PROLACTIN STIMULATION OF AMINO ACID-^{14}C UPTAKE AND INCORPORATION INTO PROTEIN IN THE PIGEON CROP MUCOSA

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This is to certify that the

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PROLACTIN STIMULATION OF AMINO ACID-^{14}C UPTAKE AND INCORPORATION INTO PROTEIN IN THE PIGEON CROP MUCOSA

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ABSTRACT

PROLACTIN STIMULATION OF AMINO ACID-^{14}C UPTAKE AND INCORPORATION INTO PROTEIN IN THE PIGEON CROP MUCOSA

By James A. Rillema

Since the pigeon crop proliferates specifically in response to prolactin, it was intended to describe the regulatory action of exogenous prolactin on the pigeon crop mucosa.

Four to six-week old White King Squabs were administered prolactin subdermally over one side of the crop-sac; the other side was injected with a control solution. Following the hormone treatment, the uptakes into the epithelium were determined for the following radiolabeled substances injected intravenously: hydrolysate ^{14}C-amino acids, ^{14}C-leucine, ^{14}C-α-aminoisobutyric acid (AIB), ^{14}C-sucrose, ^{3}H-uridine, ^{3}HOH, and ^{22}Na. A prolactin-enhanced uptake of hydrolysate ^{14}C-amino acids began at nineteen hours following the injection. The prolactin-increased uptake of the nonmetabolizable amino acid, ^{14}C-AIB, suggests that utilization of amino acids for incorporation into protein is not essential for an increased uptake. Prolactin also stimulated the uptake and incorporation of

^{14}C-leucine, but its enhanced uptake was less than the uptake of the hydrolysate mixture.

In vivo prolactin-treated crop tissue was removed from the birds and the more superficial layers of the crop were removed. The epithelium was then placed in a Lucite chamber and was bathed on both sides with bird Ringer solutions. The prolactin-treated tissue had a greater uptake of ^{14}C-leucine and ^{3}HOH added to the bathing solution than the control "epithelium" preparation. The epithelium was therefore designated as the primary target for prolactin in the pigeon crop.

Enhanced protein synthesis in response to prolactin was demonstrated by isolating the protein from the free ^{14}C-amino acids in prolactin-treated and control crop tissues and then measuring the activity of ^{14}C in the TCA protein precipitate. The prolactin-enhanced protein synthesis and amino acid uptake (^{14}C-leucine) was inhibited by actinomycin D, thereby indicating that prolactin stimulates a DNA-RNA dependent protein synthetic mechanism. The fact that puromycin was unable to inhibit the uptake and incorporation into protein of ^{14}C-leucine indicates that either the prolactin effect is puromycin insensitive, or the puromycin dose was too low to inhibit the prolactin response, or prolactin counteracts the puromycin effect.

The uptake of ^{3}HOH was increased at four hours and following fifteen hours of prolactin stimulation. These increases are probably related to metabolite movements: the four-hour increase is accompanied by enhanced uptakes of ^{22}Na and ^{3}H-uridine while the persistent hydration following fifteen hours is probably related to the increased amino acid and other metabolite uptakes.

The ^{14}C-sucrose space, "extracellular space," in the crop-sac was increased by prolactin administration, but this enlargement was not large enough to account for the total radioactivity of the accumulated amino acids and other metabolites.

Electronmicrographs of the eighteen-hour prolactin-treated crop-sac show two regions of the epithelial layer to be enlarged: the stratum spinosum and the stratum basalae. Stimulated cell division is also indicated by the characteristic aggregation of the chromatin material in the nuclei of the prolactin-treated cells.

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By

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CHAPTER I

INTRODUCTION

The homeostatic regulation of cells and cell organelles appears to be intimately related to hormones. And the mechanism of this relationship has been the subject of much recent inquiry; it was thus shown that specific metabolites are accumulated and incorporated into cellular products in response to specific hormones. But similar responses have not as yet been clearly defined for metabolite movements regulated by prolactin.

The production of crop "milk" and the simultaneous proliferation of the pigeon crop-sac are induced by the administration of exogenous prolactin alone. For studying the mechanism of action of prolactin, this organ therefore appears to be very useful.

The enhanced uptake and incorporation of amino acids into protein and imbibition of water are integral events in the proliferative processes in many tissues; and the hormone control of some of these processes has been reported. It is the author's intent to relate prolactin stimulation of the pigeon crop to the time sequence of amino acid uptake and incorporation into protein; the

^{3}HOH uptake in response to prolactin was concurrently investigated.

Subsequently, knowing the enhanced amino acid relationship to the crop-sac as regulated by prolactin, the mechanism of prolactin's effect was studied with the use of antibiotics and the nonmetabolizable amino acid, α-aminoisobutyric acid. The description of the prolactin-induced changes in the pigeon crop may have general application to the elucidation of the mechanism(s) of action of hormones.

CHAPTER II

THE LITERATURE SURVEY

The crop-sacs of pigeons and doves (Order Columbiformes) produce a nutrient milky substance which they regurgitate and use to feed their progeny. This substance was first called crop or pigeon "milk" by Hunter (1786, from Dumont 1965) and also by Bernard (1859, from Dumont 1965). An anterior pituitary hormone, prolactin, has a stimulatory effect on milk formation in these birds as well as in mammals. Davis (1939) found the crop milk to contain the following components: 33.8% fat, 58.6% protein, 4.6% ash, and 3.9% starch in the 28% dry matter. Therefore, it is the objective of this study to see how prolactin affects the enhanced protein formation in the pigeon crop. These results may then give further insight into the regulatory role of prolactin in the mammary gland and its tumors.

The effect of anterior pituitary extracts on crop membrane proliferation was initially reported by Riddle and Braucher (1931), and prolactin was eventually isolated from pituitary extracts by Riddle et al. (1932-1933). Its molecular weight was found to be about 26,000 (Dixon and Li 1964).

Others (Bassler and Forssmann 1964, Dumont 1965, Forssmann 1965, Meites and Nicoll 1966, and Weber 1962) have shown the specificity of prolactin for crop-sac epithelium and a consequent increase in mitosis (Leblond and Allen 1937 and Lahr and Riddle 1938). The crop-sac growth has also served as a basis for some commonly used prolactin bio-assays (Bates et al. 1963, Bergman et al. 1940, Lyons 1937, Nicoll 1967, and Riddle et al. 1933).

Prolactin-Stimulated Uptakes In the Crop

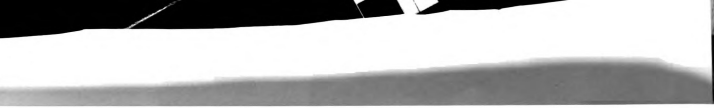
McShan et al. (1950) commenced the time sequence study of some of the biochemical changes induced by exogenous prolactin stimulation of the pigeon crop. They found prolactin to cause a progressive increase of the succinate dehydrogenase activity which they took as an index of the metabolic activity. This increase proceeded linearly for four days at which time the activity level was four times that at zero time. Also their graph seems to indicate that the activity increased within the first four hours after the prolactin administration. Pentose nucleic acid, which is a measure of the ribonucleic acid (RNA) content, was found to almost double between the first and second days of prolactin stimulation. Prior to one day, no notable increase was found. The desoxypentose nucleic acid (DNA) content and percent dry weight decreased for the first day following prolactin administration and then

progressively increased over the next forty-eight hours. The total crop-sac weight increased over the entire five-day period.

Brown et al. (1951) studied the uptake of ^{32}P into the crop as regulated by the daily intramuscular injection of prolactin. The dose-related uptake reached a maximum one hour following the radioactive nuclide injection on the fourth day; no further increase was observed. This probably means that the ^{32}P is no longer available after being in the plasma for more than one hour. They also found the prolactin-increased uptake was almost entirely in organic material, thereby indicating an increased incorporation into DNA, RNA, and other phosphorous-containing organic compounds.

By injecting prolactin daily for four days in localized subdermal injections over the crop-sac, Damm et al. (1961) essentially repeated the experiments of Brown et al. Total phosphorus content was significantly higher as was ^{32}P uptake, following a four-hour label time. The advantage of using the local injection technique is that variability among birds is eliminated from statistical consideration.

Using a subdermal injection protocol for prolactin, five times in three days, Ben-David (1967) studied its regulatory effect on the uptake of ^{3}H-methyl-thymidine in the crop epithelial cells. He reported a log-dose uptake



of the label, which was in the birds between two and three hours. These data verify the increased DNA synthesis as reported by McShan et al. (1950) and also substantiates the increased mitotic activity reported by Leblond and Allen (1937) and Lahr and Riddle (1938).

Dumont in 1965 by means of electron microscopy reported an increased fat accumulation in the crop epithelial cells twelve hours after prolactin treatment. Subsequent chemical analysis showed this fat to be primarily triglycerides. Also accompanying the enhanced accumulation of fat was an apparent increase in pinocytotic activity; this is one characteristic of the initial stages of lipid accumulation in cells. At twelve hours following the prolactin treatment, Dumont also demonstrated an increase in polysome formation; thus an increased protein synthesis is indicated.

Sherry and Nicoll (1967) studied the relationship of prolactin stimulation to protein and RNA synthesis by using the antibiotics puromycin and actinomycin D. They found an inhibition of the crop-sac response to prolactin, thereby showing that the increases in RNA (inhibited by actinomycin D) and protein (inhibited by puromycin) are integral parts of the prolactin-stimulated proliferation. These antibiotics, however, were only able to inhibit partially (20% to 30%) the response to locally injected prolactin. The inhibitions reported by these investigators

were at forty-eight hours for puromycin (200ug) and at twenty-four hours for the actinomycin D (0.5ug). The prolactin-induced increase of RNA and protein synthesis are therefore present by at least the times indicated.

Sherry and Nicoll (1967) also reported a crop-sac response to intradermal injections of RNA extracted from the crop epithelial cells of prolactin-treated birds. Although a proliferation of the crop-sac was evident, this only indicates, but does not prove, that the prolactin-induced proliferation is dependent upon the synthesis of new RNA, especially since actinomycin D was able to inhibit only 20% to 30% of the prolactin-induced crop proliferation.

Thermostability of Prolactin

In 1933 Riddle et al. first reported the stability of prolactin boiled for twenty minutes at pH 7.5 to 8.5, but they gave no data to support their conclusions. A one-hour treatment under the same conditions again showed prolactin to be thermostable (McShan and French 1937). Geschwind and Li (1955) reported that growth hormone boiled for fifteen minutes at a neutral pH had no stimulatory effect on tibial epiphysis growth in the rat. Whereas prolactin, similarly treated, had the same effect as unboiled prolactin on tibial epiphysial growth and also had its normal crop-sac stimulatory activity.

