Proc. Natl. Acad. Sci. USA
Vol. 73, No. 12, pp. 4536–4540, December 1976
Cell Biology

Hormone responsive human breast cancer in long-term tissue culture: Effect of insulin*

(growth control/macromolecular synthesis/mechanism of hormone action)

C. KENT OSBORNE, GAIL BOLAN, MARIE E. MONACO, AND MARC E. LIPPMAN†

Medicine Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by C. B. Anfinsen, September 9, 1976

ABSTRACT **The mechanisms of steroid and peptide hormone action in human breast cancer are poorly understood. We have previously characterized a cell line of human breast cancer in long-term tissue culture that possesses various steroid hormone receptors and responses, providing a model for the study of steroid hormone action. The present studies describe a human breast cancer *in vitro* that responds to physiologic concentrations of insulin with an increased rate of macromolecular synthesis and growth. Thymidine and uridine incorporation in cells in serum-free medium are stimulated by 10^{-11} M insulin and are maximal with 10^{-8} M. Leucine incorporation is stimulated by 5×10^{-11} M insulin and is maximal with 10^{-9} M. Significant stimulation of uridine and leucine incorporation is evident by 3 hr and maximal by 10 hr. A 10-hr lag period exists for insulin stimulation of thymidine incorporation, which is maximal from 14 to 24 hr. The effect of 10^{-8} M insulin on macromolecular synthesis is accompanied by a 69% increase above controls in the number of cells after 24 hr. The effect on macromolecular synthesis is observed in glucose-free medium. Insulin's effect on protein synthesis is not blocked by inhibition of RNA synthesis with actinomycin D. Glucocorticoids partially inhibit the action of insulin in these cells. This system provides a model for studying insulin action, and suggests that some human breast cancer may show growth regulation by insulin.**

We have recently characterized a model system for the study of steroid hormone action using human breast cancer cells maintained in long-term tissue culture (1, 2). One cell line in particular, MCF-7, possesses specific high affinity receptors for estradiol, progesterone, androgens, and glucocorticoids (1–4). In addition, these cells respond to estrogen and androgen with an increased rate of DNA, RNA, and protein synthesis, while inhibition of macromolecular synthesis is observed with glucocorticoids and the antiestrogen, Tamoxifen (ICI 46474) (1–3).

The effect of peptide hormones on these cells is unknown. However, insulin is known to influence mammary gland growth and development in human (5) and rodent glands in organ culture (6), and other studies suggest that insulin regulates proliferation of rat mammary carcinomata induced by the carcinogen dimethylbenz[*a*]anthracene (7, 8).

In the present study we demonstrate that the MCF-7 cell line is responsive to physiologic concentrations of insulin and propose that this cell line provides a provocative model for the study of insulin action and mechanisms of growth regulation in breast cancer.

MATERIALS AND METHODS

Materials. Porcine insulin (25.4 units/mg) containing 0.002% glucagon was the generous gift of Dr. Mary Root, Lilly Research Laboratories, Indianapolis, Ind. Crystalline trichloroacetic acid was purchased from Baker Chemical Co. [^{3}H]Uridine (5 mCi/0.027 mg) was purchased from New England Nuclear and [^{14}C]thymidine (62 mCi/mmol), [^{14}C]leucine (59 mCi/mmol), and [^{3}H]leucine (59 Ci/mmol) from the Radiochemical Centre, Amersham, England. Actinomycin D was purchased from Sigma Chemical Co., and dexamethasone from Steraloids, Inc.

Cells and Tissue Culture Techniques. The MCF-7 cell line, a cloned breast cancer line derived from a malignant pleural effusion (9), was generously provided by Dr. Marvin Rich, Michigan Cancer Foundation. The human and mammary nature of this line has been substantiated by chromosomal analysis, morphological features, and α-lactalbumin synthesis (10). The cells (at approximately the 100th passage) were maintained in monolayer cultures in Improved Eagle's Minimal Essential Medium supplemented with twice the usual concentration of glutamine, penicillin, and streptomycin (N.I.H. Media Unit) and with 10% fetal calf serum (North American Biological Co.). They were maintained at 37° in a humidified incubator with 5% CO_2. These cells were free of mycoplasma contamination on multiple determinations during the period of this study.

