performed on the same tissues of the embryos from Figure 5. Solid lines represent the tissue protein/DNA ratio, broken lines the tissue RNA/DNA ratio. Vertical lines represent the standard error of the mean, and each point the mean of 5 observations.

EFFECT OF APP ON EMBRYO GUT PROTEIN/DNA & RNA/DNA RATIOS: INJECTED DAY 12, SACRIFICE DAY 14

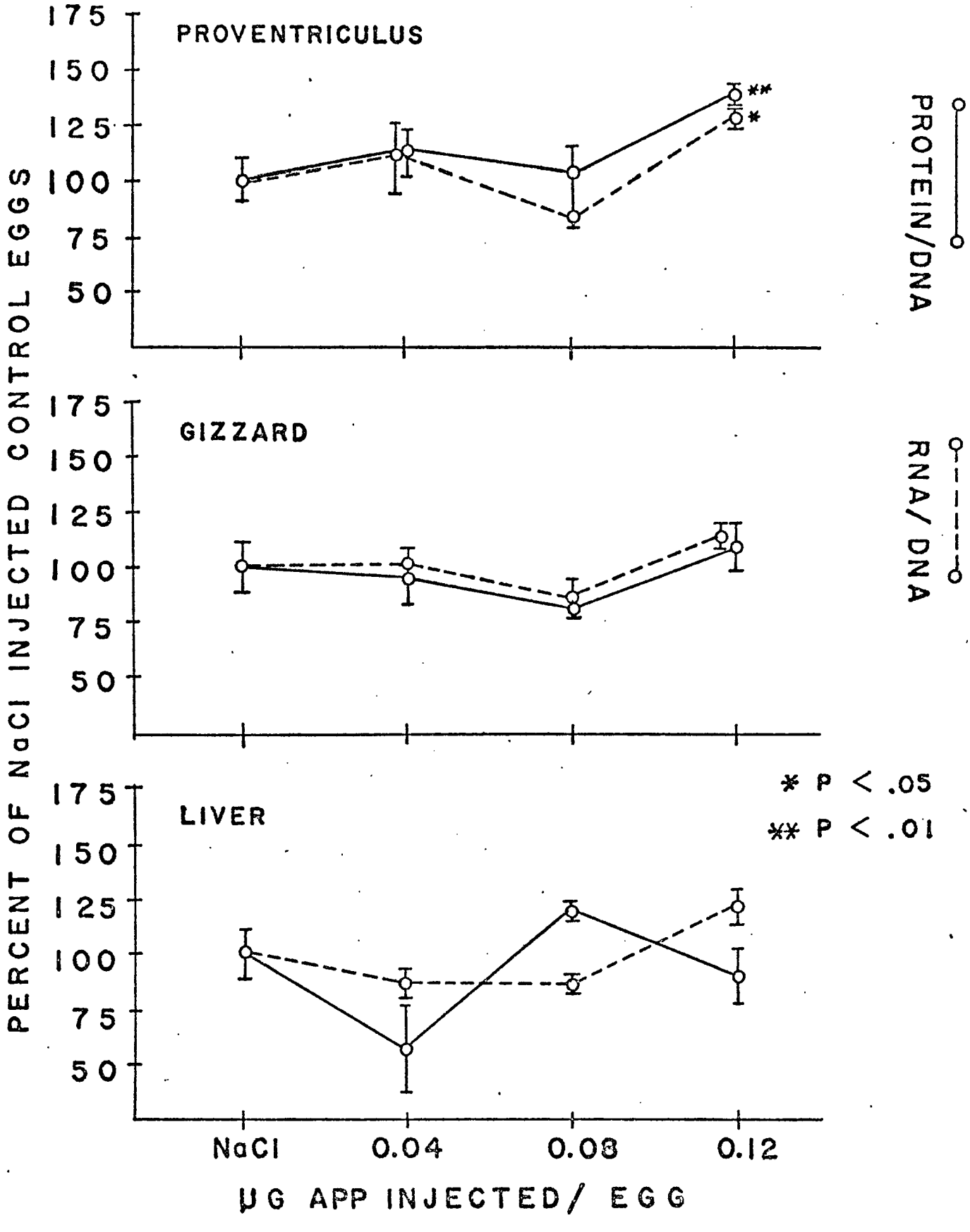
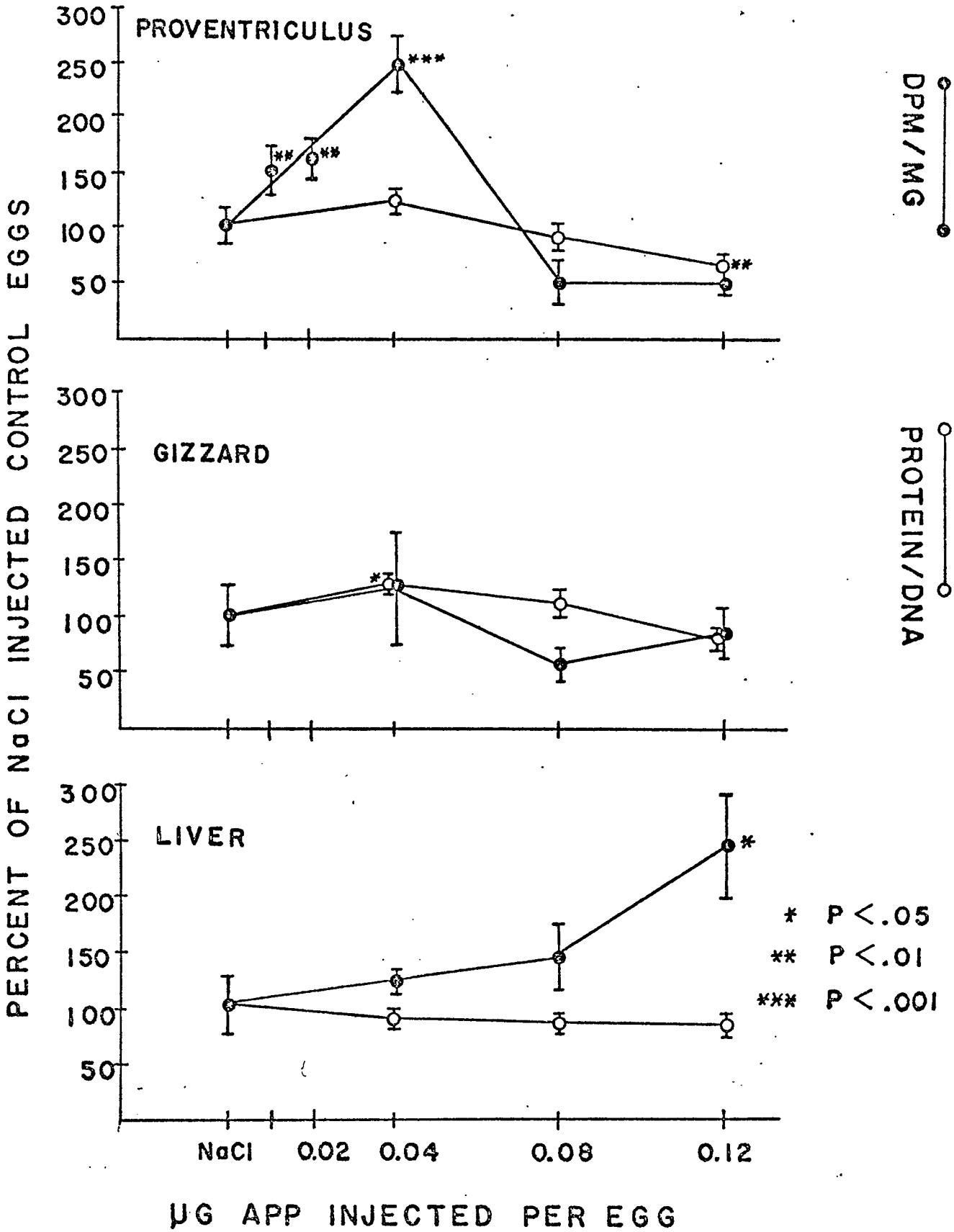


Figure 7. Effect of APP on embryo gut ^{14}C-leucine incorporation and protein/DNA ratio

Embryos were injected on day 14 and sacrificed one hour post-injection. Two separate experiments were performed on identically treated embryos; the tissues in one case were removed and incubated *in vitro* with radiolabeled leucine (incorporation represented by closed circles), the tissues in the other experiment assayed colorimetrically for protein and DNA (open circles). Proventricular incorporation in response to saline and 0.04 µg APP is, in both cases, the mean of 10 observations. All other points represent the mean of 5 observations. Vertical lines represent standard error of the mean.

