

## Effect of Somatotropin and Insulin-Like Growth Factor-I on Milk Lipid and Protein Synthesis In Vitro<sup>1</sup>

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### ABSTRACT

The objective of this research was to test the hypothesis that bST stimulates milk secretion through the action of IGF-I. Cocultures of bovine mammary, adipose, and liver tissues were incubated with increasing concentrations (0 to 1000 ng/ml) of bST, IGF-I, prolactin, or bST plus prolactin. In addition, cocultures of mammary and adipose tissues without liver tissue were incubated with IGF-I. The synthesis of milk lipids and proteins and the concentration of cellular DNA were measured. The addition of liver tissue depressed DNA concentration and the synthesis of lipids and proteins in mammary tissue. In mammary tissue, increasing bST concentration resulted in greater synthesis of FFA, total lipids, and proteins than that produced by increasing concentrations of IGF-I, and the effect on protein synthesis was linear. Conversely, in adipose tissue, increasing the concentration of IGF-I in the presence of liver resulted in greater synthesis of FFA than that obtained by bST, and the effect was linear. The results do not support the hypothesis that bST acts through the actions of IGF-I on mammary cells. Rather, bST alters the delivery of nutrients to the mammary cells from other tissues and affects milk component synthesis through a mechanism that may not involve IGF-I. (**Key words:** insulin-like growth factor-I, bovine somatotropin, milk protein, deoxyribonucleic acid)

**Abbreviation key:** bPRL = bovine prolactin.

### INTRODUCTION

Bovine somatotropin affects milk secretion by bovine mammary tissue in vivo (15) but not in vitro (8), possibly because of the lack of bST receptors on

bovine mammary tissue (13). Somatotropin is thought to act on milk secretion indirectly through the release of IGF-I from other tissues. In support of this theory, receptors for bST have been found on bovine liver tissue (13), and receptors for IGF-I have been found on ovine mammary tissue (4). The IGF-I has also been shown to affect directly DNA synthesis in bovine mammary tissue (3) and to affect growth of bovine and rabbit cartilage in vitro (23).

In order to study the relationship between bST and milk secretion, Fekry et al. (6) developed an explant culture system utilizing the coculture of bovine mammary, adipose, and liver explants. Using the coculture system, those researchers demonstrated that bST increased lipid synthesis in mammary tissue at the expense of lipid synthesis in adipose tissue (6, 12) when liver tissue was also present in the coculture. Those results suggested that bST increased milk production by partitioning nutrients away from adipose tissue and toward the mammary gland, as has been discussed by Bauman and Vernon (2). Based on the hypothesis that bST acts through the action of IGF-I, then IGF-I should also directly affect lipid and protein synthesis by mammary tissue when cocultured with adipose tissue and without liver tissue.

Although bovine prolactin (bPRL) does not stimulate milk production during lactation, bPRL does appear to be required to initiate full differentiation and milk synthesis at parturition (24). The act of removing tissue from the mammary gland, preparing explants, and placing the explants in an artificial environment could cause some of the cells to revert to a nondifferentiated state. The bPRL was added to our cocultures alone and in combination with bST to determine whether bPRL would stimulate or maintain the differentiation of the mammary epithelial cells in explant culture and thereby increase the synthesis of milk lipids and proteins.

The primary objective of the current research was to examine the hypothesis that bST stimulates milk secretion through the action of IGF-I. Secondary objectives were 1) to determine and compare the effects of bST, bPRL, and IGF-I on lipid and protein synthe-

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sis in the coculture system; 2) to determine whether changes in lipid and protein synthesis result from increases in the number of mammary epithelial cells as indicated by changes in cellular DNA content or result from increases in the expression of mRNA for the various enzymes as indicated by changes in cellular content of RNA; and 3) to examine the interaction of liver tissue in the coculture system with IGF-I.

## MATERIALS AND METHODS

### Cows

Eight Holstein cows were slaughtered by stunning and exsanguination. The cows averaged  $220 \pm 18$  d in lactation, and mean milk production was  $20.0 \pm 3.8$  kg/d. Samples of mammary and liver tissues were placed into a sterile, ice-cold Tris-sucrose buffer (25 mM Tris and 0.3 M sucrose) and were transported to the laboratory for processing. Adipose tissue from the mammary fat pad area was transported to the laboratory in sterile Tris-sucrose buffer at  $37^{\circ}\text{C}$ .