Precursor Incorporation. Cells approaching confluence were suspended in a trypsin (0.05%)-EDTA (0.02%) solution (wt/vol), and plated replicately in plastic multiwell tissue culture dishes (Linbro) in medium with 10% fetal calf serum. Cell density was adjusted to give a moderately dense subconfluent monolayer of cells. After 24 hr of incubation the medium was removed, the cells were washed once with 2 ml of serum-free medium, and 2 ml of fresh serum-free medium were added. After 24 hr in serum-free medium, hormones were added directly to the wells in concentrations as described in the figures. At various time intervals, radioactive precursors diluted in medium were added to the wells in a 25-μl volume in concentrations as described in the figures. After the cells were labeled for 2 hr, they were washed with cold Dulbecco's phosphate-buffered saline (pH 7.4, without Ca^{++} or Mg^{++}), harvested by suspending with trypsin-EDTA solution, counted with a hemocytometer, and collected by centrifugation (2 min) in a Clay-Adams serofuge (high-speed). Cell pellets were then washed once with 1 ml of phosphate-buffered saline and recentrifuged. Cell pellets were resuspended in 1 ml of water and sonicated for 5 sec in a Branson sonicator at the lowest setting. Aliquots were then taken for protein determination by the Lowry method (11) or for precipitation in 10% trichloroacetic acid. Acid-insoluble material was collected and washed with 6 ml of 10% trichloroacetic acid on 0.45 μm Millipore filters. Filters were dried and solubilized in Aquasol (New England Nuclear); the radioactivity was determined in a Packard liquid scintillation counter (efficiency for tritium ~ 39%; efficiency for ^{14}C ~ 55%).

In experiments to determine the effect of inhibition of RNA

* This is paper no. IV in a series. Paper no. III is Lippman, M. E., Bolan, G. & Huff, K. (1976) "Hormone responsive human breast cancer in long term tissue culture. III. The effect of androgens and antiandrogens," *Cancer Res.*, in press.

† To whom reprint requests should be sent.

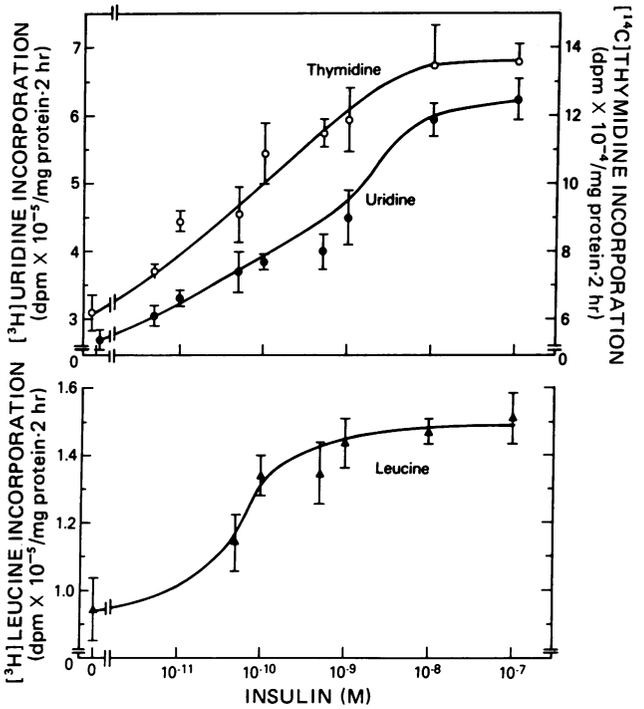

FIG. 1. (*Top*) Effect of insulin on the rates of thymidine (○) and uridine (●) incorporation in MCF-7 human breast cancer. [^{14}C]Thymidine (0.25 μCi/ml) and [^{3}H]uridine (5 μCi/ml) were added to the cells 22 hr after insulin for a 2-hr pulse. Values represent the mean of triplicate determinations ± 1 SD. (*Bottom*) Effect of insulin on the rate of leucine incorporation. [^{3}H]Leucine (2.5 μCi/ml) was added 22 hr after insulin for a 2-hr pulse. Values represent the mean of triplicate determinations ± 1 SD.

