Stimulation by Insulin of the Formation of Ribonucleic Acid and Protein by Mammary Tissues *in vitro*

BY R. MAYNE AND J. M. BARRY

Department of Agriculture, University of Oxford

AND EVELYN M. RIVERA*

National Institute for Research in Dairying, Shinfield, Reading, Berks.

(Received 1 December 1965)

1. Insulin is one of the hormones that are essential for successful tissue culture of explants of the mammary glands of pregnant mice. We report here effects of insulin on RNA and protein formation by mammary tissue from pregnant mice and rats incubated in tissue-culture medium 199. 2. The incorporation of [^{14}C]adenine over 3 hr. into the RNA of explants of the mammary glands of pregnant mice was increased by an average of 68% when the medium contained 5 $\mu\text{g.}$ of insulin/ml. Under similar conditions the incorporation into the RNA of slices of the glands of pregnant rats was increased by an average of 61%. Incorporation into the RNA of slices from lactating rats was stimulated to a smaller extent. 3. Adipose tissue was separated from the glands of pregnant mice and the effect of insulin on the incorporation of adenine into its RNA was studied. In whole explants the incorporation of adenine, both with and without insulin, is almost entirely into the RNA of the mammary parenchyma and not of the adipose tissue. 4. Insulin also stimulated by 38% the incorporation of [^{14}C]leucine over 3 hr. into the proteins of slices of the glands of pregnant rats. It had no significant effect on slices from lactating rats. 5. Actinomycin D (10 $\mu\text{g./ml.}$) decreased the incorporation of [^{14}C]adenine into the RNA of slices of the glands of pregnant rats by an average of 97%. Though it also decreased the incorporation of [^{14}C]leucine into the proteins by an average of 25%, the percentage stimulation by insulin of this incorporation remained unchanged.

When explants of the mammary glands of pregnant mice are incubated in tissue-culture medium 199, supplemented with insulin, an adrenal corticoid and prolactin, they continue to develop. After 5 days they resemble lactating tissue: lipid vacuoles appear in the alveolar cells and the alveoli become distended with a milky secretion. If prolactin is omitted the explants survive but do not develop further. If insulin, or insulin and the adrenal corticoid, are omitted necrosis occurs (Elias, 1959; Rivera, 1964). We are investigating early biochemical effects of these hormones on slices and explants of mammary gland in the same culture medium, and now report effects of insulin on the incorporation of [^{14}C]adenine into RNA and of [^{14}C]leucine into protein.

MATERIALS AND METHODS

Materials. [8- ^{14}C]Adenine (31.3 mc/m-mole) and L-[1- ^{14}C]leucine (8.5 mc/m-mole) were bought from The

Radiochemical Centre, Amersham, Bucks. Tissue-culture medium 199 was bought from Glaxo Laboratories, Greenford, Middlesex. Its composition is given by Morgan, Morton & Parker (1950). It contains among many other ingredients 0.58 mg. of adenine/100 ml., 12 mg. of DL-leucine/100 ml. and 100 mg. of glucose/100 ml. Before use in tissue-slice experiments an extra 132 mg. of NaHCO_3 /100 ml. was added; this was necessary to maintain the pH at 7.4 under the conditions of incubation. Glucagon-free insulin was a gift from Dr Otto Behrens, Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A. A solution (0.5 mg./ml. of 3 M-N-HCl) was prepared for each experiment. Actinomycin D was a gift from Dr H. D. Brown, Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A. Puromycin was bought from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Rats. These were of the Wistar strain; they were in their first pregnancy or lactation and weighed about 200 g. They were fed on Oxo 41B diet (Oxo Ltd., London, S.E. 1). In order to know the date of fertilization of the pregnant rats within 1 day, three male rats were introduced for 24 hr. into cages of eight virgin females. The size of the litters of the lactating rats was reduced to six to eight at birth.

Mice. These were from the N.I.R.D. colony of C3H mice derived from the Chester Beatty Institute inbred stock.

* Present address: Department of Zoology, Michigan State University, East Lansing, Mich., U.S.A.

They were fed on diet PRM (Christopher Hill Ltd., Poole, Dorset).

