The Effect of Prolactin on Lipogenesis in the Pigeon. *In Vitro* Studies*

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ABSTRACT: The daily injection of pigeons for 5 days with 1 mg of a bovine prolactin preparation caused the liver to double in size and markedly enhanced its lipogenic capabilities. Liver slices from birds so treated incorporated ^{14}C into fatty acids from labeled glucose (11 or 110 mM), pyruvate (40 mM), and acetate (40 mM) at rates which ranged from two to six times normal. Significant increases were seen after 1 day of prolactin treatment when glucose (11 mM) or pyruvate were the substrates. Prolactin had no effect on rates of glycogen synthesis from glucose (11 or 110 mM). Increasing the glucose concentration from 11 to 110 mM caused a three- to fourfold increase in the rate of formation of fatty acids and glycogen independent of prolactin treatment. A marked increase in the activities of malic enzyme, citrate cleavage enzyme, and malate dehydrogenase as measured in supernatants of liver homogenates was also observed after prolactin treatment. Pronounced changes in the activities of these enzymes as well as in the rate of [^{14}C]pyruvate incorporation into fatty acids occurred even when the dose of prolactin was lowered to 0.2 mg daily for 3 days. Hexokinase, glucokinase, pyruvate kinase, hexose monophosphate shunt dehydrogenases, aconitase, and isocitrate dehydrogenase activities in liver were unaltered after 3 days of treatment with 1 mg of prolactin. When birds were fasted for 3 days the rate of fatty acid synthesis from pyruvate in liver slices decreased to 6% of the rate seen in normal birds and the activities of malic enzyme, citrate cleavage enzyme, and malate dehydrogenase also decreased. Prolactin treatment, 1 mg daily for 3 days, during this fast did not prevent these changes from occurring. Injection of a growth hormone preparation, 1 mg daily for 3 days, produced the same changes in liver size and metabolism as seen with prolactin, but failed to increase crop sac size as in the case of prolactin. The action of prolactin was not mimicked by injections of insulin or triiodothyronine.

The results provide further support for the high lipogenic capabilities of pigeon liver and its role as a major site of lipogenesis. The results also suggest that the dehydrogenases of the glucose 6-phosphate shunt play a minor role in reduced nicotinamide–adenine dinucleotide phosphate production for fatty acid synthesis in pigeon liver.

Dietary carbohydrate which is consumed in excess of current needs is converted into triglycerides that are used by most animals as a future source of energy. In rats and certain other mammals this conversion process occurs in both liver and adipose tissue with the latter tissue playing a dominant role (Cahill and Renold, 1965). In pigeons the liver appears to be the major organ responsible for this process while adipose tissue serves largely as a store house for triglyceride (Goodridge and Ball, 1965, 1966, 1967a). This central role of the liver in lipogenesis in pigeons and perhaps other birds assumes significance in relation to attempts to understand the mechanisms underlying the ability of many species of bird to rapidly increase their fat stores just prior to migratory flights. It follows, therefore, that any process by which the lipogenic capabilities of the liver in birds may be enhanced is of interest. In a previous publication Goodridge and Ball (1967b) reported that daily administration of 1 mg of a prolactin preparation to pigeons for 5 days produced a three- to fourfold increase in the capacity of a unit mass of liver to convert glucose to fatty acids.[1] At the same time there occurred a doubling of liver mass so that total liver lipogenesis rates were increased six- to eightfold. In these studies the process of lipogenesis was followed *in vivo* by comparing the amount of labeled fatty acids in liver, blood, and adipose tissue of prolactin-treated and untreated pigeons at time intervals of 0.5, 7, 15, and 30 min after the injection of uniformly labeled [^{14}C]glucose.

We have now made *in vitro* studies on liver removed from prolactin-treated and untreated pigeons.

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[1] We have assumed that the incorporation of glucose and pyruvate were directly proportional to the actual rate of fatty acid synthesis and that dilution of [^{14}C]glucose and [^{14}C]pyruvate occurred to the same degree in tissue from normal and prolactin-treated birds.

Measurements in liver slices of the rate of formation of total fatty acids from ^{14}C-labeled glucose, pyruvate, and acetate support the *in vivo* findings that prolactin treatment markedly enhances the lipogenic capabilities of pigeon liver. Concomitant increases are seen in the activities in the liver of citrate cleavage enzyme, malic enzyme, and malate dehydrogenase. These increases in metabolic and enzymatic activities have then been employed to monitor the effect of changes in the dosage level and duration of administration of prolactin. Studies on the effect of starvation with and without prolactin treatment as well as liver responses to injections of insulin, growth hormone, and triiodothyronine have also been performed.