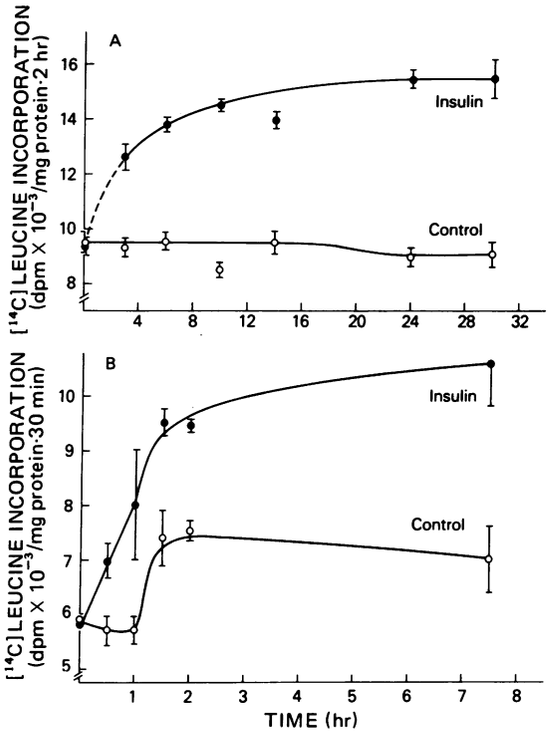

FIG. 2. (A) Kinetics of the effect of insulin on [^{14}C]leucine incorporation. Insulin (10^{-8} M) was added at time 0, and [^{14}C]leucine (0.3 μCi/ml) was added for a 2-hr pulse. Cells were harvested at the times shown. (B) Early time course of the effect of insulin on leucine incorporation. Insulin (10^{-8} M) was added at time 0 and then [^{14}C]leucine (0.5 μCi/ml) was added for a 30-min pulse. Cells were harvested at times shown. Points represent the mean of triplicate determinations ± 1 SD.

synthesis on stimulation of protein synthesis by insulin, actinomycin D (1 μg/ml) was added to one group of cells at a concentration that consistently results in greater than 90% inhibition of insulin-stimulated uridine incorporation into acid-insoluble material.

RESULTS

Physiologic concentrations of insulin stimulated macromolecular synthesis in the MCF-7 human breast cancer cells. The rates of thymidine and uridine incorporation after 24 hr of incubation with insulin were stimulated by as little as 10^{-11} M insulin and reached a plateau at 10^{-8} M with a 2- to 3-fold increase above control (Fig. 1). Similarly, the rate of leucine incorporation was also sensitive to insulin (Fig. 1). An increase was observed with 5×10^{-11} M insulin, and a maximal effect with 10^{-9} to 10^{-8} M. Thus, DNA, RNA, and protein synthesis in these cells were responsive to concentrations of insulin below the normal human fasting blood level (about 10^{-10} M). These effects are not observed in the presence of serum, and insulin cannot replace serum for long-term maintenance. The nature of these additional serum factors required for maintaining growth is not known.

Insulin's effect on macromolecular synthesis proceeded in an ordered manner. The effect of insulin on leucine (Fig. 2) and uridine incorporation was more rapid than that on thymidine incorporation (Fig 3). An increase in the rate of leucine incorporation was seen by 0.5 hr of incubation with 10^{-8} M insulin (Fig. 2B), while there was a 10-hr lag period in the stimulation of thymidine incorporation (Fig. 3). Maximal stimulation of both leucine and uridine incorporation was evident at 10 hr (Figs. 2A and 3, respectively), while the maximum for thymidine incorporation was not apparent until 14 hr. Insulin also produced an increase in the thymidine acid-soluble pool by 2 hr prior to the onset of its effect on thymidine incorporation (data not shown). We do not know whether this effect on the acid-soluble pool reflects stimulation of transport into the cell.

This stimulation of macromolecular synthesis by insulin was accompanied by stimulation of growth of the MCF-7 breast cancer cells (Fig. 4). At 72 hr after treatment with insulin a significant increase above controls in the number of cells per dish was evident with both 10^{-10} M and 10^{-8} M insulin. Similar results were obtained when total protein per dish was measured (data not shown).