Tissue-slice incubations. Rats were killed by cervical dislocation, and their abdominal mammary glands dissected out and put in chilled medium 199. The glands were then sliced in a microtome (Stadie & Riggs, 1944; bought from A. H. Thomas Co., Philadelphia, Pa., U.S.A.), and the slices kept in medium 199 at room temperature until used. They were then cut into smaller pieces, put on a damp filter paper and divided into roughly equal portions (100–150 mg.) and each portion was added to a 30 ml. beaker that contained medium 199 (3 ml.) and any additions. The beakers were placed on a shaker (60 cyc./min.) in a water bath at 37° covered with a hood through which O₂+CO₂ (95:5) was passed continuously, so maintaining the pH at 7.4.

Tissue explants. Mice between the tenth and nineteenth day of pregnancy were killed by cervical dislocation and their mammary glands exposed. Explants of mammary tissue (approx. 2.5 mm. diam. and 1 mm. thick) were removed with fine scissors under a dissecting microscope. Explants of adipose tissue free from parenchyma were taken from the edges of mammary fat pads. The watch-glass organ-culture procedure (Elias & Rivera, 1959) was modified slightly to allow more explants to be cultured. Each culture chamber consisted of a plastic Petri dish (15 mm. × 90 mm.; Sterilin Products Ltd., Richmond, Surrey) containing four circles of filter paper (Whatman no. 4) saturated with water. On the top of the filter papers lay half of a smaller plastic Petri dish (10 mm. × 35 mm.; Falcon Plastics Co. Ltd., London, S.W. 11). Medium 199 (3 ml.) and any additions were put in the smaller dish; 50–70 explants were added, stirred and left floating on the surface. The chambers were then placed, with their lids half open, in a Perspex box (125 mm. × 230 mm. × 315 mm.) in an incubator at 37°. The O₂+CO₂ (95:5) was bubbled continuously through water into the box at a rate that kept the medium at pH 7.4. After 3 hr. the explants were removed, rinsed in water, dried on filter paper, weighed and frozen until analysed.

Determination of the specific radioactivity of RNA and protein. The procedures were modifications of those of Wool & Moyer (1964). Both for RNA and protein isolations mammary tissue (100–150 mg.) was washed with ethanol (8 ml.) and separated by centrifuging. Ethanol-diethyl ether (3:1, v/v) (5 ml.) was then added and the tissue ground in a glass homogenizer with a mechanical drive. After standing for 30 min. the residue was separated by centrifuging and washed twice with ethanol-diethyl ether. All these operations were done in the cold room at 3°.

To isolate RNA, the residue was washed three times with 15% (w/w) trichloroacetic acid and four times with 96% (v/v) ethanol, all in the cold. The residue was then heated with 10% (w/v) NaCl solution (4 ml.) at 100° for 30 min. and the mixture centrifuged. The supernatant was cooled, ethanol (10 ml.) added and the mixture left overnight at –10°. The precipitate of RNA and DNA was dissolved in 0.3N-KOH (3 ml.). After 3 hr. at 37°, the solution was cooled and 15% (w/w) HClO₄ was added to give pH 2.0. After 1 hr. in the cold the precipitate of DNA and KClO₄ was separated and the supernatant neutralized with 0.3N-KOH and made to 4 ml. RNA was determined from the *E*₂₆₀ value (*E*_{1%^{1cm}} of the hydrolysed RNA was taken as 280) and sometimes checked by the orcinol method

(Mejbaum, 1939). A portion (0.7 ml.) of this solution was also pipetted on to an aluminium planchet (4.5 cm.² area) and warmed, and 3 drops of 1% Ionagar solution (Oxo Ltd.) were added. The gel was dried under an infrared lamp to give a uniform layer and ¹⁴C disintegrations were determined with a thin-windowed Geiger-Müller counter. Self-absorption was negligible.