Materials and Methods

Silver King pigeons (7–9-week old) of both sexes (420–640-g body wt) were maintained on an *ad libitum* diet of Purina pigeon grains. Male Holtzman rats (180–230-g body wt) were fed Purina laboratory chow for rats, mice, and hamsters *ad libitum*. All animals had free access to water and were kept in a temperature- (22–24°) and humidity-controlled room on a 14.5-hr daily photoperiod. Hormone injections in the pigeons were made into the pectoral muscles while in rats they were given subcutaneously in the region at the back of the neck. The amount of hormone administered and the duration of treatment varied and is stated in the results. The total volume of injected fluid was usually 0.4 ml. No measurable difference in response to the hormone injections owing to sex was noted in pigeons.

The animals were killed by decapitation in a miniature guillotine (24 hr after the last injection for treated animals). The livers were removed as rapidly as possible and pieces placed in (1) iced saline, (2) 0.25 M sucrose, and/or (3) Tris-KCl-homogenizing medium. Portions of liver from the saline were sliced with a Stadie–Riggs microtome, lightly blotted on filter paper, weighed on a torsion balance (500–750 ± 10 mg), and placed in 25-ml flasks containing 5.0 ml of medium. The flasks were gassed with 95% O_2–5% CO_2, sealed, and incubated for 1 hr at 40° in a Dubnoff metabolic shaker.

The incubation medium was the same as Krebs bicarbonate buffer with one-half the recommended amount of calcium (Umbreit *et al.*, 1964) except that K^+ was 125 mM and Na^+ was 25 mM (Flink *et al.*, 1950). One of the following radioactive substrates was added: [U-^{14}C]glucose (0.5–1.0 μc, 11 or 110 mM), [U-^{14}C]pyruvate (0.1–0.2 μc, 40 mM), or [1-^{14}C]acetate (0.2 μc, 40 mM plus unlabeled glucose, 11 mM).

At the end of the incubation period the flasks were placed in crushed ice. Tissues for glycogen extraction were placed directly in 30% KOH; those for fatty acid extraction were placed in 10% ethanolic KOH. Glycogen was precipitated from the KOH digest with one-half volume of 95% ethanol (Good *et al.*, 1933). The precipitate was extracted three times with 10% trichloracetic acid and the residue was discarded. A few drops of a saturated Na_2SO_4 solution were added to the combined trichloracetic acid extracts and one-half volume of 95% ethanol was added. The precipitate was collected by centrifugation and washed successively with 95% ethanol and acetone. The precipitate was dissolved in water and samples were pipetted directly into tared nickel-plated steel planchets, dried under an infrared lamp, cooled, reweighed, and counted in a gas-flow Geiger counter equipped with a thin window. Total fatty acids were isolated and counted as described previously (Goodridge and Ball, 1966).

For the measurement of HMP[2] shunt dehydrogenases, malic enzyme (NADP), malate dehydrogenase (NAD), citrate cleavage enzyme, aconitase, and isocitric dehydrogenase activities, homogenates (10 or 20%, w/v) were prepared in 0.25 M sucrose with a Ten Broeck homogenizer. Homogenization was performed at ice-bath temperature. Samples of the whole homogenates were removed for determination of nitrogen by a micro-Kjeldahl procedure (Frerichs and Ball, 1962). The remainder was centrifuged at 37,000*g* for 15 min at 0–3°. The resulting supernatant fraction was used for the enzyme assays (in some cases it was diluted with 0.25 M sucrose).

Malic enzyme was assayed by the method of Wise and Ball (1964), citrate cleavage enzyme by the method of Srere (1959) as modified by Kornacker and Ball (1965), and malic dehydrogenase by the method of Ochoa (1955). Total HMP shunt dehydrogenase activity was assayed by the procedure of Glock and McLean (1953) as modified by Ball and Jungas (1963). Aconitase and isocitric dehydrogenase were assayed by the method of Abraham *et al.* (1962) except that citrate was substituted for isocitrate in the aconitase assay system.

For the measurement of pyruvate kinase and glucose phosphotransferase activities, homogenates (33%, w/v) were prepared in Tris buffer (0.05 M, pH 7.4) with KCl (0.15 M), EDTA (0.05 M), $MgCl_2$ (0.005 M), and β-mercaptoethanol (0.66 ml/l.). After removing aliquots for nitrogen determination, the homogenates were centrifuged at 105,000*g* for 60 min at 0–3°. Samples of the resulting particle-free supernatant were assayed directly for glucose phosphotransferase activities (at 0.5 and 100 mM glucose) by the method of Walker and Rao (1964). The supernatant was diluted 10:1 with the homogenizing buffer prior to assaying pyruvate kinase by the method of Weber *et al.*, (1965). All enzyme assays were run at 40°.

