Effects of Hormones on Protein and Amino Acid Metabolism in Mammary-Gland Explants of Mice

By LARRY D. ANDERSON and JAMES A. RILLEMA*

Department of Physiology, Wayne State University School of Medicine, MI 48201, U.S.A.

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The effects of insulin, cortisol and prolactin on amino acid uptake and protein biosynthesis were determined in mammary-gland explants from mid-pregnant mice. Insulin stimulated [^3H]leucine incorporation into protein within 15 min of adding insulin to the incubation medium. Insulin also had a rapid stimulatory effect on the rate of aminoiso[^{14}C]butyric acid uptake, but it had no effect on the intracellular accumulation of [^3H]leucine. Cortisol inhibited the rate of [^3H]leucine incorporation into protein during the initial 4 h of incubation, but it had no effect at subsequent times. [^3H]Leucine uptake was unaffected by cortisol, but amino[^{14}C]isobutyric acid uptake was inhibited after a 4 h exposure period to this hormone. Prolactin stimulated the rate of [^3H]leucine incorporation into protein when tissues were exposed to this hormone for 4 h or more; up to 4 h, however, no effect of prolactin was detected. At all times tested, prolactin had no effect on the uptake of either amino[^{14}C]isobutyric acid or [^3H]leucine. Incubation with actinomycin D abolished the prolactin stimulation of protein biosynthesis, but this antibiotic did not affect the insulin response. A distinct difference in the mechanism of action of these hormones on protein biosynthesis in the mammary gland is thus apparent.

The initiation of the production of milk substances in mammary-gland explants from mid-pregnant mice can be accomplished by incubation with the following hormonal combination: insulin, a glucocorticoid and prolactin (Elias, 1959; Rivera & Bern, 1961; Juergens *et al.*, 1965; Stockdale & Topper, 1966). Many studies have shown that each of these hormones has multiple actions, including the stimulation of the biosynthesis of several macromolecules. Insulin stimulates DNA synthesis (Stockdale & Topper, 1966; Turkington & Hill, 1969; Oka *et al.*, 1974), the mitosis of epithelial components (Turkington, 1968*a,b*; Friedburg *et al.*, 1970; Mills & Topper, 1970), RNA synthesis (Palmiter, 1969*a*; Turkington, 1970) and protein synthesis (Wang & Amor, 1970). Cortisol enhances the formation of rough endoplasmic reticulum in the alveolar cells (Oka & Topper, 1971); it also appears to regulate polyamine biosynthesis (Oka & Perry, 1974). Prolactin, synergistically with insulin (Topper, 1970), enhances the production of RNA (Juergens *et al.*, 1965; Palmiter, 1969*a*; Topper, 1970; Turkington, 1970; Rillema, 1973, 1975*a*), casein (Palmiter, 1969*a*; Rillema, 1973, 1975*b*), lactose (Turkington *et al.*, 1968; Palmiter, 1969*b*; Topper, 1970) and several enzymes.

The precise mechanism(s) by which each of these hormones regulates the metabolism of the mammary gland are not known. To further our understanding of

these processes, the early actions of these hormones on protein and amino acid metabolism in the mammary gland were studied.

Experimental

Materials

All animals used in this study were mid-pregnant Swiss-Webster mice purchased from Spartan Research Animals, Haslett, MI, U.S.A. The animals were primiparous and were killed on the twelfth to fourteenth day of pregnancy. Day zero of pregnancy was the day when sperm were found in the vagina.

Ovine prolactin (NIH-P-S-10) was a gift from the National Institute of Arthritis and Metabolic Diseases. Porcine insulin was donated by Eli Lilly and Co., Indianapolis, IN, U.S.A. Cortisol was purchased from Chas. Pfizer and Co., Groton, CT, U.S.A. Bovine serum albumin, L-leucine, α -aminoisobutyric acid, and actinomycin D were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Nystatin was obtained from E. R. Squibb and Sons, Speke, Liverpool, U.K. Medium 199, penicillin and streptomycin were purchased from Microbiological Associates, Bethesda, MD, U.S.A. [4,5- ^3H]Leucine (50 $\mu\text{Ci}/\text{mmol}$), α -amino[1- ^{14}C]isobutyric acid (0.25 $\mu\text{Ci}/\text{mmol}$), $^3\text{H}_2\text{O}$ and [^3H]inulin were purchased from New England Nuclear Corp. Boston, MA, U.S.A.