EFFECT OF APP ON EMBRYO GUT ^{14}C-LEUCINE INCORPORATION & PROTEIN/DNA RATIO: INJECTED DAY 14, AND ANALYZED ONE HOUR POST-INJECTION



general shape as the dose response of ^{14}C-leucine incorporation; the changes in this ratio were not significant. In contrast, the ^{14}C-leucine incorporation by the proventriculus of embryos injected with 0.04 µg APP/egg was dramatically higher (250%, $p<.001$) than the saline-injected controls, while at higher doses incorporation was actually depressed. To verify this finding in the proventriculus another experiment was run using saline, and APP doses of 0.01 µg/egg, 0.02 µg/egg, and 0.04 µg/egg. The 0.04 µg APP/egg injected embryos responded identically to those of the previous experiment, hence the value for this dose represents the response of both experimental groups. The proventriculus incorporation by the 0.01 µg APP and 0.02 µg APP-injected embryos fell between the values for saline and 0.04 µg APP injected embryos, and each is significant at the level of one percent.

The gizzard protein/DNA ratio dose response is similar to that of the proventriculus, but in this case a 25% increase at 0.04 µg APP is significant. Paradoxically, this effect was not exaggerated in the presumably more sensitive incorporation technique. The liver amino acid incorporation increased with increasing amount of APP injected up to the maximum dose employed (0.12 µg/egg). The liver incorporation by these embryos was 240% of that in saline injected

control embryos, significant at the level of five percent.

V.

DISCUSSION

DISCUSSION

The response to pancreatectomy, as illustrated in Table 1, consists of a reduction in tissue protein content that is specific for the mucosa of the adult proventriculus. Protein is decreased whether expressed as milligrams per milligram of tissue weight, or as milligrams per milligram of tissue DNA. Paradoxically, there is a concomitant increase in the RNA of this tissue when compared to either protein or DNA. When measuring the incorporation of ^{14}C-labeled orotic acid into adult rat gut RNA in response to Pentagastrin, Chandler and Johnson (1972) also obtained anomalous results. Actinomycin D, added to block the transcription of new RNA, failed to prevent the incorporation of orotic acid into the stomach mucosa. Administration of actinomycin D without simultaneous Pentagastrin caused a three fold increase in orotic acid incorporation in the duodenum of saline-injected controls. A model has been proposed by Tomkins and Gelehrter (1972) to provide a mechanism for these changes, and similar ones which have been described in other work using steroids. Postulating a post-transcriptional regulation of protein synthesis, the model suggests that a labile repressor RNA, the transcription and/or action of which is blocked by trophic hormones, will inhibit the translation of messenger-

ribosome complexes in the absence of these hormones. According to Tomkins' model, removal of the hormone (APP in the present system) could at some interval increase the titre of either this labile repressor RNA or of non-translated messenger while still having the net effect of inhibiting protein synthesis. It would certainly appear that regarding the actions of trophic hormones, considerably more complicated gene regulation is involved than the original operon theory proposed by Jacob and Monod.

Injection into the yolk sac was the method of choice for administration of exogenous Pentagastrin and APP because this route has proven to be effective and reliable (Landauer, 1945). Injection into the chorio-allantoic membrane, while more difficult to perform, results in more direct absorption (Thommes, 1974). Although proteolytic activity has been demonstrated in the yolk-sac membrane (Romanoff, 1960) it should be pointed out that the function of these enzymes is the disaggregation of the colloidal yolk proteins for absorption; contrarily, experimentally injected substances are already soluble. When Pentagastrin was injected into the embryos at days 8, 12 and 14, the wet weight of the gastrointestinal tract increased in each case with the response to injection on day 14 being the greatest (Fig. 2). There was no change in proventricular length.