NADP, NADH, glucose 6-phosphate, 6-phosphogluconate, coenzyme A, adenosine diphosphate (sodium salt), adenosine triphosphate (disodium salt), and malic dehydrogenase were purchased from Sigma Chemical Co.; oxalacetic acid, sodium pyruvate, isocitric acid (trisodium salt), and 2-phosphoenol pyruvic acid (tricyclohexylammonium salt) from Cal-Biochem; and lactic dehydrogenase and glucose 6-

[2] Abbreviations used: HMP shunt, hexose monophosphate shunt; NAD, nicotinamide-adenine diphosphonucleotide; NADH, reduced NAD; NADP, nicotinamide-adenine diphosphonucleotide phosphate; acetyl-CoA, acetyl coenzyme A.

phosphate dehydrogenase from Boehringer Mannheim Corp. L-Malic acid (Pfanstiehl) was recrystallized four times from acetone–benzene. [U-^{14}C]Glucose and [1-^{14}C]acetate (sodium salt) were purchased from New England Nuclear Corp. [U-^{14}C]Pyruvic acid (sodium salt) was purchased from Nuclear-Chicago, Inc. (Solutions containing [U-^{14}C]pyruvate were prepared fresh each day as it was found that standing overnight, even when frozen, allowed the development of a substance which inhibited incorporation of the pyruvate into fatty acids.) Bovine growth hormone (1.5 U.S.P. units/mg) and bovine prolactin (13 IU/mg) were gifts of the Endocrinology Study Section, National Institutes of Health. Protamine zinc insulin (U-80) was a product of Eli Lilly and Co.

Significance of the data was tested by the Mann–Whitney test (Siegel, 1956). Standard errors are provided to indicate the degree of variance in the data.

Results

The increases produced in whole body, liver, and crop sac weight of pigeons during the course of a 5-day treatment with prolactin (1 mg/day) are shown in Table I. Body weight increased progressively over the 5-day period. Since prolactin treatment was not extended beyond 5 days, we have no information as to how long this gain would continue. Absolute liver weight increased more rapidly than body weight during the first 2 days of prolactin treatment. Subsequent to the third injection, however, the change in liver weight was proportional to the increase in body weight. It should be noted that there was a 20% decrease in liver nitrogen after 2 days of prolactin treatment ($p = 0.05$), followed by a slow increase from the third to the fifth day of treatment (see Table V). This suggests that the relative increase in liver weight observed after 1 and 2 days of prolactin may reflect an increase in water content. One injection of prolactin had no effect on the weight of the crop sac ($p > 0.05$) but three and five injections resulted in a highly significant increase in crop sac weight ($p < 0.0001$).

Table II contains the results of experiments in which fatty acid synthesis from ^{14}C-labeled glucose, pyruvate, and acetate was measured in liver slices from normal and prolactin-treated pigeons. An increase in the average rate of incorporation into fatty acids from all substrates was observed as a result of prolactin treatment. Fatty acid synthesis from 11 mM glucose was increased more than 2.5-fold ($p < 0.02$) just 24 hr after a single injection of prolactin. At the end of the 5-day treatment the increase was sixfold ($p < 0.002$). When pyruvate was the substrate, a single prolactin injection caused almost a twofold increase in fatty acid synthesis ($p < 0.05$); the difference was threefold after 5 days ($p < 0.002$). Three daily injections of saline (pH 9–10) had no effect on the conversion of pyruvate carbon to fatty acids in liver slices (data not presented). Prolactin (100 μg/ml) added *in vitro* to liver slices from normal or saline-injected pigeons was found to exert no effect upon the rate of incor-

TABLE I: The Effect of Prolactin on Whole Body, Liver, and Crop Sac Weight in the Pigeon.

	Days on Prolactin Treatment (1 mg daily)				
	0	1	2	3	4	5
Body weight[a]		3.6 ± 0.5 (8)	6.4 ± 0.8 (4)	10.5 ± 0.8 (17)	9.4 ± 1.6 (4)	15.6 ± 1.9 (8)
Liver weight	1.9 ± 0.1 (26)	2.4 ± 0.2 (8)	3.3 ± 0.3 (5)	3.1 ± 0.1 (17)	3.4 ± 0.4 (4)	3.3 ± 0.2 (8)
Crop sac weight	0.39 ± 0.02 (23)	0.38 ± 0.02 (4)		0.59 ± 0.04 (14)		0.75 ± 0.07 (8)

[a] Body weight is expressed as per cent increase plus or minus standard error. Normal pigeons had a body weight of 528 ± 8 g ($N = 26$). Average initial weight of the prolactin-treated pigeons was 536 ± 7 g ($N = 41$). Liver and crop sac values are expressed as per cent of body weight plus or minus standard error. The number of birds in each case is given in parentheses.