### Tissue Cultures

Explants were prepared from mammary, adipose, and liver tissues as described previously (6). In the first experiment 0, 1, 10, 100, or 1000 ng of GT5-3 recombinant bovine IGF-I/ml (Monsanto, St. Louis, MO) were added to media-199 (9). Cocultures of mammary and adipose tissue explants were then incubated in this media in the presence or absence of liver tissue explants. All incubations were for 24 h. The hormones and  $^{14}\text{C}$  labels discussed subsequently were added for the entire 24 h.

The second experiment consisted of comparing the addition of 0, 1, 10, 100, or 1000 ng/ml of media of the following hormones to cocultures of mammary, adipose, and liver tissue explants: 1) pituitary bST (NIH B-18, 0.81 IU/mg), 2) pituitary bPRL (USDA B-1, 13.0 IU/mg), 3) bST plus bPRL, and 4) GT5-3 recombinant bovine IGF-I. Weights of the mammary, adipose, and liver explants averaged 30, 70, and 35 mg/ml of media, respectively.

### Lipid Assays

The [ $^{14}\text{C}$ ]acetate (specific activity, 59 mCi/mmol) was added to the tissue culture plates used to study the synthesis of lipids at the concentration of  $0.5 \mu\text{Ci/ml}$ . Lipid extraction and TLC analysis were conducted as described previously (11). Lipids assayed consisted of triglycerides, diglycerides, monoglycerides, FFA, and phospholipids. The synthesis of these lipids

is reported as  $^{14}\text{C}$  disintegrations per minute per milligram of mammary or adipose tissue. The sum of all of the lipids assayed is reported and discussed as total milk lipid.

### Protein Assays

The [ $^{14}\text{C}$ ]leucine (specific activity, 300 mCi/mmol) and [ $^{14}\text{C}$ ]proline (specific activity, 250 mCi/mmol) were added to tissue culture plates to study the synthesis of milk proteins at the concentration of  $0.5 \mu\text{Ci/ml}$ . Following incubation, milk proteins were extracted from mammary explants and separated by gel electrophoresis. The  $^{14}\text{C}$  activities of  $\alpha_{s1}$ -CN,  $\alpha_{s2}$ -CN,  $\beta$ -CN, and  $\kappa$ -CN,  $\alpha$ -LA, and  $\beta$ -LG (whey) bands were counted (Bioscan System 200 Imaging Scanner; Bioscan, Inc., Washington, DC) as described previously (14). The activities of both the casein and whey bands are reported and discussed in this study as total milk protein.

### DNA and RNA Assays

After incubation, mammary explants were minced four times with a McIlwain tissue chopper (Mickle Lab. Eng. Co., Ltd., Gomshall, Surrey, England); epithelial cells were isolated from the minced tissue and fixed with 70% alcohol as described previously (14). The DNA and RNA contents of the mammary epithelial cells were measured by tagging the DNA and RNA with propidium iodide, which fluoresces when stimulated by a laser beam (13). In the assays for DNA, RNase was added to destroy any RNA that would interfere in the assay for DNA. Conversely, in the assays for RNA, DNase was added to destroy any DNA that would interfere in the assay for RNA.

### Flow Cytometer Assay

The number of mammary epithelial cells fluorescing and the intensity of their fluorescence were measured with a flow cytometer (EPICS Profile; Coulter Corp., Hialeah, FL) equipped with a 488-nm argon ion laser. Laser power was set at 15 mW. The sheath pressure was 7.5 psi. The degree of fluorescence in individual cells was measured through a 525-nm band pass filter in electronic channels from 0 to 1023. The higher that the channel number was, the greater was the concentration of DNA or RNA per cell. For example, cells in channel 400 on a linear scale would have twice the concentration of DNA or RNA as cells in channel 200. The DNA and RNA concentrations were expressed in terms of the mean channel of the fluorescing cells. Single cells were separated from

