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Does the Third Pancreatic Hormone (APP) Play a Trophic Role in the Growth of the Embryonic Chick Proventriculus? (40407)

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Avian pancreatic polypeptide (APP) was reported originally as a contaminant present during the isolation of insulin from fresh chicken pancreas (1). Subsequently, APP has been found in the pancreata of many avian species at concentrations equal to, or greater than, insulin within the same pancreas (2). Other work has shown APP to be a polypeptide of 36 residues, MW of 4250 daltons, and to have a distinctive structure which does not resemble other known pancreatic or gut hormones (3). Biological evaluation of APP indicates that it originates only from the avian pancreas, circulates at levels of 6–8 ng/ml plasma which decreases with fasting, only to increase greatly (within minutes) upon refeeding, and causes both metabolic and secretogogic effects when injected into chickens and rats (4–6).

Of the several actions reported for APP, the gastric (proventricular) secretogogic action is most prominent and consistent. This response occurs in birds and rats at extremely small doses of the injected peptide (7, 8). In many ways, the effects of injected APP in Aves are quite similar to those documented for the hormone gastrin in mammals. In addition to secretogogic effects in mammals, both gastrin and a synthetic C-pentapeptide analog of gastrin, Pentagastrin, exert a trophic effect on the mammalian digestive tract. Thus, increases in fundic parietal cell density (9), labelled amino acid incorporation into gastric mucosa protein (10), and increases in precursor incorporation into RNA (11), all have been observed when rats are injected with gastrin or Pentagastrin. Comparable studies have not been carried out in birds. However, with the now documented similarity in action of gastrin and APP as "gastric" secretogogues, it appeared desirable to study possible trophic effects of injected APP on the avian gastrointestinal tract.

Materials and methods. Preliminary study. Total pancreatectomy (including the splenic lobe) was carried out in adult chickens and the birds allowed to recover for 4 days without replacement therapy. After this period of time the proventricular and duodenal mucosa were scraped off and analyzed for RNA, DNA and total protein concentrations. Table I compares these pilot study data with data obtained from sham-operated control chickens. Such a comparison indicates that the gastric (proventricular) mucosa decreased 39.3% in protein, decreased 23.1% in protein/DNA (with protein decreasing more than DNA), increased 32.8% in RNA/DNA (with DNA levels decreasing much more than the RNA levels), and increased 71.8% in RNA/protein. Duodenal mucosa constituents remained unchanged. Because removal of the pancreas deprives the organism of at least four peptides (glucagon, insulin, somatostatin and APP), it would be presumptuous to ascribe the data of Table I to the singular absence of APP. However, careful review of the literature indicated that none of the mammalian pancreatic hormones have been shown to "maintain" gastric structural integrity. Rather, only gastrin, which shares many physiological effects in mammals with the action of APP in birds, appears to act in a trophic manner on the gastric mucosa. Limited supplies of APP prevented undertaking the obvious sequel experiment, namely, replacement therapy of APP in depancreatized chickens with reevaluation of the gut mucosa four days later. Thus, the approach presented below on the embryonic chick gut was adopted to pursue the question of whether or not APP aids in the development/maintenance of the developing proventriculus.

Animals, incubation, and injections. Fertile chicken (SCWL) eggs were obtained from a local source and placed in a Superior Model incubator at 99.3/4°F. On day 12, eggs were removed from the incubator, an area of approximately 0.5 cm of the meridial shell removed with a dental burr, and injections

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TABLE I. RESPONSE OF ADULT PROVENTRICULAR AND DUODENAL MUCOSA TO PANCREATECTOMY