These crude indications of trophic activity were used in order to establish a dosage range together with a suitable time of development for injection. Though not statistically significant, the response to the lower dose at day 12 indicated that the embryo may be most sensitive at this stage of development. The A_1-cell can be recognized in the pancreatic islets of embryos by the sixth day of development (Fujita, 1968) and morphological evidence from electron micrographs indicates that this cell begins secretory activity at 11-14 days of incubation (Machino and Sakuma, 1967). The onset of embryonic proventricular acidity occurs between the eleventh and thirteenth days (Hill, 1971). For these reasons, further studies were carried out on embryos sacrificed on day 14, either two days or one hour post-injection.

Embryos injected with Pentagastrin on day 12 and sacrificed on day 14 were used to establish a trophic response in the embryo system and to compare this response to the trophic action of Pentagastrin on mammalian gastric mucosa as taken from the literature. The results of this experiment (Figures 3 and 4) indicate that while the embryonic proventriculus and gizzard are sensitive to Pentagastrin, the liver is not. Total organ protein serves as the most consistent parameter, exhibiting a linear increase with increas-

ing amounts of Pentagastrin injected. Protein to DNA and RNA to DNA ratios were relatively unresponsive by comparison, though those changes which did occur (RNA/DNA in all tissues) were increases as would be expected in a trophic response. In mammals, *in vitro* protein synthesis was stimulated by Pentagastrin in the stomach and duodenum but not in the liver (Johnson, Aures and Hakanson, 1969). Chronic administration (21 days) of Pentagastrin in rats caused an increase in total parietal cell count and in the mucosal thickness of the gastric fundus, which would increase this tissue's total protein (Crean, *et al.*, 1969). RNA and DNA synthesis by the stomach and duodenum increases after Pentagastrin administration, as evidenced by stimulation of precursor incorporation (Chandler, *et al.*, 1972; Enochs and Johnson, 1973). The maximum stimulation of RNA synthesis in rats occurs from two to three hours after injection, the maximum increase in protein synthesis six hours after injection, and the maximum increase in DNA synthesis sixteen hours after injection (Johnson and Enochs, 1973). Thus, changes in the protein to DNA and RNA to DNA ratios in the embryo may return to normal values 48 hours after injection, while a permanent increase in total protein has been effected.

APP administered according to the same regimen as above yields results similar in many respects to that seen with

Pentagastrin injection, the major exception being that APP causes a significant increase in liver wet weight (Figures 5 and 6). Total protein in the proventriculus again responds linearly to increases in dosage. The organ wet weight and total protein of the gizzard increases as well (though in contrast to the response to Pentagastrin only the change in wet weight is significant) indicating that APP may be more specific in its action than is Pentagastrin. The gizzard is destined to become keratinized on its inner surface and exists embryonically as well as in adulthood as a muscular organ with no secretory activity. Possibly these differences in the gizzard when compared to those in the proventriculus are carried over in the susceptibility difference of the tissues to a growth promoting effect by APP.

At the highest dose attempted, APP significantly increases the ratio of proventricular protein to DNA, and RNA to DNA, further demonstrating this tissue's sensitivity (Figure 6). When these results are compared to the increases in organ protein seen in Figure 5, it is obvious that total DNA is elevated but not to the extent as is protein. Following this reasoning, RNA increases, yet is matched by, the increase in DNA (within 48 hours after injection) until the amount of APP injected reaches 0.12 µg/egg.

The time of sacrifice after injection was reduced from

two days to one hour so that a more immediate response might be detected. Figure 7 shows that one hour following injection of APP the *in vitro* incorporation of ^{14}C-leucine by the proventriculus during a fifteen minute incubation period increases with increasing dosages of APP up to 0.04 µg/egg. This portion of the graph actually represents two separate experiments each of which yielded a 250% increase by 0.04 µg APP injected embryos over saline-injected controls. By comparison, 250 µg Pentagastrin/kg administered to rats caused a 200% increase in ^{14}C-leucine incorporation by the gastric mucosa one hour post-injection using the identical *in vitro* technique (Johnson, *et al.*, 1969). If APP were similarly specific for the mucosa, the stimulation of radiolabel incorporation would be expected to be quantitatively less when expressed as the amount incorporated per milligram of entire organ weight. It should be noted that while in the bird the gizzard possesses the grinding properties of the two-part stomach, the proventriculus retains the secretory function and much of the thickness of its wall is due to the proventricular glands, consisting of rounded lobules containing the tubular alveoli (Hill, 1971). The large increase in protein precursor uptake and incorporation together with the response of wet weight and total organ protein to APP, indicate that growth

of the entire embryonic proventriculus is stimulated by APP.