Later Kostyo and Schmidt (1962) showed that prolactin boiled for twenty minutes at pH 8 maintained its crop-sac stimulatory activity while its effect on the AIB uptake of the rat diaphragm decreased. The authors therefore indicated that growth hormone, which is destroyed by the boiling procedure, probably contaminated the prolactin and was responsible for the enhanced amino acid uptake. Hjalmarson and Ahren (1967) found that prolactin and growth hormone, boiled for fifteen minutes and administered in vivo, did not inhibit the in vitro growth hormone-stimulated AIB uptake in the rat diaphragm, whereas the non-heat-treated hormones did inhibit this response. But the heat-treated prolactin retained its ability to stimulate the mammary glands. Here again growth-hormone contamination of prolactin was probably responsible for the non-boiled-prolactin response.

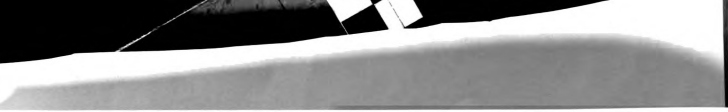
The thermostability of prolactin boiled for fifteen to twenty minutes at pH 8 is therefore clearly suggested by the available literature. Positive proof, however, that all the effects of prolactin remain following the heat treatment is lacking. That boiled growth hormone has less activity is well substantiated, but further experiments are necessary to verify the complete inactivation of growth hormone by the heat treatment.

Antibiotics

Actinomycin D and puromycin have been used rather extensively for showing how certain biological changes are effected. Actinomycin D apparently inhibits the transcription process (Hawamata and Imanishi 1960, Reich et al. 1961, and Rounds et al. 1960) by inhibiting RNA polymerase activity and forming a complex with DNA (Reich 1963). Puromycin appears to selectively prevent the amino acid transfer from t-RNA to protein, thereby preventing translation (Nathans and Lipmann 1961, Rabinovitz and Fisher 1962, and Yarmolinski and De La Haba 1959) for protein formation.

AIB

Extensive use of AIB as a substitute for metabolizable amino acids has been made. It is actively transported across the human erythrocyte by the same processes as the neutral amino acids (Winter and Christensen 1963). Growth hormone increases the accumulation of AIB in the rat diaphragm cells (Kostyo et al. 1962) in a manner similar to an enhanced accumulation of the metabolizable amino acids. Other hormones, including thyroxine on embryonic rat bone (Adamson and Ingbar 1967), epinephrine on the rat uterus (Noall et al. 1957), estrogen on the rat uterus and rat excretion (Noall et al. 1957 and Riggs and Walker 1963), insulin on rat liver (Chambers et al. 1965), TSH on bovine



thyroid slices (Debons and Pittman 1966), and hydrocortisone on rat liver (Chambers et al. 1965) also increase AIB accumulation in their respective target tissues.

Prolactin-Induced Structural Changes In the Pigeon Crop

In 1963 Forssmann by means of electron microscopy reported increased folding, cell number, and cell volume in response to prolactin. Also, fat vacuole content of the crop cells was enhanced as were the numbers of mitochondria, ergastoplasm membranes, ribosomes, and desmasome openings. Bassler and Forssmann (1964) reported similar findings, but they did not look at the initial twenty-four hours following prolactin administration.

Folds of the epithelium into the lamina propria were reported by Masahito and Fujii (1965 from Dumont 1965) twelve hours after the prolactin treatment. These folds increase with time following the hormone injection.

Dumont in 1965 took electron micrographs of twelve-hour, two-day, and four-day prolactin treated crop tissues. The stratum spinosum contained more lipid dropletts and had an increased pinocytotic activity following a twelve-hour prolactin treatment; polysome formation was also considerably enhanced. Intracellular cannals enlarged two days after the injection of prolactin, while the lamina propria contained many more vesicles. Also, after two days, the stratum basalae was enlarged. The stratum

spinosum was shown to triple in thickness four days following the prolactin treatment. Dumont also reports an increased vascularity of the crop in response to prolactin, but he does not report when this process begins.

Sherry and Nicoll (1967) show photographs of crop tissues treated with RNA extracts from other crop tissues. The proliferation of the RNA-treated tissues is obvious, but the relation of prolactin to this RNA stimulation is not clear.

CHAPTER III

MATERIALS AND METHODS

The Experimental Animals

Four to six-week old White King Squabs (Cascade Squab Farm, Grand Rapids, Michigan) of both sexes were maintained on a normal diet, at room temperature (27°C), and were exposed to fourteen hours of light daily. The birds were maintained under these conditions three to ten days prior to experimentation; food and water were available ad libitum for the duration of this "equilibration" period.

The In Vivo Experimental Protocol

Twenty-four hours prior to experimentation, the feathers of the squabs were removed from the entire crop-sac surface and from the axilla. Following this preparation the birds received intradermally 0.1 ml of a NIH prolactin solution (Table 2), thereby forming a bleb over one lateral aspect of the crop-sac. The other half of the crop-sac received an equal volume of bird Ringer solution (Table 2), distilled water, or fetal calf serum (Table 2). The prolactin used was mixed in distilled water and kept frozen until injected into the birds. Since the author

Table 1

Radioactive Nuclides Used

Radioactive Nuclide	Specific Activity	Lot Number	Company
^{3}HOH	----	----	New England Nuclear
^{14}C-protein hydrolysate amino acids	172 uC/mM	6606 6701 6702	Schwartz Bioresearch Inc.
^{14}C-leucine	165 mC/mM	22	Nuclear Chicago
^{3}H-uridine	5.0 C/mM	19	Nuclear Chicago
^{22}Na	1.13 mC/mM	103	Abbott Laboratories
^{14}C-sucrose	480 mC/mM	6701	Schwartz Bioresearch Inc.
^{14}C-α-aminoisobutyric acid	10 mC/mM	6701-P	Schwartz Bioresearch Inc.

Table 2

Drugs and Hormones Used

Substance	Source	Lot # or Activity
Prolactin PB-1*	NIH Endocrin. Research Section	12.8 IU/mg
Prolactin PB-2*	NIH Endocrin. Research Section	19.6 IU/mg
Actinomycin D*	Merck, Sharp, and Dohme Research Laboratories	L55465l-0-10
Puromycin	Nutritional Biochemicals Corp.	Control #4695
Fetal Calf Serum	Grand Island Biological Inc.	Control #419088
Bird Ringer Solution	(Frantz and Rose 1968)	

* - Donated by the above named institutions

found decreased activity of the hormone when the prolactin was in solution and kept frozen for longer than two weeks, prolactin solutions kept longer than this time were discarded. Prolactin activity also appeared to decrease more rapidly when the hormone was frozen in bird Ringer solution, probably due to the salt concentration (Riddle et al. 1933).

In preliminary experiments, it was ascertained that the effect on the crop epithelium of a single, intradermal injection of 25 ug of PB-2 prolactin remained unilateral. At specified time intervals subsequent to the hormone treatment, 5uC/Kg of each of the following

radio-labeled substances in combinations of two were injected into the wing vein: $^{22}Na^{+}$ ^{3}H-uridine, tritiated water, protein hydrolysate ^{14}C-amino acids, ^{14}C-α-amino-isobutyric acid, ^{14}C-leucine, and ^{14}C-sucrose. (See Table 1 for specific activities and sources of the radioactive nuclides.) At predetermined times, from one to six hours after the label administration, the birds were killed by cervical dislocation, and two to three cm^2 discs of the prolactin and control-treated areas of the crop were excised, weighed, in most cases sonically homogenized, and counted. Simultaneously from each bird, heart-puncture blood samples were drawn, centrifuged, and a 100ul aliquot of the plasma was transferred into the counting medium. The radioactivity within all the samples was determined in a modified Bray's (Bray 1960) dioxane scintillation solution with a Nuclear Chicago Model 6860 liquid scintillation counter. Background counts were automatically subtracted, and quench correction was done by use of an external ^{133}Ba standard; this technique allows for simple disintegration per minute determinations from dual labeled samples (see Appendices I and II for quench correction counting procedures).

The data from membrane counts was expressed as a ratio of treatment over control, or T/C, where T represents the disintegrations per minute per milligram (DPM/mg) for the prolactin-treated tissues and C, the DPM/mg of the

control-injected counterpart. The use of T/C ratios, rather than gross counts of treated and control tissues, eliminates statistical variability among birds. The statistical test for the T/C ratios being significantly greater than 1.0 was the student t-test (Ostile 1963). When comparing the uptakes of the labeled amino acids (hydrolysate ^{14}C-amino acids, ^{14}C-AIB, and ^{14}C-leucine), it was of concern that the specific activities of the various labeled substances (Table 1) differed markedly. But with respect to the endogenous amino acid concentration in the plasma, which is about 3.0-7.0mM (Spector 1956), their circulating specific activities were nearly identical, ca. 1.666 x 10-9C/mM.

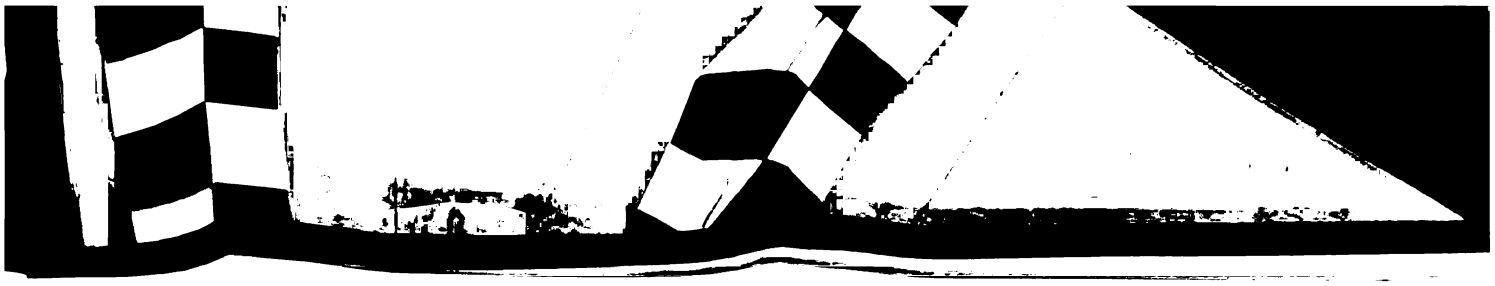
Sonicated Vs. Intact Membranes

In some experiments, counts were determined and T/C ratios were calculated from whole membranes placed in the scintillation medium. And in order to evaluate the validity of using this procedure, a series of membrane pairs was ultrasonically homogenized (Heat Systems Co., Sonifier Model W-185-c) for five minutes at 300 watt-minutes and then counts were determined in the manner described above. Although the total counts were greater, the resulting T/C ratios for hydrolysate ^{14}C-amino acids were not different from those of the intact membrane samples (Table 4). In all cases, a twenty-four hour prolactin

pretreatment and four-hour label time were used with 5uC/Kg of each labeled substance injected. In homogenized samples about ten times more DPM/mg were counted for the ^{14}C-labeled protein hydrolysate, and three times more were counted for the ^{3}HOH. Counts within the prolactin-treated and control tissues increased proportionately with the result that the T/C ratios between intact and sonicated tissues were not different. All tissues except those from the dual labeled studies of ^{3}HOH and hydrolysate ^{14}C-amino acid uptakes were sonically homogenized prior to activity determinations.

