BILIARY EXCRETION AS A MECHANISM OF AVIAN RESISTANCE TO INSULIN

A Dissertation

Presented to

The Faculty of the Department of Biology

College of Arts and Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Stewart Demond Turner

December 1972

ACKNOWLEDGEMENTS

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ABSTRACT

The role of avian biliary excretion and that of a new pancreatic polypeptide in the reported "resistance" of Aves to insulin injection was studied.

Bile was collected in 15-minute collection periods from adult chickens after the injection of beef insulin, ^{14}C-inulin, or beef insulin and ^{14}C-inulin. The volume of bile was noted as well as its ^{14}C-activity (when appropriate). It was found that beef insulin injections increase the bile flow and appearance of ^{14}C-activity in the bile.

Using the rat hemi-diaphragm bioassay, pooled 30-minute bile samples were assayed for insulin-like action after the injection of beef insulin or tolbutamide *in vivo*. After either of these injections an increase in biliary insulin-like action was noted; however, after tolbutamide injection a double peak in insulin-like action was noted as well.

Radioimmunoassay was employed to determine what percentage of insulin excreted in the bile was in a degraded form. Fifteen-minute bile samples were incubated with anti-insulin binding reagent after the injection of ^{125}I-ox insulin *in vivo*. The precipitated insulin-anti-insulin complexes were measured for ^{125}I-activity and compared to the total ^{125}I-activity of the samples. The results obtained indicated that the liver excretes less than 0.5% of the total labelled hormone pre-

sented to it and of this amount only 4.5% was immunoreactive insulin.

Avian pancreatic polypeptide (APP) had no effect on bile flow or excretion rate. However, it was found that APP was a potent stimulator of proventricular volume flow. Analysis of 10-minute collection samples of proventricular secretion indicated that APP also markedly increased the content of pepsin, H^+ ion, and total protein. Species specificity studies indicated that APP was more potent in the chicken than in the rat. It was also shown that pentagastrin was a more potent "gastric" secretogogue in the rat than in the chicken; however, the difference was not as marked as that produced by APP.

This study indicates that the liver and biliary excretion route play a minor role in the "resistance" of chickens to exogeneous insulin. It was also shown that while APP appears not to be involved in this "resistance" it is a potent "gastric" secretogogue in chickens.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

INTRODUCTION

Over the last 90 years it has been established that the metabolism of carbohydrate, or regulation of carbohydrate metabolism, differs greatly in most (if not all) avian species when compared with that known to exist in mammals. Since the classic report of Minkowski in the late 1800's, the observation that pancreatectomy in birds leads merely to a transitory hyperglycemia and glucosuria (lasting less than 7-10 days) has been verified in a wide variety of avian species (Nelson et al., 1942; Hazelwood, 1958; Koike et al., 1964; Stamler et al., 1964; and Lepkovsky et al., 1967). Additionally, other peculiarities (as compared with mammals) in carbohydrate metabolism have been reported and include the following:

1) The presence and physiological importance of the avian glycogen body. This structure is a profound storage depot of glycogen (up to 60 - 80% of its lipid-free weight) and lies within the vertebral column dorsal to the spinal cord at the level of emergence of the sciatic plexis. Despite normal structural configuration of the depot glycogen, presence of glycogenic and glycogenolytic enzymes, and a moderate vascularity the glycogen moiety of this structure appears unaffected by dietary, hormonal or pharmacological perturbation (Szepzenwol and Michalski, 1951; Snedecor and King, 1960; Hazelwood et al., 1962; Hazelwood, 1965). Furthermore,

chicken insulin added to this structure *in vitro* (Hazelwood and Barksdale 1970) or directly to the cerebrospinal fluid *in vivo* (Anderson and Hazelwood, 1969) fails to alter its glycogen levels.

2) Alloxan, a potent and rapid beta cytotoxic agent in mammals, causes only minor histological damage to the avian pancreas and decreases circulating insulin levels in chickens only to a slight extent (Langslow *et al.*, 1970). Alloxan-treated pigeons, chicks and chickens are essentially normal from a metabolic point of view.

3) Depancreatized birds respond to the hypoglycemic action of the sulfonylureas. Such sulfanilamide-substituted agents as sodium tolbutamide (Orinase) provoke the release of preformed insulin from the mammalian pancreas. Additionally its hypoglycemic action is unobtunded in depancreatized chickens (Hazelwood, 1958), in depancreatized ducks (Mirsky and Gitelson, 1957), in depancreatized-hepatectomized chickens (Hazelwood, 1958) and in enterectomized ducks (Mirsky and Gitelson, 1957). A possible species exception to these observations is the goose, a species which is unresponsive to tolbutamide after pancreatectomy (Mirsky and Gitelson, 1957). Overall, however, these reports indicate either existance of a second source of endogenous insulin or a tenacious binding of the hormone by peripheral sites which are susceptible to the releasing action of sulfonylureas.

4) Shortly after the discovery of mammalian insulin in 1922 it was found that birds tolerate pharmacological doses of insulin (10-500 U/kg body weight) whereby plasma glucose levels may be depressed from normal levels of 200-250 mg % to 20 mg % without obvious comatose or convulsive states being precipitated. Comparative studies establishing the LD-50 convulsive doses indicate chickens to be 20 - 200 times more "resistant" to exogenous mammalian insulin than are mammals (Chen et al., 1945; Hazelwood and Lorenz, 1957; and Shao et al., 1966). Such "resistance" has been documented in geese (Mirsky and Gitelson, 1957) and lizards (Miller and Wurster, 1958). It is worthy of note, however, that until 1968, only mammalian insulin was available for such studies; chicken insulin was first isolated in 1968 by Kimmel et al.

The mechanism of "avian resistance to exogenous (mammalian) insulin" has received considerable attention over the last 10 years. Among the more plausible explanations are 1) Structural differences between chicken and beef insulin 2) Avian tissue receptor sites do not "recognize" non-avian exogenous insulin, thus the latter has less intrinsic intracellular activity in chickens and is not as effective as the endogenous hormone 3) Compensatory endocrine secretions of an anti-insulin nature, and 4) The reduction of the plasma insulin concentration quantitatively or functionally.

Structural differences between chicken and beef insulin molecules are modest, indeed, and are not any more severe than those reported to exist among different mammalian insulins. Also, Hazelwood et al. (1968), demonstrated that crystalline avian insulin is as effective in promoting glucose uptake by the rat diaphragm as preparations of crystalline beef, pork and sheep insulins. These results indicate that, despite the differences of six residues in the amino acid sequence (Kimmel et al., 1968), the mammalian tissue receptor site does not discriminate among these four insulins. However, there are no data available on the comparative effect of these insulins on the avian tissue receptor site. Also it has been shown that the central nervous system of the chicken is not protected from the stress of hypoglycemia during an insulin challenge. This was demonstrated by Anderson and Hazelwood (1969) who reported a depression in the glucose levels of chicken cerebrospinal fluid after administration of beef insulin (i.v.). Pittman and Hazelwood (1971) reported subtle cardiovascular changes in birds (after administration of pharmacological doses of beef insulin), alterations which were indicative of catecholamine release. However, they concluded that these could not account for the total insulin resistance observed. Finally, Hazelwood et al. (1971) reported the presence of heat labile factors in the avian plasma which severely inhibit beef insulin activity in vitro and thereby

account for a major portion of the oft-reported "resistance" to insulin.

Although these observations account for most "resistance" they do not account for it completely. An area which has not been investigated by workers interested in this problem is the effective reduction of circulating insulin such as the excretory role of the liver in regulating plasma insulin levels. The fact that the venous drainage of the avian pancreas passes directly to the liver suggests this structure as a likely organ capable of inactivating/removing insulin. Also, it has been shown that intravenously injected ^{131}I-insulin is accumulated rapidly in the liver and kidneys of the rat (Elgee et al., 1953 and 1954) and man (Croughs et al., 1965) and is excreted in the urine. Investigators have also shown in other species that insulin is excreted in the bile of rabbits (Lopez-Quijada et al., 1967; Boyns et al., 1969; Daniel and Henderson 1968), swine (Lopez-Quijada and Goni, 1967; Jeffcoate, 1968), monkey (Daniel and Henderson, 1968), mice (Jeffcoate, 1968), man, cat, dog, ox, rat, sheep and chicken (Lopez-Quijada and Goni, 1967). The amount of immunoreactive insulin excreted in the bile of rabbits increases to a maximum 30 minutes after injection of labelled insulin (Lopez-Quijada et al., 1968). By comparing the activity of TCA soluble and insoluble bile fractions these workers determined that 6.5 to 7% of the total injected radioactivity was

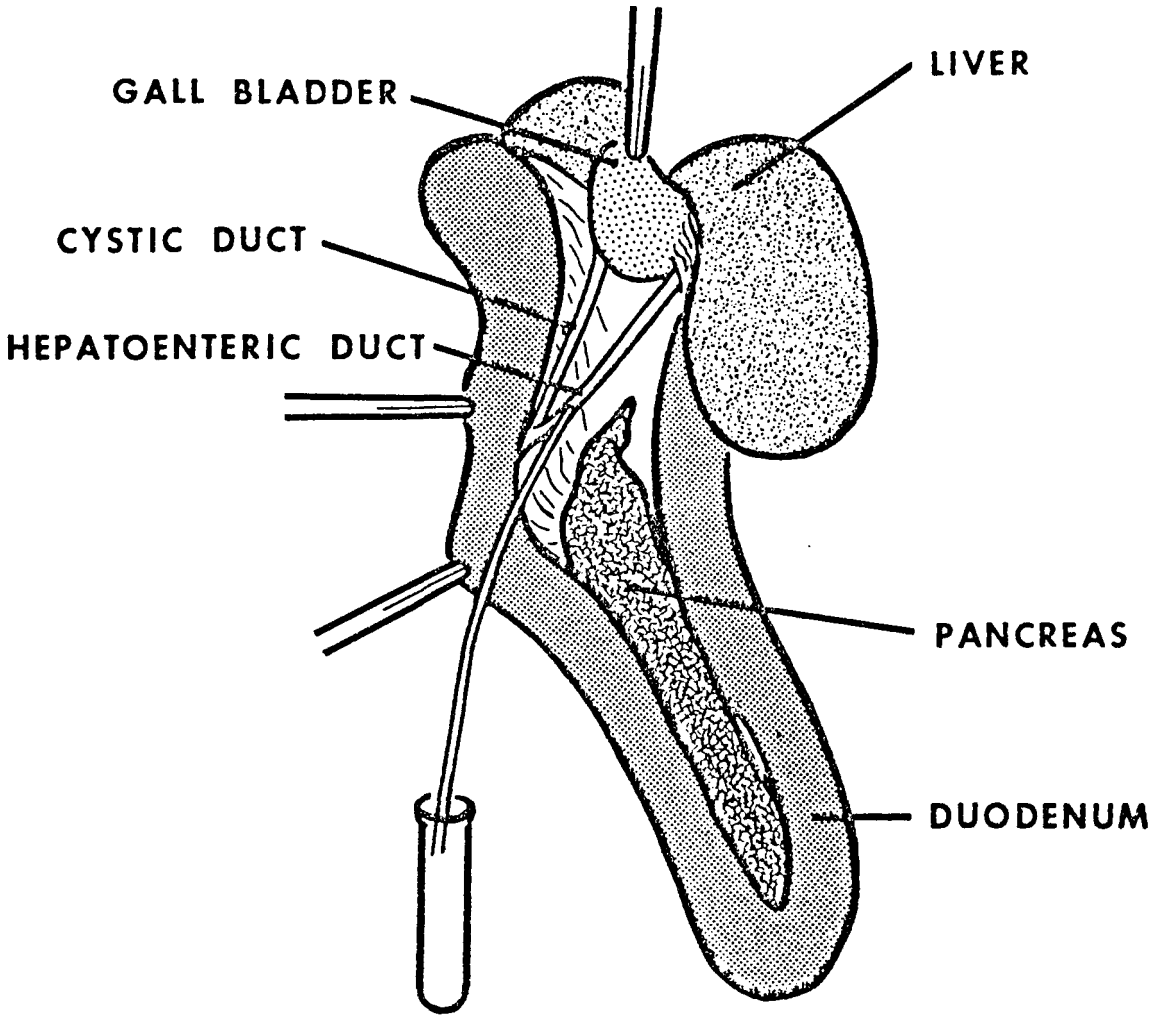
cleared into rabbit bile during 5.5 hours, and of this amount, from 15 to 51% of the activity in each sample was considered undegraded or biologically active insulin. The greatest percentage of undegraded insulin appeared in the samples taken immediately after injection of the tracer. However, Boyns et al., (1969) found that only 3% of the injected labelled insulin reached the bile of normal rabbits, one tenth of which could react with specific antisera. The marked differences between these two observations may be due to the assumption by Lopez-Quijada that TCA insoluble radioactivity represented only that radioactivity bound to whole insulin molecules.

The biliary system of the domestic fowl is particularly suited for the study of the clearance of insulin. Bile flow from the chicken liver is partitioned between two routes, one of which (the cystic duct) leads directly to the gall bladder for storage before continuing to the duodenum (Figure 1). The other route (the hepato-enteric duct) is direct from the liver to the gut, thereby bypassing the gall bladder. Collecting bile from the hepato-enteric duct enables the investigator to collect (fresh) hepatic bile which has not been subjected to the concentration and possible degradative processes that may occur during storage in the avian gall bladder. If this excretion route does, indeed, play a role in removing exogenous insulin from the circulation one would expect to find a marked increase in the biliary insulin content after injection of labelled insulin.

Figure 1

SCHEMATIC DIAGRAM OF THE CHICKEN BILIARY TREE

A schematic diagram of the chicken biliary tree showing the method of cannulation. Note: Cannulation is performed on the hepatoenteric duct allowing the collection of fresh bile rather than stored, concentrated cystic bile.


GALL BLADDER
LIVER
CYSTIC DUCT
HEPATOENTERIC DUCT
PANCREAS
DUODENUM

Parallel to the above line of investigation (yet to be done) is the role which a new avian pacreatic polypeptide may have on bile flow and possibly biliary clearance of insulin. Hellman and Lernmark (1969) suggest that the α_1 cell (also known as D-cell) of the pigeon pancreas produces a substance which has an inhibitory effect on the beta cell secretory activity. Subsequently they reported that synthetic gastrin I in low concentration (0.15 µg/ml) inhibited insulin release from microdissected pancreatic islets of mice (Lernmark et al., 1969). Gastrin has been shown to be secreted by the pathological mammalian pancreas, giving rise to the Zollinger-Ellison syndrome and characterized by gastric hyperactivity and hyper-acidity (Zollinger and Ellison, 1955, and Gregory et al., 1967).

Using modifications of established procedures for the isolation of insulin, significant quantities of a polypeptide has been separated from the insulin fraction of fresh chicken pancreas. This polypeptide (tentatively designated "Avian Pancreatic Polypeptide," or APP) has been purified by gel filtration, ion-exchange chromatography, and counter-current distribution in Dr. J. R. Kimmel's laboratory. Its homogeneity was established by thin layer chromatography and polyacrylamide gel electrophoresis. APP has been shown to contain 36 amino acids, to be a straight-chain polypeptide, and to have a molecular weight of 4250 (Kimmel et al., 1971).

These observations of a possible third pancreatic endocrine secretion, and its effect on insulin release, indicate that APP may be important in the control of insulin levels in the chicken. Also, the role which APP may play in regulating biliary flow and/or excretion of endogenous insulin into the gut for subsequent proteolytic degradation is of interest and concern. Therefore, investigation of the physiological role of APP may shed further light on the avian "resistance" to insulin which cannot be attributed to interfering plasma factors.

STATEMENT OF PROBLEM

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The foregoing summary indicates that a better understanding of the role which the chicken liver and a new pancreatic component may play in regulating plasma insulin levels would be gained by

1) Determining the effectiveness of the chicken liver and biliary excretion route in decreasing plasma insulin levels.
2) Determining what portion of an exogenous insulin injection is excreted in chicken bile as a degraded product as opposed to what part is excreted as a biologically intact or immunoreactive molecule.
3) Determining whether or not Avian Pancreatic Polypeptide (APP) is biologically active by a spectrum approach and, in doing so, ascertain if it alters bile excretion and is distinct from other avian pancreatic endocrine secretions.

METHODS AND MATERIALS

METHODS AND MATERIALS

I. Experimental Animals:

1) Adult female Single-Comb-White leghorn (SCWL) chickens weighing between 1.3 and 1.8 kg were used for the insulin clearance and Avian Pancreatic Polypeptide (APP) work. They were given food and water ad libitum.

2) Immature female Holtzman rats weighing between 120 and 150 gm were used for the rat hemi-diaphragm bioassay of insulin-like action (ILA) in avian bile. These animals were fasted from 18 to 24 hours (water ad libitum) prior to use.

3) Mature male Sprague-Dawley rats between 190 and 270 gm were used in the determination of the effect of APP of gastric secretion. These rats were fasted for 40 to 50 hours with water and sugar cubes given ad libitum. This was done to eliminate obstructing food particles from the perfused stomach while at the same time supplying the animals' caloric needs.

All animals were kept in an environment of 24°C, a light day of 12D:12L, with light provided by artificial illumination.

II. Procedure:

A. Bile Studies

1) Effect of insulin on bile flow, biliary clearance of insulin, and insulin-like action of bile.

Fed female SCWL chickens were anesthetized with injections (i.v.) of Nembutal (sodium pentobarbitol). An incision was then made on the right side, just posterior to the rib cage. The hepato-enteric bile duct (see Figure 1) was cannulated with intramedic polyethylene tubing (P.E. 60) and bile was allowed to flow continuously into collecting tubes. After two 15-minute control collection periods, the injection was made and post-injection samples were collected for three five-minute periods for the first 15 minutes and in 15-minute periods for the remaining 120 minutes. Injections were either inulin carboxyl-^{14}C, insulin, insulin and inulin carboxyl-^{14}C, or tolbutamide. The injections were made into the right alar vein at all times. For the assay of insulin-like action (ILA) of bile, 30-minute pooled samples were used. A blood sample of 0.75 ml was taken by cardiac puncture every 15 minutes after injection of inulin;^{14}C and/or insulin to monitor plasma glucose and ^{14}C-activity. The amount of radioactivity present in the samples was measured using a Packard liquid scintilation counter. The samples were prepared by placing 0.2 ml of sample (bile or plasma) in 20 ml of scintilation cocktail prepared by mixing 850 ml toluene, 150 ml Beckman Biosolv

Solubilizer (BBS-3) and 6.0 gm Packard Premix-P (98% PPO and 2% POPOP). A quench correction curve was generated for both bile and plasma and the recorded sample activity was corrected using external standard channels ratio.

To determine the role which the vagi might play in the insulin stimulation of bile flow, a group of animals were subjected to bilateral vagotomy prior to insulin injection. The vagi were isolated via an incision on the ventral surface of the neck. A pair of loose ligatures were placed around each vagus so that at the time vagotomy was scheduled (after a 30-minute control period and 30 minutes prior to injection of insulin) the nerves could be quickly retrieved, tied and sectioned.

2) Ratio of degraded to non-degraded insulin in the bile.

Fed, female SCWL chickens were treated surgically as in the above experiments. One 15-minute control bile sample was taken prior to injection (i.v.) of 1.5 μCi beef insulin-^{125}I (0.5 μU). In one group of animals the injections were made into the alar vein, in another they were made into the pancreatic vein. After injection, 15-minute collection periods were obtained for 90 additional minutes. Concomitant femoral blood samples were also taken, centrifuged and frozen for subsequent analysis.

Plasma and bile samples assayed for insulin were treated identically. The immunoassay procedure used was based on that of Hales and Randle (1963), method C. A 0.1 ml aliquot was placed in a gamma counting vial and the activity measured with a Nuclear-Chicago auto-gamma counter to determine the total radioactivity present. Another 0.1 ml aliquot of bile was diluted with 0.1 ml of buffered albumin solution and incubated for 18 hours with 0.1 ml of anti-beef insulin binding reagent from an Amersham-Searle insulin immunoassay kit. This dilution of bile with buffered albumin solution was necessary to reduce the significant inhibitory effect which bile salts have on the binding of insulin to antibodies (Jeffcoate, 1966; Boyns et al., 1969; Lopez-Quijada, 1971). The insulin-antibody complex was then filtered from the mixture using milipore filters and counted with the Nuclear-Chicago auto-gamma counter.

In an effort to determine the fate of the injected label (^{125}I), terminal liver samples were taken from three birds of the pancreatic-vein injected group. The weight of the sample was determined to the nearest 0.1 mg using a Roller-Smith torsion balance and then radioactivity of the sample was determined. Comparing this activity to the total mass of the liver allows one to estimate the amount of label that was sequestered by the liver.

3) Rat hemi-diaphragm bioassay for insulin-like action in the bile.