		Sham operated (3)[a]	Pancreatecto-mized (4)	% Change	*P*-Value
		Whole organ			
Wet Wt	Proventr.	4.7 ± 0.3	5.3 ± 0.4	+14.1	NS
	Duodenum	7.3 ± 0.5	7.0 ± 0.6	−2.9	NS
		Mucosa only			
% Protein	Proventr.	7.6 ± 0.5	4.6 ± 0.4	−39.3	0.01
	Duodenum	7.9 ± 0.8	7.7 ± 0.4	−2.0	NS
MG Protein/MG DNA	Proventr.	48.0 ± 1.8	36.9 ± 2.5	−23.1	0.02
	Duodenum	41.8 ± 6.8	44.3 ± 10.6	+5.2	NS
MG RNA/MG DNA	Proventr.	3.4 ± 0.3	4.5 ± 0.4	+32.8	0.05
	Duodenum	3.2 ± 0.8	2.9 ± 0.8	−9.3	NS
MG RNA/MG Protein	Proventr.	7.1 ± 0.6	12.2 ± 0.4	+71.8	0.001
	Duodenum	7.5 ± 1.3	6.6 ± 0.9	−12.3	NS

[a] ( ) = Number of Observations.

made into the yolk sac (0.1 ml vol) with a sterile syringe and 27 gauge needle. The eggs were then returned to the incubator (after placing a drop of warm paraffin over the injection site) for 2 days. On day 14, the embryo was removed, the entire alimentary tract isolated quickly, rinsed in saline and the proventriculus and gizzard excised. At this embryonic age both organs are readily discernible by eye.

Analyses. Tissue homogenates were extracted with alcohol: ether (3:1) and assayed for RNA and DNA using the phloroglucinol and diphenylamine reactions, respectively, as described by Schneider (12). Protein determinations were made by the method of Lowry *et al.* (13). [^{14}C]Leucine incorporation was measured by a modification of the technique of Johnson *et al.* (10). Whole pieces of embryonic tissue (~50 mg) were weighed after blotting, and homogenized with a glass pestle in 0.9 ml cold incubation medium (100 m*M* KCl, 10 m*M* MgCl, 40 m*M* NaCl, 20 m*M* Tris at pH 7.9, 6 m*M* mercapto-ethanol, and 250 m*M* sucrose). Immediately before initiation of the incubation period, 0.1 of stock solution containing 25 m*M* ATP, 1.0 m*M* GTP, and 0.5 μCi of uniformly [^{14}C] labelled L-leucine (SA = 240 mCi/mole) was added. Incubation was carried out for 15 min at 42° and terminated by addition of 2 ml of 5% TCA containing 14 m*M* unlabelled L-leucine. After centrifugation, the precipitate was washed twice with 5% TCA to which was added 14 m*M* leucine, once in 5% TCA at 90° for 15 min, and twice in ethanol:ether (3:1, vol/vol). The pellet was then dried, digested in 1 ml Soluene (Beckman Co.)/100 mg tissue weight, and quantitatively transferred to counting vials using a toluene base scintillation cocktail containing 4 g PPO and 50 mg POPOP/liter. All [^{14}C]counting was done on a Beckman LS-150 liquid scintillation counter. Results are expressed as disintegrations/min/mg tissue as a percent of saline-injected control eggs. Pilot studies indicated recovery of isotope exceeded 95%.

Statistics. Analysis was accomplished using Student's two-tailed *t* test for difference between two means; probability values of $p < 0.05$ were considered significant.

Results. Pentagastrin, the C-pentapeptide analog of gastrin, was used in initial experiments to establish an effective injection regimen (volume, dosage, and time schedule) for the embryo. Multiple injections (Pentagastrin or APP) resulted in an unacceptably high rate of mortality. A single Pentagastrin injection of 0.04–0.12 μg per egg on day 12 resulted in enlargement of the 14-day old proventriculus (Fig. 1). (This day was chosen as a desirable time for injection (14) because of the rapidly developing functional status of the embryonic proventriculus (15) and the fact that pancreatic APP content at this time is low (16). Plasma APP levels on day 12–14 are unknown.) The proventricular wet weight increased to approximately 150% of saline-injected control values at the 0.04 and 0.08 μg/egg range, with a maximum significance

level of $p < .01$. Total proventricular protein levels tended to increase with each level the pentapeptide employed but was significantly higher only at the 0.12 μg/egg dose. The liver, which was included as a non-digestive mucosal (control) tissue, was not affected by the injection of Pentagastrin.