TABLE II: Incorporation of ^{14}C-Labeled Substrates into Total Fatty Acids *in Vitro* in Liver Slices from Normal and Prolactin-Treated Pigeons.[a]

Substrate (mM)	Days on prolactin treatment (1 mg daily)				
	0	1	2	3	4	5
[U-^{14}C]Glucose (11)	110 ± 20 (10)	280 ± 40 (8)	350 ± 70 (5)	320 ± 110 (6)	470 ± 100 (4)	660 ± 140 (8)
[U-^{14}C]Glucose (110)	440 ± 130 (6)	660 ± 170 (4)		990 ± 340 (6)		
[U-^{14}C]Pyruvate (40)	340 ± 50 (11)	600 ± 70 (4)		870 ± 80 (8)		1020 ± 100 (8)
[1-^{14}C]Acetate (40)	170 ± 50 (4)			290 ± 30 (4)		

[a] Values are per milligram of liver nitrogen per hour and are expressed as millimicroatoms of carbon for glucose and pyruvate but in millimicromoles for acetate. Unlabeled glucose (11 mM) was also present in the acetate experiments. The number of animals in each group is indicated in parentheses.

poration of glucose carbon (11 mM glucose) or pyruvate carbon into fatty acids.

Pyruvate carbon was incorporated into fatty acids faster than 11 mM glucose carbon both prior to and following treatment of the pigeons with prolactin. In liver slices from normal pigeons the difference was about threefold; after 5 days of prolactin the difference had decreased to 1.5-fold. When the glucose concentration in the medium was increased to 110 mM there was a consistent increase in the rate at which glucose was converted to fatty acids, the average being about threefold for tissue from both normal and prolactin-treated pigeons. When determined at a high concentration of medium glucose, the incorporation of [^{14}C]-glucose into fatty acids was much more variable than it was at the lower concentrations, and no significant effect of prolactin treatment was demonstrated ($p > 0.05$). At this higher glucose concentration the mean rate at which glucose carbon was converted to fatty acids was almost identical with the rate for pyruvate carbon.

Incorporation of acetate into fatty acids was increased only 70% after three prolactin injections. The groups were small and variability large, so that the difference was not statistically significant ($p > 0.05$). Unlabeled glucose at a concentration of 11 mM was present in the acetate experiments. Since prolactin caused a greater effect on the conversion of 11 mM glucose carbon to fatty acids than on the conversion of pyruvate carbon to fatty acids, the presence of the unlabeled glucose probably resulted in a greater dilution of the acetate label in slices from prolactin-treated pigeons.

As shown in Table III glycogen synthesis from glucose in liver slices was not affected by the prolactin treatment (3 days) at either of the glucose concentrations employed. However, increasing the glucose concentration from 11 to 110 mM did cause a four-fold increase in the conversion of glucose carbon to glycogen in both normal and prolactin-treated pigeons.

An examination of the effect of prolactin treatment upon the activities of nine enzymes known to

TABLE III: Incorporation of [U-^{14}C]Glucose into Glycogen *in Vitro* in Liver Slices from Normal and Prolactin-Treated Pigeons.[a]

Glucose Concn (mM)	Untreated	Prolactin (1 mg/day for 3 days)
11	770 ± 230	730 ± 190
110	3100 ± 750	2700 ± 580

[a] Values are expressed as millimicroatoms of glucose carbon incorporated per milligram of nitrogen per hour plus or minus standard error. Each group contained six birds.

be involved in carbohydrate metabolism was made. The activities of three of these, citrate cleavage, malate dehydrogenase, and malic enzyme, were found to be markedly increased as shown by the data presented in Table IV. The greatest increase was seen with the citrate cleavage enzyme, 2.8-fold ($p < 0.002$), and the activity of this enzyme reached its peak after only 2 days of prolactin treatment. The changes in malate dehydrogenase activity, though not as marked, were significant at 3 days ($p < 0.02$). Its activity continued to rise, though more slowly, to the 5th day. The response of the malic enzyme appeared to be slightly slower than that of the other two. At 3 days the change was highly significant ($p < 0.02$) and had reached 78% of that seen at 5 days. In all three cases, however, the major change had occurred prior to the fourth day of prolactin treatment. In considering these results it must be remembered that they are expressed in terms of liver nitrogen. As shown in Table I the total liver weight increases fairly steadily over the 5-day period of treatment and reaches about twice its normal size at 5 days with only a slight decrease in nitrogen content. Hence in terms of increase in total liver enzyme activity the values given at 5 days in Table IV must be multiplied by 2. Thus a 5–6-fold increase has occurred in 5 days in the case of the citrate cleavage enzyme. Three days of saline injections had no effect on the activity of these enzymes. An examination of the malic enzyme activity of adipose tissue showed it was unaffected by the prolactin treatment. Data on these last two observations are not presented.