cellular debris and doublets on a forward scatter versus log side scatter histogram and gated to a second histogram to measure log fluorescence. The forward scatter gain was set at 1.5, and the photomultiplier tube for side scatter was set at 200 V. The photomultiplier tube for DNA or RNA fluorescence was set at 850 V. Results of the DNA and RNA assays were expressed in histograms. Mean channels of the fluorescing peaks in DNA and RNA histograms and the percentage of cells containing DNA in the  $G_0G_1$ , S, and  $G_2M$  phases of the mitotic cycle were determined (Cytologic Computer Program; Coulter Corp.).  $G_0G_1$ , S, and  $G_2$  are substages in the interphase stage of mitosis.  $G_0$  is the hypothetical state in which cells are no longer dividing,  $G_1$  is the pre-DNA synthesis period, S is the period during which DNA synthesis occurs, and  $G_2$  is the post-DNA synthesis period. The metaphase stage of mitosis is M, and  $G_2M$  represents the period from  $G_2$  to M.

### Statistical Analysis

Data were analyzed in two analyses using PROC MIXED of SAS (19). The variables tested were DNA mean channel of  $G_0G_1$  and  $G_2M$  cells, RNA mean channel, synthesis of milk lipids (total and FFA), and synthesis of total milk proteins. All variables were measured in mammary tissue. Total lipid and FFA synthesis were also measured in adipose tissue.

The first analysis compared the effect of increasing the concentration of IGF-I incubated with mammary, adipose, and liver tissue with the effect of increasing IGF-I incubated with mammary and adipose tissue only. Cow, the interaction of cow and liver tissue, and the interaction of cow and hormone concentration were random factors. To correct for variance heterogeneity, the mammary and adipose tissue total lipid and FFA data and the mammary total protein data were  $\log_{10}$ -transformed and used in the analyses. When concentration or the interaction of liver tissue and concentration was significant at  $P < 0.10$ , linear through cubic orthogonal polynomial contrasts were performed to determine the shape of the response over the levels of concentration. When the interaction of liver tissue and concentration was significant at  $P < 0.10$ , comparisons between the two liver tissue treatments were made.

The second analysis compared the effects of increasing the concentrations (1 to 1000 ng/ml) of bST, bPRL, bST + bPRL, and IGF-I incubated with mammary, adipose, and liver tissue. Random effects were cow, interaction of cow and hormone, and interaction of cow and hormone concentration. To correct for heterogeneity of variance, total lipid and FFA data for

the mammary and adipose tissues and the total protein data for mammary tissue were  $\log_{10}$ -transformed. The transformed variables were used in the analyses. When concentration or the interaction of hormone and concentration was significant at  $P \leq 0.10$ , linear through cubic orthogonal polynomial contrasts were performed to determine the shape of the response over the levels of concentration. When hormone or the interaction of hormone and concentration was significant at  $P \leq 0.10$ , the hormone control was included in the analysis. The data were recoded for 17 treatments and analyzed as a mixed one-factor model. Random effects were cow and the interaction of cow and treatment. The hormone control and the hormone treatments were compared.

The general shape of polynomial trends up to cubic were interpreted according to a table generated from the sign of the contrast statements, which are the same as those of the corresponding polynomial coefficients (W. Potts, 1994, Biometrical Consulting Service, USDA-ARS, Beltsville, MD).

## RESULTS

### DNA and RNA Histograms

Examples of the histograms created for DNA and RNA by the flow cytometer are presented in Figure 1. The DNA histograms consist of two peaks (Figure 1A). The first peak consists of cells in the ( $G_0G_1$  phase), and the second peak consists of cells in the  $G_2M$  phase, which has twice the diploid number of chromosomes as do cells in the  $G_0G_1$  peak. Cells in S phase, in the valley between the two peaks, are in the process of doubling the number of chromosomes. The RNA histograms consisted of one peak (Figure 1B), and the mean channel represents a measurement of the relative RNA content per cell examined.