* To whom reprint requests should be addressed.

Methods

Incubation procedures. Procedures for the preparation and incubation of mammary-gland explants were described in detail by Rillema (1973). In general, explants (3–5 mg each) were prepared, and two or three of these were placed on silicone-treated lens paper floating on 2 ml of Medium 199. The tissues were then 'preincubated' for 1 day in medium containing no hormones, 1 day in medium containing 2.5 μ g of insulin/ml or for 2 days in medium containing 2.5 μ g of insulin/ml plus 2.5 μ g of cortisol/ml; these preincubation periods were used when the effects of insulin, cortisol and prolactin respectively on protein synthesis and amino acid uptake were to be studied. After the preincubation period, the appropriate hormone (2.5 μ g/ml) was then added to certain culture flasks, and incubations were continued for specified times.

Leucine incorporation into protein. When the rate of protein synthesis was to be studied, 0.25 μ Ci of [3 H]leucine/ml was added to the bathing medium 30 min before termination of the incubations. The extent of [3 H]leucine incorporation into protein was then assessed by the methods of Kostyo & Rillema (1971). The tissues were blotted, weighed and homogenized in 3 ml of 10% (w/v) trichloroacetic acid. After centrifugation at 2000g for 10 min, the pellet was suspended in 5 ml of 10% trichloroacetic acid containing 0.1% leucine; this mixture was maintained overnight at 4°C. The tubes were then centrifuged at 2000g for 10 min, and the pellet was again suspended in 5 ml of 10% trichloroacetic acid. This mixture was heated to 85°C and centrifuged at 2000g for 10 min to remove DNA and RNA. Lipids were then removed by washing the pellet twice with 5 ml of ether/ethanol/chloroform (2:2:1, by vol.) and once with 5 ml of acetone. The pellet was heated to 45°C for 15 min during each wash and then centrifuged for 10 min at 2000g. The final pellet was suspended in 2–3 ml of 1M-NaOH and the protein was solubilized by boiling the mixture for 30–60 min. Radioactivity in this mixture was then determined by liquid-scintillation spectroscopy of a 1 ml sample in 10 ml scintillation mixture [of composition 52.5 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), 4.2 g of 2,5-diphenyloxazole (POP), 900 ml of toluene and 300 ml of Triton X-100], and protein content was assessed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Results are expressed as d.p.m./mg of protein.

Amino acid uptake. When the rate of [3 H]leucine uptake was to be studied, radioactivity in the trichloroacetic acid-soluble fraction of tissues incubated with [3 H]leucine (see above) was measured by liquid-scintillation spectroscopy. The intracellular accumulation of [3 H]leucine was then calculated by methods described by Rillema (1973). By the same methods, the uptake of aminoisobutyric acid was

determined after adding 0.25 μ Ci (1 mM) to the incubation medium 30 min or 60 min before termination of the experiments. Total tissue water and extracellular volumes were determined by equilibrating the explants with 3 H $_2$ O and [3 H]inulin respectively (Rillema, 1973). The volumes of distribution of 3 H $_2$ O ($50.6 \pm 2.1\%$ S.E.M.) and [3 H]inulin ($24.5 \pm 1.0\%$ S.E.M.) were not different from those reported earlier (Rillema, 1973). Also, the hormones used in these studies did not affect these distribution values.