Proventricular protein did not increase significantly in proportion to tissue DNA; neither did RNA levels when compared to DNA (Fig. 2). The liver protein/DNA ratio increased to 120% of the saline-control value in response to 0.04 μg of Pentagastrin per egg, but no other dose produced a change in either protein/DNA or RNA/DNA ratios.

Injections of APP at the same dosage as employed with Pentagastrin and at identical times caused no observable effects on the total organ weight and protein of either the proventriculus or the liver. Rather, the results were highly variable with large standard errors (Fig. 3). The protein/DNA ratio of both proventriculus and liver increased markedly at 0.12 APP μg per egg, however, as did the RNA/DNA ratio of the liver at this same dose (Fig. 4).

The response to APP injection could be

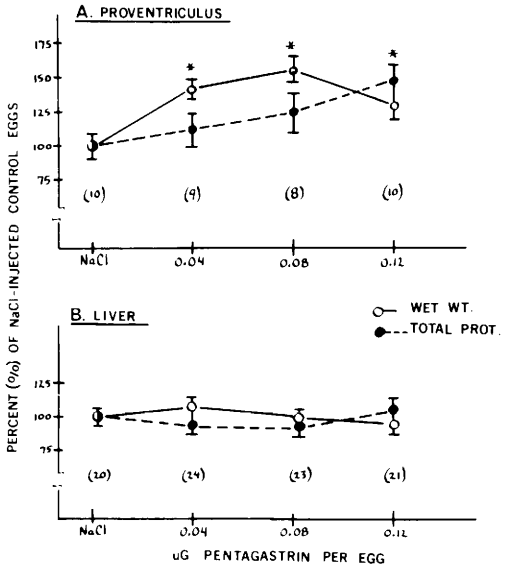

FIG. 1. Effect of Pentagastrin on chick embryo tissue wet wt (solid line) and total organ protein (dashed line). Injections were made on day 12 and tissues assayed on day 14 of development. Vertical lines are SEM; ( ) = number of observations. * $p < .01$ when compared with saline-injected control embryos.

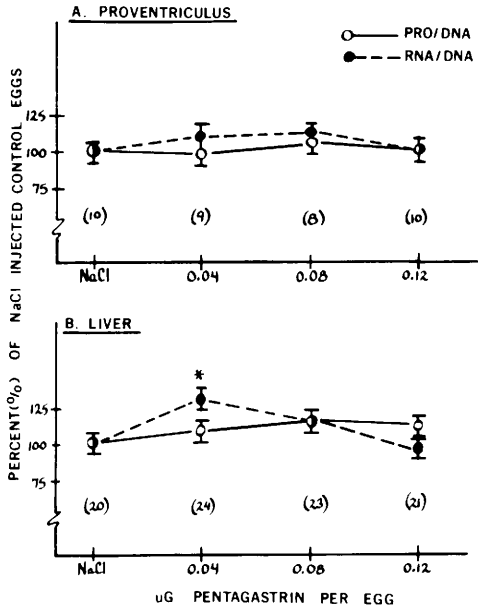

FIG. 2. Effect of Pentagastrin on chick embryo tissue protein/DNA ratio (solid line) and RNA/DNA ratio (dashed line). Injections were made on day 12 and tissues assayed on day 14 of development. Vertical lines are SEM; ( ) = number of observations. * $p < .01$.

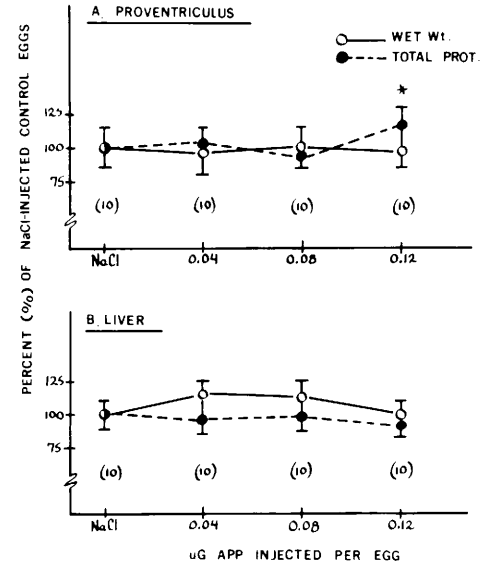

FIG. 3. Effect of APP on chick embryo tissue wet wt (solid line) and total organ protein (dashed line). Injection/assay/data protocol identical with that of Fig. 1.