The decrease in amino acid incorporation by the proventriculus observed at higher doses of APP is very pronounced and does not correlate with the continued increase in total organ protein observed two days post-injection. The inhibitory effects of high hormone concentrations in contradistinction to the stimulatory effects of lower concentrations was first discovered in experiments with plant auxins but has since been shown to occur also with animal hormones, most notably thyroxin (Rupp, Paschkis and Cantarow, 1949). The precise genetic regulatory mechanism responsible for these actions has yet to be elucidated, however. This mode of decremental action may be the cause of the decrease in incorporation with higher doses of APP, while the prolonged absorption of APP from the yolk after it has been diluted to a stimulatory concentration could result in the continued stimulation of growth by the same higher doses extended over a longer period of time (48 hours).

There was no significant change in amino acid incorporation by the gizzard in response to APP, though the general shape of the curve mirrors the response in the proventriculus. The increase in incorporation of ^{14}C-leucine by the liver reaches its maximum at an APP dosage level twice that which

maximally stimulated the proventriculus. Recalling that the hepatic glycogenolytic and plasma hypoglycerolemic effects were seen only with injections of exogenous APP greater than those producing gastric secretagogic effects in adult birds (Hazelwood, et al., 1973), and that pancreatic hormones are presented to the liver at levels higher than those which reach the stomach, it need not be surprising that the embryonic liver responds only to the higher doses employed. APP has also been shown to decrease circulating free fatty acids without alteration of immunoreactive insulin and increasing glucagon levels (Langslow and Hazelwood, 1975). Pentagastrin has not been shown to have these metabolic effects, which may correlate with the lack of trophic action by Pentagastrin on the rat liver (Johnson, et al., 1969). In these respects, APP function covers a broader spectrum of effects than does gastrin. In the absence of an increase in total liver protein two days after APP injection, it remains possible that the increase in amino acid incorporation represents a transitory response to APP caused by an increased synthesis of the enzymes mediating these metabolic responses in the liver.

While the absence of gastrin bioactivity in chickens (Ruoff and Sewing, 1969) suggests that APP acts alone in hormonal regulation of gastric secretion in Aves, subse-

quent investigations using immunofluorescent labeling techniques have demonstrated "gastrin cells" in the gizzard-duodenum junction (Larsson, Sundler, Hakanson, Rehfeld and Stadil, 1974). This finding is yet to be confirmed by other investigators; no circulating gastrin can be found in the plasma of chickens (Ketterer, Ruoff and Sewing, 1973). Thus, APP may prove to be the major functional secretagogic hormone in birds and its trophic action the sole source of tissue maintainance for the secretory stomach (proventriculus).

VI.

SUMMARY

SUMMARY

1. The removal of the pancreas, the only known source of APP, from adult chickens causes a depletion of tissue protein content that is specific for the proventriculus (secretory stomach) mucosa four days post-operative. Mucosa from the duodenum showed no deviations from sham-operated controls.

2. The proventriculus and gizzard of Pentagastrin-injected two week old embryos demonstrate a response that is similar to that seen in Pentagastrin-injected rat stomach mucosa (ie., increase in protein synthesis), indicating that embryo injection serves as a valid technique for demonstrating trophic effects on gut tissues.

3. Precocious administration of APP to the developing chick causes an increase in protein synthesis by the proventriculus (increased total protein and protein precursor incorporation). At higher doses, amino acid incorporation is stimulated in liver tissue.