Six additional liver enzymes were examined for the effect of prolactin on their activities. In these studies the prolactin was administered for a 3-day period since the previous results had indicated that marked changes in liver metabolism and the enzymes listed in Table IV had occurred by this time. The results presented in Table V indicate that the activity of none of these enzymes was significantly altered by the hormone. Most worthy of note is the fact that no increase was seen in the dehydrogenases of the hexose monophosphate shunt.

The effect of varying the amount of prolactin administered over a 3-day period has been examined and these results are given in Table VI. Lowering the dose of prolactin from 1.0 to 0.2 mg daily caused a significant decrease ($p < 0.02$) in the response as measured by liver or crop sac weight. Enzyme activities and metabolic processes sensitive to prolactin treatment showed increases that were unaltered ($p > 0.05$) by lowering the dose. The number of experiments run at 5.0 mg daily was restricted because of a limited supply of the prolactin preparation. In the two experiments run, however, there was only a small increase in the response with respect to a daily dose of 1 mg.

An answer was sought to the question of whether the effects of prolactin treatment would be seen in the absence of food intake. The results of these experiments are given in Table VII. First it may be seen that 3 days of fasting caused the rate of incorporation of pyruvate carbon into fatty acids by liver slices to fall to a rate

TABLE IV: The Effect of Prolactin on the Activity of Citrate Cleavage Enzyme, Malic Dehydrogenase, and Malic Enzyme of Pigeon Liver.

	Days of Prolactin (1 mg/day)				
	0	1	2	3	4	5
Citrate cleavage enzyme[a]	8.7 ± 1.1	10.7 ± 1.4	24 ± 4.4	20 ± 1.9	22 ± 4.4	23 ± 4.5
Malate dehydrogenase[b]	1600 ± 160	1900 ± 100	2400 ± 190	2700 ± 260	2700 ± 290	2900 ± 200
Malic enzyme[c]	81 ± 10	85 ± 9	120 ± 12	150 ± 17	160 ± 27	170 ± 19
Nitrogen[d]	2.76 ± 0.09	2.47 ± 0.10	2.24 ± 0.20	2.59 ± 0.07	2.43 ± 0.14	2.49 ± 0.07
Number of experiments	10	8	5	9	4	8

[a] Micromoles of citrate cleaved per milligram of nitrogen per hour plus or minus standard error. [b] Micromoles of NADH oxidized per milligram of nitrogen per hour plus or minus standard error. [c] Micromoles of NADPH formed per milligram of nitrogen per hour plus or minus standard error. [d] Nitrogen (mg)/100 mg wet wt plus or minus standard error.

TABLE V: The Effect of Prolactin on the Activity of Certain Carbohydrate-Metabolizing Enzymes in Pigeon Liver.

Enzyme	Untreated	Prolactin (1 mg/day for 3 days)
Hexokinase[a]	4.2 ± 0.7 (4)[b]	2.4 ± 0.6 (4)
Glucokinase[a]	1.0 ± 0.3 (4)	1.4 ± 0.2 (4)
Total HMP shunt dehydrogenase[c]	8.0 ± 1.0 (6)	5.6 ± 1.1 (6)
Pyruvate kinase[d]	110 ± 10 (8)	110 ± 20 (4)
Aconitase[e]	24 ± 0.5 (6)	23 ± 1.0 (6)
Isocitrate dehydrogenase[c]	110 ± 10 (6)	140 ± 10 (6)

[a] Micromoles of NADPH formed (*via* added glucose 6-phosphate dehydrogenase) per milligram of nitrogen per hour plus or minus standard error. [b] Number of birds. [c] Micromoles of NADPH formed per milligram of nitrogen per hour plus or minus standard error. [d] Micromoles of pyruvate formed per milligram of nitrogen per hour plus or minus standard error. [e] Micromoles of aconitate formed per milligram of nitrogen per hour plus or minus standard error.

6% of that observed in liver slices from pigeons fed normally ($p < 0.002$). At the same time there was a fall in activity of about 50% for citrate cleavage enzyme and malic enzyme and 30% for malate dehydrogenase per mg of liver nitrogen. The weight of the liver in relation to total body weight was not significantly changed by the fast ($p > 0.05$). Three daily injections of 1 mg of prolactin did not alter the pattern of these changes induced by fasting. Thus as measured by these criteria there is no response of pigeon liver to prolactin in the absence of food intake.

It is of interest to note, however, that the crop sac did respond to prolactin treatment during fasting. As shown in Table VII a 68% increase was seen in the weight of crop sac relative to total body weight. In fed pigeons the same prolactin treatment produces a 51% increase in this value (*cf.* Table I). In addition, as shown in Table VIII the same increase in total fatty acid content of the crop sac occurs after prolactin treatment whether pigeons are fed or fasted.