detected in 14-day embryos one hour later by measuring *in vitro* incorporation of [^{14}C]leucine into TCA precipitable protein during a

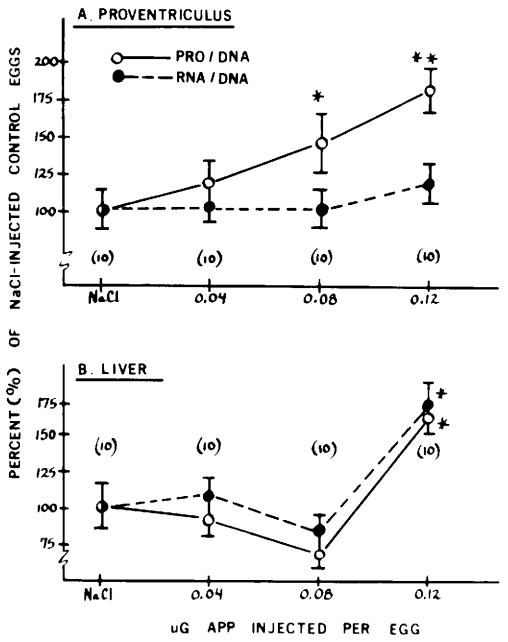

FIG. 4. Effect of APP on chick embryo tissue protein/DNA ratio (solid line) and RNA/DNA ratio (dashed line). Injection/assay/data protocol identical with that of Fig. 2. * $p < .01$, ** $p < .001$.

15-min incubation (Fig. 5). At this time of post-APP injection the proventricular amino acid incorporation increased linearly (in proportion to dose) to a maximum at 0.04 μg per egg, while the liver incorporation was significantly only at 0.12 μg APP per egg. Protein levels did not increase in proportion to DNA in either tissue within one hour of injection.

Discussion. Preliminary data (see Methods section) from our laboratory indicated definite structural "tissue wasting" of the proventriculus in depancreatized chickens (Table I). Such evidence suggested that the avian pancreas may, indeed, exert a trophic effect over the gastrointestinal tract and that of the four known (endocrine) pancreatic peptides, APP would be the most likely candidate for such a role.

Previous results (14) using Pentagastrin indicated that the proventriculus (glandular stomach) of the avian embryo was most sensitive during the second week of development to the trophic activity of this gastrin analog. This period of embryo-genesis coincides with the appearance of proventricular acid secretion (15) and, therefore, the probable onset of