### IGF-I Incubated with and Without Liver Tissue

Figure 2 presents the effects of IGF-I on total protein synthesis, total lipid synthesis, mean  $G_0G_1$ , and  $G_2M$  DNA channel in mammary tissue cocultured with adipose tissue in the presence and absence of liver tissue. The addition of liver tissue to the cocultures depressed DNA content in  $G_0G_1$  ( $P < 0.007$ ) and  $G_2M$  cells ( $P < 0.001$ ) and depressed the synthesis of total protein ( $P < 0.018$ ) in mammary tissue but had no effect ( $P > 0.17$ ) on total lipid synthesis.

The main effect of IGF-I concentration was not significant ( $P > 0.10$ ) for any of the variables measured in mammary tissue; however, the interaction of

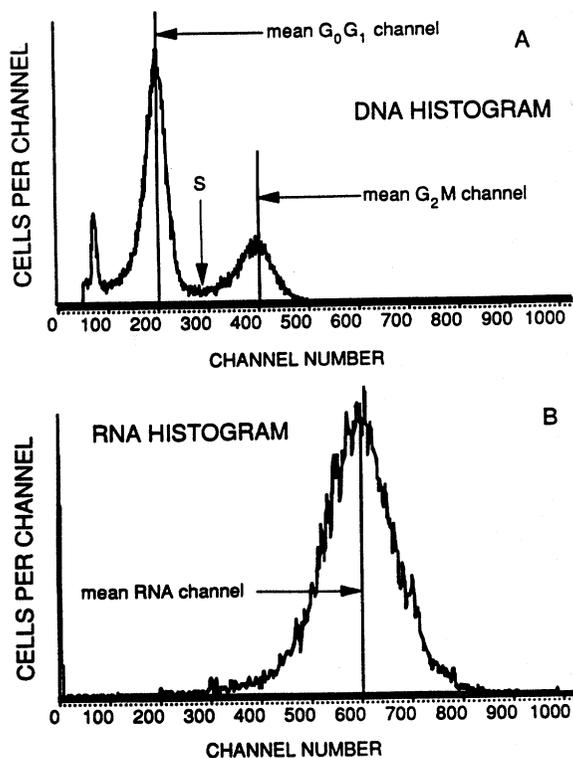


Figure 1. Example of a DNA (A) and RNA (B) histogram from the Epics profile flow cytometer (Coulter Corp., Hialeah, FL).  $G_0G_1$ , S, and  $G_2$  are substages in the interphase stage of mitosis.  $G_0$  is the hypothetical state in which cells are no longer dividing,  $G_1$  the pre-DNA synthesis period, S the period during which DNA synthesis occurs, and  $G_2$  the post-DNA synthesis period. M is the metaphase stage of mitosis, and  $G_2M$  represents the period from  $G_2$  to M.

liver tissue with IGF-I concentration was significant for RNA mean channel and  $G_2M$  cell DNA mean channel. The RNA content of mammary epithelial cells was depressed by increasing IGF-I concentration in the presence of liver tissue, and the effect was linear ( $P < 0.016$ ). The RNA content was not affected by IGF-I in the absence of liver tissue. The  $G_2M$  cell DNA content increased as IGF-I concentration increased in the presence of liver tissue, and the effect was quadratic ( $P < 0.002$ ). The  $G_2M$  cell DNA content decreased in the absence of liver tissue as IGF-I content increased, and the effect was linear ( $P < 0.092$ ).

The addition of liver tissue to the cocultures also depressed FFA synthesis in mammary ( $P < 0.005$ ) and adipose tissues ( $P < 0.003$ ). Increasing the IGF-I concentration had no effect on FFA synthesis in mammary tissue ( $P > 0.726$ ) but increased FFA synthesis ( $P < 0.023$ ) in adipose tissue. The effect on FFA synthesis by IGF-I concentration in adipose tissue was quadratic ( $P < 0.03$ ).

## Hormone Effects in the Presence of Liver Tissue

Figure 3 contains line graphs of the effects of hormone concentration on total protein synthesis, total lipid synthesis, mean  $G_0G_1$ , and  $G_2M$  DNA channel in mammary tissue. The DNA and RNA mean channels were not affected by the main effect of hormones ( $P > 0.10$ ), but total lipid ( $P < 0.03$ ) and protein synthesis ( $P < 0.001$ ) were affected. Mean comparisons indicated that IGF-I depressed ( $P < 0.10$ ) lipid synthesis, and bST enhanced ( $P < 0.01$ ) lipid synthesis, relative to the control.