We have tested the effect of some other hormones on the hepatic processes stimulated by prolactin. Insulin was chosen because of the role of food in eliciting the prolactin effects and because of its importance in the regulation of ligogenesis in mammals (Cahill and Renold, 1965). One unit per day of protamine zinc insulin administered for a total of 3 days had no effect on liver size or function (Table IX). When the dose was increased to 8 units daily, given in two divided doses, the pigeons became quite lethargic and failed to eat properly. These birds lost weight, and slices prepared from their livers incorporated

TABLE VI: The Effect of Different Doses of Prolactin (3 days) on Prolactin-Sensitive Processes in the Pigeon.

Measurement[a]	Untreated	0.2 mg	1.0 mg	5.0 mg
Body weight		+7.9 ± 1.3	+10.5 ± 0.8 (17)	+13.0 (±1)[b]
Liver weight	1.9 ± 0.1 (26)[c]	2.4 ± 0.1	3.1 ± 0.1 (17)	3.9 (±0.5)
Crop sac weight	0.39 ± 0.02 (23)	0.44 ± 0.01	0.59 ± 0.04 (14)	0.80 (±0.3)
Pyruvate to fatty acids	340 ± 50(11)	920 ± 100	870 ± 80 (8)	
Citrate cleavage enzyme	8.7 ± 1.1	20 ± 1.0	20 ± 1.9	31 (±3)
Malate dehydrogenase	1600 ± 160	2600 ± 100	2700 ± 260	2500 (±100)
Malic enzyme	81 ± 10	180 ± 10	150 ± 17	220 (±10)
Liver nitrogen	2.76 ± 0.09	2.75 ± 0.12	2.59 ± 0.07	2.44 (±0.05)
Number of birds	10	4	9	2

[a] Data are expressed as indicated in the footnotes to previous tables. [b] Since only two birds were used at the 5.0-mg dose, the range is provided. [c] The number of birds in each group is given at the bottom of each column except where noted as in this manner.

TABLE VII: The Effect of Prolactin in Fasted Pigeons.

Measurement[b]	Normal	Fasted	Fasted and Prolactin[a] (1 mg/day)
Body weight		−11.4 ± 0.5	−10.6 ± 0.9
Liver weight	1.9	1.8 ± 0.1	2.0 ± 0.1
Crop sac weight	0.39	0.53 ± 0.03	0.89 ± 0.07
Pyruvate to fatty acid[c]	340	21 ± 3	27 ± 2
Citrate cleavage enzyme[d]	8.7	4.5 ± 0.5	4.7 ± 0.6
Malate dehydrogenase	1600	1100 ± 70	1300 ± 130
Malic enzyme	81	42 ± 6	36 ± 3
Nitrogen	2.76	3.17 ± 0.03	3.14 ± 0.08
Number of birds		6	6

[a] The first prolactin injection was given at the same time that food was withdrawn. [b] Data are expressed as indicated in the footnotes to previous tables. [c] Incorporation of [U-^{14}C]pyruvate into fatty acids in liver slices *in vitro*. [d] Enzyme and nitrogen data refer exclusively to liver.

pyruvate into fatty acids at a significantly lower than normal rate ($p = 0.02$). This dose of insulin also caused a significant decrease in the activity of liver malic enzyme ($p < 0.05$). The decrease in the activity of liver citrate cleavage enzyme was of borderline significance ($0.05 < p < 0.1$) and liver malic dehydrogenase was not decreased significantly ($p > 0.1$). These changes resemble those seen in starvation and probably reflect a lowered food intake. Hexokinase and glucokinase activities were not affected by insulin at 1 unit/day and pyruvate kinase activity was unaffected by insulin at this dosage level or at 8 units daily (data not presented).

We tested the effect of growth hormone on pigeon liver lipogenesis because this hormone is reported to increase food consumption, body weight, and liver weight in hypophysectomized pigeons (Bates *et al.*, 1962). In our intact pigeons growth hormone (1 mg/day for 3 days) caused a significant increase in body weight ($p = 0.028$) and relative liver weight ($p < 0.002$) (Table IX). Slices prepared from the livers of the growth hormone treated pigeons incorporated pyruvate into fatty acids at a significantly higher rate than slices from normal pigeons ($p = 0.02$). There were also significant increases in the hepatic activities of citrate cleavage enzyme ($p < 0.002$), malic enzyme ($p < 0.002$), and malate dehydrogenase ($p < 0.02$). The relative weight of the crop sac was not increased by growth hormone ($p > 0.05$). Like prolactin, growth hormone *in vitro* had no effect on the incorporation of pyruvate into fatty acids in liver slices prepared from saline-injected pigeons (data not presented).