Results

Insulin effects

Table 1 shows the time-course for the effect of insulin on the rate of [3 H]leucine incorporation into protein in mammary-gland explants. A stimulation was observed after a 15 min incubation with insulin; this effect persisted during exposure periods of up to 2 h. An early effect of insulin on [3 H]leucine uptake

Table 1. Time-course of insulin stimulation of [3 H]leucine incorporation into protein

Mammary-gland explants were preincubated for 1 day in medium containing no hormones. Insulin was then added to certain flasks and incubation was continued for the times indicated. [3 H]Leucine (0.5 μ Ci/ml) was added to each flask for the final 30 min of incubation; when the incubation period was 15 min, the label was present only for that time-period. Values are means $\pm$ S.E.M. of 21 observations.

Incubation time (min)	3 H]Leucine incorporation into protein (d.p.m./mg of protein)		
	Control	Insulin	P
15	2928 $\pm$ 156	4113 $\pm$ 184	<0.05
30	9526 $\pm$ 409	12726 $\pm$ 522	<0.05
60	8438 $\pm$ 230	11307 $\pm$ 276	<0.05
120	9830 $\pm$ 350	13958 $\pm$ 336	<0.05

Table 2. Effect of insulin on the uptake of amino [14 C]isobutyric acid and [3 H]leucine

Mammary-gland explants were preincubated for 1 day in medium containing no hormones. Insulin was then added to certain flasks, and incubation was continued for 1 h. [3 H]Leucine (50 μ M, 0.2 μ Ci/ml) or amino [14 C]isobutyric acid (1 mM, 0.2 μ Ci/ml) was added to each flask for the final 30 or 60 min of incubation. Values are means $\pm$ S.E.M. of 14 observations. N.S., Not significant.

Amino acid	Amino acid uptake (mmol/30 or 60 min per litre of cell water)		
	Control	Insulin	P
Aminoisobutyric acid	1.56 $\pm$ 0.10	2.23 $\pm$ 0.10	<0.05
Leucine	0.069 $\pm$ 0.015	0.052 $\pm$ 0.006	N.S.

into the mammary tissues, however, was not observed, despite the fact that the uptake of aminoisobutyric acid was enhanced under the same experimental conditions (see Table 2). It is thus unlikely that an altered specific radioactivity of ³H in the leucine pools of the mammary-gland cells accounts for the enhanced rate of [³H]leucine incorporation into protein observed in response to insulin. Additional experiments were carried out to determine whether the early effect of insulin on protein metabolism requires on-going RNA synthesis. Incubation of mammary-gland explants with a concentration of actinomycin D that was previously shown to lower the rate of RNA synthesis to low values (Rillema, 1975*b*) did not attenuate the magnitude of the insulin stimulation of [³H]leucine incorporation into protein (see Table 3).

Cortisol effects

In contrast with insulin, cortisol inhibited the rate of [³H]leucine incorporation into protein during the initial 4h of exposure to this hormone (Table 4).

Table 3. Effect of actinomycin D on insulin stimulation of [³H]leucine incorporation into protein

Mammary-gland explants were preincubated for 1 day in medium containing no hormones. Insulin and/or actinomycin D (5 µg/ml) was then added to certain flasks and incubation was continued for 1 h. [³H]Leucine (0.5 µCi/ml) was added to each flask for the final 30 min of incubation. Values are means ± S.E.M. of 14 observations.

Incubation conditions	[³ H]Leucine incorporation into protein	
	(d.p.m./mg of protein)	P
No hormones	7814 ± 558	<0.05
Insulin	9804 ± 670	
Actinomycin D	6867 ± 334	
Insulin + actinomycin D	9234 ± 388	

At subsequent times through 48h, however, cortisol had no effect on the rate of [³H]leucine incorporation. It, like insulin, had no effect on the rate of [³H]leucine uptake into the mammary tissues. The rate of aminoisobutyric acid uptake was inhibited by cortisol when the tissues were exposed to this hormone for a period of 4h (Table 5).