The main effect of hormone concentration was observed for the  $G_2M$  mean DNA channel ( $P < 0.021$ ) and total lipid synthesis ( $P < 0.045$ ). Increasing the concentrations of all hormones increased the  $G_2M$  DNA mean channel and total lipid synthesis. The effect was linear ( $P < 0.007$ ) for DNA and for total lipid synthesis.

The interaction of hormone and hormone concentration was significant ( $P < 0.059$ ) for total protein synthesis. Mean comparisons determined that all concentrations of bST increased ( $P < 0.01$ ) total protein synthesis compared with the control. The effect of increasing bST concentration was linear ( $P < 0.034$ ). The 100 ng/ml ( $P < 0.01$ ) and 1000 ng/ml ( $P < 0.10$ ) of IGF-I depressed total protein synthesis compared with the control. Mean comparisons determined that bPRL, bST + bPRL, and the control were similar with respect to total protein synthesis.

## FFA Synthesis in Mammary and Adipose Tissue

Line graphs of FFA synthesis are presented in Figure 4 for mammary (A) and adipose tissue (B) cocultured with liver tissue and bST or IGF-I. The main effect of hormones on FFA synthesis was significant in mammary ( $P < 0.035$ ) and adipose tissue ( $P < 0.012$ ). Mean comparisons determined that the effects of bST, bPRL, and bST + bPRL on FFA synthesis in mammary tissue were not different ( $P > 0.10$ ) from the effect of the control, but IGF-I depressed ( $P < 0.10$ ) FFA synthesis.

The main effect of hormone concentration on FFA synthesis was significant in mammary ( $P < 0.009$ ) and adipose tissue ( $P < 0.03$ ). The effect of increasing the hormone concentrations on FFA synthesis by mammary tissue was linear ( $P < 0.001$ ).

There was also a significant effect for the interaction of hormone, and concentration ( $P < 0.053$ ) on FFA synthesis by adipose tissue. Mean comparisons determined that the 1, 10 ( $P < 0.10$ ), and 1000 ng/ml