Triiodothyronine (100 μg/day for 2 or 5 days) had no effect on body weight or liver malic enzyme activity (data not presented).

As shown in Table X we were unable to detect any increase in the size and enzyme activity of male rat liver after injection of 0.5 mg of prolactin daily for 3 days. The rats employed were in a weight range of 200 g; hence this dose was slightly higher on a "per kilogram of body weight basis" than the 1 mg/500 g used for pigeons.

TABLE VIII: The Effect of Prolactin on Crop Sac Total Fatty Acid Content[a] in Normal and Fasted Pigeons.

Treatment	Normal	Prolactin[b]
Normally fed	2.1 ± 0.1	5.5 ± 0.2
Fasted	1.8 ± 0.3	4.3 ± 0.2

[a] Microequivalents per milligram of nitrogen. [b] For normally fed animals the prolactin (1 mg/day) was administered for 5 days; for the fasted animals, 3 days.

Discussion

The response of pigeon liver to injections of the prolactin preparation is prompt and precedes any change seen in the weight of the crop sac. Pronounced changes are seen in liver weight and in the ability of liver slices to convert both glucose and pyruvate to fatty acids (*cf.* Tables I and II) within 1 day following the injection of 1 mg of the hormone preparation. Though there is a hint at this time of an increase in the activities of citrate cleavage enzyme, malic enzyme, and malate dehydrogenase, significant changes in the activity of these enzymes occur 24 hr later. The manner in which prolactin elicits these liver responses is not clear. Its action would seem to be dependent upon food intake since the responses are not seen during starvation. The crop sac, on the other hand, responds regardless of food intake. This suggests that the liver changes are not due to a direct action of prolactin upon the liver but are perhaps the resultant of a hyperphagia induced in some manner by prolactin. If this

TABLE IX: The Effect of Growth Hormone and Insulin on Prolactin-Sensitive Processes in the Pigeon.

Measurement	Hormone[a]			
	Untreated	Insulin 1 unit/day	Insulin 8 units/day	Growth Hormone (1 mg/day)
Body weight[b]		−0.4 ± 0.7	−9.3 ± 1.1	+11.9 ± 0.8
Liver weight	1.9 ± 0.1 (26)[c]	2.0 ± 0.05	2.3 ± 0.2	3.3 ± 0.3
Crop sac weight	0.39 ± 0.02 (23)			0.43 ± 0.03
[U-^{14}C]Pyruvate–fatty acids[d]	340 ± 50 (11)	450 ± 80	100 ± 10	740 ± 30
Citrate cleavage[e] enzyme	8.7 ± 1.1	9.2 ± 0.7	5.8 ± 0.6	27 ± 3
Malic dehydrogenase	1600 ± 160	2000 ± 130	1300 ± 160	2700 ± 210
Malic enzyme	81 ± 10	69 ± 8	42 ± 9	190 ± 20
Liver nitrogen	2.76 ± 0.09	2.74 ± 0.07	2.79 ± 0.12	2.64 ± 0.0
Number of birds	10	4	4	4

[a] The hormones were administered for 3 days; the 8-unit dose of insulin was actually 4 units given twice daily. [b] Data are expressed as indicated in the footnotes to Tables I, IV, and V. [c] The number of birds in each group is given at the bottom of each column except as noted in parentheses. [d] Incorporation of [U-^{14}C]pyruvate into fatty acids in liver slices *in vitro*. [e] Enzyme and nitrogen data refer exclusively to liver.

is the case, then the changes would be somewhat analagous to the increased lipogenic activity seen in the liver and adipose tissue of rats when they are fasted and refed.

The results in hand do not permit one to pinpoint the primary metabolic step or enzymatic reaction which is first stimulated, so that increased lipogenesis ensues. The fact that the rate of conversion of both glucose and pyruvate to fatty acids in liver slices rises early and concomitantly suggests that some step or steps in the conversion of pyruvate to triglycerides is first affected. Support for such a conclusion is provided also by the finding that the rate of glucose carbon incorporation into fatty acids, even at a glucose concentration of 110 mM, never exceeds that from pyruvate carbon. The acetate data are not very helpful in deciding whether the step affected lies between pyruvate and acetyl-CoA or in the pathway between the latter compound and triglyceride. Following this initial change, whatever it may be, it would appear that there ensues what may be termed secondary changes. One such change perhaps occurs in the utilization of glucose at a concentration of 11 mM. This is suggested by the fact that after 5 days of prolactin treatment, the rate of conversion of glucose to fatty acids at this glucose concentration increases sixfold as compared to a threefold increase in the rate of pyruvate conversion to fatty acids. Even then the rate of conversion of 11 mM glucose to fatty acids is well below that seen with 110 mM glucose after only 3 days of prolactin treatment. A logical explanation for these results would be that an increase in hexokinase activity had occurred. None, however, is seen after 3 days of prolactin treatment (*cf.* Table V) so if this is the answer, then the change occurs between the third and fifth day of treatment.