Prolactin Vs. Calf Serum

Since prolactin is a proteinaceous compound, it is germaine to question whether a nonspecific protein stimulation of metabolite uptake in the pigeon crop would occur. Lahr et al. (1943) demonstrated a crop-sac proliferation with oils, bile, and many other substances. To establish its specificity, therefore, prolactin was administered as previously described, and fetal calf serum (700ug/ml) was used in the control injection. Its protein content, molecule per molecule, was the same as that of the injected prolactin. Calf serum was used for a control in all experiments except the dual label studies where ^{3}HOH and hydrolysate ^{14}C-amino acids were injected. Therefore, the various comparisons using the same time sequence for injections and sampling establish the specificity of prolactin



for causing the enhanced uptake and other changes which are reported (^{3}HOH ratios in Figure 4 and Table 6).

Growth Hormone Effects

The amount of growth hormone present in NIH PB-2 prolactin is reported by NIH to be less than one percent. But, since growth hormone is able to cause an increased amino acid accumulation in other tissues, (Kostyo and Schmidt 1962), it was decided to test whether growth hormone is also a causative agent in the various enhanced uptakes observed with the prolactin solution. It is fairly well established, but not conclusively, that boiling at pH 8 for twenty minutes destroys growth hormone activity whereas prolactin retains its activity. Using a twenty-four hour prolactin treatment and a three-hour label time (5uC/Kg hydrolysate ^{14}C-amino acid and 5uC/Kg ^{3}HOH), a comparison was made between a 25 ug normal PB-2 prolactin injection and a 25 ug boiled PB-2 dose. The results (Table 3) show no difference between the T/C ratios of ^{3}HOH and the hydrolysate ^{14}C-amino acids of the two groups. It is concluded that there is no effective activity of growth hormone in NIH PB-2 prolactin. And also the stability of prolactin under the conditions listed above is further verified.

Table 3.--The effect of boiled prolactin on the uptakes of hydrolysate ^{14}C-amino acids and ^{3}HOH into the pigeon crop.

	PROLACTIN	
	NORMAL	BOILED
^{14}C Amino Acid T/C	1.23±0.10 (9)	1.28±0.07 (10)
^{3}HOH T/C	1.26±0.06 (19)	1.35±0.13 (10)

Mean ± Std. Error (N)

In Vitro Uptakes in In Vivo Treated Crop-Sac Epithelium

For in vitro studies the bird's feathers were plucked twenty-four hours prior to a 25 ug intradermal injection of PB-2 prolactin over one side of the crop-sac; the control side received an equimolar injection of fetal calf serum protein. Twenty-four hours later, the hormone and control-treated membrane portions were excised, and the epithelia were isolated by teasing away with a forceps the more lateral layers of the crop. The membranes were then put between identical halves of Lucite bathing chambers. The tissue preparation and modified Ussing bathing apparatus are described by Frantz and Rose (1968) and Rose (1967). Subsequent to a one-half to two-hour perfusion time for equilibration, where the membranes had bird Ringer solutions on both sides with constant stirring, 1.0 uC of ^{14}C-leucine and 1.0 uC of tritiated water were placed in the serosal bathing fluid. A 100 ul sample of the "hot"

bathing fluid was taken and put in the scintillation counting medium. After a one or ten-minute label time, the fluid was removed from the bathing chambers, and the membranes were thoroughly rinsed once with cold bird Ringer solution. Then the bathed portion of the crop tissue was removed from the Lucite blocks and cooled immediately to stop metabolic activity. The tissues were then weighed, placed in 1.0 ml water, sonically homogenized, and counted in the liquid scintillation fluid.

Protein Synthesis Determination

The procedures involved in the demonstration of an enhanced protein synthesis in response to prolactin are described primarily in the results section, thereby eliminating some unnecessary repetition. Briefly, the proteins from prolactin and control-treated crop tissues, sonically homogenized, obtained from birds injected with hydrolysate ^{14}C-amino acids, were precipitated with 10% TCA and the activity of the protein then determined. The activities in the prolactin-treated and control tissues were then compared. The effect of t-RNA binding of amino acids on the protein precipitate counts was eliminated by washing the precipitates with 1N NaOH for less than two minutes. Then the protein was reprecipitated with 10% TCA and washed once more with the TCA. After the protein was again isolated by centrifugation, the activity in the

protein precipitate was determined and compared to that in the tissue homogenate.

Inhibition Studies

The manner by which prolactin exerts its influence was examined with the use of two antibiotics, actinomycin D and puromycin (Table 2). Puromycin (200 ug) was injected intradermally over one lateral aspect of the crop-sac; the other surface received an equal volume of bird Ringer solution, the same medium used to mix the antibiotics. One hour later, one-half of these birds received 0.1 ml PB-1 prolactin (0.5 IU) into the bleb where the puromycin had been injected; the control side received simultaneously a fetal calf serum injection. Seven other birds received prolactin and calf serum only, i.e. they received no antibiotics. A protocol similar to that for the puromycin experiments was used for actinomycin D except that the dose was 0.5 ug. Twenty-four hours after the antibiotic or hormone treatments, the birds received intravenously 5 uC/Kg ^{3}HOH and 5 uC/Kg ^{14}C-leucine. Three hours later the treated and control tissues were excised, halved, weighed, and sonicated. One of the sonicated halves was then used for a total count determination by liquid scintillation counting; the other half was used for determining the counts in the protein fraction. The procedure for the protein isolation and ^{14}C counting was as previously described.

Both the puromycin and actinomycin D solutions were premixed in bird Ringer solution and kept frozen until used. Both were used for experimentation within a week, thus minimizing the loss of activity of these antibiotics.

Electron Microscopy

Eighteen-hour prolactin (25 ug of PB-2) and calf-serum-treated membranes were prepared for electron microscopy following an *in vivo* hormone and control treatment. The tissues were excised and fixed in glutaraldeheyde buffered to pH 7.5 with phosphate. Then two hours later the tissues were post-osmicated for ninety minutes in phosphate-buffered osmiun tetroxide. As outlined by Dumont (1965), the tissues were further prepared by Mr. Gordon Spink of the Biological Research Center, Michigan State University.

CHAPTER IV

RESULTS

Hydrolysate ^{14}C-Amino Acid Uptakes

The uptake of hydrolysate ^{14}C-amino acids into the piegon crop epithelium in response to a 25 ug injection of prolactin is plotted in Figure 1. Zero time represents the beginning of a single PB-2 prolactin treatment, and the arrows on the baseline (T/C = 1.0) represent the subsequent intravenous radiolabel injections. The data points are mean T/C values ($N \geq 5$); the vertical bars are the standard errors of the means. Hourly ratios for the hydrolysate ^{14}C-amino acids show an enhanced uptake beginning at nineteen hours after the prolactin treatment. The dotted lines represent best fit curves (visual) for average data points corresponding to separate isotope injections; the solid line represents a composite curve for the significant ratios up to thirty hours.

Following a twenty-four hour PB-2 prolactin treatment, a dose-response relationship (Figure 2) of the hydrolysate ^{14}C-amino acid T/C ratios is evident twenty-eight to thirty hours after the prolactin was injected into the birds. The plateau in the 25 ug curve can be

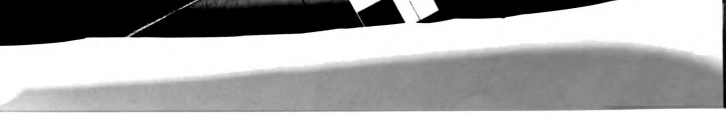


FIGURE 1

T/C ratios for hydrolysate ^{14}C-amino acids as a function of time after a single 25 ug intradermal injection of prolactin at 0 hour. Each arrow represents the injection of 5 uC/kg (173 uC/mMole) of amino acids. Each open circle, which corresponds to the immediately preceding arrow, is the average T/C ± SE ($N \geq 5$) which is significantly greater than 1.0 ($P<0.05$). The solid circles are insignificant ones. The dotted lines are best-fit curves for the mean values derived from the 18 and 24 hour label injections. The solid line is the best-fit curve for the maximal uptake ratios of the two individual curves.

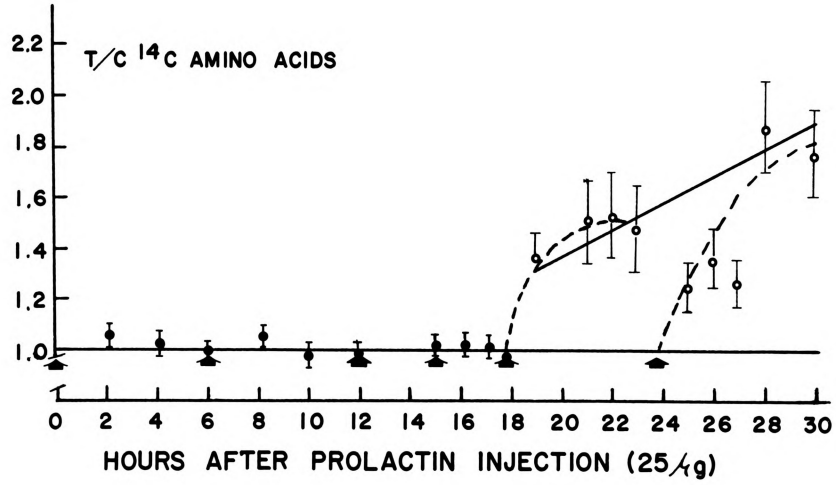
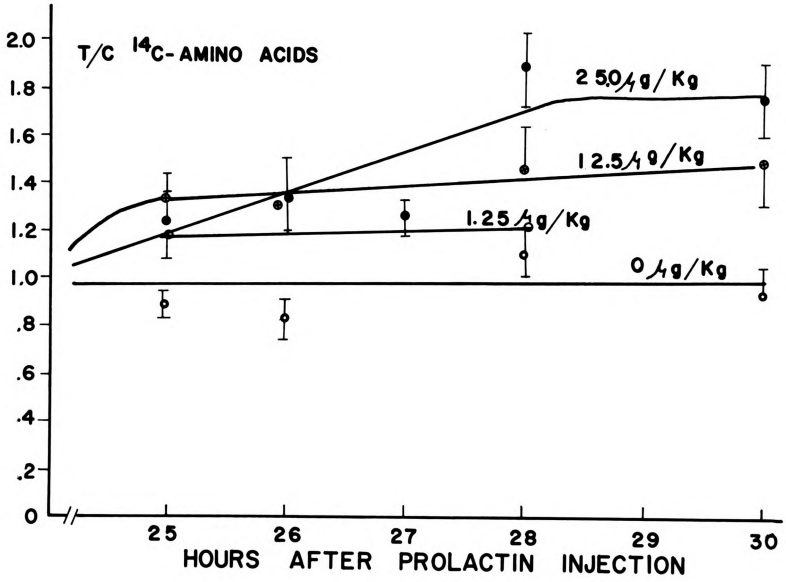