Young adult male Holtzman rats were fasted for 24 hours, decapitated and the diaphragm with one attached rib quickly removed and placed on an iced, inverted petri dish. The diaphragm was then trimmed of fat, connective tissue and excess muscle leaving only one rib attached to each side of the diaphragm. Special care was taken to avoid or minimize the damage to the diaphragm which was then rinsed for 10 to 15 minutes in bicarbonate-buffered Krebs-Ringer solution (KRB) (Umbriet et al., 1964). After a final inspection for excess tissue and/or damage, the diaphragm was bisected and each half placed in separate 25 ml erlynmeyer flasks, one hemi-diaphragm serving as the control for the other. Prior to the addition of the hemi-diaphragms, the flasks were filled with one ml each of either control or experimental media.

Initially, buffered Krebs-Ringer glucose solution which had been previously gassed with 95% oxygen-5% carbon dioxide was used as the control media. Preliminary work, however, indicated that normal bile had an inhibitory effect on the glucose uptake of the rat diaphragm muscle. Therefore it was decided to add 0.9 ml KRB containing 2.5 mg glucose to 0.1 ml normal control bile. The experimental media contained 0.1 ml experimental bile added to 0.9 ml gassed KRB-glucose solution.

All flasks were gassed briefly with 95% oxygen-5% carbon dioxide, stoppered and placed in a Dubnoff metabolic shaker for one hour at 38°C and 90 oscillations per minute. At the end of one hour the hemi-diaphragms were removed blotted dry, weighed, dried at 107°C for two hours in an oven and re-weighed. A sample of each of the two media was deproteinized with equal volumes of 10% sodium tungstate and 2/3 N sulfuric acid, and analyzed for glucose content using the Nelson-Somogyi technique (Somogyi, 1952). The results are presented as percent glucose uptake per gm of tissue per hour above control hemi-diaphragm levels. This method is a modification of the insulin bioassay method first described by Kipnis and Cori (1957).

B. Avian Pancreatic Polypeptide Studies

1) Spectrum effect(s) of APP in chickens.

Fed, female SCWL chickens were anesthetized with Nembutal and an incision made in the abdomen on the left side of the animal, just posterior to the rib cage. The proventriculus was then isolated as well as the arteries supplying both the proventriculus and the gizzard. Retracting loops were placed around these vessels. A ligature was then placed below the proventriculus just superior to the gizzard, and a polyethylene cannula (0.6 mm O. D. and 0.45 mm I. D.) was inserted anterior to the ligature into the proventriculus. Via an incision in the neck, the esophagus was isolated and separated from the crop by a ligature placed just inferior to the latter.

In some experiments a cannula was also inserted into the gizzard at its junction with the duodenum. The gut segment was then washed of food particles by slow injection of 10 ml of warm physiological saline into the esophagus by a hypodermic syringe and 18 gauge needle. If more flushing was required it was repeated until the effluent was clear. The effluent was collected and the percent recovery noted (92 - 98%). In all experiments the chicken was tilted upward 30 degrees to facilitate the gravitational flow from the cannulae.

Three 10-minute control collections were made prior to any injection. The first of these collections was discarded because of possible dilution of the gastric juice by residual "flush" volume. Injections of test substances were made either into the left alar vein, the proventricular artery or the gizzard artery depending upon the experimental objective.

Avian Pancreatic Polypeptide (APP) was injected into the alar vein in doses of 12.5, 25.0 or 50 µg APP/kg body weight. The proventricular responses to these doses were compared to the effects of pentagastrin (1.0 µg/kg) and physiological saline.

The effect of APP on related organs in the digestive tract of the bird was studied by the injection of APP (6.25 µg/kg) into the isolated arterial supply of first the proventriculus, then the gizzard. Injections of pentagastrin

(1.0 µg/kg) and 25 µg APP/kg were also made into the arterial supply of the gizzard.

The volume and pH (Instrumentation Lab pH/P_{CO_2} electrometer with glass electrodes) of the samples were immediately determined upon collection and then were subsequently frozen for later determination of pepsin content by the hemoglobin degradation method of Anson as modified by Bucher et al. (1945), and total protein content by the Lowry method (Lowry et al., 1951).

To elucidate further the biological effects of APP the effect of bilateral vagotomy was determined. The surgical and experimental procedure followed was the same as that used in the bile studies. Alar vein injections of beef glucagon at doses of 23 and 100 µg/kg body weight were administered in studies to compare the volume response with that observed with APP. These doses were chosen to represent an amount of protein equal to the APP injected and pharmacological doses, respectively. These experiments were repeated with chicken glucagon (when it became available very late in these studies) using doses of 23 and 50 µg chicken glucagon/kg body weight.

The possibility that the cardiovascular system may have been involved in the effect APP had on the proventriculus was studied using an E and M Physiograph equipped with linear core blood pressure transducers and an impedance pneumograph. Heart rate, respiratory rate, systolic pressure, diastolic

pressure and mean pressure were measured (and the pulse pressure calculated) after the injection of 25 µg APP or 1.0 µg pentagastrin/kg body weight. A small number of animals were tested also at doses of 12.5 and 50 µg APP, and 0.5 and 25.0 µg pentagastrin/kg body weight. At no time were animals used in the computation of the data in which it was necessary to use supplemental injections of anesthetic. This was done to avoid the depressant effects of the barbiturate anesthetic on cardiovascular parameters.

2) The possible role of histamine in the biological action of APP.

Fed adult female chickens were prepared surgically identically to those used in the previously described proventricular studies. The birds were then divided into four groups of five animals each. One group was injected subcutaneously with 100 µg histamine base/kg body weight. The second group was injected with the same dose of histamine base ten minutes after an intravenous injection of 100 µg glycopyrrolate bromide/kg body weight. Glycopyrrolate bromide (Robinul, A. H. Robins) blocks the gastric stimulatory effect of histamine in mammals (Abbott et al., 1962; Foss et al., 1964; and Ruffin et al., 1966). The third group was injected with 25 µg APP/kg body weight intravenously and the fourth group was injected with the same dose of APP as the third group ten minutes following an intravenous injection of 100 µg

glycopyrrolate/kg body weight. Each run consisted of a glycopyrrolated-blocked bird (those in Group two and four) and a corresponding bird without the blocking agent (Group one and three). Therefore, the effect of the blocking agent (glycopyrrolate) could be compared to the normal response under identical conditions. In all histamine and APP bird groups, three 10-minute control collection periods were made previous to six 10-minute experimental periods which followed the injection. In the glycopyrrolate injected birds three 10-minute control collections were made followed by one 10-minute collection period after the injection of the glycopyrrolate. Six 10-minute collection periods were then made after the injection of the histamine or APP. In all groups the first control sample was discarded for reasons previously described. The volume and pH of the samples were determined immediately after collection and the remainder of the samples were then frozen for protein and pepsin determinations later.

3) Effect of APP in rats.

Adult, male Sprague-Dawley rats were anesthetized with injections (i.p.) of Nembutal. The sample collection procedure used in this phase of the work was a modification of a procedure described by Lai (1964). Initially the trachea was cannulated and the introduction of a segment of P.E. 160 intramedic tubing was made into the esophagus at the neck and passed posteriorly to the cardiac end of the stomach. The

stomach was then cannulated by passing a tube through the pyloris from an incision in the proximal end of the duodenum. At this time the posterior vena cava was isolated preparatory to injection of the test substance(s). Prior to closing the abdominal incision a gauze pad, soaked in physiological saline, was placed against the "ruminal portion" of the stomach to prevent passive distention during the course of the lavage. After the abdominal incision was closed the esophageal cannula was connected to a syringe and flushed with 20 ml of warm physiological saline. The esophageal cannula was then connected to a gravitational infusion apparatus as described by Lai (1964). The samples were collected in 10 ml graduated cylinders and the perfusion rate of warm saline maintained at 8.0 ml per ten minutes (7.9 ± 0.15, n = 344) through two control and six subsequent experimental 10-minute collection periods. The pH of the samples was determined immediately and the samples then frozen for later determination of pepsin and total protein content. In these experiments the animals were grouped in sets of three: a saline control and two experimental animals. The experimental animals were injected either with APP (25 µg/kg) or pentagastrin (25 or 230 µg/kg) and the control rats with 1.0 ml saline per kg/body wt.

III. Statistical Analysis:

Statistical evaluation of the data was performed, when appropriate, using the Student's "T" test calculated on an

IBM 1108 computer located in the University of Houston computing center.

IV. Equipment:

1) Packard Tri-carb Spectrometer liquid scintillation counter. Window settings: A: 50, B: 310, C: 50, D: 1000. Gain: 8%.

2) Nuclear-Chicago 1185 Series Automatic Gamma Counting System, 200 sample capacity.

3) Instrumentation Laboratory Inc. Duo-matic pH/P_{CO_2} electrometer, Model 123, with constant temperature bath, Model number 127.

4) Bausch and Lomb Spectronic 20 Spectrophotometer.

5) Nuclear-Chicago Model 188a Geiger-Muller counter.

6) Roller-Smith Torsion Balance, Model L6.

7) E and M Physiograph -Four- with a linear core pressure transducer model P-1000A and impedance pneumograph.

V. Chemicals:

1) Avian Pancreatic Polypeptide (APP):
Isolated and purified by Dr. J. R. Kimmel, University of Kansas Medical Center. Lot numbers CH-III-287 and JRK-F286.

2) Glucagon: (Chicken)
Isolated and purified by Dr. J. R. Kimmel, University of Kansas Medical Center. Lot number CH-Pen-1-236.

3) Glucagon: (Beef)

Eli Lilly and Co., as a hydrochloride (Crystalline) Reconstituted to 1.0 mg/ml, gift of Dr. Mary A. Root.

4) Glycopyrrolate Bromide:

A. H. Robins, Robinul injectable. 0.2 mg/ml., Compliments of the A. H. Robins Company.

5) Heparin:

Sodium heparin, Riker Co.

6) Histamine:

Eli Lilly and Co., histamine phosphate, USP, Ampoule No. 328, 0.2 mg histamine base/ml.

7) Insulin:

Eli Lilly, Iletin, regular, beef-pork insulin, U-40 (40 IU/ml).

8) Inulin carboxyl-^{14}C:

New England Nuclear Corp., Lot number 334-122, crystalline, 2.7 μCi/mg. Radiochemical purity verified upon receipt by chromatography.

9) Pentagastrin:

Ayerst Laboratories, Peptavalon, AY-6608, in crystalline form. Generously supplied by Dr. T. Robitscher, M.D., Associate Medical Director, Ayerst Laboratories Medical Department, New York.

10) Radioimmunoassay Kit:

Amersham/Searle kit for mammalian insulin, human insulin standard, ^{125}I-ox insulin; binding reagent consisted of a combination of guinea-pig anti-insulin sera and rabbit anti-guinea-pig sera.

11) Sodium pentobarbitol:

Nembutal, Abbott, 50 mg/ml.

12) Tolbutamide:

Orinase, Upjohn, as a sodium salt. Reconstituted to 0.1 mg/ml of physiological saline.

RESULTS

RESULTS

I. Biliary Flow Studies in Adult Chickens

Injection of 10 U beef insulin/kg body weight into anesthetized SCWL chickens produced an immediate and marked increase in the bile flow rate (see Methods for surgery and cannulation details). Biliary flow rates increased from 0.39 ± 0.06 ml to 0.56 ± 0.08 ml/10 minutes within ten minutes after insulin injection. A peak of 0.64 ± 0.09 ml/10 minutes occurred 30 minutes after hormone injection. Saline injected birds exhibited a continuous decrease in flow rate with time (Fig. 2). The total bile volume excreted during the 140 minutes of the experiment was 6.88 ± 0.81 ml in the insulin-injected birds as compared with 3.59 ± 0.60 ml in the saline-injected birds. Simultaneous measurement of plasma glucose levels indicate that large doses of insulin decreased plasma glucose over 100 mg % from pre-injection levels. There was no significant change in the plasma glucose of birds injected with saline.

Inulin carboxyl-^{14}C injected intravenously (alar vein) could be detected in the bile within five minutes after injection (Fig. 3). Bile radioactivity reached a maximum between 10 and 15 minutes after injection and then rapidly fell off. When pharmacological amounts of beef insulin (10 U/kg) were injected simultaneously with ^{14}C-inulin there

Figure 2

EFFECT OF BEEF INSULIN ON BILE FLOW IN CHICKENS

The effect of beef insulin on bile flow in adult female chickens. The vertical bars are standard errors of the mean (S.E.M.) at each point. The two isolated points at 140 minutes represent the 140-minute accumulative bile flow as indicated by the legend at the right of the figure.

EFFECT OF BEEF INSULIN ON BILE FLOW IN CHICKENS

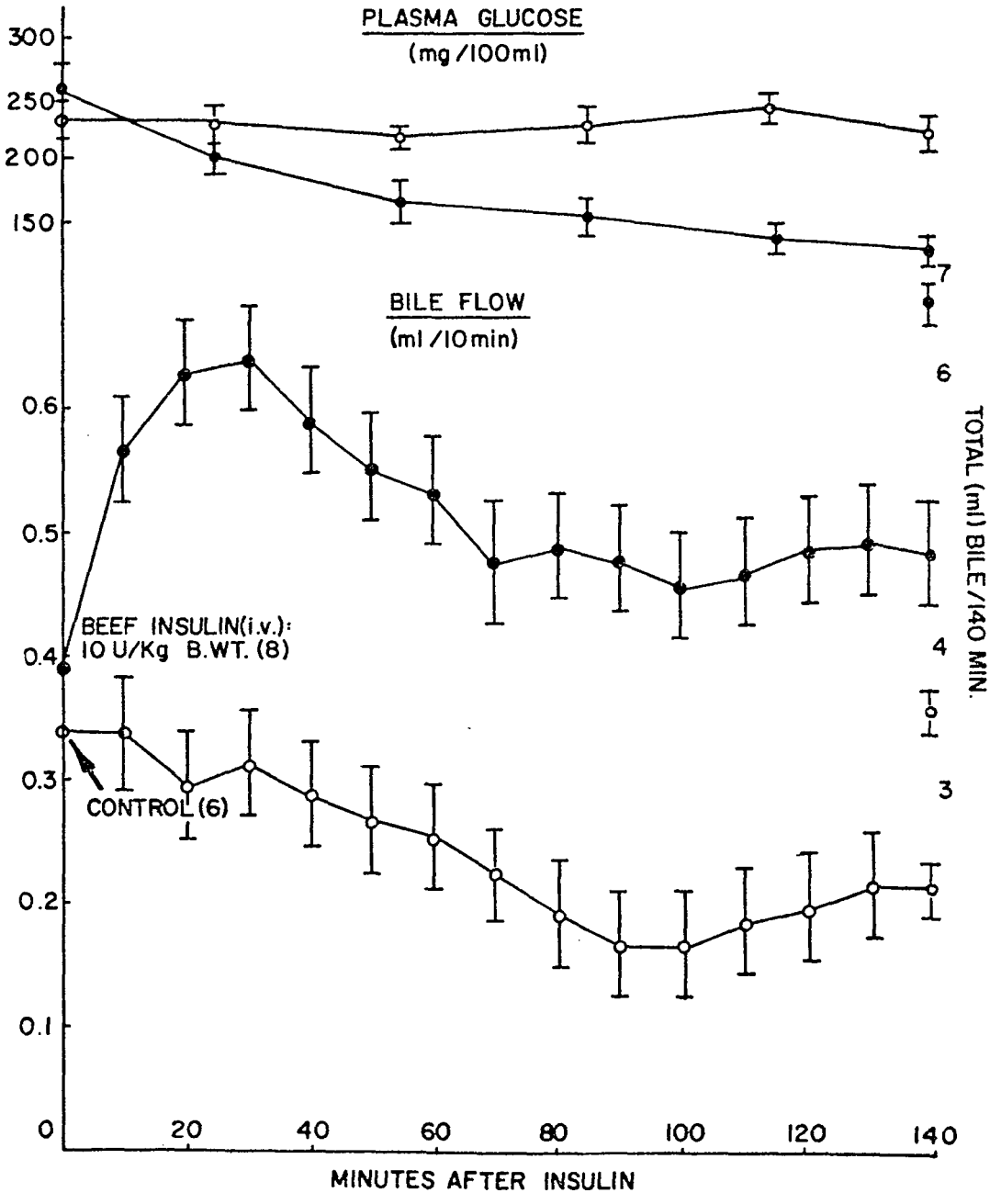
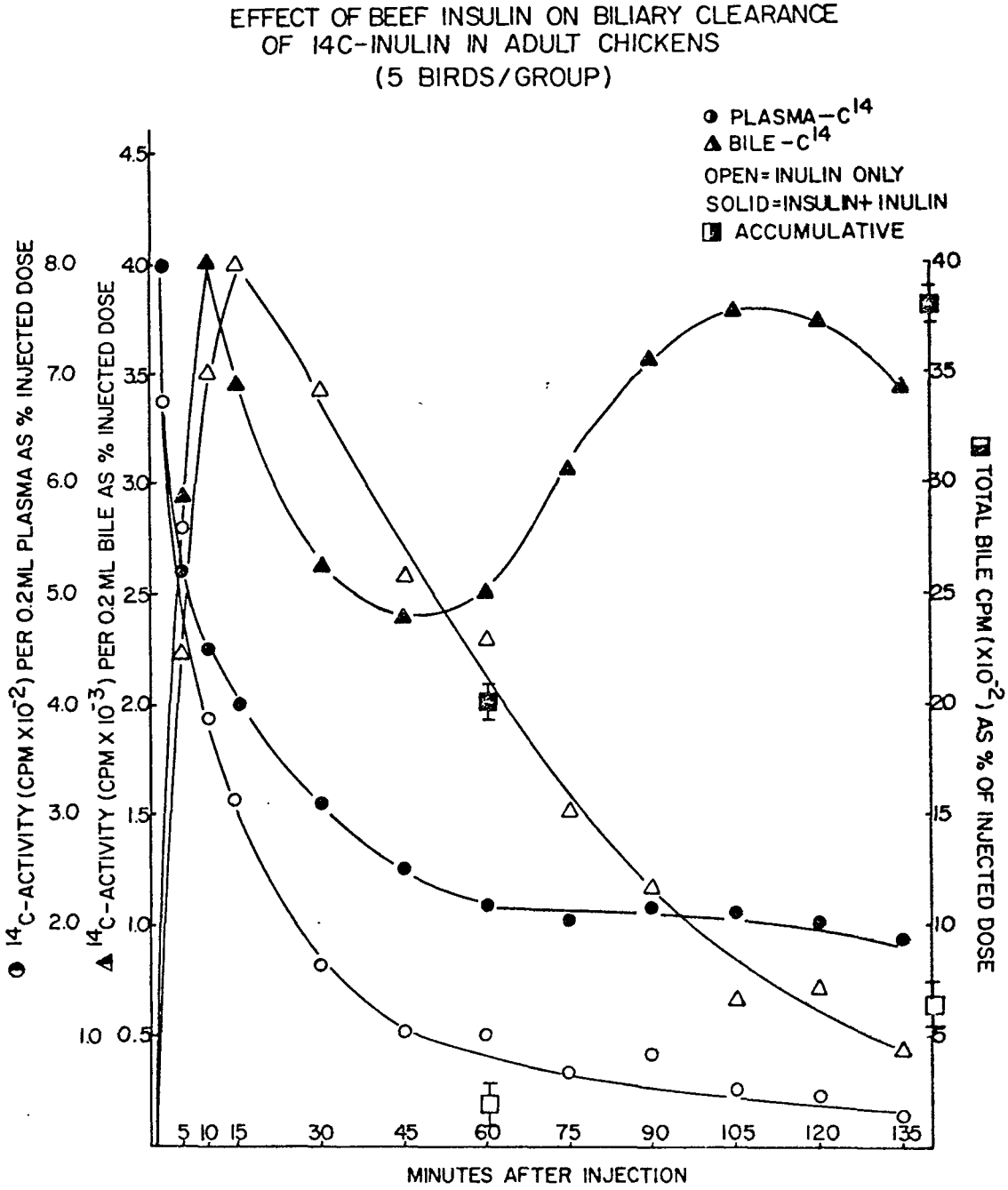


Figure 3

EFFECT OF BEEF INSULIN ON BILIARY CLEARANCE OF ^{14}C-INULIN IN ADULT CHICKENS

The effect of beef insulin on biliary clearance of ^{14}C-inulin in chickens. The isolated points at 60 and 140 minutes indicate the accumulative amounts of ^{14}C-inulin excreted at 60 and 140 minutes, respectively. S.E.M.'s were omitted from the other points for clarity. In each animal 1.8 μCi of inulin carboxyl-^{14}C (2.7 μCi/mg) and 10 U beef insulin/kg body weight were injected simultaneously. Each group contained five adult chickens.