Prolactin effects

The time-course for the effect of prolactin on the rate of protein synthesis was also examined. Fig. 1 shows that prolactin stimulated the rate of [³H]leucine incorporation into trichloroacetic acid-precipitable protein at 4h and at all subsequent times after exposure of these tissues to prolactin. It is apparent, however, that there is a 3–4h lag period before this effect of prolactin on protein synthesis becomes manifest. The uptake of [³H]leucine and amino[¹⁴C]-

Table 4. Time-course of cortisol inhibition of [³H]leucine incorporation into protein

Mammary-gland explants were preincubated for 2 days in medium containing insulin. Cortisol was then added to certain flasks and incubation was continued for the times indicated. [³H]Leucine (0.5 µCi/ml) was added to each flask for the final 30 min of incubation. Values are means ± S.E.M. of (a) 28 and (b) 14 observations. N.S., Not significant.

Incubation time	[³ H]Leucine incorporation into protein (d.p.m./mg of protein)		
	Control	Cortisol	P
30 min	3974 ± 202 (a)	3337 ± 171 (a)	<0.05
60 min	7641 ± 490 (a)	5872 ± 271 (a)	<0.05
4 h	8340 ± 402 (a)	6729 ± 343 (a)	<0.05
8 h	9859 ± 637 (b)	10078 ± 621 (b)	N.S.
12 h	7372 ± 665 (b)	7444 ± 887 (b)	N.S.
24 h	7335 ± 878 (b)	7449 ± 339 (b)	N.S.
48 h	7388 ± 874 (b)	6784 ± 636 (b)	N.S.

Table 5. Effect of cortisol on the uptake of [³H]leucine and amino[¹⁴C]isobutyric acid

Mammary-gland explants were preincubated for 2 days with insulin. Cortisol was added to certain flasks and incubation was continued for the times indicated. [³H]Leucine (50 µM, 0.2 µCi/ml) or amino[¹⁴C]isobutyric acid (1 mM, 0.2 µCi/ml) was added to each flask for the final 30–60 min of incubation. Values are means ± S.E.M. of (a) 21 and (b) 7 observations. N.S., Not significant.

Amino acid	Incubation time (h)	Amino acid uptake (mmol/30 or 60 min per litre of cell water)		P
		Control	Cortisol	
Leucine	0.5	0.010 ± 0.004 (a)	0.013 ± 0.003 (a)	N.S.
Leucine	1.0	0.019 ± 0.003 (a)	0.020 ± 0.004 (a)	N.S.
Leucine	4.0	0.019 ± 0.003 (a)	0.022 ± 0.004 (a)	N.S.
Aminoisobutyric acid	0.5	1.32 ± 0.20 (b)	1.56 ± 0.18 (b)	N.S.
Aminoisobutyric acid	1.0	2.22 ± 0.23 (b)	2.29 ± 0.14 (b)	N.S.
Aminoisobutyric acid	4.0	2.08 ± 0.21 (b)	1.15 ± 0.19 (b)	<0.05

Fig. 1. Time-course of prolactin stimulation of [^3H]leucine incorporation into protein

Points are means $\pm$ S.E.M. of 21 observations. Mammary-gland explants were preincubated for 2 days in medium containing insulin and cortisol. Prolactin was then added to certain flasks and incubation was continued for the times indicated. [^3H]Leucine (0.5 $\mu\text{Ci/ml}$) was added to each flask for the final 30 min of incubation.

isobutyric acid into the mammary-gland explants was also measured in tissues incubated with prolactin. Table 6 shows that prolactin had no effect on the uptake of either of these amino acids. In further experiments, the effect of prolactin on [^3H]leucine incorporation into protein was tested in the presence and absence of on-going RNA synthesis. Table 7 shows that incubation with actinomycin D nearly abolished the prolactin stimulation of protein synthesis.

Discussion

Several facets of the actions of insulin on protein and amino acid metabolism in the mammary gland have been explored. First of all, it was shown that insulin stimulates the rate of [^3H]leucine incorporation into protein within minutes of adding insulin to medium bathing mammary-gland explants. Insulin similarly has a rapid action on protein biosynthesis in

Table 7. Effect of actinomycin D on prolactin stimulation of [^3H]leucine incorporation into protein

Mammary-gland explants were preincubated for 2 days in medium containing insulin + cortisol. Prolactin and/or actinomycin D (5 $\mu\text{g/ml}$) was then added to certain flasks and incubation was continued for 6 h. [^3H]Leucine (0.5 $\mu\text{Ci/ml}$) was added to each flask for the final 30 min of incubation. Values are means $\pm$ S.E.M. of 14 observations.