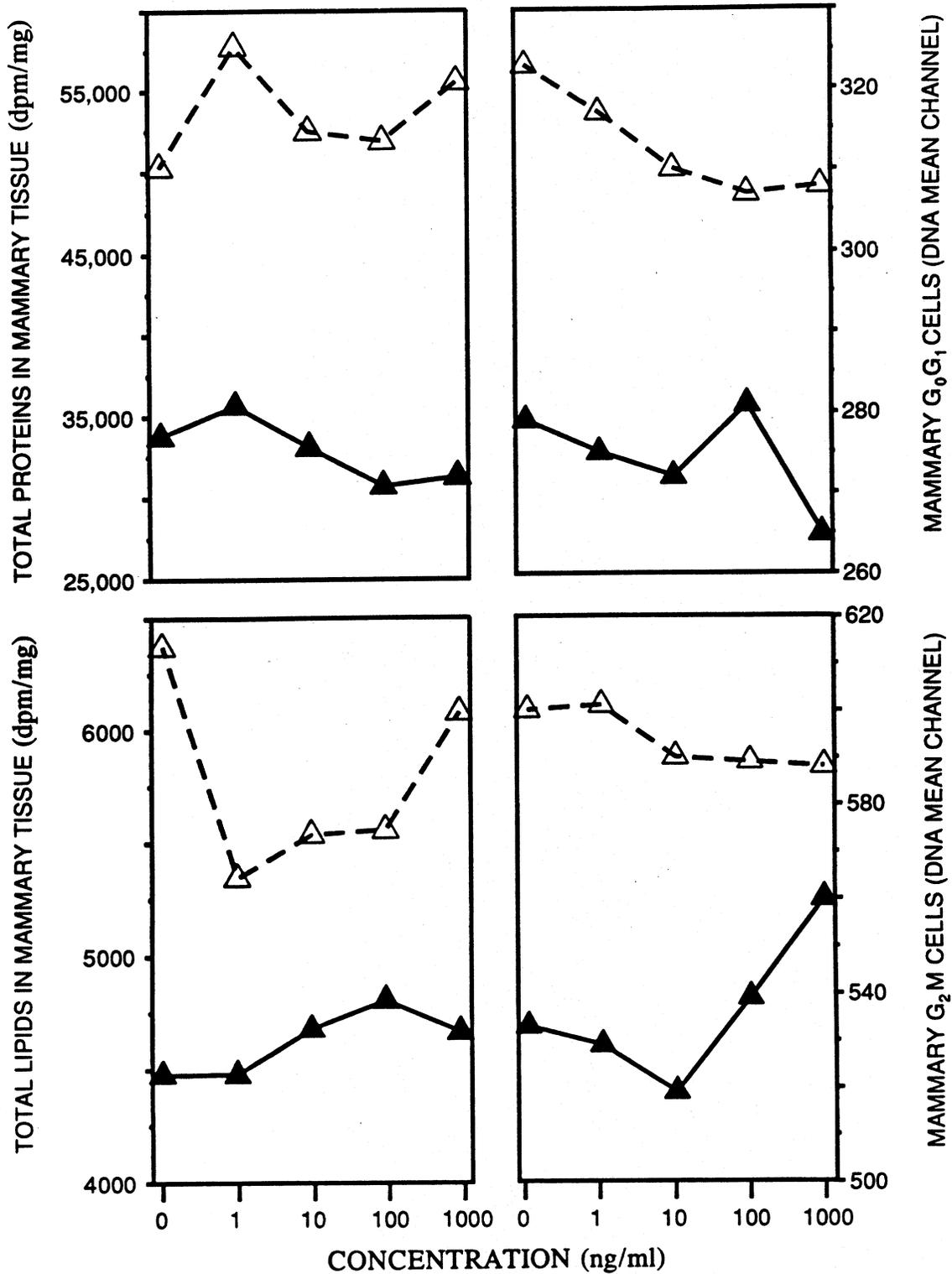


Figure 2. Comparison of the effects of IGF-I on total protein synthesis, total lipid synthesis, and DNA concentration in G<sub>0</sub>G<sub>1</sub> and G<sub>2</sub>M cells extracted from mammary tissue cocultured with adipose tissue in the presence (▲) and absence (△) of liver tissue. Synthesis was measured as the number of disintegrations per minute of <sup>14</sup>C-labeled amino acids or acetate incorporated into milk proteins or lipids per unit of tissue mass. The addition of liver tissue significantly depressed protein synthesis ( $P < 0.08$ ) and G<sub>0</sub>G<sub>1</sub> ( $P < 0.02$ ) and G<sub>2</sub>M ( $P < 0.001$ ) mean channels.