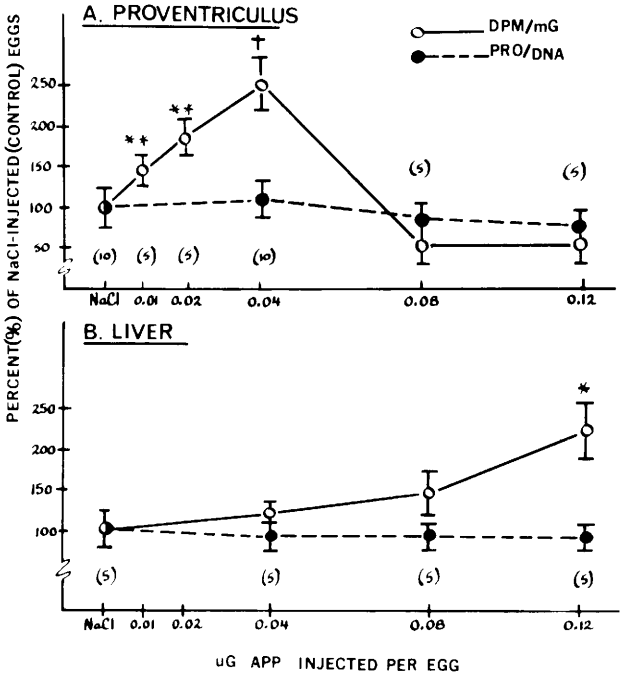

FIG. 5. Effect of APP on chick embryo incorporation of [^{14}C]leucine into TCA precipitable protein as DPM/mg (solid line) and protein/DNA ratio (dashed line). Injections of APP were made on day 14 and tissues assayed 1 hr later. Vertical lines are SEM; ( ) = number of observations. * $p < .05$, ** $p < .01$, + $p < .001$.

significant *in vivo* APP release as suggested by the potent secretogogic effect of APP in adult chickens (7) as well as by the low pancreatic content of APP at this embryonic age (16). The latter report may indicate a reciprocal relationship exists at this time between organ content and plasma levels of APP. Further work is needed to clarify this point. Further, characterization of the trophic response to Pentagastrin (Figs. 1 and 2) indicates that while the increase in embryonic proventricular weight is not due solely to an increased water uptake (evidenced by the trend in total tissue protein), there are no changes in the relative amounts of RNA and DNA; this result is contrary to that demonstrated for the rat gut mucosa in response to the same pentapeptide (17).

The response of the embryonic proventriculus to APP injection (Figs. 3 and 4) may not argue favorably for hyperplasia and/or trophism because the protein/DNA increased (Fig. 4) while total protein level increased significantly only at the highest dose employed (0.12 μg; Fig. 3). The ratio of protein to DNA increased linearly in response to the APP doses up to a maximum (at 0.12 μg/egg) of 185% of the saline-injected control value and was due largely to the fact that virtually no change in DNA levels was observed. Similarly, the resistance of this parameter to the trophic effect of Pentagastrin in antrectomized rats has been reported (17).

The results of amino acid incorporation studies using APP (Fig. 5) demonstrate the sensitivity of this technique compared to less sophisticated biochemical analyses, furthering the observed comparison between the avian response to APP and the mammalian response to Pentagastrin. One hour after injection of 0.04 μg APP/egg, proventricular amino acid incorporation into precipitable protein increased to a maximum of 250% above that of saline-injected control eggs. Repetition of the experiment within this APP dose range indicated response to be linear with the dosage of APP (14). This response to APP in the embryo is to be compared with the 190% increase in [^{14}C]leucine incorporation over control values observed in oxyntic mucosa one hour after injection of similar doses of Pentagastrin into rats (10). The fact that APP does encourage uptake and incorporation of amino acids within one hour of application, but does not (apparently) alter total protein levels greatly 48 hours later; suggests that multiple doses of the peptide may be necessary to affect protein levels. The doses chosen herein are 9–30 μM per egg and thus much greater than embryonic pancreatic content. Also, this observation encourages future studies on the evaluation of DNA synthesis during the first 24 hours after APP injection. The increase in [^{14}C]incorporation by the liver once again indicates the apparent dual function of APP in birds, acting both on the proventriculus and on this accessory digestive organ. Recalling the hepatic glycogenolytic effects reported in response to relatively high doses of APP in adult birds (7), it need not be unexpected that the embryonic liver responds to the higher APP dose (0.12 μg/egg) with an increase in the protein/DNA and RNA/DNA ratios (Fig. 4), as well as in [^{14}C]leucine uptake and incorporation (Fig. 5). Overall, however, the results suggest favorably that APP may be trophic in the system employed and that future experiments should be directed to time-lapse studies at the molecular level.

Summary. Avian pancreatic polypeptide (APP) is a recently discovered pancreatic peptide with hormone-like characteristics which acts as a gastric (proventricular) secretogogue in chickens. When injected into 12–14 day old fertile eggs, APP was shown to:

(a) cause a marked increase in proventricular protein/DNA ratio within two days, and (b) cause incorporation of labelled [^{14}C] leucine into proventricular, but not into liver, protein unless very high doses were employed. The results suggests that APP may be an anabolic and secretogogic regulator of the developing avian gut.

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