EFFECT OF BEEF INSULIN ON BILIARY CLEARANCE
OF 14C-INULIN IN ADULT CHICKENS
(5 BIRDS/GROUP)
● PLASMA-C14
▲ BILE-C14
OPEN= INULIN ONLY
SOLID=INSULIN+ INULIN
ACCUMULATIVE
● 14C-ACTIVITY (CPM X 10-2) PER 0.2ML PLASMA AS % INJECTED DOSE
▲ 14C-ACTIVITY (CPM X 10-3) PER 0.2ML BILE AS % INJECTED DOSE
TOTAL BILE CPM (X10-2) AS % OF INJECTED DOSE
MINUTES AFTER INJECTION
5 10 15 30 45 60 75 90 105 120 135

was an increased rate of appearance of ^{14}C-inulin in bile and therefore a reduction in the time elapsed before the peak in ^{14}C excretion occurred. Also, the clearance rate was maintained at high levels during the remainder of the collection periods. Thus, a higher total amount of ^{14}C-inulin was cleared both at 60 minutes and at 15 minutes in the insulin-treated animals than was cleared in the control birds (Fig. 3). When the data on biliary appearance of radioactivity was recast in terms of total (accumulative) amount of the activity present in the bile (Fig. 4) the increase in inulin clearance can be seen clearly. Comparison of the areas under the two curves reveals that injection of insulin increases inulin-^{14}C clearance approximately 1.8 times.

After establishing the insulin effect on biliary flow and ^{14}C-inulin clearance, it was felt necessary to determine if these effects were due to the hormone per se (as opposed to the presence of a foreign protein) and to determine what role the vagi play, if any, in the increased biliary flow observed. Bovine serum albumin (0.43 mg, Sigma, crystalline and lyopholized/kg body weight) was administered (i.v.) to adult female chickens in amounts equal to the protein contained in the insulin used in the previous study. Such injections resulted in no significant changes in bile flow rate (Table 1) from three ten-minute control periods.

Figure 4

BILIARY CLEARANCE OF ^{14}C-INULIN IN ADULT CHICKENS

Biliary clearance of ^{14}C-inulin in adult chickens. Data presented here are the same as in Figure 3; however, the data are presented here in terms of <u>total amount</u> appearing in the bile rather than as a concentration. See Figure 3 for ^{14}C doses administered. Vertical bars are S.E.M.'s for five observations in each group.

BILIARY CLEARANCE OF ^{14}C-INULIN IN ADULT FEMALE CHICKENS: EFFECT OF INSULIN

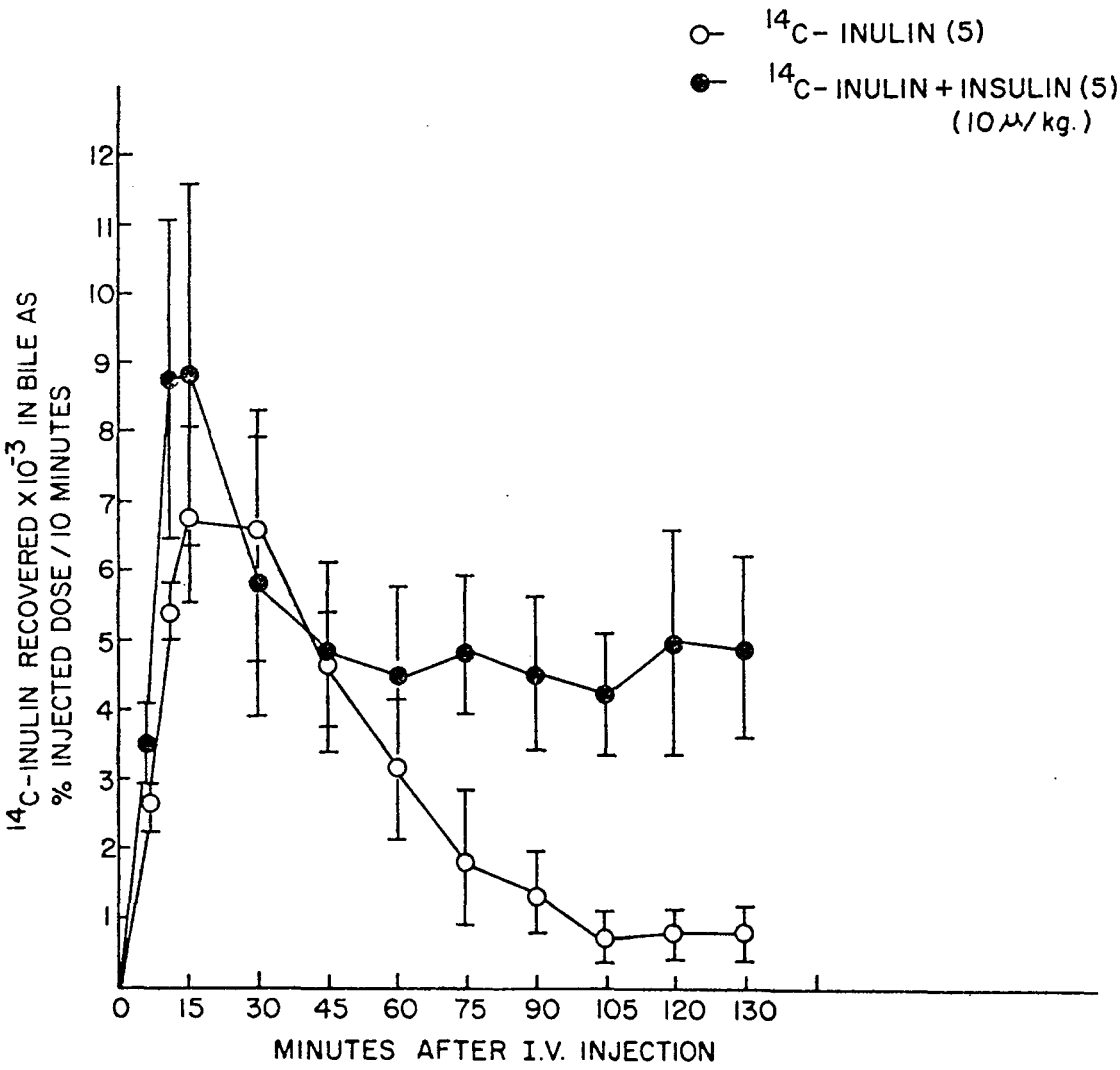


TABLE 1

EFFECT OF BOVINE ALBUMIN INJECTION (IV) ON BILE FLOW OF ADULT CHICKENS*

(ml/10 min)

CONTROL PERIOD			MINUTES AFTER ALBUMIN INJECTION (I.V.)												
-20**	-10	0	+10	+20	30	40	50	60	70	80	90	100	110	120	130	140
0.26+ ± 0.02	0.20 ± 0.04	0.21 ± 0.03	0.22 ± 0.03	0.22 ± 0.03	0.21 ± 0.04	0.20 ± 0.01	0.21 ± 0.01	0.21 ± 0.02	0.19 ± 0.02	0.20 ± 0.01	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.03	0.25 ± 0.03	0.22 ± 0.03	0.24 ± 0.02

*Albumin injected: 0.43 mg/kg body weight.
**Time in minutes
+Mean ± S.E.M. for N=6.

Another group of birds was bilaterally vagotomized (cervical region) 30 minutes prior to the injection of insulin (10 U/kg). Respiratory rates of these birds decreased from 42 ± 3 to 10 ± 1 breaths/minute after vagotomy indicating completeness of the surgical maneuver. The effects of insulin injection on bile flow are shown in Figure 5 along with concomitant changes in plasma glucose. It can be seen that despite the depression in plasma glucose, no increase in biliary flow above control levels was observed after insulin injection. There was evidence, however, of a tonic vagal effect on bile flow prior to bilateral vagotomy.

Once the insulin effect on biliary flow was established it was of interest to determine if the magnitude of insulin appearance in bile was dependent upon injection of large doses of insulin. Figure 6 presents results of a rat hemi-diaphragm bioassay for the insulin-like action (ILA) of chicken bile which was collected after injection of bovine insulin (10 U/kg). In such a bioassay, the isolated diaphragm is bisected and each hemi-diaphragm is incubated in separate flasks, one half serving as control for the other half. Control flasks contained KRB only; experimental flasks contained bile (taken at various times after <u>in vivo</u> injection of insulin) in addition to the KRB media. The absence of glucose-uptake in the presence of bile was unexpected. The last 30-minute sample (taken 120 minutes after the

Figure 5

EFFECT OF BILATERAL VAGOTOMY ON INSULIN STIMULATED BILE FLOW IN ADULT CHICKENS

The effect of bilateral (cervical) vagotomy on insulin-stimulated bile flow. Vertical bars are S.E.M. The top tracing presents plasma glucose changes; the bottom tracing is change in bile flow. Vagotomy was performed at 30 minutes and insulin injection (10 U/kg) 30 minutes later, i.e., 60 minutes after the start of the experimental period. Completeness of vagotomy was observed by a profound decrease in respiratory rate from 42 ± 3 to 10 ± 1 breaths/minute.

EFFECT OF BILATERAL VAGOTOMY ON INSULIN STIMULATED BILE FLOW IN ADULT CHICKENS

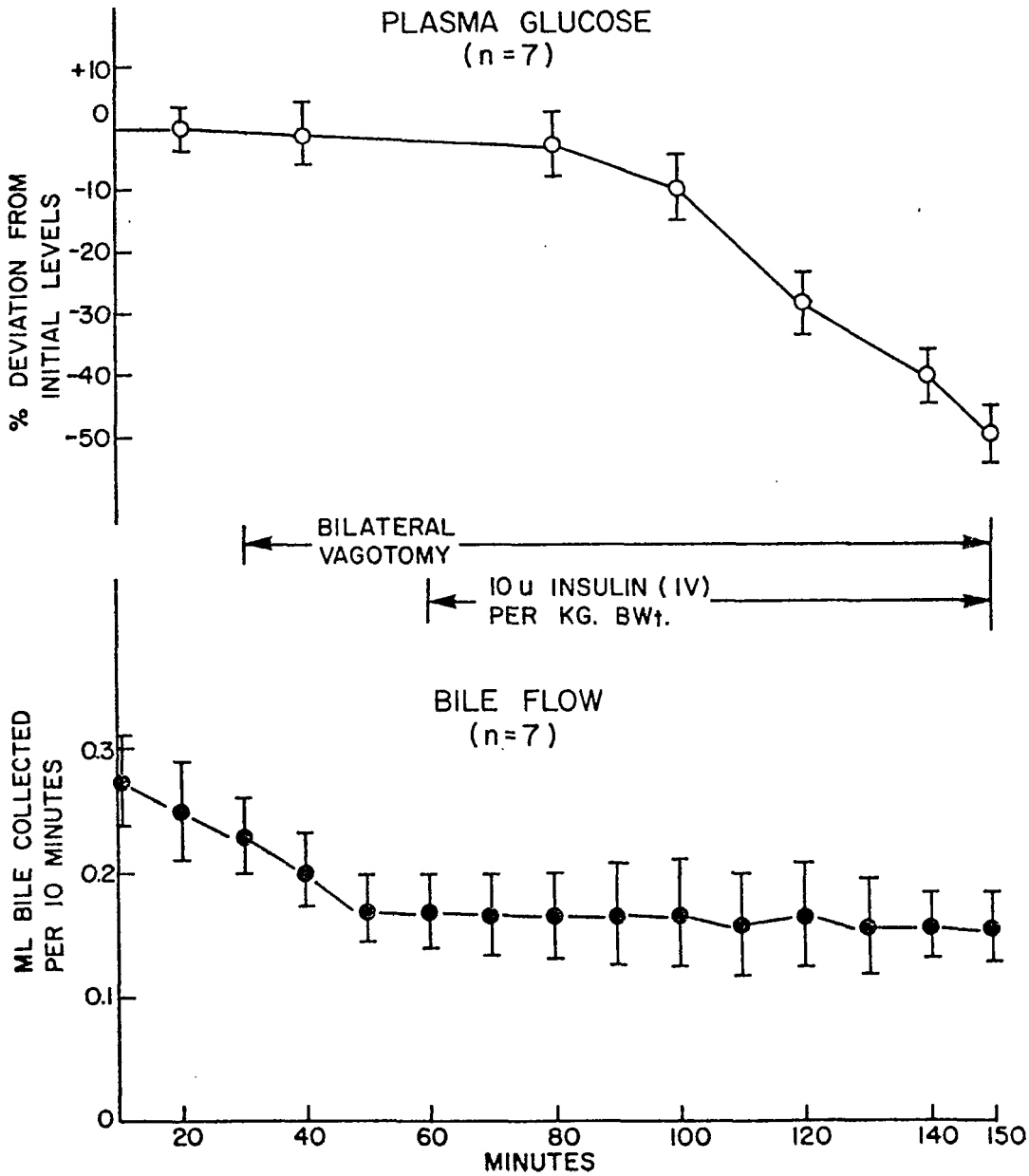
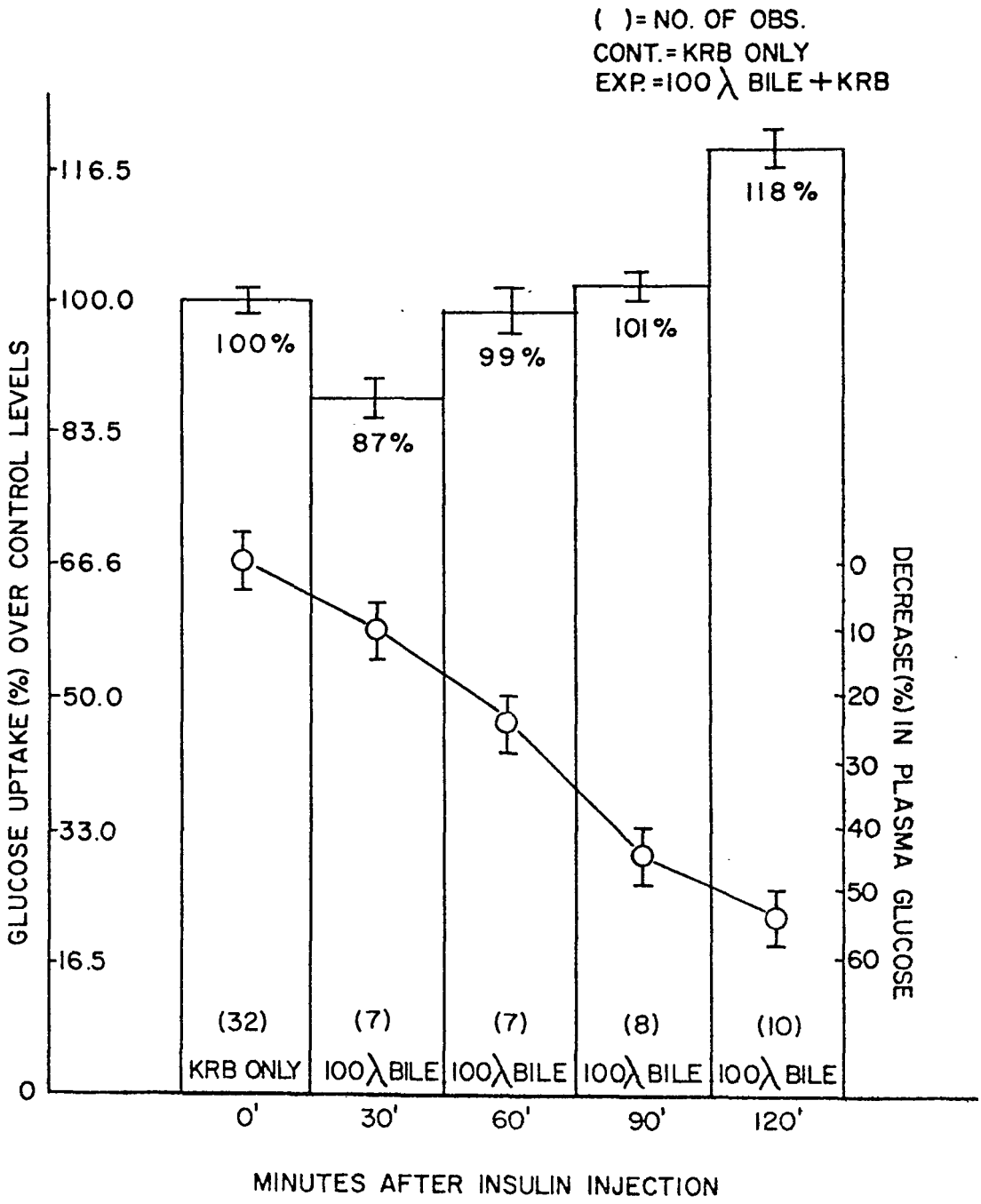


Figure 6

INSULIN-LIKE ACTION OF CHICKEN BILE AFTER THE INJECTION OF BEEF INSULIN (10 U/kg) INTO ADULT CHICKENS

The insulin-like action (ILA) of chicken bile after the injection of beef insulin (10 U/kg). The line graph within the histogram indicates changes in plasma glucose after insulin injection; the ordinate is at the right. Vertical bars are S.E.M. ( ) along abscissa represent number of observations at each timed-collection. Note: The control hemi-diaphragm flasks contained only KRB media. Accounting was taken in calculations for the dilution effect (of initial glucose levels) by adding bile to the experimental flasks.

ILA OF CHICKEN BILE AFTER INJECTION OF BEEF INSULIN (10 U/KG) INTO ADULT SCWL CHICKENS



insulin injection) was the only one indicating significant ILA above control levels ($P<.001$). The possible inhibitory effect of normal bile on glucose uptake by skeletal muscle then was considered a possible explanation of these results. To test this possibility, varying amounts of normal bile collected from uninjected chickens were added to the incubation media prior to the incubation of the hemi-diaphragm. The results of this bioassay when compared to KRB (non-bile) controls are shown in Figure 7. It can be seen that as the amount of bile was increased, the glucose uptake by the hemi-diaphragm decreased ($P = .10$ for 50 lambda of bile). Addition of 100 lambda of bile significantly decreased glucose uptake from the 50-lambda group ($P<.025$) and the control group ($P<.01$). The 150-lambda group significantly differed from the 100-lambda group ($P<.05$). These results indicated that perhaps this inhibitory effect of normal bile on glucose uptake masked any simultaneous stimulatory effect of insulin present in the same bile sample. Therefore, it was necessary to repeat the experiment presented in Figure 6 using control media containing known amounts of normal bile per control flask. This would compensate for the 100 lambda of experimental bile added to the experimental flasks. The results of such a study are shown in Figure 8. These data indicate an increase ($P<.01$) in insulin-like action 30 to 60 minutes after hormone injection. Other data, not presented

Figure 7

INFLUENCE OF CHICKEN BILE ON GLUCOSE UPTAKE BY THE RAT HEMI-DIAPHRAGM

The influence of normal chicken bile on glucose uptake by the rat hemi-diaphragm. The amount (lambda) of bile added to the incubation media is indicated at the bottom of each histogram. In calculation, account was taken of the glucose dilution effect by adding bile to the media. Vertical bars are S.E.M. The mean plasma glucose of the chickens used as a bile source was 257 mg%.