To isolate protein, material prepared in the same way as for RNA isolation was heated with 10% (w/v) trichloroacetic acid (5 ml.) at 100° for 10 min. The residue was washed twice with 5% (w/v) trichloroacetic acid and then dissolved in 2 ml. of 90% (v/v) formic acid–30% (w/v) H₂O₂ (5:1, v/v). After 20 min. 10% (w/v) trichloroacetic acid (10 ml.) was added. After a further 1 hr. the protein precipitate was separated by centrifuging and washed twice in acetone and once in light petroleum (b.p. 60–80°)–diethyl ether (3:1, v/v). It was then dissolved in a little 90% (w/v) formic acid and the solution was spread evenly on a weighed aluminium planchet and dried under an infrared lamp. The protein (2–4 mg.) was determined by weighing and the ¹⁴C disintegrations were measured in a Geiger-Müller counter. Self-absorption was negligible. That the protein isolated was free from labelled amino acids unincorporated into protein was tested by incubating slices of the mammary glands of rats in a medium containing [¹⁴C]leucine with and without puromycin (500 µg./ml.). With puromycin the specific radioactivity of the isolated protein was only 2% of that without.

RESULTS

Table 1 shows that the incorporation of [¹⁴C]-adenine into the RNA of explants of the mammary glands of pregnant mice over 3 hr. was increased by an average of 68% when the medium contained insulin at the concentration used in organ culture. Table 2 shows that insulin also stimulated

Table 1. *Influence of insulin on the incorporation of [¹⁴C]adenine into RNA of explants of mammary glands of pregnant mice*

In each experiment explants were taken from three mice and mixed. Equal numbers (between 50 and 70) of explants were cultured for 3 hr. in medium 199 (3 ml.) containing [8-¹⁴C]adenine (1.5 µc) and, where indicated, insulin (5 µg./ml.).

Expt. no.	Specific radioactivity of RNA (µmc/mg.)		Increase with insulin (%)
	Control	+ Insulin	
1	104	173	64
2	71.1	123	73
3	61.3	97.1	59
4	100	176	76
5	37.6	49.3	32
6	61.2	114	87
7	81.0	134	65
8	92.7	173	87
			Mean 68

Table 2. *Influence of insulin on the incorporation of [14 C]adenine into RNA of slices of mammary glands of pregnant rats*

In each experiment the abdominal mammary glands of a rat between the twelfth and seventeenth day of pregnancy were sliced. Six portions of the slices (100–150 mg. each) were incubated at 37° in medium 199 (3 ml.) containing [14 C]adenine (0.5 μ C) for 3 hr. under $O_2 + CO_2$ (95:5). Six similar incubations contained insulin (5 μ g./ml.). Each specific activity is the mean $\pm$ s.e.m. of six separate incubations.

Expt. no.	Specific radioactivity of RNA (μ C/mg.)		Increase with insulin (%)	P
	Control	+ Insulin		
1	41.0 $\pm$ 0.9	73.7 $\pm$ 2.0	80	< 0.001
2	65.9 $\pm$ 3.9	98.1 $\pm$ 3.0	49	< 0.001
3	72.5 $\pm$ 4.1	113.7 $\pm$ 2.7	57	< 0.001
4	60.8 $\pm$ 2.2	105.1 $\pm$ 2.6	73	< 0.001
5	44.1 $\pm$ 1.4	72.5 $\pm$ 3.3	64	< 0.001
6	74.3 $\pm$ 1.2	105.0 $\pm$ 2.6	42	< 0.001
			Mean 61	

Table 3. *Influence of insulin on the incorporation of [14 C]adenine into RNA of slices of mammary glands of lactating rats*

In each experiment the abdominal mammary glands of a rat between the twelfth and fifteenth day of lactation were sliced. Conditions were otherwise as given in Table 2. N.S., Not significant.

Expt. no.	Specific radioactivity of RNA (μ C/mg.)		Increase with insulin (%)	P
	Control	+ Insulin		
1	6.73 $\pm$ 0.41	7.35 $\pm$ 0.32	9	N.S.
2	13.8 $\pm$ 0.5	16.7 $\pm$ 0.7	21	< 0.01
3	11.3 $\pm$ 0.4	13.5 $\pm$ 0.7	21	< 0.05
4	11.0 $\pm$ 0.4	13.9 $\pm$ 0.6	26	< 0.01
5	13.5 $\pm$ 1.0	13.9 $\pm$ 0.7	3	N.S.
6	13.5 $\pm$ 0.5	19.1 $\pm$ 0.6	41	< 0.001

($P < 0.001$) the incorporation of [14 C]adenine into the RNA of slices of the mammary glands of pregnant rats in the same tissue-culture medium. Table 3 shows that with slices from lactating rats the stimulation by insulin was smaller and not always significant.