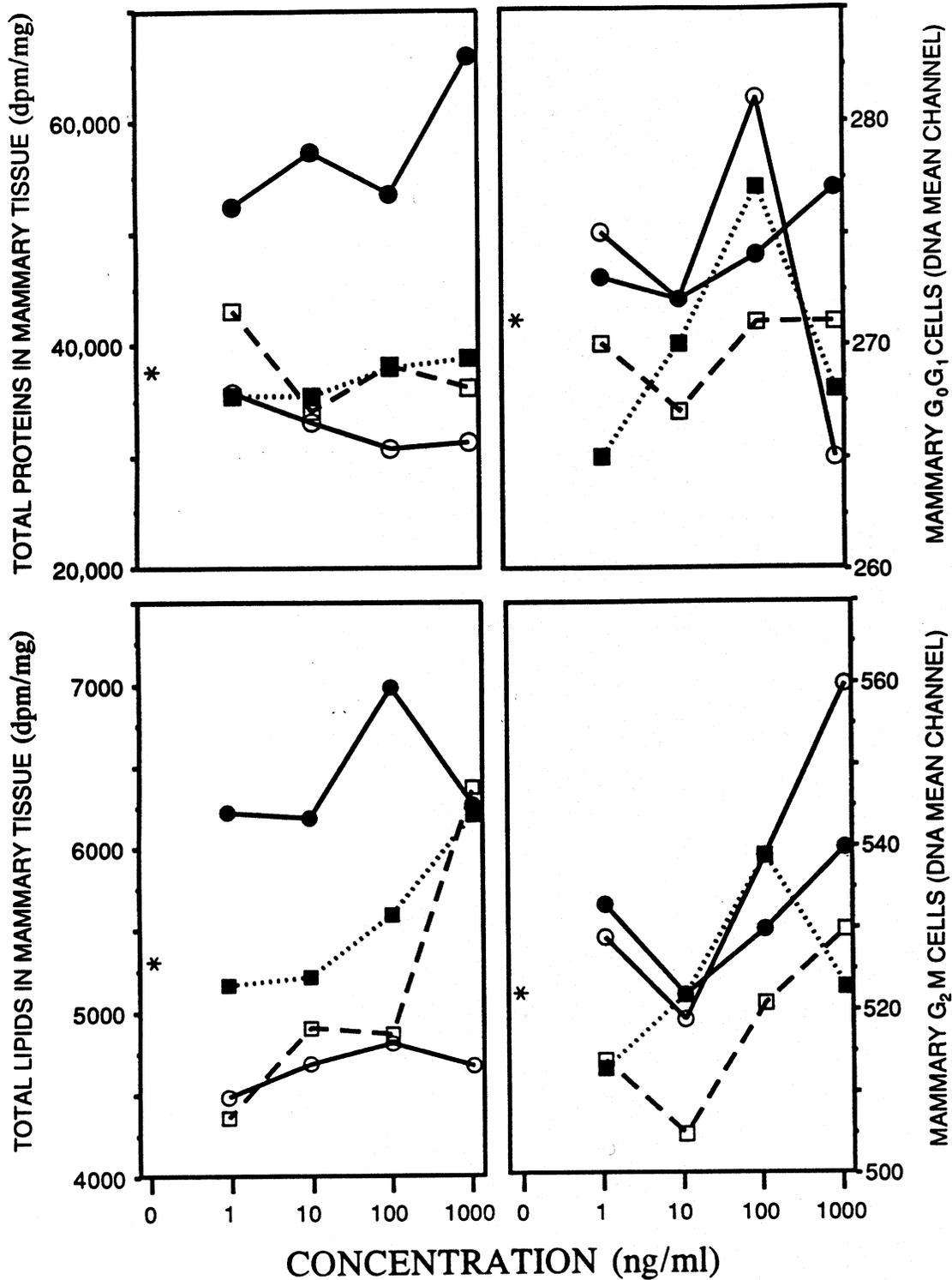


Figure 3. The effects of 0, 1, 10, 100, or 1000 ng/ml of media of pituitary-derived bST (●), bPRL (□), bST + bPRL (■), or recombinant bovine IGF-I (○) on total protein synthesis, total lipid synthesis, and DNA concentrations in G<sub>0</sub>G<sub>1</sub> and G<sub>2</sub>M cells extracted from mammary tissue cocultured with adipose and liver tissue. Synthesis was measured as the number of disintegrations per minute of <sup>14</sup>C-labeled amino acids or acetate incorporated into milk proteins or lipids per unit of tissue mass. The main effect of hormones was significant for total lipids ( $P < 0.03$ ) and proteins ( $P < 0.001$ ). Hormone concentration was significant for G<sub>2</sub>M mean channel ( $P < 0.021$ ) and total lipids ( $P < 0.045$ ). The interaction of hormone and hormone concentration was significant for total protein ( $P < 0.059$ ). \* = Control.

synthesis (Figure 3) in the presence of liver tissue as previously shown for lipid synthesis (6, 12). Conversely, except for its positive effect on FFA in adipose tissue, IGF-I had no effect on total milk lipid synthesis or total milk protein synthesis (Figures 2, 3, and 4).

In our experiment, the cellular concentration of DNA was depressed by IGF-I in the absence of liver tissue and was stimulated in the presence of liver tissue during the 24-h incubation. Others (3, 17) have also demonstrated an effect of IGF-I on DNA synthesis in bovine mammary tissue after a 3- to 6-d incubation. Given that IGF-I alone did not elicit the same responses on lipid and protein synthesis as did bST, it is difficult to conclude that bST is acting through the action of IGF-I.