INFLUENCE OF CHICKEN BILE ON GLUCOSE UPTAKE
BY THE RAT HEMIDIAPHRAGM

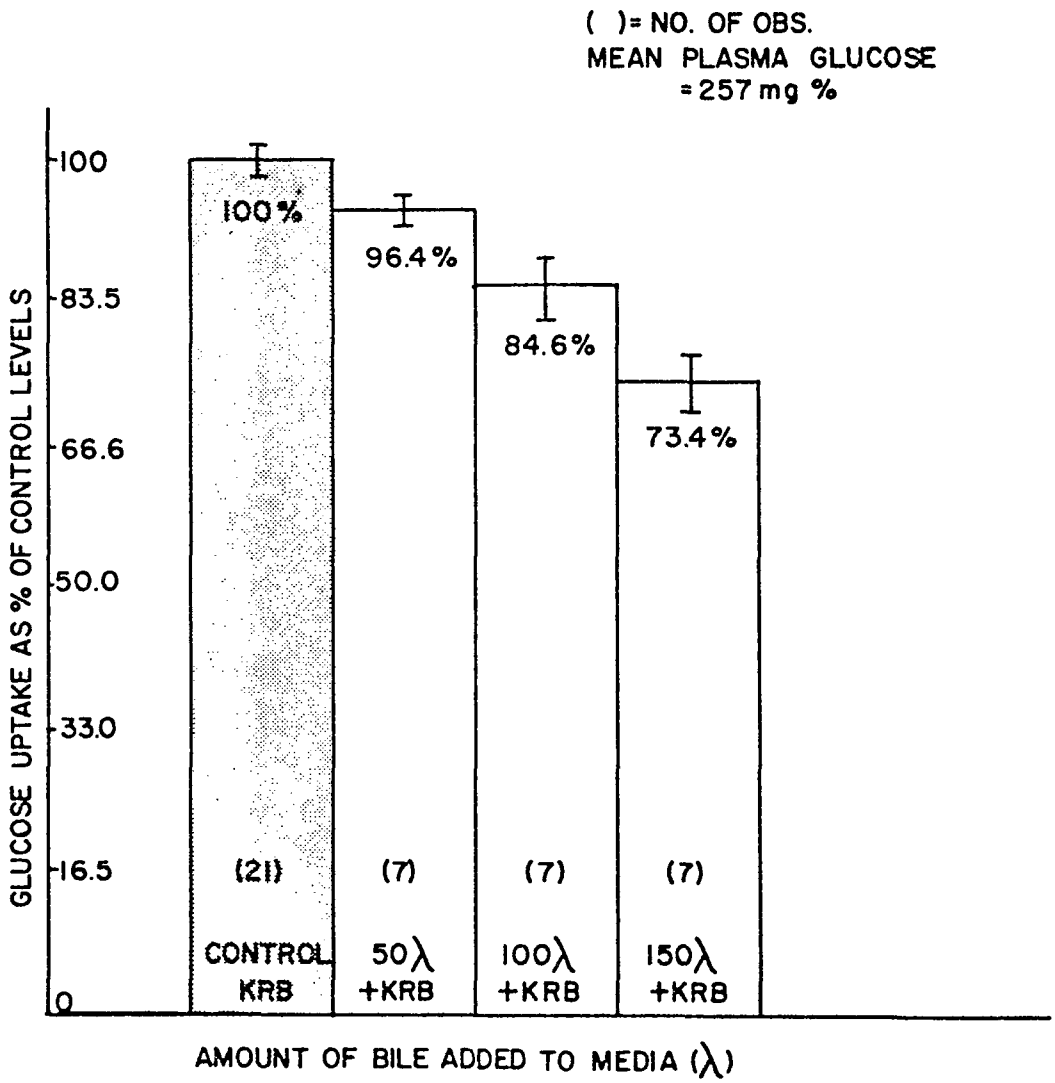
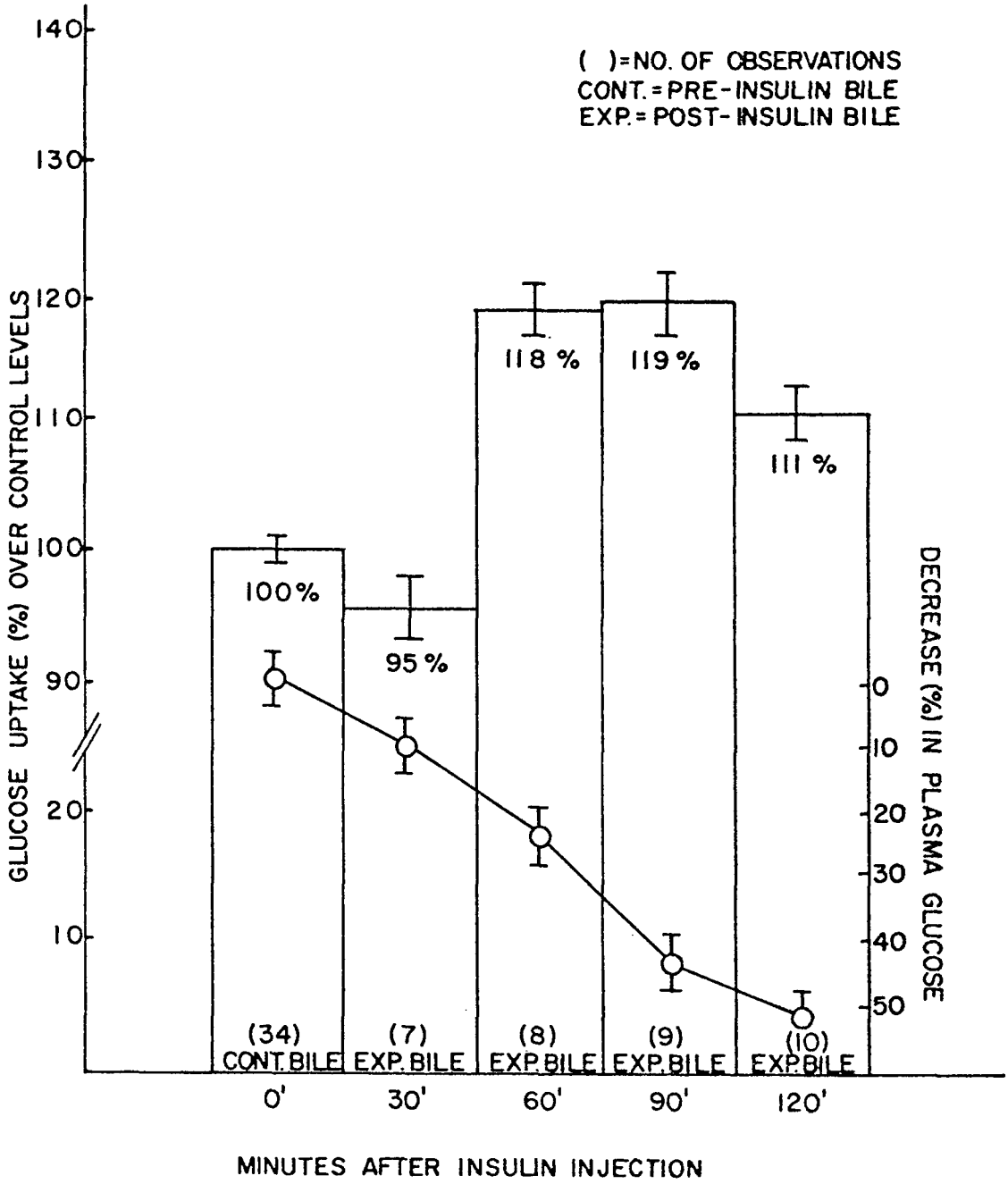


Figure 8

INSULIN-LIKE ACTION OF CHICKEN BILE AFTER THE INJECTION OF 10 U BEEF INSULIN/KG BODY WEIGHT IN ADULT CHICKENS

Insulin-like action of bile after injection of beef insulin (10 U/kg, i.v.) in adult chickens. The procedure followed is the same presented in Figure 6; however, here, 100 lambda pre-insulin (control) bile was added to the control flasks. Plasma glucose changes of the experimental animals are known as a line graph within the ILA histogram. Vertical bars are S.E.M. ( ) represent the number of observations at each timed-collection.

ILA OF CHICKEN BILE AFTER INJECTION OF 10 U BEEF INSULIN / KG BODY WEIGHT INTO ADULT CHICKENS



in this figure, suggest that the ILA was 118 ± 2% of control levels from 120 to 150 minutes after injection, and 115 ± 2% of control levels 150 to 180 minutes after insulin injection.

To complete this evaluation of biologically active insulin in chicken bile it appeared necessary to determine whether or not endogenous insulin affects the ILA content of bile. Figure 9 presents the results when tolbutamide was injected (i.v.) causing release of pre-formed insulin from the avian pancreas. An increase in biliary ILA was apparent in 30 minutes ($P<.001$) followed by a second peak starting between 60 and 90 minutes after tolbutamide injection ($P<.001$). The second peak was maintained at 130 ± 3% of control at 120 to 150 minutes and 120 ± 3% at 150 to 180 minutes after tolbutamide injection.

Collectively, the foregoing studies indicated that biliary excretion of endogenous and exogenous insulin is a plausible physiological mechanism in Aves for ridding the animal of "excess" hormone. Further, large doses of insulin increase biliary excretion of the hormone.

II. What proportion of biliary excreted insulin is degraded or inactive?

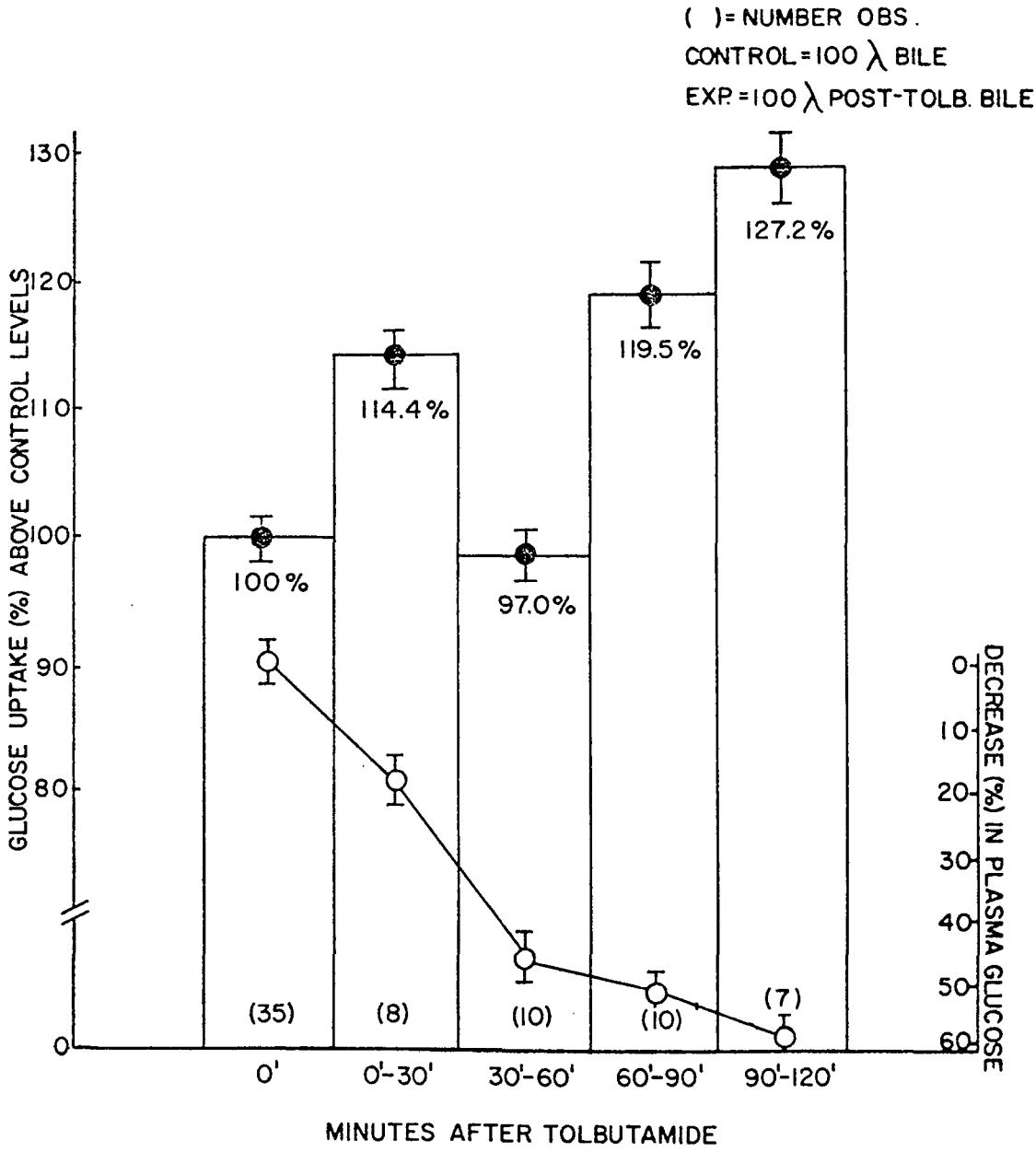
The above studies provided information on the existence of biologically active insulin excreted in the bile. Further investigation was carried out to quantitate how much degraded, therefore biologically inactive, insulin was excreted in bile.

Figure 9

INSULIN-LIKE ACTION OF CHICKEN BILE AFTER SODIUM TOLBUTAMIDE (30 mg/kg BODY WEIGHT)

Insulin-like action (ILA) of bile after injection of sodium tolbutamide (30 mg/kg). The procedure followed was the same as in Figure 8. Plasma glucose changes are shown as a line graph within the histogram. Vertical bars are S.E.M.

ILA OF CHICKEN BILE AFTER SODIUM TOLBUTAMIDE
(30 MG/KG BODY WT.)



This was accomplished by the injection of trace amounts (0.5 μU) of ^{125}I-labelled ox insulin (i.v.) and measuring the amount of immunoreactive label as compared to total label recovered in the bile of adult chickens. The results of this study are presented in Table 2. The amount of insulin injected into the wing vein (0.02 μg, 0.5 μU) was not enough to alter plasma glucose levels of the bird significantly (225 vs 231 mg%); neither was there any observed increase in biliary flow rate. The results indicated that ^{125}I activity in the plasma rapidly falls during the first 15 minutes after injection. This decrease was followed by much slower loss of activity from the plasma for the remainder of the observation period.

Despite the high total plasma ^{125}I activity in the first 15-minute sample only a small percent of this label was immunoreactive (9.33 ± 0.11%). This trend persisted throughout the experiment despite the fact that approximately 92% of the activity in the initial injectant was shown to be bound in insulin by TCA precipitation and immunoassay.

After ^{125}I injection, the radioactivity of the bile rapidly increased in the first 15 minutes to a level of about one-half that of the plasma. Following this period the biliary radioactivity fell off rapidly, though at a rate slower than that of plasma. The total amount of ^{125}I removed during each 15-minute collection period decreased at a more

TABLE 2

BILIARY CLEARANCE OF ^{125}I-INSULIN FROM PLASMA OF ADULT CHICKENS

(WING VEIN INJECTION)

PARAMETER	UNITS	MINUTES AFTER ^{125}I-INSULIN INJECTION					
		15*	30*	45*	60	75	90
Plasma CPM (Total)	cpm/ml	26,093** ± 761	5614 ±411	4506 ± 216	2365 ± 174	1922 ± 94	1683 ± 81
Plasma CPM (Bound)	cpm/ml	2420 ± 52	1153 ± 35	2215 ± 43	1552 ± 20	396 ± 4	351 ± 5
^{125}I Bound in Plasma	%	9.33 ±0.11	21.0 ± 9.2	49.6 ±17.1	66.2 ±15.2	20.9 ± 5.2	21.0 ± 3.6
Bile Flow	ml/15 min	0.49 ±0.03	0.46 ±0.02	0.43 ±0.07	0.35 ±0.05	0.29 ±0.02	0.27 ±0.07
Bile CPM	cpm/15 min	5936 ± 52	4526 ± 25	2441 ± 31	1287 ± 36	581 ± 20	745 ± 20
Bile CPM (Total)	cpm/ml	12,115 ± 106	9838 ± 86	5677 ± 42	3677 ± 33	2004 ± 20	2741 ± 1
Bile CPM (Bound)	cpm/ml	488 ± 2	309 ± 6	235 ± 4	197 ± 1	153 ± 3	81 ± 1
^{125}I Bound in Bile	%	4.03 ±0.1	3.19 .0.1	4.14 ±0.1	5.36 ±0.2	7.64 ±0.2	3.04 ±0.1
Plasma Vol. Cleared by Liver	ml/min	0.018 ±0.009	0.054 ±0.009	0.036 ±0.003	0.036 ±0.006	0.020 ±0.002	0.027 ±0.004

** Mean ± SEM *No. Obs. = 6 at these times; all other data based on 5 obs.

Note: 1.5 μCi of ^{125}I-insulin (75 μCi/μg bovine insulin) injected at time '0' into alar vein.

constant rate than the specific activity of the bile, reflecting the gradual decrease in bile flow with time. The percentage of immunoreactive radioactivity as compared to the total radioactivity in the bile per 15-minute bile sample remained very small throughout the observation period (3.19 - 7.64%). Calculation of the rate of which ^{125}I was cleared from the plasma indicates an initial high clearance rate, followed by a much lower, though relatively constant, rate.

Observation of the rapid disappearance of ^{125}I activity from the plasma, which was not reflected by the appearance of a high radioactivity in the bile, indicated that the ^{125}I-label may have extravasated and was diluted in extracellular fluid. To evaluate this possibility, injections of the same ^{125}I-insulin dose and specific activity were made into the pancreatic vein. This approach was indicated for two reasons: the pancreatic vein leads directly to the liver via the hepatic-portal vein, reducing the possibility of major dilution before the bolus of ^{125}I reached the liver; also, the pancreatic vein is the route normally followed by endogenous insulin after it is released from the pancreas. As with the previous study, the amount of insulin injected was not enough to cause significant alterations in the plasma glucose or bile flow.

The results of this study are shown in Table 3 and indicate that a more rapid initial loss of plasma activity

TABLE 3

BILIARY CLEARANCE OF ^{125}I-INSULIN FROM PLASMA OF ADULT CHICKENS

(PANCREATIC VEIN INJECTION)

PARAMETER	UNITS	MINUTES AFTER ^{125}I-INSULIN INJECTION					
		15*	30	45	60	75	90
Plasma CPM (Total)	cpm/ml	4219** ± 252	3676 ± 196	3188 ± 188	2955 ± 101	2209 ± 96	2508 ± 103
Plasma CPM (Bound)	cpm/ml	1539 ± 100	1559 ± 102	898 ± 54	723 ± 49	653 ± 28	644 ± 35
^{125}I Bound in Plasma	%	32.2 ± 8.0	43.2 ± 9.3	28.0 ± 7.1	24.0 ± 3.2	30.0 ± 4.6	25.0 ± 5.9
Bile Flow	ml/15 min	0.35 ±0.07	0.34 ±0.03	0.27 ±0.02	0.25 ±0.09	0.23 ±0.01	0.20 ±0.02
Bile CPM	cpm/15 min	1667 ± 42	1963 ± 52	1263 ± 19	1025 ± 46	907 ± 20	720 ± 20
Bile CPM (Total)	cpm/ml	4763 ± 76	5774 ± 52	4678 ± 56	4102 ± 21	3942 ± 20	3599 ± 26
Bile CPM (Bound)	cpm/ml	481 ± 27	601 ± 32	433 ± 25	344 ± 20	324 ± 43	305 ± 30
^{125}I Bound in Bile	%	10.2 ± 1.1	10.7 ± 0.9	9.5 ± 0.7	8.4 ± 0.8	8.1 ± 0.5	8.5 ± 5.9
Plasma Vol. Cleared by Liver	ml/min	0.027 ±0.003	0.036 ±0.003	0.027 ±0.001	0.023 ±0.002	0.027 ±0.002	0.019 ±0.002

** Mean ± SEM *No. Obs. = 6 at all time-samples.

Note: 1.5 μCi of ^{125}I-insulin (75 μCi/μg bovine insulin) injected at time '0' into pancreatic vein.

occurred than when the injection was made into the alar vein; however, the loss of plasma radioactivity was slower during the remaining experimental periods. More of the activity present in the plasma was in an immunoreactive form than in the alar vein-injected birds. It also can be seen that the ^{125}I-activity appeared more slowly in the bile than in the peripherally injected birds but was maintained at a higher level and with a greater percentage of the label in the immunoreactive form. Except for the elimination of the initially high clearance rate, the clearance of ^{125}I from the plasma remained relatively unchanged as compared to peripherally injected birds. It may be concluded that while biliary clearance of insulin probably occurs *in vivo*, this system does not excrete more than 0.25% of the total available hormone.

III. Does a newly isolated avian pancreatic polypeptide influence biliary excretion?

In an effort to determine what possible effect the recently isolated pancreatic polypeptide (APP) might have on insulin excretion, preliminary studies were performed on small groups of adult chickens. Adult SCWL chickens were anesthetized and the bile ducts and the pancreatic ducts cannulated. Due to the presence of the proventriculus on the opposite side of the animal the proventriculus was cannulated in a separate group of birds. Typical results of this study are

shown in Table 4. It can be seen that no effect could be observed on the secretion (or lack of it) from the two biliary excretion routes, nor were there any observed responses by the exocrine pancreas. There was, however, a marked increase in the volume of secretion from the proventriculus ("secretory stomach") of the adult chicken.

Quantitation of the proventricular secretion effect was pursued by determining the response of the proventriculus to graded doses of avian pancreatic polypeptide (APP). The results presented in Figure 10 indicate an immediate increase in proventricular secretion which was maintained for a minimum of 80 minutes after APP injection. Increasing the injected dose (from 12.5 to 25.0 or 50.0 ug APP/kg) resulted in a related increase in the proventricular flow rate. However, no significant changes in plasma glucose levels were observed regardless of the APP dose level. A reference standard of 1.0 ug pentagastrin/kg body weight was used to check the viability of the surgical preparation. Pentagastrin (Pentavalon, Ayerst) is a synthetic gastric secretagogue (an agent which promotes secretion) modeled after the essential terminal five amino acid residues of mammalian gastrin.

In order to determine whether APP acts directly on the proventriculus to increase flow rate or whether it acts via a vagal mechanism, a group of chickens was subjected to bilateral vagotomy prior to APP injection (25 µg/kg). The results

TABLE 4

THE EFFECT OF APP ON THE VOLUME FLOW OF SELECTED GASTROINTESTINAL SECRETIONS IN ADULTS CHICKENS (25 µg/kg i.v.)