T/C 14C AMINO ACIDS
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
HOURS AFTER PROLACTIN INJECTION (25γg)

FIGURE 2

T/C ratios for hydrolysate ^{14}C-amino acids (filled circles, mean ± SE) in response to single, intradermal injections at 0 hour of prolactin in the amounts indicated on the curves. For the control ratios (open circles) bird Ringer solutions were injected at 0 hour over both halves of the crop. Into all the pigeons 5 uC/kg (173 uC/mMole) of hydrolysate amino acids were injected iv at 24 hours; tissue samples were taken at intervals for six hours. Each point represents an average T/C ± SE ($N \geq 5$).


T/C 14C-AMINO ACIDS
250 µg/Kg
12.5 µg/Kg
1.25 µg/Kg
0 µg/Kg
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
25
26
27
28
29
30
HOURS AFTER PROLACTIN INJECTION

attributed to the limiting amount of labeled amino acids available from the plasma.

^{3}HOH Uptakes

Figure 3 shows the uptake of ^{3}HOH in the pigeon crop epithelium in response to a 25 ug injection of prolactin. The ratios are calculated and plotted in a manner similar to those of the hydrolysate ^{14}C-amino acids (Figure 1). Between zero and fifteen hours following the prolactin administration no ratios were significantly greater than 1.0 except for the four-hour response. Ratios obtained from a repetition of the experiment covering the initial six hours, in which both prolactin and ^{3}HOH were given at zero time and tissue samples obtained from one to six hours later, were not different from the original data; i.e. in only the four-hour samples were the T/C ratios significantly greater than 1.0. Following a fifteen-hour prolactin treatment, the T/C ratios for ^{3}HOH are significantly greater than 1.0 for at least up to thirty hours. The ratios also appear to increase progressively for the duration of the fifteen to thirty-hour time increment.

Following a twenty-four hour prolactin treatment, a dose-response relationship of ^{3}HOH uptake is evident from twenty-eight to thirty hours after the label administration (Figure 4). The rate at which the T/C ratios fall toward 1.0 in the 1.25 and 12.5 ug curves appears to be a function

FIGURE 3

T/C ratios for ^{3}HOH as a function of time after a 25 ug intradermal injection of prolactin at 0 hour. Each arrow represents an iv injection of 5 uC/kg of ^{3}HOH. Each open circle, which corresponds to the immediately preceeding arrow, is the average T/C ± SE ($N \geq 5$) which is significantly greater than 1.0 ($P<0.05$). The filled circles are for the T/C ratios which are not significantly different from 1.0. C = 77.7 ± 6.4 DPM/mg, N = 164.

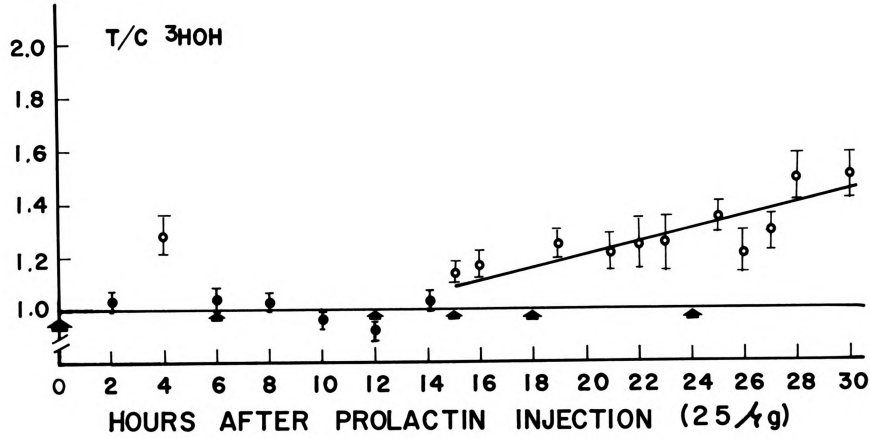
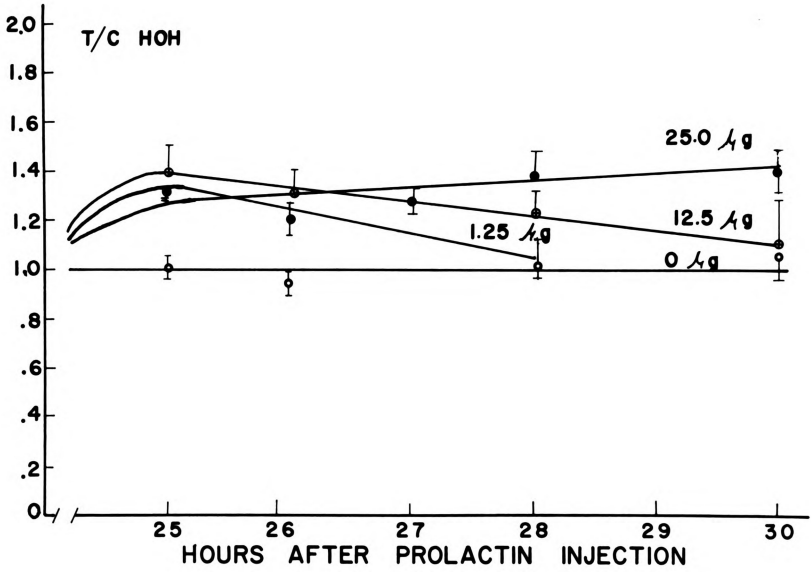

T/C 3HOH
2.0
1.8
1.6
1.4
1.2
1.0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
HOURS AFTER PROLACTIN INJECTION (25 μg)

FIGURE 4

T/C ratios for ^{3}HOH (filled circles, mean ± SE, N $\geq$ 5) in response to the amounts of prolactin indicated on the curves. A single 5 uC/kg dose of ^{3}HOH was injected at 24 hours after the single, intradermal injection of prolactin. For the control ratios (open circles) bird Ringer solution was injected intradermally at 0 hour over both halves of the crop, then 5 uC/kg of ^{3}HOH was given iv at 24 hours. C = 77.7 ± 6.4 DPM/mg, N = 164.


T/C HOH
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
25.0 µg
12.5 µg
1.25 µg
0 µg
25
26
27
28
29
30
HOURS AFTER PROLACTIN INJECTION

of prolactin titre; only the sufficiently stimulated epithelium (25 ug) is able to accumulate ^{3}HOH for up to six hours.

^{14}C-AIB Uptake

The relationship of the increased uptake of amino acids to their utilization was evaluated in the following manner. Using a twenty-four hour 25 ug PB-2 prolactin treatment, a comparison was made between the accumulation of the hydrolysate ^{14}C-amino acids and ^{14}C-α-aminoisobutyric acid (a non-metabolizable amino acid) in intact and sonicated membranes taken three hours after the label injections. The resultant T/C ratios (Table 4) are not different from one another, but both are significantly greater than 1.0. Since prolactin enhances the uptake of this non-metabolizable amino acid, it can be concluded that increased utilization is not essential for an enhanced accumulation of amino acids.

^{3}H-Uridine and $^{22}Na^{+}$ Uptakes

Whether the four-hour prolactin-stimulated-tritiated water uptake is related to some metabolite movement was examined with the use of ^{3}H-uridine and $^{22}Na^{+}$. The tracers (5 uC of each/Kg bird weight) were injected simultaneously with PB-2 prolactin (25 ug) at zero time. The samples were taken from one to six hours later. As

Table 4.--The effect of prolactin on the uptakes of hydrolysate ^{14}C-amino acids and ^{14}C-amino acids and ^{14}C-α-aminoisobutyric acid; the effect on the hydrolysate ^{14}C-amino acids into protein; and a comparison of sonicated vs. intact membranes of the pigeon crop.

		INTACT MEMBRANES		SONICATED MEMBRANES	
WASH	^{14}C LABEL	$\frac{T}{C}$	CONTROL $\frac{DPM}{mg}$	$\frac{T}{C}$	CONTROL $\frac{DPM}{mg}$
NONE	AA	1.23±0.10 (20)	4.56±0.41	1.41±0.16 (7)	43.3±4.0
TCA	AA	1.45±0.37 (5)	2.60±0.30	1.41±0.17 (7)	26.2±1.6
NONE	AIB	1.28±0.13 (10)	3.65±0.60	1.47±0.16 (10)	21.6±3.5
TCA	AIB	(insig.) (5)	0.88±0.005	(insig.) (5)	1.9±0.32

AA = HYDROLYSATE AMINO ACIDS

AIB = α-AMINOISOBUTYRIC ACID

MEAN±SE(N); T/C>1.0, P<0.05

can be seen in Table 5, the T/C ratios for both isotopes were significantly greater than 1.0 only at four hours following the prolactin treatment. The interdependence of the increased ^{3}HOH, $^{22}Na^{+}$, and ^{3}H-uridine uptakes cannot be explained from this data. It is also pertinent to note here that T/C ratios (Figure 1) for the hydrolysate ^{14}C-amino acids, simultaneously injected, were insignificant at the four-hour time following the prolactin stimulation.

^{14}C-Sucrose Uptake

In order to evaluate the effect of prolactin on extracellular space, the distribution of ^{14}C-sucrose was determined twenty-eight hours following a PB-1 prolactin (0.5 IU) treatment. ^{14}C-sucrose (5 uC/Kg) and ^{3}HOH (5 uC/Kg) were injected four hours prior to sampling; the ^{3}HOH ratios thus served for comparative purposes with previously obtained ratios. As can be seen in Table 6, the T/C ratios for ^{14}C-sucrose and ^{3}HOH are both significantly greater than 1.0. And the population of ^{3}HOH T/C ratios was not different from that of previously obtained ratios using a similar treatment (Figure 4). An enlarged ^{14}C-sucrose space, "extracellular space," (Fenstermacher and Bartlett 1967) in response to prolactin is thereby indicated by these data.