Incubation conditions	[^3H]Leucine incorporation into protein	
	(d.p.m./mg of protein)	P
Control	8142 $\pm$ 452	<0.05
Prolactin	10052 $\pm$ 561	
Actinomycin D	6292 $\pm$ 216	
Prolactin + actinomycin D	6826 $\pm$ 531	<0.05

skeletal muscle (Wool & Moyer, 1964). Since neither insulin nor other hormones used in these studies affected the rate of [^3H]leucine uptake into the mammary gland, it is unlikely that an altered specific radioactivity of ^3H in the intracellular leucine pool is responsible for the observed changes in the rates of [^3H]leucine incorporation into protein. Thus the enhanced rate of [^3H]leucine incorporation in response to insulin probably reflects an enhanced rate of protein biosynthesis. In contrast with the lack of effect on the uptake of [^3H]leucine, insulin enhanced the uptake of amino[^{14}C]isobutyric acid into the mammary gland. This effect occurred within 30 min of exposing mammary-gland explants to insulin; Oka *et al.* (1974) previously reported a stimulation by insulin of amino[^{14}C]isobutyric acid uptake into the mammary gland during a 4 h incubation period. It is thus apparent that insulin has a rapid action on the uptake of only certain amino acids into the mammary gland.

Table 6. Effect of prolactin on the uptake of [^3H]leucine and amino[^{14}C]isobutyric acid

Mammary-gland explants were preincubated for 2 days with insulin and cortisol. Prolactin was added to certain flasks and incubation was continued for the times indicated. [^3H]Leucine (50 $\mu\text{Ci/ml}$) or amino[^{14}C]isobutyric acid (1 mM, 0.2 $\mu\text{Ci/ml}$) was added to each flask for the final 30 or 60 min of incubation. Values are means $\pm$ S.E.M. of seven observations. N.S., Not significant.

Amino acid	Incubation time (h)	Amino acid uptake (mmol/30 or 60 min per l of cell water)		
		Control	Prolactin	P
Leucine	4.0	0.015 $\pm$ 0.004	0.025 $\pm$ 0.009	N.S.
Leucine	6.0	0.064 $\pm$ 0.005	0.067 $\pm$ 0.010	N.S.
Aminoisobutyric acid	4.0	2.50 $\pm$ 0.60	2.70 $\pm$ 0.20	N.S.
Aminoisobutyric acid	6.0	4.40 $\pm$ 0.30	4.10 $\pm$ 0.70	N.S.
Aminoisobutyric acid	8.0	2.60 $\pm$ 0.20	2.70 $\pm$ 0.20	N.S.

Further studies were carried out to assess whether the effect of insulin on protein biosynthesis requires on-going RNA biosynthesis. Incubation of explants with actinomycin D, an inhibitor of RNA synthesis, failed to suppress or attenuate the insulin effect on the rate of [^3H]leucine incorporation into protein. Thus, although insulin stimulates the rate of [^3H]uridine incorporation into RNA within minutes of exposing mammary-gland explants to insulin (Rillema, 1975b), the effect of insulin on protein biosynthesis appears to occur independently of the early effect of insulin on RNA metabolism. It is therefore likely that the early effect of insulin on protein biosynthesis occurs via an activation of the translocation processes rather than by an RNA-DNA-dependent mechanism. Further studies will be required to clarify this matter.

Since cortisol reportedly (Oka & Topper, 1971) stimulates an elaboration of the rough endoplasmic reticulum in the alveolar cells of the mammary gland, it was somewhat surprising that a stimulatory effect on the rate of [^3H]leucine incorporation into protein was not observed. In fact, a transient decrease in the rate of protein biosynthesis was observed during the initial 4h of exposure to cortisol. It is possible, however, that this decrease reflects a decreased rate of protein biosynthesis in cells other than the alveolar cells in this tissue preparation. Since the rate of uptake of [^3H]leucine was unaffected by cortisol, the lower rate of [^3H]leucine incorporation into protein is apparently not related to an altered rate of its uptake. Amino[^{14}C]isobutyric acid uptake, however, was decreased in response to cortisol; the glucocorticoids similarly attenuate the rate of uptake of aminoisobutyric acid into the rat diaphragm (Kostyo & Schmidt, 1963).