Source of Secretion	CONTROL		MINUTES AFTER APP INJECTION								
	-10	0	10	20	30	40	50	60	70	80	90
Bile (Hepatic)	0.81*	0.80	0.74	0.67	0.63	0.65	0.62	0.66	0.65	0.55	0.48
Bile (Cystic)	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Exocrine Pancreas**	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Proventriculus	0.26*	0.25	0.60	0.51	0.35	0.32	0.30	0.28	0.28	0.25	0.26

Note: These data are from two adult chickens.

*Milliliters collected in 10 minutes.

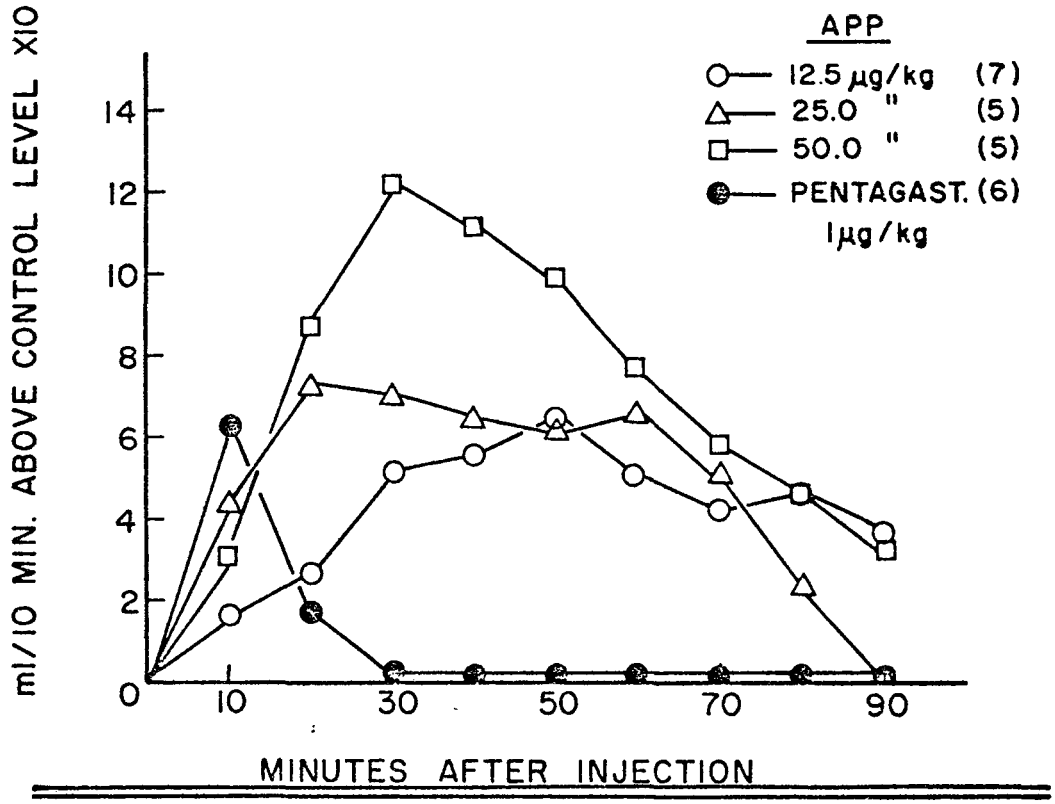
**Total (simultaneous) secretion from three pancreatic ducts.

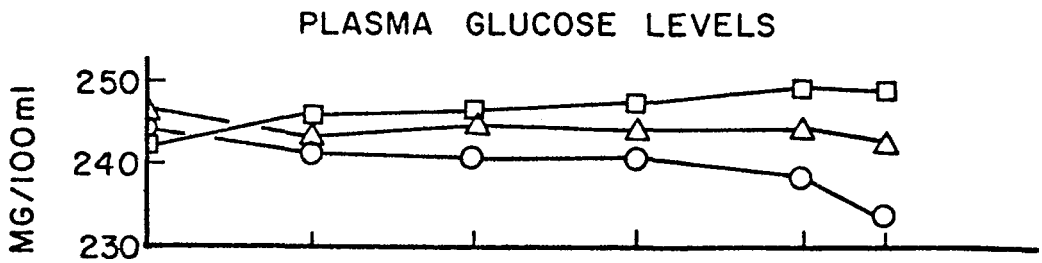
Figure 10

PROVENTRICULAR (SECRETORY) RESPONSE TO APP

Proventricular secretory response to graded doses of APP (Avian Pancreatic Polypeptide). The pentagastrin (Pentavalon, Ayerst) response is shown as a viability reference. Plasma glucose levels obtained during the course of the experiment are shown at the bottom of the figure. Standard errors of the mean (S.E.M.) were very small and are omitted for clarity. They were small enough, however, to indicate significant differences in proventricular flow for the three dose levels of APP up to 60 minutes after injection. No significant differences or changes were observed in the plasma glucose levels. ( ) represent the number of observations in each group.

PROVENTRICULAR (SECRETORY) RESPONSE TO APP





are shown in Figure 11. Immediately after vagotomy ($\bar{V}$) there was an increase in proventricular secretion. This is attributable to the surgical manipulation of the vagi during ligature and section. The small but rapid decrease in proventricular secretion observed following vagal sectioning was probably the result of the release of tonic vagal activity. After injection of APP (25 µg/kg) there was a rapid but slightly depressed increase in proventricular secretion. The respiratory rate of the birds decreased from 39 ± 4 to 9 ± 1 breaths per minute after vagotomy, indicating completeness of the surgery.

Prior to the very recent (1972) isolation and purification of chicken glucagon by Dr. J. R. Kimmel, there existed the distinct possibility that APP was indeed chicken glucagon. Also, the possibility that APP was contaminated with glucagon existed. Therefore, it was necessary to observe the effect of mammalian glucagon on proventricular secretion to establish similarity or differences with that obtained with APP. It can be seen in Figure 12 that when either equimolar (23 µg/kg) or large doses (100 µg/kg) of mammalian glucagon were injected (i.v.) into birds there was no increase in proventricular flow, despite plasma glucose increases of 150 mg% at the higher level. Very recently chicken glucagon became available in purified form. It was advisable to repeat the above experiment using the isologous hormone.

Figure 11

PROVENTRICULAR SECRETION AFTER BILATERAL VAGOTOMY

The effect of APP on proventricular secretion after bilateral (cervical) vagotomy ($\bar{V}$). Note: The small increase in proventricular flow occurring at -20 minutes was attributed to stretch, ligation and section of both vagi. Increase in flow thus observed invariably occurred immediately after vagal section and did not persist during the remainder of the 10-minute collection period. APP was administered (i.v.) 30 minutes after bilateral vagotomy. The success of the vagotomy was observed by a decrease in the respiratory rate from 30 ± 4 to 9 ± 1 breaths/minute. Vertical bars are S.E.M. and were obtained on six adult chickens.

PROVENTRICULAR SECRETION AFTER BILATERAL VAGOTOMY : EFFECT OF APP

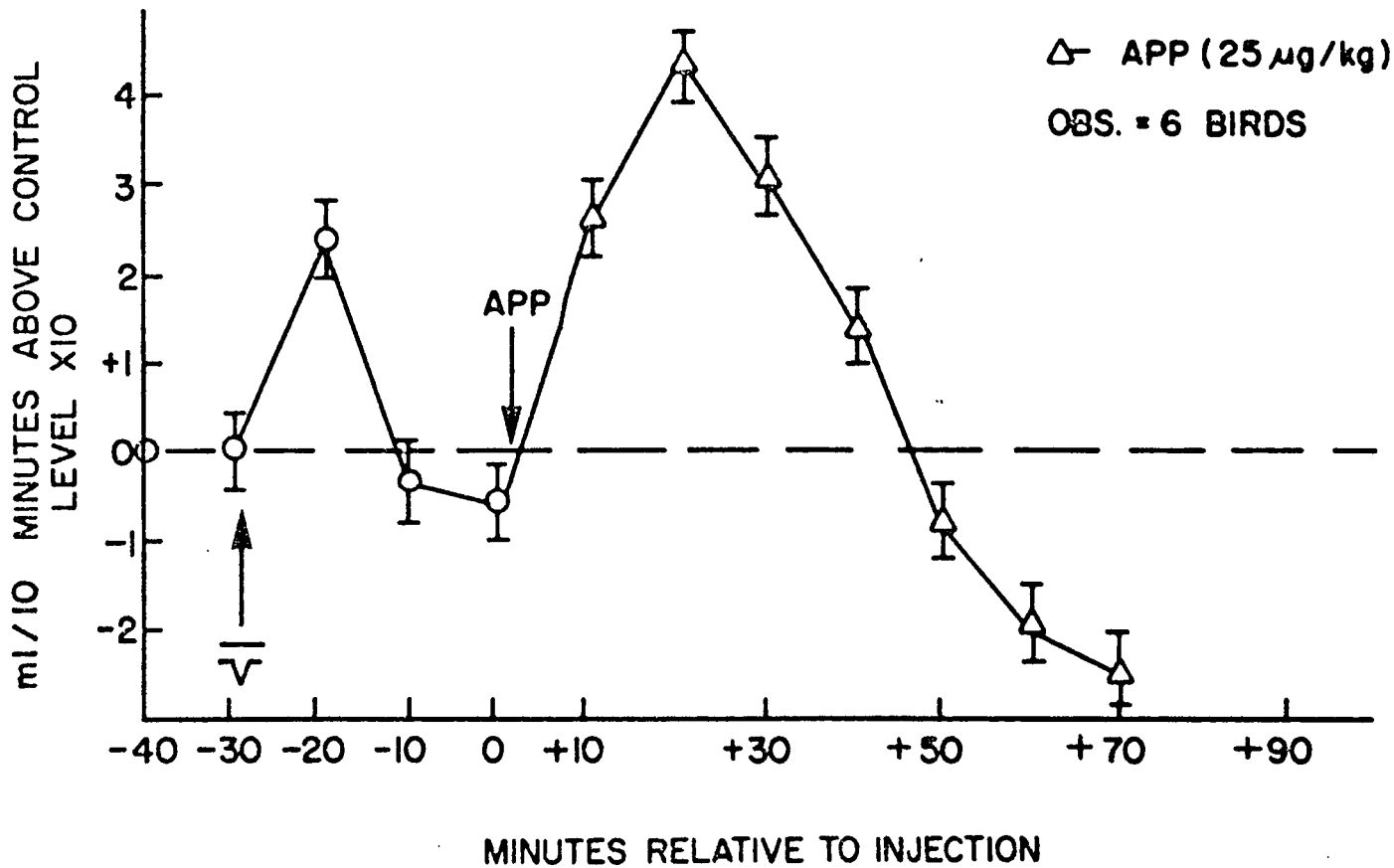
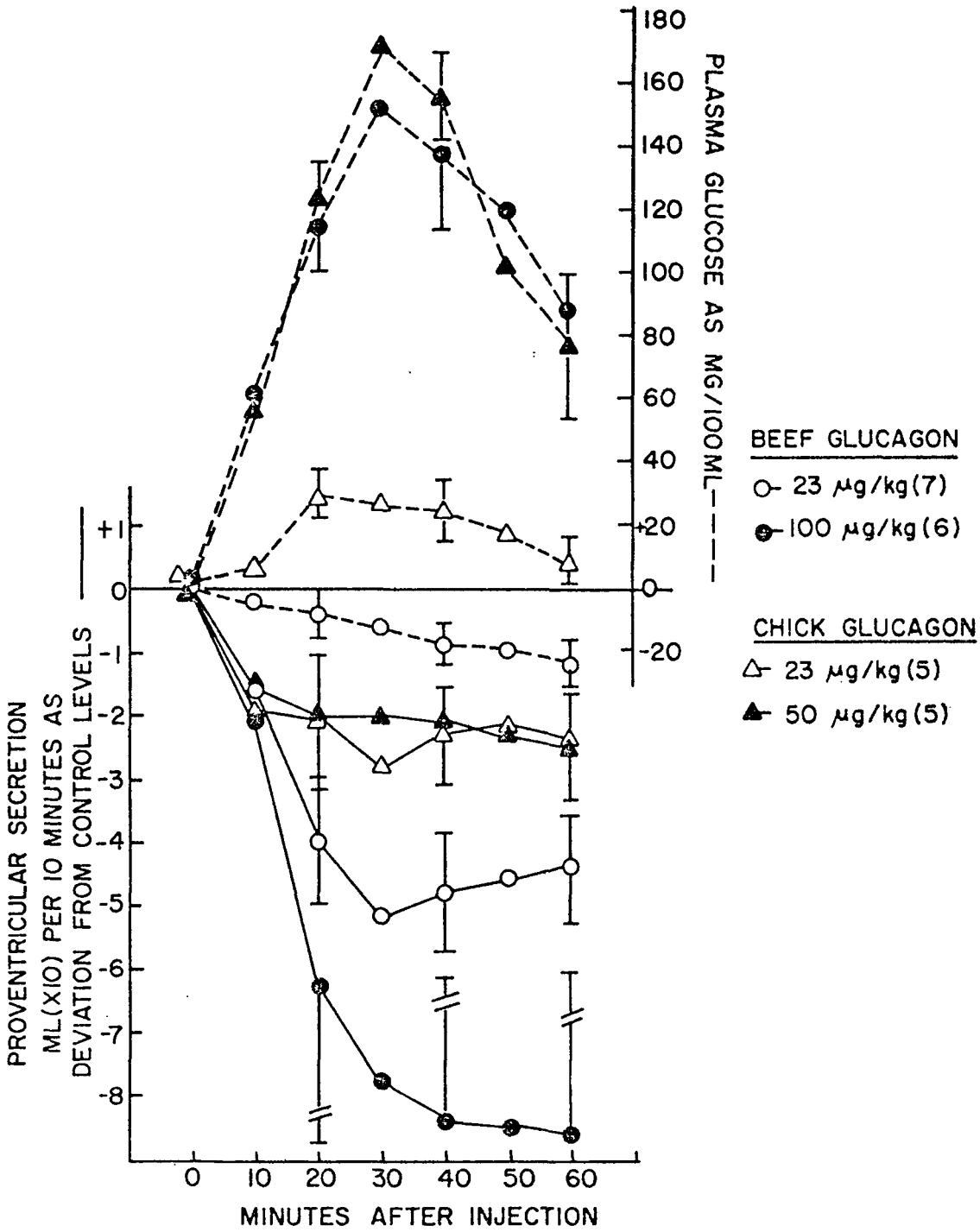


Figure 12

EFFECTS OF GLUCAGON INJECTION ON PROVENTRICULAR SECRETION IN CHICKENS

The effect of glucagon injection on proventricular flow in chickens. The upper half of the figure represents changes in plasma glucose concomitant with depressions in proventricular secretion which are shown in the lower half of the figure. Vertical bars are representative S.E.M. Number of observations are in ( ).

EFFECTS OF GLUCAGON INJECTION ON
PROVENTRICULAR SECRETION IN CHICKENS



The only change necessary was the reduction of the larger dose of glucagon to 50 µg/kg due to the greater sensitivity of the bird to a isologous substance. The results (Fig. 12) once again indicated no increase in proventricular secretion despite the perturbation in plasma glucose produced by both glucagon doses.

Examination of the possible role played, if any, by the circulatory system was made in an effort to separate any proventricular response to APP from generalized, systemic cardiovascular changes. Heart rate, respiratory rate, systolic pressure, diastolic pressure, pulse pressure and mean pressures were measured on an E & M Physiograph equipped with linear core pressure transducers and an impedence pneumograph. Adult chickens were injected with APP (25µg/kg) or pentagastrin (1 µg/kg) intravenously. The results obtained (Tables 5 and 6) indicated no significant alteration of these parameters by either of the test substances. Further examination of the effect of 12.5 µg and 50.0 µg APP/kg body weight on these parameters (not presented here) produced equally negative results, as did doses of 0.5 µg and 25.0 µg pentagastrin/kg body weight.

Additional specificity of action studies were carried out. Determination of the gastrointestinal specificity of APP action was achieved by injecting it into various vessels selected in their distribution to restricted gastro-intestinal

TABLE 5

CARDIOVASCULAR RESPONSES OF ADULT FEMALE CHICKENS TO APP

APP (i.v.) at 25 µg/kg body wt.

Time	mm Hg				Per Minute	
	Systolic Pressure	Diastolic Pressure	Pulse Pressure	Mean Pressure	Heart Rate	Respiratory Rate
0 min	267 ± 24.0*	169 ± 14.3	98 ± 15.6	202 ± 16.1	369 ± 7	31 ± 3
10 min	275 ± 24.3	174 ± 25.4	101 ± 16.2	203 ± 19.5	354 ± 5	30 ± 3
20 min	275 ± 22.7	178 ± 20.1	97 ± 19.5	209 ± 24.7	353 ± 5	31 ± 3
30 min	287 ± 25.8	185 ± 18.4	102 ± 14.0	224 ± 24.0	341 ± 3	30 ± 3
40 min	285 ± 26.4	184 ± 21.1	101 ± 12.6	223 ± 24.0	349 ± 5	28 ± 3
50 min	280 ± 24.8	182 ± 20.0	98 ± 11.7	216 ± 21.9	356 ± 7	26 ± 2
60 min	283 ± 18.6	183 ± 11.0	110 ± 12.6	204 ± 15.0	348 ± 9	32 ± 3

* Mean ± S.E.M. No. obs. = 5 for all parameters at all time intervals.

TABLE 6

CARDIOVASCULAR RESPONSES OF ADULT FEMALE CHICKENS TO PENTAGASTRIN

Pentagastrin (i.v.) at 1.0 µg/kg body wt.

	mm Hg				Per Minute	
Time	Systolic Pressure	Diastolic Pressure	Pulse Pressure	Mean Pressure	Heart Rate	Respiratory Rate
0 min	265 ± 17.4*	177 ± 19.7	88 ± 5.2	212 ± 21.2	340 ± 15	28 ± 2
10 min	269 ± 18.4	178 ± 20.4	91 ± 7.1	211 ± 15.7	334 ± 17	28 ± 2
20 min	273 ± 10.6	181 ± 15.1	92 ± 7.2	224 ± 19.2	337 ± 10	28 ± 2
30 min	271 ± 15.8	182 ± 16.6	89 ± 6.4	215 ± 37.7	338 ± 9	28 ± 2
40 min	278 ± 20.3	186 ± 41.0	92 ± 12.7	226 ± 12.4	342 ± 8	28 ± 3
50 min	287 ± 15.3	188 ± 21.0	99 ± 13.0	220 ± 19.9	340 ± 9	27 ± 3
60 min	295 ± 13.9	196 ± 20.1	99 ± 16.2	239 ± 19.6	338 ± 11	27 ± 3

* Mean ± S.E.M. No. obs. = 5 for all parameters at all time intervals.