The mammary glands of pregnant mice and rats are composed principally of mammary parenchyma (alveoli and ducts) and adipose tissue. Our explants from mice appeared, under the microscope, to be roughly half parenchyma and half adipose tissue. (Lymph-node, capillary and nervous tissue were absent from all explants or present only in small amounts.) It therefore seemed possible that insulin was stimulating the formation of RNA by adipose tissue (see Carruthers & Winegrad, 1962) rather than by the mammary parenchyma, a possibility that could be tested if the two kinds of tissue could be separated. We found it impossible to separate the mammary parenchyma from the adipose tissue, but we were able to separate, under

the microscope, adipose tissue free from parenchyma from the edges of the mammary fat pads of mice. This tissue is continuous with the adipose tissue in which the parenchyma is embedded and there is no reason to believe that the two differ in their metabolism. Table 4 compares the rates of incorporation of [14 C]adenine into the RNA of adipose tissue and of whole explants. To demonstrate the relative activities of the two tissues the results have been expressed as [14 C]adenine incorporated per unit weight of tissue, and not per unit weight of RNA as before. (Eight samples each of adipose tissue and whole explants from four mice contained an average of 0.64 and 2.65 μ g. of RNA/mg. wet wt. respectively.) The rates of incorporation into the whole explants were many times greater than into adipose tissue. It is clear that RNA synthesis in the parenchyma is far more rapid than in the adipose tissue and that the additional RNA synthesis in the presence of insulin occurs almost exclusively in the parenchyma.

Table 4. *Relative rates of incorporation of [14 C]-adenine into RNA of adipose tissue and of whole explants from the mammary glands of pregnant mice*

Adipose tissue was separated as described in the Materials and Methods section. Conditions were otherwise as given in Table 1.

Expt. no.	[14 C]Adenine incorporated (m μ C incorporated into RNA/g. wet wt. of tissue)			
	Adipose tissue		Whole explants	
	Control	+ Insulin	Control	+ Insulin
1	33.6	40.3	282	471
2	25.1	31.2	167	290
3	24.9	39.7	185	294
4	27.0	33.8	247	435

Fig. 1 shows the incorporation of [14 C]adenine into the RNA of slices from a pregnant rat at various times after the start of incubation with and without insulin. Differences first appear between 30 and 60 min.

Table 5 shows that insulin also stimulated the incorporation over 3 hr. of [14 C]leucine into the proteins of slices from pregnant rats by an average of 38%. No consistent stimulation could be detected in slices from lactating rats under the same experimental conditions.

Stimulation by insulin of the incorporation of labelled amino acids into protein has been found in other tissues and it has been suggested that it is the consequence of a primary stimulation of RNA formation, although experiments in which RNA synthesis has been inhibited with actinomycin D have made this unlikely (Wool & Moyer, 1964; Eboué-Bonis, Chambaut, Volfin & Clauser, 1963). We found that the incorporation of [14 C]adenine into the RNA of slices of glands of pregnant rats is virtually eliminated if the slices are preincubated for 1 hr. in a medium containing 10 μ g. of actinomycin D/ml. Thus, in two experiments, six portions (100–150 mg. each) of slices of the abdominal mammary glands of a rat between the fifteenth and sixteenth day of pregnancy were incubated under the usual conditions and six more were similarly incubated but with actinomycin D (10 μ g./ml.) in the medium. After 1 hr. [8- 14 C]-adenine (0.5 μ C) was added to each vessel and the incubation continued for a further 3 hr. The mean specific radioactivities of the RNA in the vessels with actinomycin were 3 and 4% of those without actinomycin in the two experiments. Table 6 shows that, although the incorporation of [14 C]-leucine into proteins is lowered by about 25% by this treatment, the percentage stimulation of the incorporation is nevertheless unaffected, strongly