Flow cytometers are able to detect changes in the concentration of DNA within individual cells. As seen in Figure 1, cells in the G<sub>2</sub>M peak have twice the concentration of DNA as those cells in the G<sub>0</sub>G<sub>1</sub> peak. If cells in the G<sub>2</sub>M peak are not arrested during this stage, they divide and become two G<sub>0</sub>G<sub>1</sub> cells. The flow cytometer in our experiment observed a slight shift to the right on the X-axis of both the G<sub>0</sub>G<sub>1</sub> and G<sub>2</sub>M peaks. This shift indicates an increase in the concentration of DNA within individual cells that does not appear to be related to mitosis.

Increases in the DNA concentration within individual cells, observed when the concentration of bST (Figure 3) was increased, were concurrent with increases in the synthesis of total milk lipids and total milk proteins (Figure 3). The decrease in the DNA concentration within individual cells, observed when the media concentration of IGF-I was increased in the absence of liver tissue (Figure 2), was similar to results of an earlier experiment (14) in which protein synthesis also decreased concurrently. As discussed by Keys et al. (14), these positive and negative changes in cellular DNA concentration might be a result of changes in the amplification of individual genes. Others (1, 21, 22) have previously observed changes in cellular DNA content associated with changes in protein synthesis that did not appear to be related to mitosis. Banerjee et al. (1) postulated that the changes in DNA concentration were a result of changes in the number of copies of specific genes, or gene amplification, rather than to changes in total chromatic DNA. Whatever the mechanism responsible for the changes in DNA concentration, the DNA changes appeared to be directly related to changes in lipid and protein synthesis and should be the focus of future study.

Evidence by others (7, 11, 20) also does not support the hypothesis that bST stimulates milk secre-

tion through the release of IGF-I. Flint et al. (7) found that injecting IGF-I into rat dams that were depleted of somatotropin and prolactin had no effect on pup weight, but injection of bST restored pup weight gain to 40% of control values. Flint et al. (7) suggested that bST might stimulate IGF-I-binding proteins, which could affect the action of IGF-I. If so, injection of IGF-I without bST to modify the IGF-I-binding proteins could render the additional IGF-I ineffectual. That hypothesis is supported by evidence of Pell and Bates (16), who found that the effects of bST and IGF-I on skeletal muscle and weight gain by dwarf mice were additive.

Bovine somatotropin and IGF-I may also act on milk production independently of one another. For cows, plasma IGF-I concentration falls dramatically around parturition and increases thereafter until the end of lactation (11, 20). Conversely, plasma bST increases dramatically at parturition and gradually falls thereafter through the rest of the lactation cycle as does daily milk production (20). Therefore, an indirect relationship exists between serum IGF-I and bST over the course of lactation in cows. This indirect relationship between bST and IGF-I is shown in their action on FFA synthesis (Figure 4). The addition of bST stimulated FFA synthesis in mammary tissue but had no effect on FFA in adipose tissue. This effect would occur during the earlier part of the lactation cycle when milk production and plasma concentration of bST were highest. Conversely, IGF-I had no effect on FFA synthesis in mammary tissue but stimulated FFA synthesis in adipose tissue (Figure 4). This effect would occur near the end of lactation when milk production was lowest, plasma IGF-I concentration was highest, and the cow was building body reserves in preparation for calving and the next lactation cycle. These results duplicate results reported in an earlier study (12) with bST and support the theory of nutrient partition (2).

In conclusion, the evidence of this study does not support the hypothesis that bST stimulates milk secretion through the release of IGF-I. The results also do not indicate that bST or IGF-I act on milk production by increasing the number of secretory cells or by increasing the expression of mRNA as measured by total concentration of RNA within individual cells. The results do suggest, however, that bST and IGF-I act independently and inversely from each other and affect milk production through the partitioning of nutrients between mammary and adipose tissue. The evidence with bST and the addition of liver tissue to cocultures suggests a relationship between the synthesis of milk components and the concentration of DNA within the mammary cell; however, the exact nature of this relationship remains to be investigated.

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