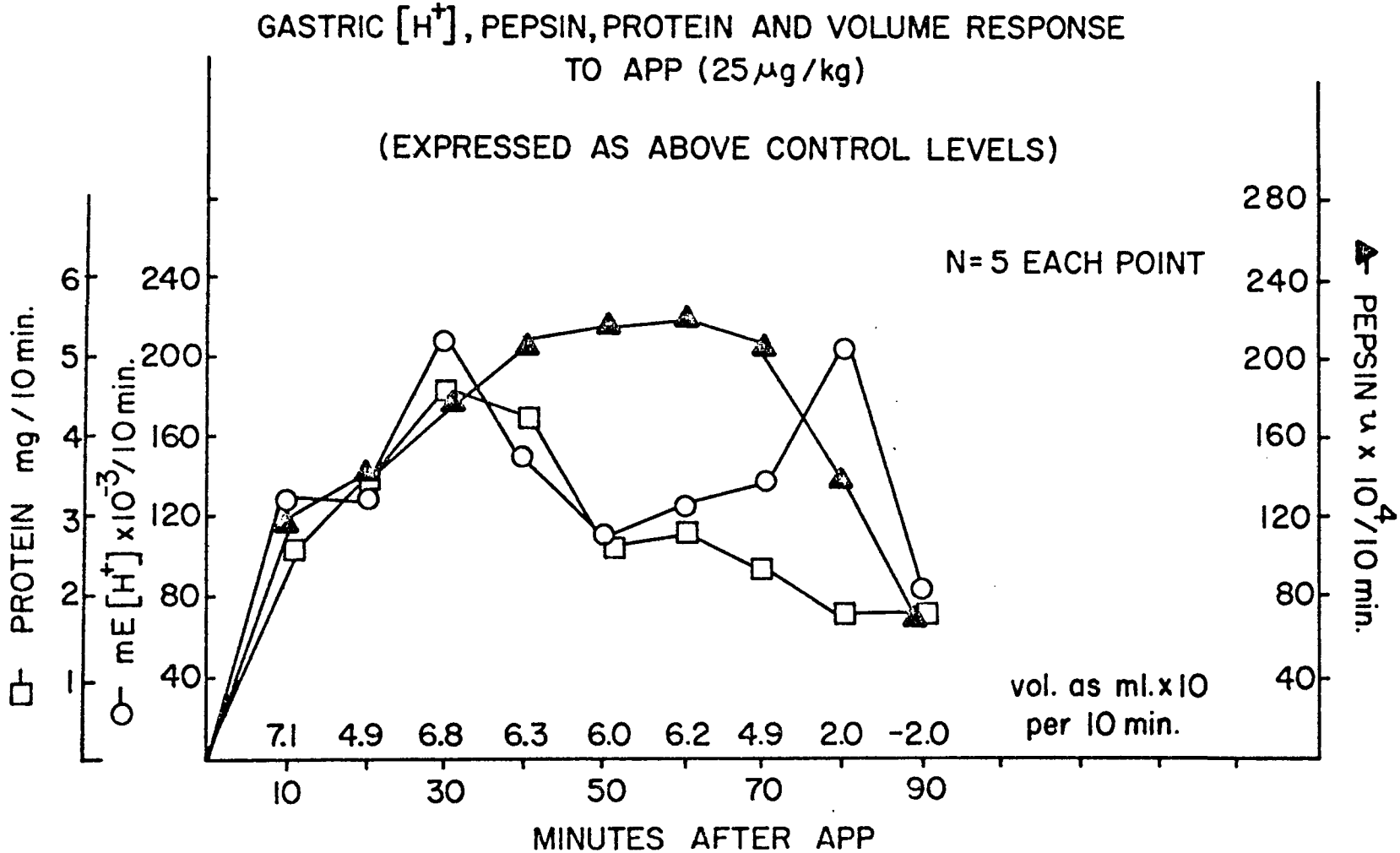
areas. Also, levels of APP were employed which would approximate those when injected via alar vein and complete vascular mixing had occurred. Injection of 6.25 µg APP/kg body weight into the arterial supply of the proventriculus resulted in a minimal increase in the secretory volume of the proventriculus and no change in the basal secretory rate of the gizzard. When this same dose was injected into the arterial supply of the gizzard neither organ responded. When the dose injected into the gizzard arterial supply was increased to 25 µg/kg the gizzard again did not respond; however, the proventriculus showed a markedly delayed and somewhat less secretory response when compared to alar vein injections of the same dose. The peak in proventricular secretion rate occurred 80 minutes after injection, at which time it was at approximately basal levels. Pentagastrin injected at 1.0 µg/kg into the gizzard artery was without effect on this organ. However, a typical response was observed in the chicken proventriculus; that is, a rapid initial increase in secretory activity followed by a rapid decline slightly below control levels.

Next, attention was turned to determining the constituent composition in the APP-induced proventricular secretions. The results of injection of 25.0 µg APP/kg body weight are shown in Figure 13 where volume, H^+ ion, pepsin and total protein levels were determined. It can be seen that there was an early marked increase in all four of these

Figure 13

GASTRIC H^+, PEPSIN, PROTEIN AND VOLUME RESPONSE TO APP IN CHICKENS

Proventricular H^+ ion, pepsin, protein and volume responses to APP in chickens. All data are expressed as changes above control levels. The S.E.M. are omitted for clarity. The numbers above the abscissa are the increases in the secretory volume at the indicated times. Each point represents five observations. Control values for protein, H^+ ion and pepsin were 2.21 ± 0.10 mg/10 min., 137.7 ± 21.6 mEq/10 min., and 79.2 ± 15.4 PU_{Hb} x 10^4/10 min., respectively.


GASTRIC [H+], PEPSIN, PROTEIN AND VOLUME RESPONSE
TO APP (25 μg/kg)
(EXPRESSED AS ABOVE CONTROL LEVELS)
N= 5 EACH POINT
PROTEIN mg / 10 min.
mE [H+] x10-3/10 min.
PEPSIN u x 10^4/10 min.
vol. as ml. x 10
per 10 min.
7.1 4.9 6.8 6.3 6.0 6.2 4.9 2.0 -2.0
10 30 50 70 90
MINUTES AFTER APP

parameters. These increases occurred within the first 10 minutes after injection and were maintained at levels greater than control levels for 90 minutes. When a lower (12.5 µg/kg) or a higher (50 µg/kg) dose was used, there was a lesser and greater response observed, respectively, than the response shown in Figure 13.

It was then thought necessary to determine the potency of APP in a mammalian system in order to evaluate any possible species specificity. It was advantageous, therefore, to establish a means of comparing APP in birds and mammals to a mammalian gastric secretatogue under identical conditions. To do this cannulated chickens were injected with different doses of pentagastrin. Data representative of this study are shown in Figure 14. The proventricular response to pentagastrin increased with increasing size of dose; unlike APP, however, the secretory response induced by pentagastrin in chickens lasts only 30 minutes after injection. It was decided from these data that a dose of 25.0 ug pentagastrin/kg could be used to compare rat and chicken "gastric" secretory response to that induced by APP.

The comparison of the above preparations and doses as stimulators of rat gastric secretion are shown in Table 7. For this study it was necessary to perfuse the rat stomach (by gravity) to obtain sufficient gastric volume for analysis; therefore, a constant lavage volume was maintained at 8.0 ml/

Figure 14

PROVENTRICULAR SECRETORY RESPONSE TO VARIOUS DOSES OF PENTAGASTRIN

The proventricular secretory response to graded doses of pentagastrin (Peptavalon, Ayerst) in adult chickens. These are representative data, one bird for each dose level. The response to 25 µg APP/kg body weight is presented for comparison.

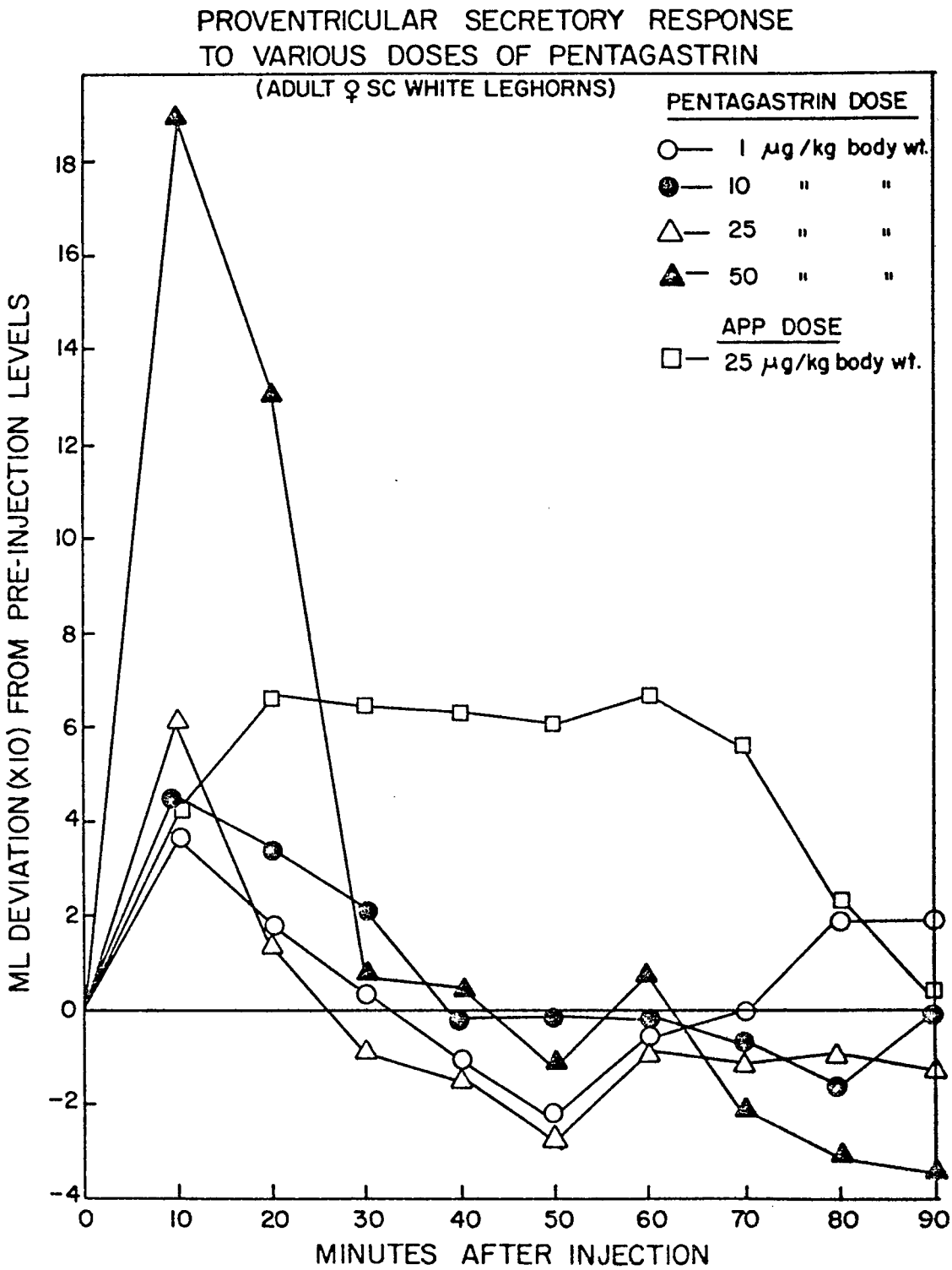

PROVENTRICULAR SECRETORY RESPONSE
TO VARIOUS DOSES OF PENTAGASTRIN
(ADULT ♀ SC WHITE LEGHORNS)
PENTAGASTRIN DOSE
1 µg/kg body wt.
10 " "
25 " "
50 " "
APP DOSE
25 µg/kg body wt.
ML DEVIATION (X10) FROM PRE-INJECTION LEVELS
18
16
14
12
10
8
6
4
2
0
-2
-4
0
10
20
30
40
50
60
70
80
90
MINUTES AFTER INJECTION

TABLE 7

COMPARISON OF EFFECTS OF APP AND PENTAGASTRIN IN PERFUSED RAT STOMACH PREPARATIONS*

ACID ($H^+ \times 10^{-6}$ mEq):

Group	No. Obs.	DEVIATION FROM CONTROL LEVELS							TOTAL
		Control	10	20	30	40	50	60	per 60 min
Saline	16	1.05** ±0.28	-0.15 ±0.13	-0.17 ±0.09	-0.09 ±0.09	-0.06 ±0.07	-0.35 ±0.21	-0.10 ±0.20	-0.92 ±0.60
APP (25 µg/kg)	15	0.85 ±0.19	0.12 ±0.08	0.25 ±0.09	0.30 ±0.15	0.70 ±0.21	1.17 ±0.25	1.55 ±0.35	4.20 ±0.75
Penta (25 µg/kg)	5	0.95 ±0.17	0.25 ±0.15	0.42 ±0.20	0.99 ±0.16	0.98 ±0.19	0.90 ±0.25	0.89 ±0.17	4.42 ±0.96
Penta (230 µg/kg)	9	0.96 ±0.14	0.43 ±0.12	0.86 ±0.52	1.00 ±0.22	0.94 ±0.21	1.53 ±0.46	2.45 ±1.02	6.61 ±1.57

PEPSIN ($P.U._{Hb} \times 10^4$):

Group	Obs.	Control	10	20	30	40	50	60	TOTAL
Saline	16	75.4 ± 9.2	-2.3 ±0.8	-4.5 ±0.2	-7.4 ±1.2	-9.4 ±1.4	-12.1 ± 3.7	-12.9 ± 3.1	-47.9 ± 8.7
APP (25 µg/kg)	15	82.4 ± 6.4	1.2 ±0.9	2.5 ±0.2	6.6 ±1.3	11.9 ±1.4	12.1 ± 2.3	11.0 ± 1.5	41.9 ± 9.5
Penta (25 µg/kg)	5	88.9 ± 9.3	11.0 ±5.6	15.2 ±6.4	31.3 ±5.6	30.4 ±6.8	32.3 ± 5.9	30.6 ± 6.5	146.8 ±25.4
Penta (230 µg/kg)	9	79.3 ± 2.9	11.2 ±1.2	23.3 ±2.1	33.1 ±4.3	38.1 ±7.9	38.1 ± 4.6	37.5 ± 5.8	184.4 ±21.8

*Due to the normal low basal gastric secretion rate in rats, perfusion volume was maintained at 8.0 ml/10 min (7.97 ± 0.15 ml/10 min, n = 344).

**Mean ± S.E.M.

TABLE 7 - CONTINUED

COMPARISON OF EFFECTS OF APP AND PENTAGASTRIN IN PERFUSED RAT STOMACH PREPARATIONS*

TOTAL PROTEIN (µg):

Group	No. Obs.	Deviation From Control Levels							Total per 60 min
		Control	10	20	30	40	50	60	
Saline	16	795** ± 34	-3.4 ± 0.6	-50.6 ±11.6	-102.3 ± 11.6	-103.2 ± 19.2	-120.1 ± 20.5	-120.4 ± 59.2	-419 ± 52
APP (25 µg/kg)	15	665 ± 38	2.4 ± 0.3	10.8 ± 2.9	18.5 ± 3.5	33.7 ± 4.9	32.3 ± 5.1	29.6 ± 5.0	126 ± 11
Penta (25 µg/kg)	5	697 ± 39	54.4 ± 9.9	37.6 ± 8.6	42.7 ± 7.7	61.8 ± 9.8	63.8 ±10.7	64.7 ± 9.9	325 ± 47
Penta (230 µg/kg)	9	696 ± 34	23.1 ± 8.2	30.2 ± 8.7	31.0 ± 6.5	29.0 ± 7.3	33.0 ± 5.5	32.0 ± 7.5	165 ± 9

*Due to the normal low basal gastric secretion rate in rats, perfusion volume was maintained at 8.0 ml/10 min (7.97 ± 0.15 ml/10 min, n = 344).
**Mean ± S.E.M.

10 minutes and, as a result, only H^+ ion, pepsin and total protein could be determined. It can be seen that the rat responded greater to 25 µg pentagastrin (pepsin and protein data) than it did to equivalent amount of APP ($P<.005$ and $<.025$, respectively). However, no difference was observed in H^+ ion secretion. Elevating the dose of pentagastrin to 230 µg/kg body weight caused a marked increase in all parameters over control levels, significant differences from the lower dose of pentagastrin being present for the pepsin content ($P<.02$) and total protein content ($P<.001$).

When these results obtained in rats were compared with results obtained with the same doses in chickens (Table 8) it was seen that chickens were markedly more responsive to APP than are rats. It can also be seen that rats responded better to pentagastrin than chickens, although the difference was not as striking.

The gastric-stimulatory effect of histamine is well documented in mammals. Other investigators have established that chickens injected with histamine respond in much the same manner as mammals, i.e., an increase in secretory volume, H^+ ion, pepsin and total protein content occur. It was necessary, therefore, to establish whether it causes a response by stimulating the release of "gastric" histamine. Table 9 and Figure 15 present the results of a study in which the "gastric" effect of APP is compared to that of histamine in normal birds

TABLE 8

COMPARISON OF 60-MINUTE ACCUMULATIVE "GASTRIC" RESPONSES OF ADULT RATS AND CHICKENS

APP (25 μg/kg)

PARAMETER	CHICKENS* (n = 9)	RATS+ (n = 15)
"GASTRIC" Vol, ml	2.42 ± 0.37	——**
FREE $[H^+]$, mEq	9.52 ± 0.49 ($x10^{-4}$)	4.20 ± 0.75 ($x10^{-6}$)
Pepsin, units	1179 ± 95 ($x10^4$)	42 ± 10 ($x10^4$)
Total Protein, mg	16.01 ± 2.42	0.13 ± 0.01

PENTAGASTRIN (25 μg/kg)

PARAMETER	CHICKENS* (n = 5)	RATS+ (n = 5)
"GASTRIC" Vol, ml	0.22 ± 0.06	——**
FREE $[H^+]$, mEq	4.03 ± 0.90 ($x10^{-6}$)	4.42 ± 0.96 ($x10^{-6}$)
Pepsin, Units	1.06 ± 0.35 ($x10^4$)	147 ± 25 ($x10^4$)
Total Protein, mg	0.025± 0.010	0.33 ± 0.05

NOTE: ALL VALUES ARE EXPRESSED AS ABOVE CONTROL LEVELS

*Adult, female, nonfasted.
+Adult, male, fed sugar cubes.
**See Methods or Table 7 footnote for rationale.

TABLE 9

EFFECT OF GLYCOPYRROLATE BROMIDE* ON APP AND HISTAMINE STIMULATED PROVENTRICULAR SECRETION

VOLUME:

Group	Dose	ml/10 min AS DEVIATION FROM CONTROL LEVELS								Total per 60 min
	µg/kg	Control	Gp*	10	20	30	40	50	60	
Hist	100	0.62** ±0.25	-----	0.79 ±0.15	0.85 ±0.19	0.90 ±0.19	0.81 ±0.14	0.75 ±0.17	0.25 ±0.18	4.35 ±0.21
Hist + Gp	100+100	0.65 ±0.29	-0.05 ±0.10	0.06 ±0.10	0.08 ±0.09	0.10 ±0.09	0.05 ±0.05	0.02 ±0.05	-0.05 ±0.01	0.36 ±0.09
APP	25	0.58 ±0.25	-----	0.75 ±0.16	0.74 ±0.14	0.68 ±0.17	0.62 ±0.10	0.55 ±0.10	0.42 ±0.13	4.06 ±0.21
APP + Gp	25+100	0.55 ±0.21	-0.09 ±0.10	0.70 ±0.17	0.76 ±0.22	0.70 ±0.19	0.61 ±0.17	0.50 ±0.21	0.41 ±0.32	3.68 ±0.43

ACID:

Group	Dose	mEq H^+/10 min AS DEVIATION FROM CONTROL LEVELS								Total per 60 min
	µg/kg	Control	Gp*	10	20	30	40	50	60	
Hist	100	138 ± 30	-----	90 ±11	115 ± 11	140 ± 16	138 ± 17	119 ± 19	92 ±12	694 ± 25
Hist + Gp	100+100	125 ± 21	-15 ± 9	5 ±3	15 ± 8	25 ± 8	2 ±3	1 ±6	-15 ± 5	33 ±11
APP	25	140 ± 25	------	143 ± 11	195 ± 16	219 ± 21	185 ± 12	125 ± 10	126 ± 12	948 ± 32
APP + Gp	25+100	120 ± 21	-21 ±11	195 ± 23	227 ± 18	225 ± 19	179 ± 15	130 ± 21	119 ± 22	1075 ± 48

* Glycopyrrolate bromide (Robinul, A. H. Robins, Inc.) injected i.v. at time - 10 min.
** Mean ± S.E.M. for five (5) observations each group, each time.
Note: Histamine (SQ) and APP (i.v.) were injected at time '0', i.e., 10 minutes after glycopyrrolate injection when combination studies were performed.

TABLE 9 (CONTINUED)

EFFECT OF GLYCOPYRROLATE BROMIDE* ON APP AND HISTAMINE STIMULATED PROVENTRICULAR SECRETION

PEPSIN:

Group	Dose µg/kg	P.U.HbX10^4/10 min. ABOVE CONTROL LEVELS							Total Per 60 min.
		Control	Gp*	10	20	30	40	50	60	
Hist	100	55** ±12	----	120 ±16	121 ±19	220 ±19	200 ±21	142 ±21	120 ±18	923 ±41
Hist + Gp	100+100	69 ±16	-3 ±9	11 ±10	9 ±11	12 ±10	8 ±11	5 ±10	6 ±12	52 ±22
APP	25	70 ±16	----	115 ±21	250 ±18	175 ±17	175 ±20	150 ±20	143 ±21	961 ±35
APP + Gp	25+100	66 ±13	-1 ±8	112 ±16	256 ±13	200 ±20	196 ±20	138 ±18	126 ±16	1028 ±65

TOTAL PROTEIN:

Group	Dose µg/kg	mg/10 min. ABOVE CONTROL LEVELS							Total Per 60 min.
		Control	Gp*	10	20	30	40	50	60	
Hist	100	1.85** ±0.28	----	2.28 ±0.38	2.50 ±0.30	3.90 ±0.48	3.75 ±0.29	2.10 ±0.35	2.00 ±0.35	16.53 ±0.95
Hist + Gp	100+100	1.99 ±0.25	-0.10 ±0.30	0.10 ±0.39	0.11 ±0.25	0.25 ±0.24	0.15 ±0.19	0.15 ±0.28	0.09 ±0.30	0.85 ±0.65
APP	25	2.19 ±0.20	----	2.38 ±0.45	3.10 ±0.45	3.85 ±0.61	3.25 ±0.21	1.21 ±0.20	1.00 ±0.09	14.79 ±0.98
APP + Gp	25+100	2.10 ±0.21	-0.06 ±0.25	1.90 ±0.29	2.25 ±0.35	3.50 ±0.29	2.10 ±0.29	2.10 ±0.16	2.09 ±0.31	12.94 ±0.99

*Glycopyrrolate bromide (Robinul, A. H. Robins, Inc.) injected i.v. at time -10 min.
**Mean ± S.E.M. for five (5) observations each group, each time.