Fig. 1. Rate of incorporation of [14 C]adenine into RNA of slices from a pregnant rat. The abdominal mammary glands of a rat at the fifteenth or sixteenth day of pregnancy were sliced. Twelve portions of slices (100–150 mg. each) were incubated at 37° in medium 199 (3 ml.) containing [8- 14 C]adenine (0.5 μ C) under O₂+CO₂ (95:5). Twelve similar incubations contained insulin (5 μ g./ml.). At intervals after the start of incubation two pairs of samples were withdrawn and kept frozen until analysed. Each point is the average of two separate incubations. ○, Without insulin (control); ●, with insulin.

suggesting that it cannot be a consequence of the stimulation of RNA formation.

DISCUSSION

This work aims eventually to discover how hormones induce differentiation in the mammary gland and the onset of lactation. Hormones may act by inducing the formation of specific messenger RNA, with the consequent formation of specific proteins. For this reason the incorporation of precursors into the RNA and proteins of mammary tissue with and without each of the hormones required for successful tissue culture has been studied. Only insulin has so far given marked effects; but, as reported above, the percentage stimulation by insulin of the incorporation of [14 C]leucine into the proteins of slices from pregnant rats was unaffected by virtual inhibition of RNA synthesis with actinomycin D. Hence it appears unlikely that the effects of insulin on protein and RNA synthesis are interrelated, or that insulin plays a primary part in inducing the differentiation of the gland. [Other evidence that various effects of insulin are not a consequence of stimulation of RNA synthesis has been provided by

Table 5. *Influence of insulin on the incorporation of [^{14}C]leucine into protein of slices of mammary glands of rats*

In each experiment the abdominal mammary glands of a rat between the twelfth and sixteenth day of pregnancy were sliced. Six portions of the slices (100–150 mg. each) were incubated as before in medium 199 containing L-[1- ^{14}C]leucine (0.25 μC). Six similar incubations contained insulin (5 $\mu\text{g./ml.}$). Each specific radioactivity is the mean $\pm$ s.e.m. of six separate incubations.

Expt. no.	Specific radioactivity of protein ($\text{m}\mu\text{C/mg.}$)		Increase with insulin (%)	P
	Control	+ Insulin		
1	2.12 $\pm$ 0.07	2.94 $\pm$ 0.09	39	< 0.001
2	2.18 $\pm$ 0.06	3.10 $\pm$ 0.18	42	< 0.01
3	2.28 $\pm$ 0.07	2.80 $\pm$ 0.12	23	< 0.01
4	2.35 $\pm$ 0.09	3.57 $\pm$ 0.14	52	< 0.001
5	1.99 $\pm$ 0.13	2.79 $\pm$ 0.12	40	< 0.01
6	2.31 $\pm$ 0.13	3.00 $\pm$ 0.21	30	< 0.05
			Mean 38	

Table 6. *Effect of actinomycin D on the stimulation by insulin of the incorporation of [^{14}C]leucine into mammary-gland slices*

In each experiment the abdominal mammary glands of a rat between the fifteenth and sixteenth day of pregnancy were sliced. Twelve portions of the slices (100–150 mg. each) were incubated at 37° in medium 199 (3 ml.) under $\text{O}_2 + \text{CO}_2$ (95:5). Twelve similar incubations contained actinomycin D (10 $\mu\text{g./ml.}$). After 1 hr. L-[1- ^{14}C]leucine (0.25 μC) was added to all vessels, and insulin (15 $\mu\text{g.}$) to six control vessels and six that contained actinomycin D. Incubation was continued for another 3 hr. Each specific radioactivity is the mean $\pm$ s.e.m. of six separate incubations.