TABLE X: The Effect of Prolactin on the Size and Enzyme Activity of Rat Liver.

Measurement[b]	Untreated	Prolactin[a] (0.5 mg/day)
Liver weight	4.5 ± 0.1	4.6 ± 0.1
Total HMP shunt dehydrogenase	15 ± 0.5	14 ± 0.5
Citrate cleavage enzyme	3.4 ± 0.6	1.9 ± 0.5
Malic dehydrogenase	1100 ± 40	1100 ± 50
Malic enzyme	5.4 ± 0.5	4.0 ± 0.4
Nitrogen	2.66 ± 0.20	2.49 ± 0.02

[a] Treatment was for 3 days. [b] Data are expressed as indicated in the footnotes to Tables I, IV, and V.

The marked increases in the activities of citrate cleavage, malate dehydrogenase, and malic enzyme may also be classified as secondary changes. At least 2 days of prolactin treatment were required before any significant increase occurred in the activities of these enzymes, and the extent of the change was nearly complete after 3 days. Evidence has been presented that these three enzymes are involved in the process of lipogenesis in rat liver and adipose tissue (Young *et al.*, 1964; Ball, 1966). In conjunction with pyruvate carboxylase they are capable of forming a metabolic cycle which would serve to transport acetyl-CoA from inside the mitochondria to the outside and to convert NADH to extramitochondrial NADPH for use in reduction to fatty acids of the acetyl-CoA so transported. The activity of all three of these enzymes increases in both rat liver and adipose tissue when lipogenesis in these tissues is stimulated by hormonal treatment or dietary

manipulations (*cf.* Ball, 1966). The changes observed in the activities of malic enzyme and citrate cleavage enzyme in rat tissues under such conditions are very much greater than those observed here for pigeon liver. It should be noted, however, that the activities of these two enzymes is much higher to start with in normal pigeon liver than in rat liver. In the case of malic enzyme it is 16–19 times and for citrate cleavage enzyme about five times higher in pigeon liver than in rat liver. This fact prompted Goodridge and Ball (1966) to suggest that malic enzyme may play a more prominent role in furnishing NADPH for lipogenesis in pigeon liver than in rat liver. In this regard it is of interest to note that the activities of the dehydrogenases of the glucose 6-phosphate shunt in pigeon liver do not increase but tend to decline as a result of prolactin treatment. This behavior is in marked contrast to that seen in rat liver when lipogenesis is accelerated. In this case, as shown by Tepperman and Tepperman (1964), the activities of the shunt dehydrogenases and malic enzyme rise markedly and concomitantly.

In considering the results reported here it is important to emphasize that for convenience we have used the term prolactin preparation and prolactin as synonymous. In so doing we are fully cognizant of the fact that the effects described may not be due to prolactin *per se*, but to some factor present as a contaminant in the preparation. The preparation employed, NIH-P-B_1, was supplied by the Endocrinology Study Section of the National Institutes of Health. It was reported by the suppliers to contain 13 IU of prolactin/mg as assayed by the systemic pigeon crop weight method of Riddle *et al.* (1933). The growth hormone content of this preparation is of interest in view of the fact that the injection of 1 mg of growth hormone preparation (1.5 U.S.P. units) daily produced effects on pigeon liver comparable to that observed with 1 mg of the prolactin preparation. This content is reported to be less than 0.01 U.S.P. unit of growth hormone/mg. Thus, on this basis if the effects of the prolactin preparation are due to its content of growth hormone then the purified growth hormone preparation (1.5 units/mg) should be fully effective at daily doses of less than 0.01 mg. We have not tested the action of growth hormone at levels below 1.0 mg daily. The reverse of this coin is the possibility that the action of growth hormone preparation is due to its content of prolactin. The supplier reports the prolactin content to be less than 0.1 IU/mg of growth hormone preparation. Thus if the effects of the growth hormone preparation are due to its content of prolactin it should be possible to elicit a response with 0.01 mg daily of the prolactin preparation equal to that seen with 1.0 mg. We have lowered the dosage of prolactin to 0.2 mg daily. The metabolic and enzymatic changes at this level are about the same as with the higher dose but the increase in total body weight and liver weight are somewhat diminished (*cf.* Table VI). The question raised thus cannot be resolved at this time. The best conclusion at the moment seems to us to be that both prolactin and growth hormone are capable of producing the changes observed in pigeon liver or that the preparations of both of these hormones employed here contain a substance capable of this action.

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