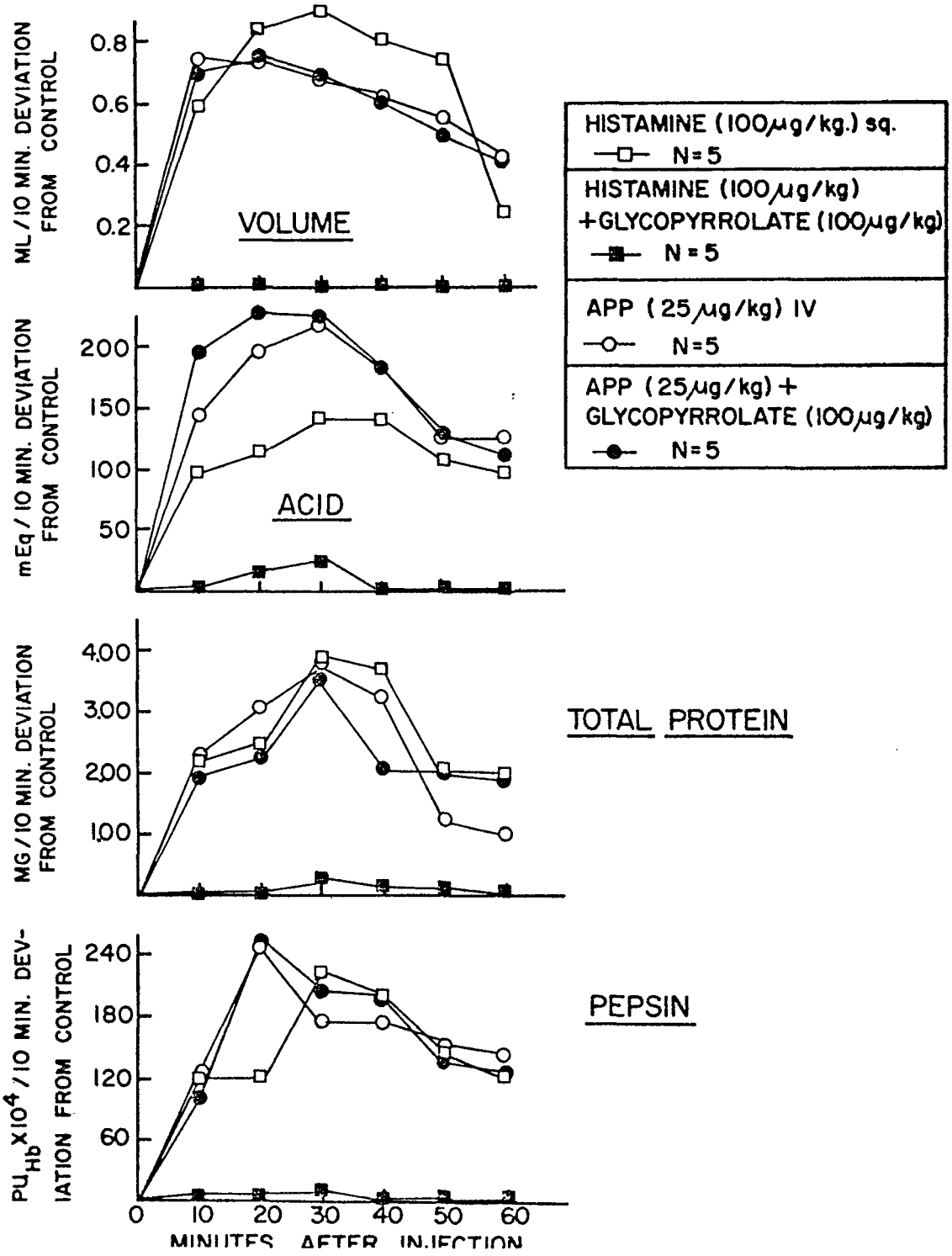
NOTE: Histamine (SQ) and APP (iv) were injected at time '0', i.e., 10 minutes after glycopyrrolate injection when combination studies were performed.

Figure 15

EFFECT OF GLYCOPYRROLATE BROMIDE ON APP AND HISTAMINE STIMULATED PROVENTRICULAR SECRETION

The effect of glycopyrrolate bromide on APP and histamine stimulated proventricular secretion. APP and glycopyrrolate were administered intravenously while histamine was administered subcutaneously. Glycopyrrolate was allowed to equilibrate 10 minutes prior to the injection of histamine or APP. Data presented here are the means of five observations for each point. Standard errors of the mean (see Table 9) are omitted for clarity.

EFFECT OF GLYCOPYRROLATE BROMIDE ON APP AND HISTAMINE STIMULATED PROVENTRICULAR SECRETION



and in birds that have been previously treated with a powerful anticholinergic, glycopyrrolate bromide (Robinul, A. H. Robins). Glycopyrrolate is unique in that it blocks the gastric stimulatory effect of histamine without affecting cardiovascular receptors to histamine. The results indicated that at the doses chosen, glycopyrrolate markedly suppressed the increased volume ($P<.001$), acid ($P<.001$), protein ($P<.001$) and pepsin ($P<.001$) effect of histamine in normal chickens but had no such effect in suppressing the action of APP on these parameters.

This last series of experiments indicated that APP is distinct from chicken glucagon and insulin, does not influence bile or exocrine pancreatic flow rate, and has a powerful gastric secretagogic effect in chickens. APP does not act *via* release of histamine and in many respects is similar to mammalian gastrin.

DISCUSSION

DISCUSSION

I. Biliary flow studies in adult chickens.

The resistance of chickens to large doses of insulin is well documented in the literature (Chen et al., 1945; Hazelwood and Lorenz, 1959; and Shao and Hill, 1966). Investigation of the basis for this resistance has involved the study of plasma binding factors (Hazelwood et al., 1971), cardiovascular (Pittman and Hazelwood, 1971) and catecholamine responses (Pittman and Hazelwood, in press, 1972). Although the importance of the mammalian liver in the removal of insulin from the blood stream is established (Elgee et al., 1953, 1954; and Crough et al., 1965), to this date attention has not been turned to the avian liver for a similar, plausible explanation of the avian "resistance" to insulin. It should be indicated, however, that whatever role the avian liver could play, it would be relatively less than that of plasma-complexes or compensatory catecholamine release. Plasma interference of insulin action accounts for 50-60% and epinephrine release during hypoglycemia for at least 10-20% of "resistance" observed in chickens (Hazelwood, 1971; and Pittman, Ph.D. thesis, 1970). Glucagon secretion is not altered by insulin in Aves (Hazelwood and Lorenz, 1959).

An economical way for the liver to remove large amounts of insulin from the body would be via the biliary path. This excretion route would present the hormone directly to

proteolytic enzymes of the gut, removing it quickly from the body. This excretion route, therefore, appeared to be a likely candidate as a contributor to the chicken "resistance" to insulin as immunoreactive insulin has already been reported in the bile of many animals including the chicken (Lopez-Quijada and Goni, 1967).

A marked increase in the production of hepatic bile was observed (Fig. 2) during induced hypoglycemia, demonstrating that, as in mammals, a physiologically effective dose of insulin causes an increase in bile flow. Insulin injections were shown also to increase the hepatic clearance of ^{14}C-inulin into the bile as well as to shorten the delay period between the time of injection and the first appearance of the bile label (Fig. 3). This effect was more dramatically seen when the data were presented in terms of the total amount excreted (Fig. 4). Thus, it was evident that biliary excretion may indeed play a role in the "resistance" of Aves to insulin. Further investigation demonstrated that this effect was not caused merely by the injection of a foreign protein (Table 1) and that it appears to be mediated by the central nervous system (CNS), particularly via the vagus nerves (Fig. 5). Such endocrine-CNS loops are common in endocrine physiology.

In an attempt to observe how the appearance of insulin itself in the bile was affected by large doses of bovine insulin, insulin-like action (ILA) of bile from birds which

were previously injected with a large dose of insulin was bioassayed. At first, the lack of observed effect (Fig. 6) was surprising considering the amount of insulin reported normally to be in chicken bile (Lopez-Quijada and Goni, 1967). Examination of possible reasons underlying this observation led to the finding that normal bile exerts an inhibitory effect on the glucose uptake of the rat diaphragm muscle (Fig. 7). Increasing the amount of added bile caused a progressive decrease in the glucose uptake of this sensitive tissue. Figure 8 demonstrates that adding normal control bile to the incubation media negates the possible masking of biliary ILA. It also demonstrates that large doses of insulin increased the insulin-like action of bile over and above the inhibitory effect of normal bile. The peak of biliary ILA coincides with a significant depression of the plasma glucose (approximately 20%).

At the time the above experiments were conceived sufficient quantities of chicken insulin were not available to employ in a study to eliminate any differences due to variations in hormone structure. It was necessary, therefore, to use tolbutamide to release preformed "natural" insulin from the chicken pancreas (Fig. 9). It was seen that endogenous insulin, when released by tolbutamide in quantities large enough to cause a marked decrease in plasma glucose, also caused an increase in biliary ILA. The steep decrement in

plasma glucose reflected a greater sensitivity of the chicken to its own hormone. It was interesting to note the apparent "double peak" in biliary ILA. Similar results were reported by Hunzicker and Hazelwood (1970) who also observed a double peak in ILA of chicken cerebrospinal fluid after injection of the same dose of tolbutamide as employed herein. These findings (that the second peak occurs 60 minutes after injection) are complemented by the data presented here. Such results, along with reports that depancreatized and/or enterectomized birds respond to tolbutamide normally (Mirsky and Gitelson, 1957; and Hazelwood, 1958), suggest an extrapancreatic source of insulin in chickens.

It can be concluded that when chickens are challenged with large doses of either endogenous or exogenous insulin, they respond by increasing the insulin content of the bile as well as its flow rate. These data indicate that in addition to other possibilities biliary excretion of insulin is a plausible physiological mechanism in birds for ridding the body of "excess" hormone.

II. What proportion of biliary excreted insulin is degraded or inactive?

It is likely to expect that a powerful insulinase system exists in the avian liver, a suggestion based upon extensive reports of such a system in the mammalian liver (Segal, 1964). If such is the case, hepatic breakdown products of insulin,

as well as the intact molecule, would be excreted into bile. This problem was approached using two routes of injection. The first involved the injection of the ^{125}I-insulin into the alar vein, resulting in considerable hemodilution of the hormone prior to its encounter with the hepatocyte. The second approach involved the injection of ^{125}I-insulin into the pancreatic vein, resulting in a minimal dilution of the injected bolus prior to its arrival at the liver. Thus, it was possible to compare the effect of high hormone doses at the level of the liver with that of very low doses. The unavailability of labelled chicken insulin at the time of these experiments warranted the use of labelled ox insulin.

Results obtained from employing both injection routes (Tables 10 and 11) indicated that a very small amount of the label appeared in the bile during any given 15-minute collection period. During the course of the experiments a total of 0.46% of the peripherally injected label and 0.23% of the "centrally" injected label appeared in the bile. An average of 4.5% (3.0 - 7.6%) of this label was excreted attached to immunoreactive insulin when the injection was made into the wing vein (Table 2) and 9.2% (8.1 - 10.7%) when made into the pancreatic vein (Table 3). In both cases the volume of plasma cleared of label per minute approximated that reported for rabbits (Lopez-Quijada, 1971). Comparison of the two routes of injection indicated that more radioactivity

TABLE 10

SUMMARY OF HEPATIC, PLASMA AND ECV DISTRIBUTION OF ^{125}I-INSULIN IN ADULT CHICKEN: ALAR VEIN INJECTION*

		MINUTES AFTER ^{125}I-INSULIN INJECTION						
PARAMETER	UNITS	0	15	30	45	60	75	90
Total Activity Injected	CPM	3.3×10^6	----	----	----	----	----	----
Plasma Activity	CPM/0.75ml	----	19,570**	4,211	3,380	1,774	1,452	1,262
	% of ^{125}I-inj.	----	0.59	0.13	0.10	0.05	0.04	0.04
Bile Activity	CPM/15 min	----	5,936	4,526	2,441	1,287	581	745
	% of ^{125}I-inj.	----	0.18	0.13	0.07	0.04	0.02	0.02
Pla/Bile	CPM/CPM	----	3.30	0.93	1.38	1.38	2.50	1.69
Estimated Ext. Cell Fluid †	CPM/Bird	3.3×10^6	9.6×10^6	2.1×10^6	1.7×10^6	0.9×10^6	0.7×10^6	0.7×10^6
Non-ECF Activity	CPM/Bird	0	"0"	1.2×10^6	1.6×10^6	2.4×10^6	2.6×10^6	2.6×10^6

* 1.5 µCi of bovine ^{125}I-insulin injected via wing vein

** Mean of 5-6 observations each; see also Table 2

† Calculated as 24.6% body weight as documented by Pittman and Hazelwood (1971) and Hunsaker (1965)

TABLE 11

SUMMARY OF HEPATIC, PLASMA AND ECV DISTRIBUTION OF ^{125}I-INSULIN IN ADULT CHICKENS: PANCREATIC VEIN INJECTION*

		MINUTES AFTER ^{125}I-INSULIN INJECTION						
PARAMETER	UNITS	0	15	30	45	60	75	90
Total Activity Injected	CPM	$3.3x10^6$	----	----	----	----	----	----
Plasma Activity	CPM/0.75ml	----	3,164**	2,757	2,391	2,216	1,657	1,681
	% of ^{125}I-inj.	----	0.09	0.08	0.07	0.07	0.05	0.06
Bile Activity	CPM/15 min	----	1,667	1,963	1,263	1,025	907	720
	% of ^{125}I-inj.	----	0.05	0.06	0.04	0.03	0.03	0.02
Pla/Bile	CPM/CPM	----	1.91	1.37	1.83	2.18	1.82	2.53
Liver (Agonal)	Total CPM	----	----	----	----	----	----	18,632
Estimated Ext. Cell Fluid†	CPM/Bird	$3.3x10^6$	$1.6x10^6$	$1.4x10^6$	$1.2x10^6$	$1.1x10^6$	$0.8x10^6$	$0.9x10^6$
Non-ECF Activity	CPM/Bird	0	$1.7x10^6$	$1.9x10^6$	$2.1x10^6$	$2.2x10^6$	$2.5x10^6$	$2.4x10^6$

* 1.5 μCi of bovine ^{125}I-insulin injected via pancreatic vein.

** Mean of 6 observations; also see Table 3

† Calculated as 24.6% body weight as documented by Pittman and Hazelwood (1971) and Hunsaker (1965)

was excreted in the bile of birds injected by the alar vein than in the bile of birds injected via the pancreatic vein. In all samples (except one) the plasma/bile activity ratio was greater than one, indicating that at all times more insulin label was in the plasma than in the bile. Despite this small amount of biliary activity, a large portion of the injectant was removed from the plasma (99.1% with alar vein injections and 99.7% with pancreatic vein injections). Such comparisons indicated that avian biliary excretion probably plays a minor role in removal of exogenous insulin and that the label is readily distributed in other (non-plasma) extracellular fluids (Tables 10 and 11).

It is well documented that the addition of radioisotopes to molecules alters (in some cases significantly) the biological effect of that molecule. This is particularly true when a radioactive "foreign" atom, such as iodine, is added to insulin. Even though it has been demonstrated in our laboratory that the number of ^{125}I atoms added to the insulin preparation used did not significantly affect its immunological properties, it is possible that the labelling affected the saturation kinetics of the hepatic insulin "transport" and/or insulinase system. These alterations can be envisioned in terms of a change in the conformation of the insulin molecule such that its affinity with receptor sites is altered.

Account should also be taken of the disposition of the ^{125}I-label after its injection into the blood stream. It is apparent from the data presented that it is not being excreted in large quantities into the bile. It is also apparent that no significant amount of the injected label is sequestered by the liver parenchyma (Table 11). Even though data were not collected from thyroid tissue, it is quite possible that a reasonable amount of ^{125}I was sequestered preferentially there as a result of the iodine trapping capabilities of this tissue. Another area of possible iodine concentration is in the kidneys and urine. It has been shown that the kidney cortex of rats accumulates ^{131}iodine in amounts three times that of the liver as soon as fifteen minutes after injection of labelled insulin (Elgee et al., 1954). This accumulation is followed by rapid iodine excretion in the urine. Further investigation in chickens is necessary in order to establish the relative importance of these possible iodine excretion pathways.

It is felt, however, that additional information could be gained here first by use of labelled chicken insulin, both in large and small doses. This approach would eliminate any differences which might exist as a result of different insulin structures. Second, the use of insulins with different degrees of iodination may be necessary to pin-point any isotope effects. Finally, it may be important to study the

avian liver *in vitro* in order to obtain a clearer picture of the mechanism of removal of insulin from the plasma to the bile and the importance of the avian hepatic "insulinase" system in the regulation of insulin levels.

III. Does a newly isolated avian pancreatic polypeptide influence biliary excretion?

When APP was initially isolated it was thought that it may play a role in control of insulin levels in birds. Such an assumption was not unprecedented. Hellman and Lernmark (1969) reported that a substance from the α_1 (delta cell) of the pigeon pancreas exerted an inhibitory effect on mammalian beta cell secretory activity. Possibly, APP might also affect the rate of bile flow and thereby increase the removal of insulin from the circulation. It can be seen from Table 4 that no effect of APP was observed on either of the bile secretory channels or on exocrine pancreatic secretions. However, a marked increase in secretion volume by the proventriculus ("secretory stomach") was observed. Concomitant with this preliminary work, other investigators were studying metabolic facets of the biological properties of APP. Hazelwood *et al.* (1971) reported that when 0.1 mg APP/kg body weight was injected into SCWL chickens there was a marked early decrease in plasma glycerol and hepatic glycogen content without simultaneous changes in plasma glucose. It has been shown also that APP elevates the circulating levels of non-esterified

free fatty acids in birds, suggesting that APP could be involved in lipid utilization.

APP can be detected by radioimmunoassay in the pancreas of birds but not in extracts of other tissues such as the proventriculus and small intestine. The pancreas of chickens contains APP in amounts of 8 mg/100 g tissue. Analysis of serum levels indicates that circulating levels vary from 0.5 to 27 ng/ml. It was also found that these serum levels increased with feeding and decreased with fasting (Kimmel et al., 1971). Using the same immunoassay procedure, APP-like compounds can be detected in duck, pigeon, goose, guinea fowl, spoonbill, great horned owl, red-tailed hawk, alligator and turtle pancreas, the greatest amount being found in the alligator. However, using the same assay, APP-like compounds could not be detected in crude extracts of dog, bullfrog, rattlesnake, marine toad, guinea pig, rabbit, cat, and human pancreas (Kimmel, personal communication). This, however, could be merely a manifestation of the highly species-specific nature of this assay.

The tentative amino acid sequence of APP, as suggested by Kimmel et al. (1971), is as follows:

Gly-Pro-Ser-Glx(Pro,Thr)Tyr-Pro-Gly-Asx(Asx,Pro,Ala)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

This structure bears no similarity to chicken insulin or any

fraction thereof. It also differs from glucagon by the presence of an isoleucine residue, an N-terminal glycine, four proline residues and the cluster of basic amino acids at the C-terminal end.

(Independent of the above work, the Eli Lilly Research laboratories have been working on a similar 36 amino acid polypeptide isolated from porcine, bovine, ovine and human pancreata. An immunoassay developed for these polypeptides, while sensitive, indicates poor cross-reactivity between and among the four different mammalian preparations (Personal communication, W. W. Bromer to J. R. Kimmel.))

Collectively, these observations support the previously reported biochemical evidence that APP was a distinct substance present in the chicken pancreas which possesses definite biological properties.

It is apparent from Figure 10 and 13 that when increasing amounts of APP were administered to a viable preparation there was a corresponding increase in the volume of secretant and its contents without any changes in plasma glucose levels. These findings are in accord with immunological data indicating that blood levels of APP vary with circulating glucose levels but the converse is not true. Thus, APP might have a regulatory function in the avian digestive system. (It should be noted that the response of chickens to pentagastrin (1.0 µg/kg) was markedly different from that to APP. Admittedly,

this could be merely a reflection of the doses selected; however, it could be the result of molecular species differences as pentagastrin is modelled after a mammalian hormone. Work clarifying this is discussed below.)

At the time these studies were performed, chicken glucagon had not been isolated. It was felt necessary, therefore, to provide physiological evidence, along with the previously mentioned biochemical evidence, to establish firmly that APP was not in fact chicken glucagon or even contaminated with it. Evidence has already been presented (Fig. 10 and Hazelwood, 1971) indicating that APP had no effect on plasma glucose levels. Figure 12 demonstrates that even when large increases in plasma glucose occur as a result of glucagon injection there is no increase in proventricular secretion; in fact, an inhibitory effect is observed. This indicates that mammalian glucagon does not alter plasma glucose or proventricular secretion in the same manner as APP. When chicken glucagon became available (late 1972) repetition of the above experiments produced similar results. The greater response in terms of plasma glucose levels produced by the isologous substance was not surprising. The depressive effect of both species of glucagon was predictable in view of the depressive effect on gastric motility and gastric secretion by glucagon observed in mammals (Davenport, 1966).