Table 5.--The effect of two to six hours of prolactin stimulation of the pigeon crop on the uptakes of ^{3}H-uridine and ^{22}Na.

	TIME (Hours)				
	2	3	4	5	6
T/C ^{3}H Uridine	1.08±.06	1.05±.07	1.27±.10	1.08±.06	.97±.03
C ^{3}H Uridine	@20.9±.63	19.8±.97	20.4±1.71	19.2±1.36	19.07±.70
T/C ^{22}Na	1.05±.03	.99±.04	1.22±.09*	1.06±.07	.93±.02
C ^{22}Na	4.59±.27	4.54±.24	4.30±.26	4.61±.18	4.92±.19

N = 5 @(DPM/mg)

*P<.05 Mean±S.E.

Table 6.--The effect of prolactin on the ^{3}HOH and ^{14}C-sucrose spaces in the pigeon crop.

	RADIOACTIVE NUCLIDE	
	^{3}HOH	^{14}C SUCROSE
T/C	1.21±.10* (5)	1.13±.04** (5)
C =	32.5±2.7 DPM/mg	2.77±3.6 DPM/mg

Mean S.E. *P<.05 **P<.01

^{14}C-Leucine Uptake

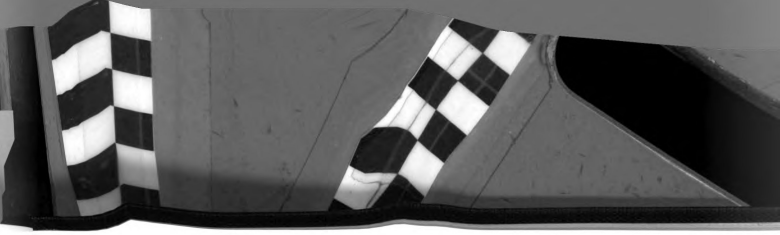
The *in vivo* uptake of ^{14}C-leucine, which is one of the constituents of the ^{14}C-amino acid hydrolysate mixture, provided a basis for comparison with *in vitro* labeled membranes. Using a twenty-four hour, PB-1 prolactin (0.5 IU) treatment, the birds received 5 uC/Kg ^{14}C-leucine and 5 uC/Kg ^{3}HOH; the samples were taken four hours later. The T/C ratios for the ^{14}C-leucine and ^{3}HOH are both greater than 1.0 (Tables 8 and 9). The ratios for the tritiated water being in the same statistical population as those obtained when hydrolysate ^{14}C-amino acids were used indicate constant experimental conditions. The ratio for the ^{14}C-leucine (1.13) was greater than 1.0 but less than those obtained for the hydrolysate ^{14}C-amino acids (1.41) (Figures 1 and 2). A preferential uptake of other amino acids in the hydrolysate ^{14}C-amino acid mixture

also appears evident since the average increased uptake of the hydrolysate mixture was 41%.

In Vitro Uptakes

The demonstration of an enhanced uptake *in vitro* of ^{3}HOH and ^{14}C-leucine in prolactin-stimulated crop tissues was attempted for two reasons. First, to show that endogenous factors are not immediately essential for the enhanced accumulation of the above two substances, once prolactin has affected the crop tissue. And second, to show that the prolactin-enhanced accumulating capacity for amino acids is in the crop epithelium and not in the muscle or other tissue. The crop-sacs were 25 ug PB-2 prolactin-treated for twenty-four hours; the control side received an equimolar concentration of fetal calf serum. The membranes were placed in identical bathing chambers and the isotopes (^{3}HOH and ^{14}C-leucine) placed in the Ringer solution bathing the serosal surface of the crops. Then the tracer-exposed area of the crop was excised, blotted, weighed, and sonicated, and the radioactivity was determined.

Following a ten-minute radioactive nuclide exposure *in vitro*, no difference in counts was evident in the prolactin and calf-serum-treated tissues. But using a one-minute exposure time, T/C ratios were significantly greater than 1.0 for ^{14}C-leucine ($p<.10$) and ^{3}HOH ($p<.05$) (Table 7). This demonstrates that the pigeon-crop mucosal epithelium,



stimulated *in vivo*, is able to accumulate amino acids and ^{3}HOH *in vitro* in the absence of submucosal layers. But this does not mean that prolactin has no effect on the tissues which were removed from the bathed membrane portion.

Table 7.--The effect of prolactin on the uptakes of ^{3}HOH and ^{14}C-leucine *in vitro* into the crop-sac epithelium.

		RADIOACTIVE NUCLIDE	
TIME	UNITS	^{3}HOH	^{14}C LEUCINE
1 min.	T/C	2.13±.27** (7)	1.75±.38* (7)
1 min.	$\frac{\text{C DPM/mg. memb.}}{\text{DPM/mg. ser. fl.}}$	0.474±.057 (7)	0.612±.090 (7)
10 min.	T/C	0.93±.37 (7)	0.97±.08 (7)
10 min.	$\frac{\text{C DPM/mg. memb.}}{\text{DPM/mg. ser. fl.}}$	2.366±.559 (7)	2.714±.354 (7)

Mean±S.E. *P<.10 **P<.01

Amino Acid Availability and Incorporation Into Protein

To better understand the manner in which labeled substances are available from the blood, a series of plasma samples from the radiolabeled birds were counted, centrifuged, the dioxane supernatants removed, and both fractions were counted. The counts remaining in the supernatant provided an estimate of the radiolabeled fraction that was not bound to plasma proteins. As determined by this dioxane

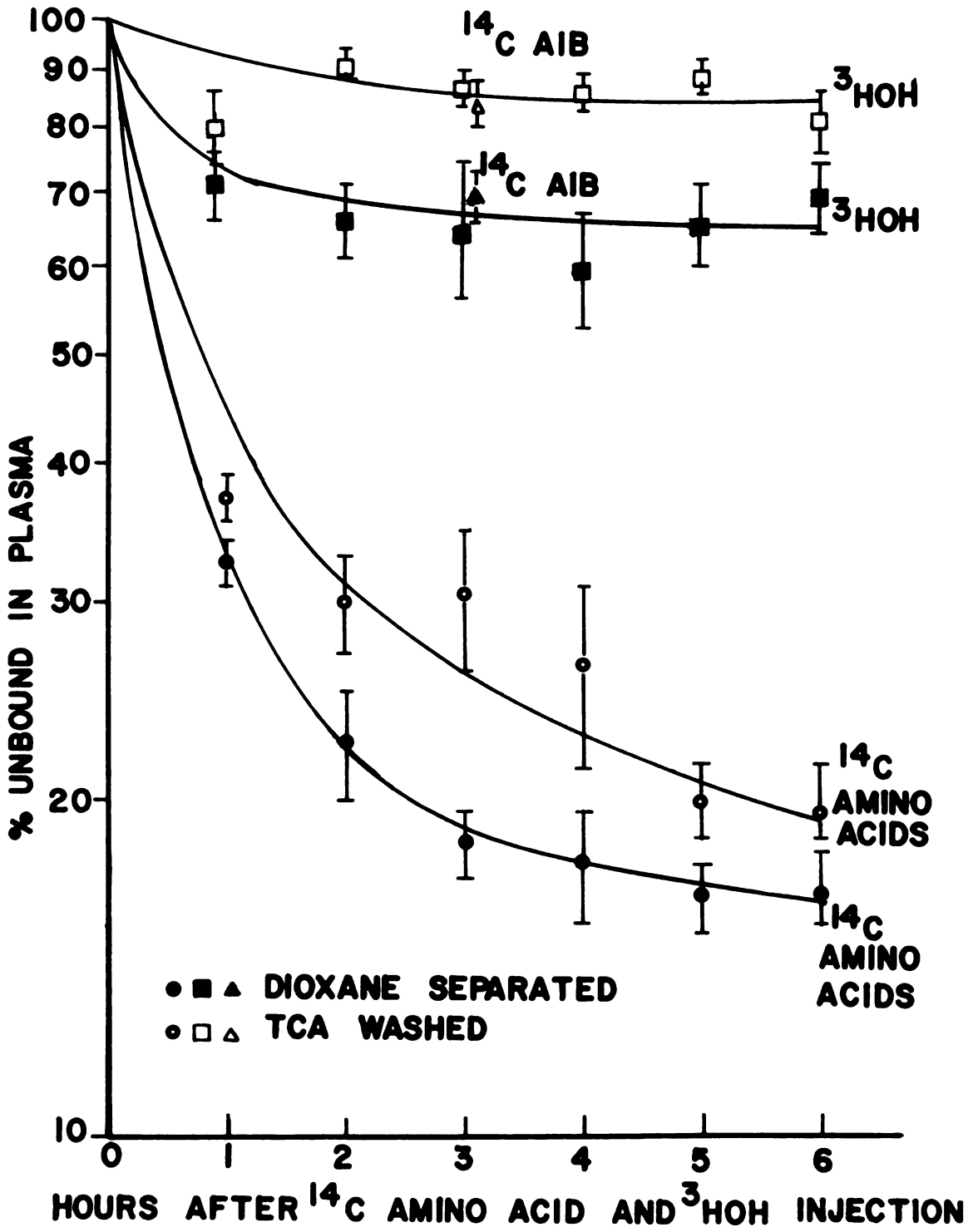
partitioning, the fraction of unbound ^{14}C-amino acids in the plasma decreased exponentially in four hours to about 17% (lowest curve of Figure 5, filled circles). A plot of the accumulated count in the precipitated plasma protein produces a reciprocal curve which when added to the curve for counts in the supernatant totals 100% of the initial counts.

To determine the affinity of nonpeptide-linked amino acids for the plasma precipitates, the dioxane-separated precipitates were centrifuged and shaken in 0.5 ml of a 10% TCA solution for one hour. These TCA precipitates were then washed twice with water and counted in fresh scintillation fluid. Washing with TCA increases the yield of the protein fraction by about 7% (open circles, Figure 5).