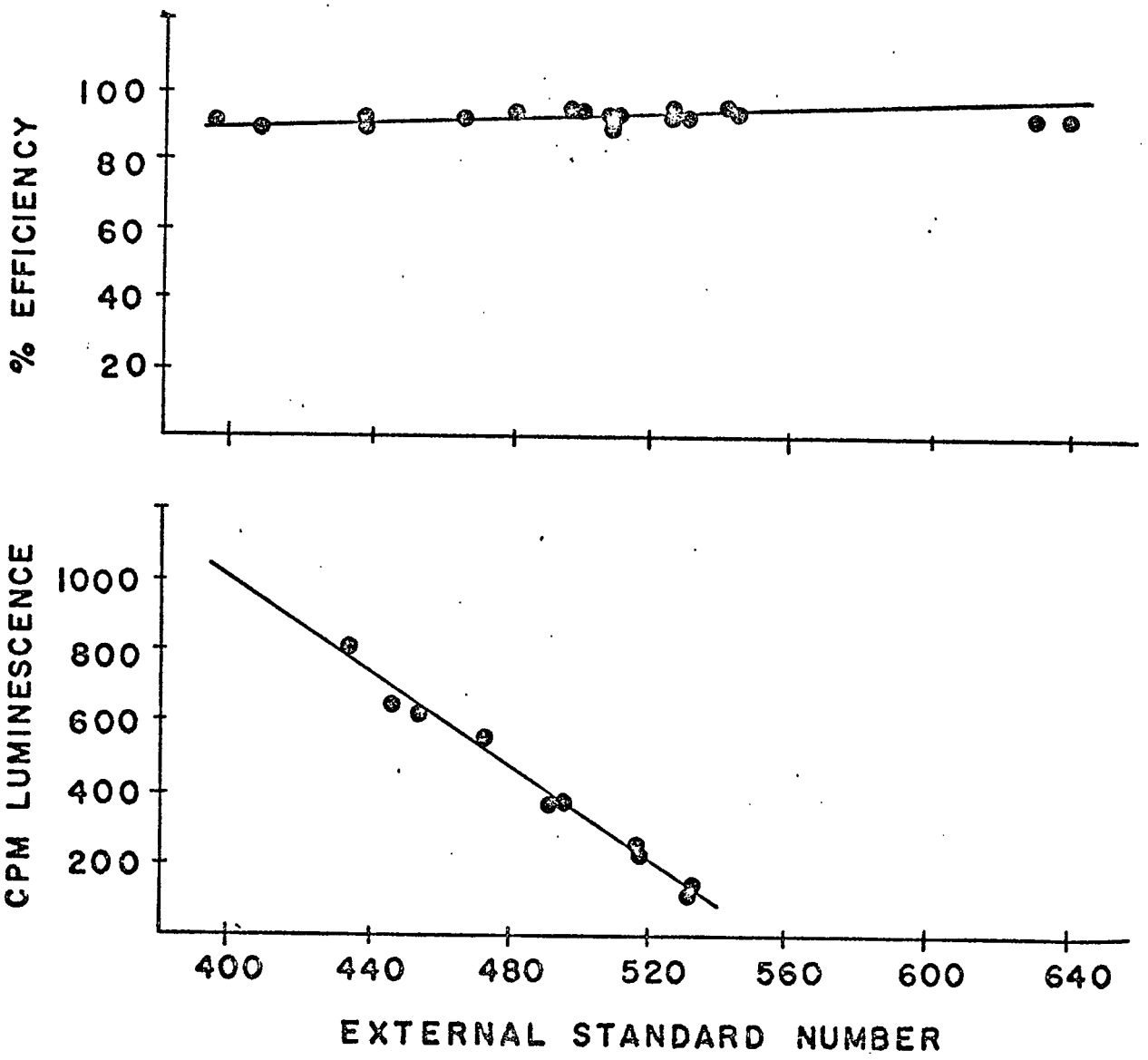
Conclusions from this evidence are that APP is trophic for the proventriculus and possibly the liver of the chicken embryo.

APPENDIX

Figure 8. Standard curves for calculation of disintegrations per minute per milligram of tissue

The top graph represents the counting efficiency determined by counting 22,000 uCi ^{14}C-toluene with various concentrations of tissue. The bottom graph represents the luminescence of the tissue with no isotope present. The CPM's of luminescence were subtracted from the experimentally obtained CPM's on the basis of external standard number, and this value divided by the counting efficiency at the corresponding number on the upper graph to determine the actual disintegrations per minute for that sample.

STANDARD CURVES FOR CALCULATION OF DISINTEGRATIONS/MINUTE/MG TISSUE



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