A stimulation of the rate of [^3H]leucine incorporation into protein by prolactin was detectable after a 3–4h lag period. Similarly, there is a 3–4h lag period before the rate of [^3H]uridine or $^{32}\text{P}_i$ incorporation into RNA is enhanced by prolactin (Rillema, 1973). The fact that these effects of prolactin may be related in a causal manner is suggested by the experiment in which the prolactin stimulation of protein biosynthesis was decreased to an insignificant level by actinomycin D (see Table 1). It thus seems apparent that on-going RNA synthesis is required for prolactin to manifest its early stimulatory action on the rate of [^3H]leucine incorporation into protein. An enhanced rate of synthesis of casein, the primary milk protein

in the mammary gland, is not detectable until 8–10h after incubation with prolactin (Juergens *et al.*, 1965; Stockdale *et al.*, 1966; Rillema, 1973). The early 4h effect of prolactin on protein biosynthesis therefore appears to be on the synthesis of proteins other than casein. Presumably, these proteins are subsequently associated with the production of milk substances.

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References

- Elias, J. J. (1959) *Proc. Soc. Exp. Biol. Med.* **101**, 500–502
- Friedburg, S. H., Oka, T. & Topper, Y. J. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1493–5000
- Juergens, W. G., Stockdale, F. E., Topper, Y. J. & Elias, J. J. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 629–634
- Kostyo, J. L. & Rillema, J. A. (1971) *Endocrinology* **88**, 1054–1062
- Kostyo, J. L. & Schmidt, J. E. (1963) *Am. J. Physiol.* **204**, 1031–1038
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mills, E. S. & Topper, Y. J. (1970) *J. Cell Biol.* **44**, 310–328
- Oka, T. & Perry, J. W. (1974) *J. Biol. Chem.* **249**, 7647–7652
- Oka, T. & Topper, Y. J. (1971) *J. Biol. Chem.* **246**, 7701–7707
- Oka, T., Perry, J. W. & Topper, Y. J. (1974) *J. Cell Biol.* **62**, 550–556
- Palmiter, R. D. (1969a) *Endocrinology* **85**, 747–751
- Palmiter, R. D. (1969b) *Biochem. J.* **113**, 409–417
- Rillema, J. A. (1973) *Endocrinology* **92**, 1673–1679
- Rillema, J. A. (1975a) *Endocrinology* **96**, 1307–1311
- Rillema, J. A. (1975b) *Am. J. Physiol.* **228**, 1531–1534
- Rivera, E. M. & Bern, H. A. (1961) *Endocrinology* **82**, 340–353
- Stockdale, F. E. & Topper, Y. J. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1283–1289
- Stockdale, F. E., Juergens, W. G. & Topper, Y. J. (1966) *Dev. Biol.* **13**, 261–288
- Topper, Y. J. (1970) *Recent Prog. Horm. Res.* **26**, 287–308
- Turkington, R. W. (1968a) *Endocrinology* **82**, 540–546
- Turkington, R. W. (1968b) *Experientia* **24**, 226–228
- Turkington, R. W. (1970) *J. Biol. Chem.* **245**, 6690–6697
- Turkington, R. W. & Hill, R. L. (1969) *Science* **163**, 1458–1460
- Turkington, R. W., Brew, K., Vanamon, T. C. & Hill, R. L. (1968) *J. Biol. Chem.* **243**, 3382–3387
- Wang, D. Y. & Amor, V. (1971) *J. Endocrinol.* **50**, 241–249
- Wool, I. G. & Moyer, A. N. (1964) *Biochim. Biophys. Acta* **91**, 248–256