The effectiveness of TCA in releasing absorbed amino acids and ^{3}HOH in the absence of protein synthesis (Figure 5, zero hours) was demonstrated by mixing 0.25 uC/ml (equivalent to the 5 uC/Kg *in vivo*) of ^{3}HOH and either hydrolysate ^{14}C-amino acids or ^{14}C-AIB with pigeon plasma in a test tube for one hour; the plasma proteins of the separated samples were washed with TCA in the above manner and counted. After the TCA wash, 92.0 ± 1.3% (N = 5) of both the ^{14}C-amino acids and ^{14}C-AIB, and 96.0 ± 1.3% (N = 10) of the ^{3}HOH introduced *in vitro* was released from the plasma protein fractions. Therefore, the fraction of

FIGURE 5

The separation of unbound label recovered in the plasma vs. time following a single 5 uC/kg injection iv at 0 hr. (mean percent of the total count ± SE, N $\geq$ 5). Hydrolysate ^{14}C-amino acids are represented by circles, ^{14}C-AIB by triangles, and ^{3}HOH by squares.

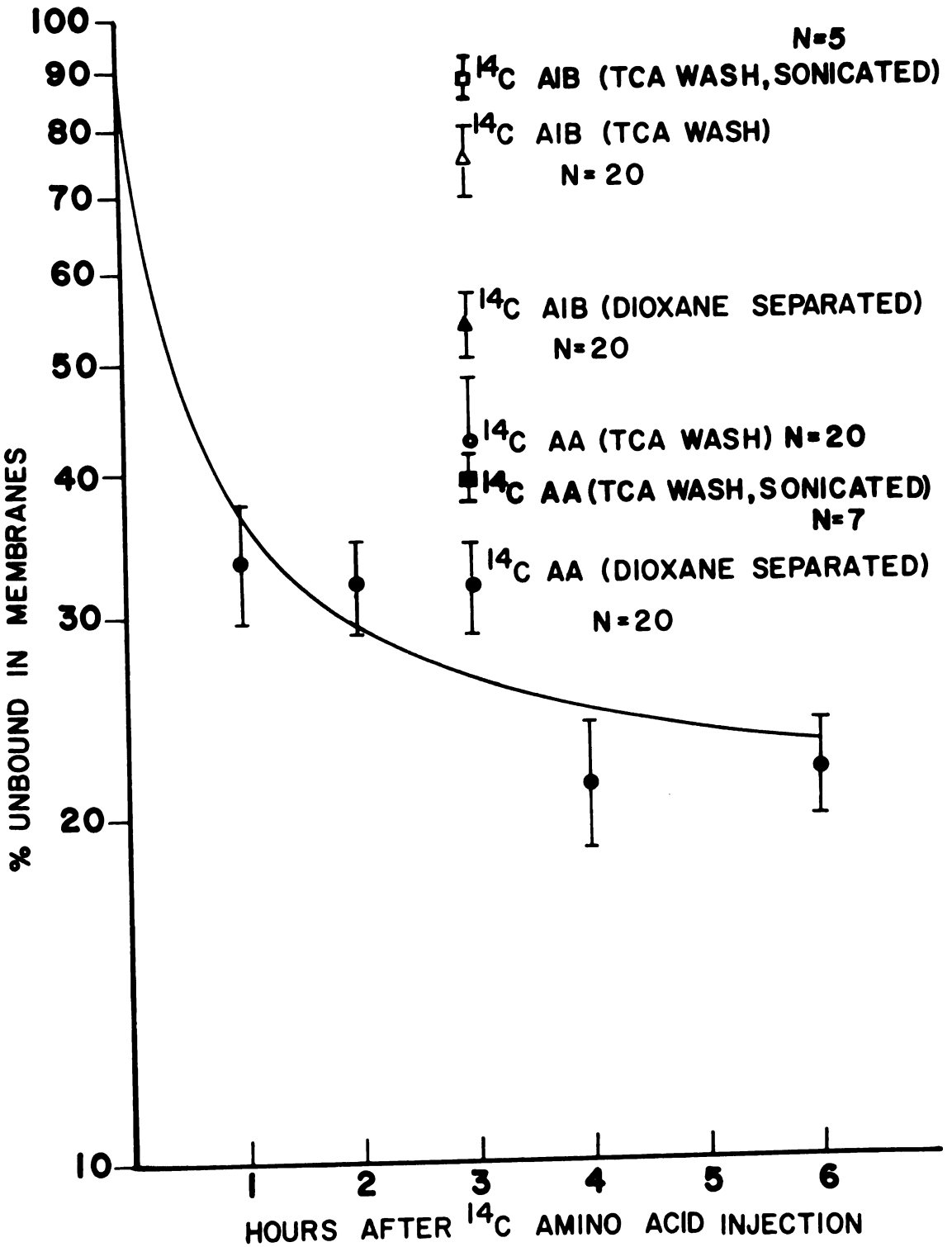

100
90
80
70
60
50
40
30
20
10
% UNBOUND IN PLASMA
^{14}C AIB
^{3}HOH
^{14}C AIB
^{3}HOH
^{14}C AMINO ACIDS
^{14}C AMINO ACIDS
● ■ ▲ DIOXANE SEPARATED
○ □ △ TCA WASHED
0
1
2
3
4
5
6
HOURS AFTER ^{14}C AMINO ACID AND ^{3}HOH INJECTION

^{14}C-amino acid that remained in the plasma protein after the TCA wash (open circles, Figure 5), minus the 8% not removed at zero time, is inferred to be peptide-linked; this is approximately 60%. After a two-hour equilibration time *in vivo*, 69% of the plasma ^{3}HOH (second curve of Figure 5, filled squares) and ^{14}C-AIB (filled triangle) separated with the dioxane fraction. The TCA wash (open squares ^{3}HOH, open triangle ^{14}C-AIB of Figure 5) provides a 20% increase in both unbound water and ^{14}C-AIB; the remaining 10% presumably represents label still bound to plasma protein.

The determination of amino acid and water distribution in the two-phase system of the plasma provides a basis for interpreting their distribution in the more complex structure of the mucosal epithelium. Data for whole membrane samples, treated in a manner similar to those of the plasma, are given in Figure 6. The amount of hydrolysate ^{14}C-amino acids unbound in the dioxane-counting medium when the pigeon crop membrane was taken three hours after labeling had decreased exponentially to about 25% of the total count. When the membranes were washed with the TCA solution before they were suspended in the dioxane solution, the quantity of the unbound hydrolysate ^{14}C-amino acids was 43%. When the membranes were sonically homogenized, then washed with 10% TCA and 1N NaOH solutions, a similar proportion of the total ^{14}C counts was recovered

FIGURE 6

The unbound hydrolysate ^{14}C-amino acids and ^{14}C-AIB recovered in crop membrane vs. time following a single injection iv of 5 uC/kg of label. Each data point is the mean percent of the total counts ± SE, N $\geq$ 5.


100
90
80
70
60
50
40
30
20
10
% UNBOUND IN MEMBRANES
1
2
3
4
5
6
HOURS AFTER 14C AMINO ACID INJECTION
N=5
14C AIB (TCA WASH, SONICATED)
14C AIB (TCA WASH)
N=20
14C AIB (DIOXANE SEPARATED)
N=20
14C AA (TCA WASH) N=20
14C AA (TCA WASH, SONICATED)
N=7
14C AA (DIOXANE SEPARATED)
N=20

in the precipitated protein. Thus about 60% of the labeled amino acids, injected three hours prior to sampling, are bound in the membrane homogenate with an energy greater than can be overcome by the alkali and TCA washes.

When labeling was done by injecting ^{14}C-AIB and the membranes were treated in the same manner as those described above, a progressively greater recovery of counts was achieved with each partitioning step. As seen in Figure 6, 55% of the ^{14}C-AIB was unbound as determined by the dioxane separation, and 75% was unbound when the intact membranes were washed with the 10% TCA solution. When the membranes were sonically homogenized and then washed in the TCA and NaOH solutions, the quantity of the ^{14}C-AIB removed from the protein fraction was 90%. This value approaches the expected 100% recovery of a nonmetabolizable, but permeant, amino acid.

For a zero time binding determination 0.02 uC of ^{3}HOH and 0.02 uC of the hydrolysate ^{14}C-amino acids were mixed with sonically homogenized crop tissue. After twenty-four hours of mixing, the protein was precipitated and washed twice with 5% TCA, once with 1N NaOH, and twice with TCA again; the activity in the protein was then determined. Of the ^{3}H label, 5.35 ± 1.41% remained with the protein portion, and 0.082 ± 0.014% of the ^{14}C label was found in the precipitate. It is therefore evident that the wash

procedure removes essentially all nonpeptide-linked amino acids, but some ^{3}HOH is retained in the protein fraction.

Using membranes which were excised after a three-hour label time (^{3}HOH and hydrolysate ^{14}C-amino acids) and a twenty-four hour prolactin treatment, the enhancement of protein synthesis by prolactin was examined. The control and prolactin-treated tissues were separated into two portions and sonically homogenized. One portion was used for a total activity determination. The other portion was treated with TCA and NaOH as described in the preceding paragraph; these tissues were then examined for radioactivity. The results (Table 4) indicate an enhanced uptake of label totally, and also the precipitated protein had a significantly higher ^{14}C-label content.

The Method of Increased Protein Synthesis

The antibiotics actinomycin D and puromycin were employed to ascertain how prolactin effects an increased protein synthesis. As can be seen in Table 8, actinomycin D counteracted the prolactin-stimulated accumulation of ^{3}HOH and hydrolysate ^{14}C-amino acids. This inhibition occurred both with the total counts and the washed portion, i.e. the precipitated protein.

The puromycin data (Table 9) indicates that this antibiotic is inhibitory only on the ^{3}HOH uptake. No effect on the total ^{14}C-leucine or protein-bound ^{14}C-leucine

Table 8.--The effect of actinomycin D, prolactin, and actinomycin D with prolactin on the uptake and incorporation of ^{14}C-leucine and ^{3}HOH.

		TREATMENT		
WASH	LABEL	25µg PROLACTIN	0.5µg ACTINOMYCIN D	0.5µg ACTINOMYCIN D +25µg PROLACTIN
none	^{14}C-leucine	T/C = 1.14±.05*	T/C = 0.97±.07	T/C = 0.82±.07
		C = 20.1±1.8	C = 21.8±1.6	C = 24.0±1.1
TCA	^{14}C-leucine	T/C = 1.67±.20*	T/C = 0.98±.15	T/C = 0.79±.17
		C = 3.61±.64	C = 3.34±.43	C = 3.37±.17
none	^{3}HOH	T/C = 1.26±.07*	T/C = 1.03±.11	T/C = 0.85±.06
		C = 107±12	C = 70.6±12.7	C = 95.8±5.1

C = DPM/mg *P<.05

N = 7 Means ± Std. Errors

Table 9.--The effect of puromycin, prolactin, and puromycin with prolactin on the uptake and incorporation of ^{14}-leucine and ^{3}HOH.