Expt. no.		Specific radioactivity of protein ($\text{m}\mu\text{C/mg.}$)		Increase with insulin (%)	Inhibition of control by actino- mycin D (%)
		Control	+ Insulin		
1	No actinomycin	2.53 $\pm$ 0.09	3.64 $\pm$ 0.16	44	
	Actinomycin	1.92 $\pm$ 0.07	2.76 $\pm$ 0.11	44	24
2	No actinomycin	3.46 $\pm$ 0.15	4.17 $\pm$ 0.19	20	
	Actinomycin	2.27 $\pm$ 0.15	2.75 $\pm$ 0.12	21	34
3	No actinomycin	2.22 $\pm$ 0.07	2.91 $\pm$ 0.21	31	
	Actinomycin	1.61 $\pm$ 0.08	2.06 $\pm$ 0.10	28	18

Eboué-Bonis *et al.* (1963), Wool & Moyer (1964) and Mayne & Barry (1965).] Consequently, we have not so far studied further this action of insulin. Nor do we have any evidence whether the increased incorporation of [^{14}C]adenine and [^{14}C]leucine results from stimulation of the rates of RNA and protein synthesis, or from other effects such as stimulation of the uptake of precursors from the medium.

Nevertheless, our results do seem of interest for the following reasons. They are similar to effects of insulin on RNA and protein formation in other tissues that have been extensively studied by Wool and his colleagues (see Wool, 1965). But the effects on the mammary gland of the pregnant animal

are very pronounced, and since the tissue is available in large amounts and is easily sliced it would be good material for further study. Also, our method of demonstrating that the effect of insulin is on the mammary parenchyma and not on the adipose tissue could be useful in other studies on the mammary gland.

In the experiments reported above, slices of the mammary glands of pregnant rats were more sensitive to insulin than those from lactating rats. In some respects, however, tissue from lactating rats is more sensitive to insulin. Thus, when insulin is added to slices of the glands of lactating rats in bicarbonate buffer that contains glucose and acetate, fatty acid synthesis is stimulated. Slices

from pregnant rats do not respond (Balmain & Folley, 1951; Balmain, Folley & Glascock, 1954; Mayne & Barry, 1965). Wool & Manchester (1962) have reported a stimulation by insulin of the incorporation of [^{14}C]glycine into the proteins of slices of the mammary glands of lactating rats in bicarbonate buffer both with and without glucose or pyruvate. They did not report experiments on slices from pregnant rats. Another study of insulin related to the present work is that of Moretti & DeOme (1962). They showed that mammary-tissue cultures without insulin absorb less glucose from the medium than those with insulin, but the difference was only marked after the first day of culture.

We are grateful to Dr H. D. Brown of Merck, Sharp and Dohme Research Laboratories for the gift of actinomycin D, to Dr O. K. Behrens of the Lilly Research Foundation for the gift of glucagon-free insulin, to Mr J. H. D. Kent for help in providing rats, and to the Agricultural Research Council for a research grant. E. M. R. is grateful to Professor S. J. Folley, F.R.S., for his interest and encouragement. She was aided by American Cancer Society Grant PF-203.

REFERENCES

- Balmain, J. H. & Folley, S. J. (1951). *Biochem. J.* **49**, 663.
Balmain, J. H., Folley, S. J. & Glascock, R. F. (1954). *Biochem. J.* **56**, 234.
Carruthers, B. M. & Winegrad, A. I. (1962). *Amer. J. Physiol.* **202**, 605.
Eboué-Bonis, D., Chambaut, A. M., Volfin, P. & Clauser, H. (1963). *Nature, Lond.*, **199**, 1183.
Elias, J. J. (1959). *Proc. Soc. exp. Biol., N.Y.*, **101**, 500.
Elias, J. J. & Rivera, E. M. (1959). *Cancer Res.* **19**, 505.
Mayne, R. & Barry, J. M. (1965). *Biochim. biophys. Acta*, **107**, 160.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Morgan, J. F., Morton, H. J. & Parker, R. C. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 1.
Moretti, R. L. & DeOme, K. B. (1962). *J. nat. Cancer Inst.* **29**, 321.
Rivera, E. M. (1964). *Endocrinology*, **74**, 853.
Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.
Wool, I. G. (1965). In *Mechanisms of Hormone Action*, p. 98. Ed. by Karlson, P. Stuttgart: Georg Thieme.
Wool, I. G. & Manchester, K. L. (1962). *Nature, Lond.*, **193**, 345.
Wool, I. G. & Moyer, A. N. (1964). *Biochim. biophys. Acta*, **91**, 248.