APP activity is not mediated by vagal action (Fig. 11) and, apparently, neither APP nor pentagastrin cause any cardiovascular changes which could result in an increase in proventricular secretion (Tables 5 and 6). Any increase in pressure observed could be attributed to the gradual decrease in the depth of anesthesia. Nonetheless, it is apparent that in order to rule out the mediating effect of the cardiovascular system completely it would be necessary to measure the changes in blood flow through the proventriculus per se.

When gastric responses to different routes of APP injection were compared it became apparent that the gizzard does not respond to APP, leading one to believe that the proventriculus is a major (avian) gut target organ for APP. The fact that the proventriculus does not respond when APP is injected directly into gizzard arteries (even when the dose used, 25 µg APP/kg, was shown to be active peripherally) indicated that the liver, through which the APP must pass before reaching the proventriculus, probably had an efficient mechanism for the degradation of APP. Pentagastrin on the other hand, appears to be equally effective regardless of the route of injection. The fact that pentagastrin is modeled after the structure of mammalian gastrin indicates that any existing hepatic "APP-ase" system does not recognize other "gastrin-like" substances. The fact that pentagastrin caused a response, and APP did not, rules out hemodilution as a cause of the decreased APP effect.

In summary, it has been established that APP is physiologically distinct from mammalian and chicken glucagon in terms of its effect on the proventriculus as well as its effect on plasma glucose levels. Its activity is not mediated by the vagus nerve and probably not by the cardiovascular system. This information pin-points APP as a physiologically distinct avian substance of "hormone-like" nature. These data also indicate that APP could, indeed, be an important proventricular secretogogue. It may in fact be the avian equivalent to mammalian gastrin as no reports of a chicken gastrin have been published. To the contrary, Ruoff and Sewing (1970) reported that extracts of proventriculus, gizzard and duodenum were free from acid stimulatory activity (i.e., gastrin activity) in birds.

The presence of a gastric stimulatory substance in the mammalian pancreas has been reported, adding further support to the above hypothesis. Zollinger and Ellison (1955) described a syndrome in humans in which non-beta cell adenomas of the pancreas occurred concomitantly with marked gastric hyperacidity and duodenal ulcer. Cytological examination of these tumors have shown them to be of α_1 (delta cell) origin (Potet et al., 1966, Cavallera et al., 1967). The biological activity of these tumors appeared to result from secretion of gastrin (Gregory et al., 1967). Greider and McGuigan (1971), using selective staining techniques, demonstrated that the

delta cell of the human pancreas was a gastrin secreting cell. This observation was true of both normal pancreas and of those containing Zollinger-Ellison adenomas. Thus, precedent is set for the pancreas secreting a gastrin-like substance.

Having established the proventricular secretory properties of APP in chickens, attention was turned to the effectiveness of APP on the mammalian digestive system. The choice of 25 µg pentagastrin/kg body weight to be used for comparison with APP was based on three reasons. First, it represented an amount of protein equivalent to 25 µg APP/kg body weight. Second, the initial response to this dose of pentagastrin was equivalent to a dose of 25 µg APP/kg (Fig. 14). Finally, the 50 µg pentagastrin dose was rejected, despite the apparent equality in area under its secretion curve with that of the 25 µg APP dose (Fig. 14), because it was feared that such an abrupt increase in secretory activity would produce other undesirable and/or unobserved effects.

Data in Table 7 indicate that APP is active in the mammalian system, producing significant responses in all parameters measured as compared with saline-injected animals. However, except for the hydrogen ion secretion, the secretory response induced by pentagastrin (synthetic "mammalian gastrin") was greater than that induced by APP in the parameters measured.

Data from the above, as well as from other experiments, were grouped together (Table 8) in an attempt to establish firmly an apparent species specificity. Clearly, it is seen that APP is more potent in the chicken than it is in the rat; also, it can be seen that pentagastrin is more active in the rat than it is in the chicken. However, the difference in the latter case is not as striking as it is in the former. Again, note that in terms of hydrogen ion secretion pentagastrin induces a similar magnitude of response in both species, an observation which is complicated by the fact that in chickens both the hydrogen ion and pepsinogen are secreted by the (same) chief cell (Sturkie, 1965). Further work is, therefore, needed to clarify how chief cells produce two different secretions. Do they operate as a single population of cells or as two populations each producing a different secretion?

Finally, it was necessary to demonstrate that the observed gastric results of APP in chickens were not secondary to the release of histamine. This aspect of the study was undertaken because other investigators have reported proventricular responses as a result of histamine injections very similar to those reported here (Long, 1967; Kokas et al., 1967; and Burhol and Hirshowitz, 1970, 1972). A substance was sought that would block selectively the proventricular response to histamine. Such an agent was glycopyrrolate bromide which is known to be a powerful anti-cholinergic drug

and has been shown to block the gastric stimulatory effects of histamine in mammals. Apparently, this histamine blocking effect is the result of the interference of glycopyrrolate with the binding of histamine to its "reactive site" (Abbott et al., 1962; Foss et al., 1964; and Amure, 1969). The present study indicates that glycopyrrolate also provides an effective blockage against histamine-stimulated proventricular secretion in chickens (Table 9, Fig. 15). It also indicates that the response to APP is not affected by glycopyrrolate, thus demonstrating that the effect elicited by APP is not attributable to a release of histamine.

This last series of experiments indicated that APP has a powerful and direct gastric secretogogic effect in chickens, one which is not mediated by histamine. It has also been shown that APP is effective in the mammalian system, though to a much smaller degree than mammalian-like pentagastrin.

The literature reviewed and data presented here indicate that APP is a pancreatic secretion with two apparent unrelated effects; i.e., a gastric stimulatory effect coupled with a metabolic effect. Such effects may not be totally unrelated since there are physiological and behavioral observations in birds which may be explained by the presence of such a polypeptide. Epple (1965) reported indirect (histological) evidence indicating an increase in pancreatic α_1 cell activity just prior to migration in the blackbird, Turdus merula.

This increase in α_1 cell activity was correlated with hyperphagia and the laying down of food stores in the form of fat. Possibly this is the result of an APP-like substance emanating from the α_1 cell. Kimmel (personal communication) observed that the highest pancreatic APP content was found to be in the day-old hatch-out chick. It is at this time that the chick is using up the last of its yolk sac contents (containing large amounts of fat) and has a voracious appetite. Admittedly these reports are circumstantial; however, they are encouraging and warrant further investigation.

The apparent dual action of APP is also interesting from an evolutionary point of view. Birds can be considered to occupy, in terms of physiological mechanisms, an intermediary position on the evolutionary scale lying somewhere between the more primitive reptiles and the more advanced mammals. This position could account for the presence of a "hormone" with a dual function. Such a dual function may indicate a lesser degree of specialization, possibly resulting in some inefficiency in regulation such as the stimulation of both lipogenesis and gastric secretion when only the stimulation of one function is required. In the evolution of the more specialized mammal the gastric stimulatory function of this polypeptide was assumed by gastrin and the lipogenic effect was lost being a duplication of other hormones such as glucagon. The finding that a gastrin-like substance is

present in the α_1 cells of normal pancreas as well as in Zollinger-Ellison adenomas (Greider and McGuigan, 1971) supports this postulate, particularly if this gastrin-like substance is vestigially present in the normal pancreas and only becomes more concentrated under pathological conditions.

Much work is necessary in order to support or refute the above suggestion. For instance, why, if APP-like substances can be detected in the more primitive turtle and alligator, can't it be detected in the rattlesnake which holds a much more advanced evolutionary position? It is possible that this is only the result of immunological cross-reaction differences; only the extraction and subsequent bioassay of such a substance will establish definitely. Also, there is no reported pathological (lipid) metabolic disturbance associated with the gastric distress observed in the Zollinger-Ellison Syndrome. Perhaps this characteristic of the α_1 cell secretion was lost completely prior to the evolution of man, or perhaps it is not related at all to APP. Further investigation of lower mammals would add conclusive evidence.

Whatever the outcome of the investigation started herein, pursuit of this problem may be of great importance. Not only will it shed light on the process of biochemical evolution but the biochemical and physiological study of the vertebrate pancreas necessary to pursue this problem will no doubt open

the door to the answers of many important questions regarding pancreatic function.

SUMMARY AND CONCLUSIONS

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In the foregoing study insulin was shown to be excreted in the bile of chickens, the greater proportion being in the degraded form. Injections of large doses of mammalian insulin were shown to increase the bile flow, to increase clearance of ^{14}C-inulin from the blood into bile, and to increase the insulin-like action of bile. However, the amount of insulin removed by the bile only amounted to 0.5%, or less, of the injected dose indicating that biliary clearance of the hormone plays a less important role than plasma factors or catecholamine release in the avian "resistance" to exogenous insulin.

Avian Pancreatic Polypeptide (APP) was shown to be a biologically active pancreatic component which had no effect on bile secretion or exocrine pancreatic secretion. APP was shown, however, to be effective in increasing the volume and content of proventricular secretion. This stimulatory effect was shown neither to be mediated by the vagus nerve nor by the release of histamine. APP was found to be not as potent a "gastric" secretogogue in rats as in chickens and pentagastrin was found to have a greater effect in rats than in chickens.

Conclusions drawn from this work include: the biliary excretion route does not play a major role in the "resistance"

of chickens to exogenous mammalian insulin. APP does not play a role in this "resistance" via an action on bile secretion; it was, however, found to be a potent proventricular secretogogue in chickens. Further investigations are necessary to determine if chicken insulin has a greater effect on the above biliary excretion parameters and to quantitate further the physiological role of APP in chickens and other species.

REFERENCES

REFERENCES

Abbott, W. E., A. Sourial, H. Krieger, S. Levey. Effect of glycopyrrolate (Robanul) on basal and histamine- or insulin-induced gastric secretion. Ann. N. Y. Acad. Sci. 99: 163-173, 1962.

Amure, B. O., Effect of glycopyrronium bromide on basal and histamine- or gastrin-induced gastric secretion. J. Pharm. Pharmac., 21: 502-505, 1969.

Anderson, D. K. and R. L. Hazelwood. Chicken cerebrospinal fluid: Normal composition and response to insulin administration. J. Physiol. 202: 83-95,.1969.

Bucher, G. R., M. I. Grossman and A. C. Ivy. A pepsin method: the role of dilution in the determination of peptic activity. Gastroenterology 5: 501-511, 1945.

Burhol, P. G. and B. I. Hirschowitz, Single subcutaneous doses of histamine and pentagastrin in gastric fistula chickens. Am. J. Physiol., 218: 1671-1675, 1970.

Burhol, P. G. and B. I. Hirschowitz, Dose responses with subcutaneous infusion of histamine in gastric fistula chickens. Am. J. Physiol., 222: 308-313, 1972.

Boyns, A. R., N. Pearce and R. F. Mahler, Insulin in bile. The effect of monosaccharides and hypoglycaemic agents. Diabetologia 5: 304-308, 1969.

Cavallero, C., E. Solcia and R. Sampieto. Cytology of islet tumors and hyperplasias associated with the Zollinger-Ellison syndrome. Gut 8: 172-177, 1967.

Chen, K. K., R. C. Anderson, and N. Maze. Susceptibility of birds to insulin as compared with mammals. J. Pharm. Exp. Ther. 84; 74-77, 1945.

Croughs, R. J. M., D. Maingay, J. C. Touber, H. A. de Ruyter and R. van Aalst. The effect of insulin binding antibodies on insulin ^{131}I Metabolism. Acta Endocrinol. 50: 213-223, 1965.

Daniel, P. M. and J. R. Henderson. Insulin in bile and other body fluids. Lancet I: 1256-1257, 1967.

Davenport, H. W. Physiology of the Digestive Tract, 2nd edition. Year Book Medical Publishers, Inc., 1967.

Epple, A. Investigations on a third pancreatic hormone. Gen. Comp. Endoc. 5 : 674, 1965.

Elgee, N. J., R. H. Williams and N. D. Lee. Distribution and degradation studies with insulin-^{131}I. J. Clin. Invest. 33: 1254-1260, 1954.

Elgee, N. J., R. H. Williams, N. D. Lee, T. Wong, and J. R. Hogness. Studies of radioactive insulin. Diabetes 2 : 370-371, 1953.

Foss, D. L., L. S. Staveney, T. Haraguchi, H. N. Harkins and L. M. Nyhus. Pathophysiologic and therapeutic considerations of Curling's Ulcer in the Rat. J. Amer. Med. Assoc. 187: 592-594, 1964.

Gregory, R. A., M. I. Grossman, H. J. Tracy and P. H. Bently. Nature of the gastric secretagogue in Zollinger-Ellison Tumors. Lancet 2: 543-544, 1967.

Greider, M. H. and J. E. McGuigan. Cellular localization of gastrin in the human pancreas. Diabetes 20: 389-396, 1971.

Hazelwood, R. L. The peripheral action of tolbutamide in domestic fowl. Endocrinology 63: 611-618, 1958.

Hazelwood, R. L., Carbohydrate metabolism. Chapter 12, pages 313-371, Avian Physiology by Paul D. Sturkie, 2nd edition, Cornell University Press, 1965.

Hazelwood, R. L. Endocrine control of avian carbohydrate metabolism. Poultry Sci. L: 9-18, 1971.

Hazelwood, R. L. and B. Barksdale. Failure of chicken insulin to alter polysaccharide levels of the avian glycogen body. Comp. Biochem. Physiol. 36: 823-827, 1970.

Hazelwood, R. L., J. R. Kimmel and H. G. Pollock. Biological characterization of chicken insulin activity in rats and domestic fowl. Endocrinology 83: 1331-1336, 1968.

Hazelwood, R. L., J. R. Kimmel and H. G. Pollock. Influence of chicken and rat plasma on in vitro activity of chicken and beef insulin. Comp. Biochem. Physiol. 39B: 267-278, 1971.

Hazelwood, R. L. and F. W. Lorenz, Effects of fasting and insulin on carbohydrate metabolism of the domestic fowl. Am. J. Physiol., 197: 47-51, 1959.

Hazelwood, R. L., S. D. Turner, and J. R. Kimmel, Biological evaluation of a polypeptide (third hormone?) isolated from chicken pancreas, Endocrinology, 88(Suppl), Page XVII, Abstract Number 25, 1971.

Hales, C. N. and P. J. Randle. Immunoassay of insulin with insulin-antibody precipitate. Biochem. J. 88: 137-146, 1963.

Hellman, B. and A. Lernmark. Inhibition of in vitro secretion of insulin by an extract of pancreatic α_1 cells. Endocrinology 84: 1484-1488, 1969.

Hunsaker, W. G. Determination of Evans blue in avian plasma by protein precipitation and extraction. Proc. Soc. Exptl. Biol. Med. 120: 747-749, 1965.

Hunzicker, M. E. and R. L. Hazelwood. Chicken cerebrospinal fluid: insulin-like activity. Comp. Biochem. Physiol. 36: 795-801, 1970.

Jeffcoate, S. L. Insulin in bile: Studies on the effect of bile acids on the radioimmunoassay of insulin. Diabetologia 4: 281-285, 1968.

Kimmel, J. R., H. G. Pollock and R. L. Hazelwood. Isolation and characterization of chicken insulin. Endocrinology 83: 1323-1330, 1968.

Kimmel, J. R., H. G. Pollock, and R. L. Hazelwood. A new polypeptide hormone. Fed. Proc. 30 : Part II, Abstract Number 1550, 1971.

Kipnis, D. M. and C. F. Cori. Studies of tissue permeability III. The effect of insulin on pentose uptake by the diaphragm. J. Biol. Chem. 24: 681-693, 1957.

Koike, T., A. Nalbandov, M. K. Dimick, Y. Matsumura and S. Lepkovsky. Activity of insulin upon blood glucose levels of fasted hypophysectomized, depancreatized and normal chickens, Endocrinology 74: 944-948, 1964.

Kokas, E., J. L. Phillips, Jr., and W. D. Brunson, Jr., The secretory activity of the duodenum in chickens. Comp. Biochem. Physiol. 22: 81-90, 1967.

Lai, K. S. Studies on Gastrin. Gut 5: 327-333, 1964.

Langslow, D. R., E. J. Butler, C. N. Hales, and A. W. Pearson. The response of plasma insulin, glucose and non-esterified fatty acids to various hormones, nutrients and drugs in the domestic fowl. J. Endocrinol. 46: 243-260, 1970.

Lepkovsky, S., M. V. Dimick, F. Furuta, N. Snapir, R. Park, N. Narita and K. Komatsu. Response of blood glucose and plasma free fatty acids of fasting and to injections of insulin and testosterone in chickens. Endocrinology 81: 1001-1006, 1967.

Lernmark, A., B. Hellman and H. G. Coore. Effects of gastrin on the release of insulin in vitro. J. Endocrinol. 43: 371-375, 1969.

Long, J. F., Gastric secretion in unanesthetized chickens. Am. J. Physiol. 212: 1303-1307, 1967.

Lopez-Quijada, C., and P. M. Goni. Liver and insulin: presence of insulin in bile. Metabolism 16: 514-521, 1967.

Lopez-Quijada, C., P. M. Goni and J. L. R-Candela. Degraded and undegraded insulin in the bile of rabbits after intravenous administration of tracer amounts of labeled insulin. Proc. Soc. Explt. Biol. Med. 127: 736-740, 1968.

Lopez-Quijada, C., P. Goni and E. Blazque. Passing of insulin from plasma into bile. Proc. Soc. Explt. Biol. Med. 125: 939-941, 1967.

Lopez-Quijada, C., P. Goni and E. Blazque. Transfer of insulin into the bile in rabbit late pregnancy. Acta Diabet. Lat. 8: 469-478, 1971.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

Miller, M. R. and D. H. Wurster. Further studies on the blood glucose and pancreatic islets of lizards. Endocrinology 63: 191-200, 1958.

Mirsky, I. A. and S. Gitelson. Comparison of the hypoglycemic action of tolbutamide in the fowl pancreas. Endocrinology 71: 464-473, 1957.

Nelson, N., S. Elgart, and I. A. Mirsky. Pancreatic diabetes in the owl. Endocrinology 31: 119-123, 1942.

Pittman, R. P. and R. L. Hazelwood. Cardiovascular response of chickens to administration of nonavian insulin. Proc. Soc. Exptl. Biol. Med. 137: 1060-1065, 1971.

Pittman, R. P. and R. L. Hazelwood. Catecholamine response of chickens to exogenous insulin and tolbutamide. Comp. Biochem. Physiol. In Press. 1972.

Potet, F., E. Martin, J. P. Thiery, J. P. Bader, S. Bonfils and A. Lamkling. Etude histologique et cytologique du pancreas endocrine, tumoral et non-tumoral dans le syndrome du Zollinger-Ellison. Rev. Int. Hepat. 16: 737-761, 1966.

Ruoff, H. J. and Sewing, K. Die wirkung von histamin, carbachol, pentagastrin und huhnergastrinextrakten auf die magensekretion von nicht narkotisierten huhnern mit einer magenfistel. Arch. Exptl. Pathol. Pharmokol. 267: 170-176, 1970.

Ruffin, J. M., W. M. Roufail, R. C. Stone and J. B. Currin. The role of anticholinergic drugs in the treatment of peptic ulcer. The Stomach, Thompson et al., Ed., pages 354-359, Grune and Stratton, Inc., 1966.

Segal, B. M. Insulin inhibition and antagonism. Metabolism 13: 753-765, 1964.

Shao, T. and D. C. Hill. A comparison of the effect of dietary fat and carbohydrate on free amino acid in the blood plasma of chicks. Canad. J. Physiol. Pharm. 45: 225-234, 1966.

Snedecor, J. G. and D. B. King, Refractoriness of the chick glycogen body to hypothroidism. Anat. Rec. 137: 393, 1960.

Somogyi, M. Notes on sugar determination. J. Biol. Chem. 195: 19-23, 1952.

Stamler, J., C. Bolene, L. N. Katz, R. Harris and R. Pick. Influence of pancreatectomy on lipid metabolism and atherogensis in the chick. Fed. Proc. 9: 121, 1950.

Sturkie, P. D., Avian Physiology, 2nd ed., Cornell University Press, Ithaca, New York, 1965.

Szepsenwol, J., and J. V. Michalski. Glycogenolysis in the liver and glycogen body of the chicken after death. Am. J. Physiol. 165: 624-627, 1951.

Umbreit, W. W., R. H. Burris, and J. F. Standler. Manometric Techniques and Related Methods for the Study of Tissue Metabolism, 4th ed., Burgess Publishing Co., 1964.

Zollinger, R. M. and E. H. Ellison. Primary peptic ulceration of the jejunum associated with islet cell tumors of the pancreas. Ann. Surg. 142: 709-728, 1955.