		TREATMENT		
WASH	LABEL	25µg PROLACTIN	200µg PUROMYCIN	200µg PUROMYCIN +25µg PROLACTIN
none	^{14}C-leucine	T/C = 1.14±.05* C = 20.1±1.8	T/C = 1.09±.07 C = 11.9±1.0	T/C = 1.23±.12* C = 14.3±1.4
TCA	^{14}C-leucine	T/C = 1.67±.20* C = 3.61±.64	T/C = 1.08±.21 C = 2.70±.61	T/C = 1.40±.15* C = 3.84±.54
none	^{3}HOH	T/C = 1.26±.07* C = 107±12	T/C = 0.96±.03 C = 95.0±8.3	T/C = 1.26±.17 C = 104±12

C = DPM/mg *P<.05

N = 7 Means ± Std. Errors

as effected by prolactin was observed. Recirculation of the puromycin and its resultant equal inhibition of both lateral portions of the crop are evident by the equal decrease in accumulation of ^{14}C-leucine in the control counts (puromycin only vs. prolactin only). Prolactin appears to have, at least partially, exerted its effect when administered subsequent to puromycin.

Electron Microscopy

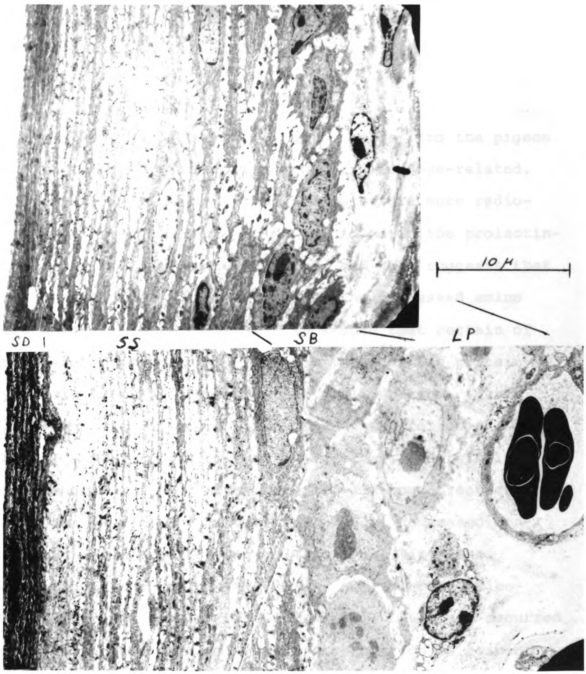
The initial changes in the pigeon crop epithelium as effected by prolactin were examined by viewing eighteen-hour treated tissues with an electron miscroscope. The prolactin influence can be observed by comparing the photomicrograph of Figure 7 to that of Figure 8. The thickening of the epithelial layers, i.e. the stratum basalae and the stratum spinosum, is clearly shown. Evident also in the spinosum layer and especially the basalae layer is the treatment effect on cellular size; i.e. a marked increase in response to prolactin is shown. Nuclear material is also accumulated into a denser mass, perhaps indicating the preparation for cell division; this is especially evident in the stratum basalae.

FIGURE 7

Photomicrograph of untreated crop mucosal epithelium, x4400. SD, stratum disjunctum; SS, stratum spinosum; SB stratum basale; LP, lamina propria.

FIGURE 8

Photomicrograph of eighteen hour, 25 ug prolactin-treated pigeon crop mucosal epithelium, x4400.


10 μ
SD 1
SS
SB
LP

CHAPTER V

DISCUSSION

The enhanced uptake of amino acids into the pigeon crop in response to exogenous prolactin is dose-related. Also, there is a nineteen-hour period before more radioactivity of the ^{14}C-amino acids was found in the prolactin-stimulated vs. the control tissues, and this suggests that other events are occurring prior to the increased amino acid uptake. It is reasonable to assume that certain of these events are prerequisites for the enhanced protein synthesis leading to the crop-sac proliferation. Tata (1963) found an enhanced amino acid uptake following thyroxine administration to the rat liver, and this increase was found only after a twenty-six-hour treatment. Increased oxygen consumption (Tata 1963), decreased content in the liver of glycogen (Tata 1963), increased phospholipid synthesis (Tata 1966), and increased ribonucleic acid synthesis (Tata and Windell 1966) all occurred prior to the enhanced amino acid accumulation in response to thyroxine. Further substantiating the view that similar events would occur prior to the increased amino acid uptake in the prolactin-stimulated crop is the

observation that actinomycin D inhibits amino acid uptake in the crop. This indicates there is an enhanced ribonucleic acid synthesis in response to prolactin, and this probably occurs prior to the increased protein synthesis.

The AIB T/C ratios greater than 1.0 (Table 4) show that prolactin also stimulates the uptake of this non-metabolizable amino acid, but it is obvious that its enhanced accumulation cannot depend upon its utilization for incorporation into protein. It is assumed (Noall et al. 1957) that the AIB enters the cells under the same influences as some of the metabolizable amino acids. But if the effect of prolactin on the mucosal epithelium is the greater utilization of metabolizable amino acids into protein, then this may account for the enhanced uptakes of both AIB and hydrolysate amino acids. The possibility also exists that prolactin causes a greater rate of amino acid transport apart from a greater rate of protein synthesis.

The case for the enhancement of protein synthesis in the pigeon-crop in response to exogenous prolactin is as follows. The maximum recovery of unbound hydrolysate ^{14}C-amino acids from TCA-washed membranes, whether sonicated or intact, was about 40% (filled square and open circle of Figure 6). This compares well with the 60% retention of the initial counts of hydrolysate ^{14}C-amino acids in the TCA-washed precipitates of sonicated, treated and untreated crop epithelia taken three hours after the

labeling (Table 4). A wash with 1N NaOH, which should have removed all the amino-acyl-t-RNA-linked amino acids, did not alter the 60% of labeled amino acids retained in the membrane precipitates. It is inferred that this 60% is peptide-linked. This is a reasonable inference since it was shown that ^{14}C-AIB, which had accumulated in a significant amount, is almost entirely eluted by the TCA and NaOH washes. Likewise, when both ^{14}C-AIB and ^{14}C-hydrolysate amino acids were added to pigeon blood *in vitro*, a condition under which no protein synthesis would be expected, over 90% of both kinds of labeled amino acids was removed from the precipitated plasma proteins. Finally, the protein precipitates from the prolactin-treated membranes contained 43% more TCA-resistant ^{14}C activity than the control membranes (Table 4). Therefore, it seems evident that prolactin stimulates protein synthesis within the pigeon-crop mucosal epithelium.

The prolactin-stimulated uptake of ^{14}C-leucine (a 13% increase) *in vivo* was considerably less than the enhanced uptake of the labeled hydrolysate amino acids (a 41% increase). Since this 41% increase is an average of the enhanced uptakes of the thirteen labeled amino acids, the uptakes of other of the hydrolysate amino acids appear to be more affected by prolactin than leucine. In the *in vivo* experiments, the amount of injected labeled amino acids was constant, and the control counts for the

leucine and hydrolysate mixtures were 2.77 DPM/mg and 43.3 DPM/mg respectively. The uptake of leucine into the pigeon crop therefore appears to be much less than other amino acids in the hydrolysate mixture. The ^{3}HOH uptake ratios were no different whether determined with the leucine or hydrolysate amino acids; a uniform response to prolactin therefore appears evident.

Since a prolactin-enhanced uptake of ^{14}C-leucine occurred in the in vitro preparation, it appears that the epithelium alone increases its amino acid accumulation when the crop-sac is exposed to prolactin in vivo. Whether an exposure of the epithelium to prolactin entirely in vitro will effect an increased amino acid accumulation has not yet been shown since the crop epithelium does not usually remain viable for more than ten hours using this particular in vitro technique. And from the in vivo studies it appears that nineteen hours is the minimal time necessary for prolactin to effect an increased amino acid accumulation. It is therefore not clear whether prolactin affects the epithelium directly or through some intermediate process, presumably through the more superficial areas of the crop-sac. But the epithelium seems the most likely target tissue since changes were noted earliest in this layer.

Actinomycin D apparently inhibits m-RNA synthesis in the nucleus (Reich 1963), and m-RNA is an integral part of the protein synthetic process. Since the

prolactin-stimulated protein synthesis is inhibited by actinomycin D, it is apparent that one of the effects of prolactin is on DNA-RNA dependent protein synthesis. Both the accumulation and incorporation of amino acids into protein were inhibited by this antibiotic, and, therefore, it seems that prolactin-stimulated RNA production is essential for both these events. Yet the cause and effect, if any exists, of the increased protein synthesis and free amino acid accumulation in the prolactin-stimulated cells remains to be delineated. Also unclear is the mechanism by which the ^{3}HOH accumulation is inhibited by actinomycin D, although this could be explained partially by the inhibition of amino acid accumulation, assuming water movement is coupled to metabolite fluxes.

Puromycin, an antibiotic which inhibits the amino acid transfer from t-RNA to protein, did not inhibit the prolactin-stimulated uptake and incorporation of ^{14}C-leucine. The fact that gross counts in the hormone and calf-serum-treated tissues were fewer indicates that puromycin reaches the non-treated crop surface, and label uptake is reduced equally for both crop-membrane surfaces. The prolactin-stimulated uptake in the puromycin-treated tissue means that either the prolactin effect on the crop is puromycin-insensitive, or puromycin was present in an insufficient quantity to inhibit the effect of prolactin. Sherry and Nicoll (1967) also found only a partial

inhibition of the crop-sac response to prolactin when they used either actinomycin D or puromycin. Therefore, it seems possible that the crop-sac epithelium has two types of protein synthesis: one, puromycin-sensitive, and the other puromycin-insensitive, but prolactin-sensitive. It is also possible that prolactin counteracts the effect of puromycin.

The mobilization of metabolites preceding the enhanced protein synthesis may explain the dose-related, time-dependent hydration of the prolactin-stimulated epithelium. Hydration responses are a well-established phenomenon in the proliferative organs of some other species. Rudolphand and Samuels (1949) demonstrated that ten hours was required before testosterone could effect a water imbibition in the seminal vesicles of castrate rats. Peak hydration levels of the estradiol-stimulated rat uterus were obtained at six hours and after twenty hours of exposure to the hormone (Astwood 1938, Szego and Roberts 1953). This compares well to the brief peak at four hours and the persistent hydration beginning at fifteen hours for the prolactin-stimulated crop mucosa. The four-hour peak was attributed by Szego to an increased capillary permeability and the twenty-hour response to proliferative activities. Within twenty-four hours of prolactin treatment, vascularization of the crop-sac wall increases (Dumont 1965). This also was observed in the present study

by gross inspection, incidental to tissue preparation. It should be emphasized that in the repeated experiments when ^{3}HOH and ^{14}C-amino acids were simultaneously injected, the T/C ratios for ^{3}HOH were greater than 1.0 at four hours, but the T/C ratios for the hydrolysate ^{14}C-amino acids were not. The uptakes of two solutes, ^{3}H-uridine and $^{22}Na^{+}$, are probably related to the four-hour hydration, but it is obvious that the amino acids are not. Recently Farese and Schnure (1967) studied the effect of ACTH on ^{3}H-uridine triphosphate uptake in the rat adrenal gland. They found a prolonged uptake beginning at about fifteen hours, but there was a brief peak uptake at four hours following the hormone administration. This, therefore, almost parallels the ^{3}HOH peak uptake in the prolactin-stimulated pigeon crop in which the four-hour ^{3}H-uridine uptake was also noted. But unlike prolactin, the ACTH was able to elicit an enhanced ^{3}H-uridine accumulation within one-half hour after the hormone treatment.

With the prolactin-stimulated crop, the persistent hydration beginning at fifteen hours precedes the enhanced accumulation of the hydrolysate amino acids, beginning at nineteen hours, by too large an interval to look for a direct relationship. However, it is an attractive possibility that the accumulation of the precursors associated with protein synthesis, i.e. nucleotide uptake leading to the formation of RNA, could explain the accumulation of

^{3}HOH beginning at fifteen hours after prolactin administration. For the times subsequent to nineteen hours, the T/C ratios are significantly greater than 1.0 for both the ^{3}HOH and hydrolysate ^{14}C-amino acids, and these could be related in a causal manner. This relationship is seen also in the dose-response T/C curves of Figures 3 and 4 when samples were taken after 25ug, twenty-four-hour prolactin treatments.

The simultaneous increased uptakes of $^{22}Na^{+}$ and ^{3}H-uridine occurred while no increased ^{14}C-amino acid uptake was noted. Although this is not proof that the $^{22}Na^{+}$ and ^{3}H-uridine uptakes are related to the four-hour ^{3}HOH peak, this explanation is plausible. Sodium and amino acid transport across the rabbit ilium (Curran et al. 1967), Ehrlich cells (Christensen et al. 1967), and rabbit red blood cells (Wheeler and Christensen 1967) have been shown to be interdependent, and a sodium-nucleic acid interdependent transport is also possible. The osmotic imbalance caused by the influx of sodium and nucleic acids could be the cause of the four-hour water imbibition.

The increased sucrose space at twenty-seven hours following prolactin administration was 13%. It is therefore evident that an increased extracellular space is responsible for at least part of the uptakes as reported in this manuscript. But the increased extracellular space does not account for the magnitude of most of the reported

uptakes. ^{3}HOH uptake was also significantly larger than the increased sucrose space, and, therefore, intracellular expansion in response to prolactin is also evident.

The electronmicrographs show quite vividly some changes induced by prolactin in the crop epithelium. As was also reported by Dumont (1965), the thickening of the stratum spinosum is clearly shown. The enlargement of the stratum basalae cells at eighteen hours was not evident in the twelve-hour photomicrographs of Dumont (1965). This could result from the water imbibition shown to be initiated fifteen hours following the prolactin treatment. The stratum disjunctum layer appears unchanged, and Dumont showed it unchanged until the fourth day of hormone stimulation. Also evident from the photomicrographs is the greater aggregation of the chromatin material, presumably in preparation for the subsequent cell division.

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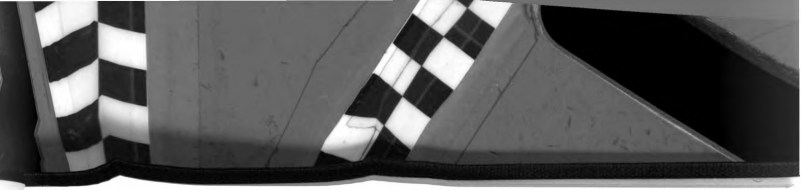

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APPENDICES

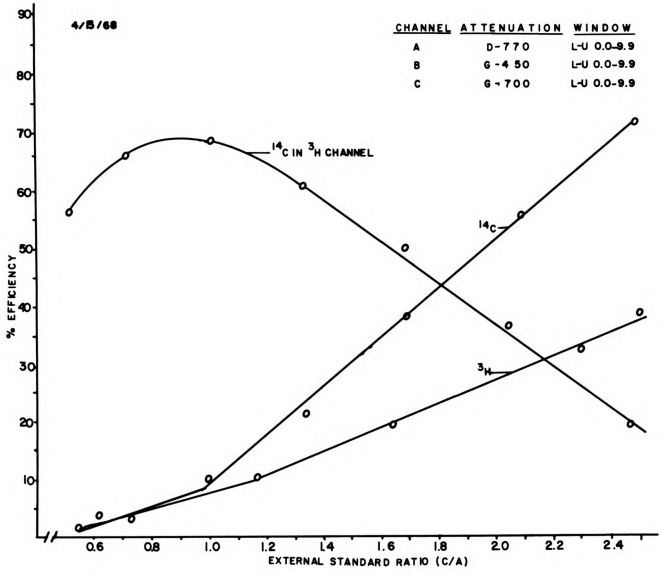
APPENDIX I: QUENCH CORRECTION IN DUAL LABEL STUDIES

Using a three channel liquid scintillation counter the procedure for dual label quench correction of ^{3}H and ^{14}C will be outlined. With the least quenched ^{3}H and ^{14}C standards, one channel is peaked for ^{3}H, one for ^{14}C, and the third for ^{3}H with the ^{133}Ba external standard. ^{3}H is eliminated from the ^{14}C channel by raising the lower window of the ^{14}C peaked channel. The ^{3}H and ^{14}C variably quenched standards are then counted and the efficiency of counts calculated; after each standard is counted, the external standard is also counted. The external standard ratio of the counts in the ^{3}H with ^{133}Ba peaked channel to the counts in the ^{3}H peaked channel is plotted against the efficiency of the corresponding standard. This procedure provides a linear ^{3}H efficiency vs. external standard ratio plot; curves for ^{14}C efficiency and ^{14}C in the ^{3}H channel are also obtained. The resulting curves and the instrument settings are illustrated in Figure 9.

After the ^{3}H and ^{14}C counts per minute are printed for an unknown sample, the external standard ratio is also printed. The efficiencies of count are then found from the quench correction plot. The total ^{14}C activity in disintegrations per minute (DPM) in the sample is calculated by dividing the ^{14}C counts per minute (CPM) by the ^{14}C

FIGURE 9

Dual label, ^{3}H and ^{14}C, quench correction curves using an external standard. Channel A is peaked for ^{3}H, channel B for ^{14}C and channel C for ^{3}H and ^{133}Ba.


4/15/68
CHANNEL ATTENUATION WINDOW
A D-770 L-U 0.0-9.9
B G-4 50 L-U 0.0-9.9
C G-700 L-U 0.0-9.9
^{14}C IN ^{3}H CHANNEL
^{14}C
^{3}H
% EFFICIENCY
EXTERNAL STANDARD RATIO (C/A)

efficiency. The total ^{3}H activity is found by the following calculations:

$$^{3}H\ DPM = \frac{^{3}H\ CPM - \frac{^{14}C\ DPM}{\%\ ^{14}C\ in\ ^{3}H\ channel}}{^{3}H\ efficiency}$$

A similar procedure as outlined above can be followed when two radioactive nuclides with differing B particle energies are used; this was done with ^{22}Na and ^{3}H.

APPENDIX II: THE COUNTING SOLUTION

Radioactive samples were counted in 15 ml of a solution made by the following recipe:

80 grams naphthalene
5 grams PPO
50 milligrams a-NPO
Add dioxane to one liter

Counting solutions were used within three months following mixing.

APPENDIX III: COMPOSITE DATA OF RADIOLABELED STUDIES

All samples were taken twenty-seven hours after prolactin treatment and three hours after the label injection.

Sonicated	TCA Wash	Label or Antibiotic	T	C	% Increase
No	No	hydrolysate ^{14}C-amino acid	5.61±.52	4.56±.41	23±10
No	Yes	hydrolysate ^{14}C-amino acid	3.77±.84	2.60±.30	45±37
Yes	No	hydrolysate ^{14}C-amino acid	61.1±8.3	43.3±4.0	41±16
Yes	Yes	hydrolysate ^{14}C-amino acid	36.9±4.8	26.2±1.6	41±17
No	No	^{14}C-AIB	4.67±.34	3.65±.60	28±13
No	Yes	^{14}C-AIB	.92±.07	.88±.05	insig.
Yes	No	^{14}C-AIB	31.8±6.7	21.6±3.5	47±16
Yes	Yes	^{14}C-AIB	2.01±.09	1.9±.3	insig.
Yes	No	^{3}HOH	134±15	107±12	26±7
Yes	No	^{14}C-sucrose	3.13±.07	2.77±.36	13±4
Yes	No	^{14}C-leucine	22.9±.08	20.1±1.8	14±5
Yes	Yes	^{14}C-leucine	6.03±1.8	3.61±.64	67±20

APPENDIX III - Continued

Sonicated	TCA Wash	Label or Antibiotic	T	C	% Increase
		actinomycin D (no prolactin) on			
Yes	No	^{14}C-leucine	21.1±2.3	21.8±1.6	insig.
Yes	Yes	^{14}C-leucine	3.27±.34	3.34±.43	insig.
Yes	No	^{3}HOH	72.7±8.4	70.6±12.7	insig.
		actinomycin D (with prolactin) on			
Yes	No	^{14}C-leucine	19.7±2.6	24.0±1.1	insig.
Yes	Yes	^{14}C-leucine	2.66±.41	3.37±.17	insig.
Yes	No	^{3}HOH	81.4±3.6	95.8±5.1	insig.
		Puromycin (no prolactin) on			
Yes	No	^{14}C-leucine	13.0±1.3	11.9±1.0	insig.
Yes	Yes	^{14}C-leucine	2.92±.34	2.70±.61	insig.
Yes	No	^{3}HOH	91.2±6.0	95.0±8.3	insig.
		Puromycin (with prolactin) on			
Yes	No	^{14}C-leucine	18.8±2.1	15.3±1.4	23±12
Yes	Yes	^{14}C-leucine	5.38±1.1	3.84±.54	40±15
Yes	No	^{3}HOH	131±16	104±12	26±17