The effect of insulin on protein synthesis was not blocked by inhibiting new RNA synthesis with actinomycin D (Table 1). Insulin alone stimulated the rate of uridine incorporation by nearly 2-fold. When actinomycin D was added, uridine incorporation was inhibited by 87% in controls and by more than 90% in cells incubated with insulin. Although actinomycin D resulted in a moderate decrease in the control rate of leucine incorporation, it did not prevent stimulation of leucine incorporation by insulin. Since the effect of insulin on protein synthesis occurred without a significant lag period and the effect was not blocked by inhibiting RNA synthesis, we suggest that insulin enhanced protein synthesis in the MCF-7 cells primarily by a post-transcriptional mechanism, as suggested by data in other target tissues (12).

To investigate the effect of glucose availability on the ability of insulin to promote the synthesis of macromolecules, we ex-

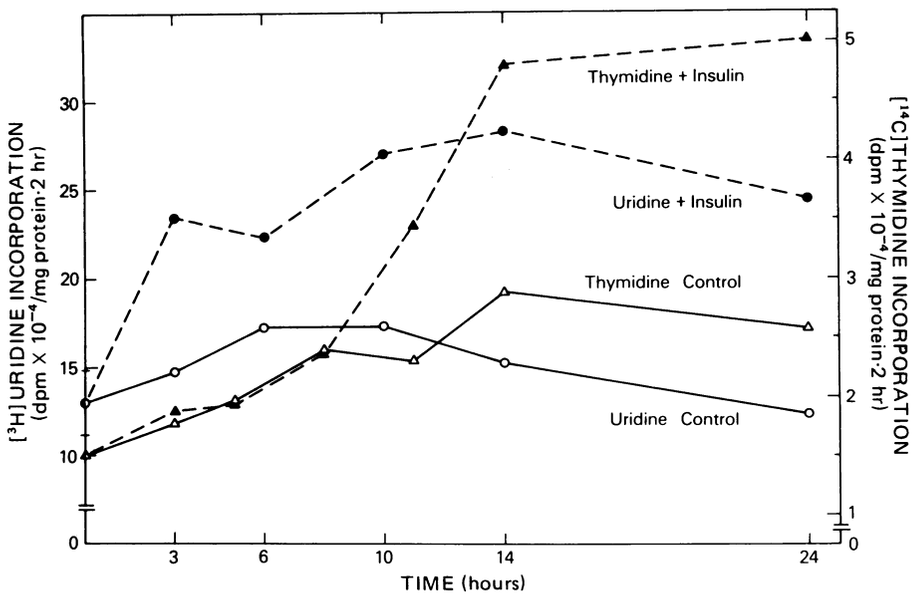

FIG. 3. Kinetics of the effect of insulin on thymidine and uridine incorporation. Insulin (10^{-8} M) was added at time 0. [^{14}C]Thymidine (0.25 μCi/ml) and [^{3}H]uridine (5 μCi/ml) were added for a 2-hr pulse and the cells were harvested at the times shown. Points represent the mean of triplicate determinations. Standard deviations were generally between 5 and 10%.

amined the rates of leucine and thymidine incorporation in MCF-7 cells in glucose-free medium as shown in Fig. 5. The rate of thymidine incorporation was significantly stimulated (about 2-fold) by insulin after 24 hr in cells incubated with and without glucose, although absolute unstimulated rates of incorporation were about 30–40% lower in cells incubated without glucose. The curves for leucine incorporation in cells with and without glucose were similar throughout the time course and revealed a 50–60% increase above controls after 24 hr. Absolute unstimulated rates of leucine incorporation were about 20% lower in cells incubated without glucose. Nevertheless, stimulation of macromolecular synthesis by insulin in these cells cannot be the result of enhanced glucose availability.

We have previously shown that the MCF-7 cells have glucocorticoid receptors and that glucocorticoids inhibit DNA synthesis and growth (3). Fig. 6 demonstrates that dexamethasone also inhibited the effect of insulin on DNA and protein synthesis in these cells. It is interesting to note, however, that dexamethasone alone had no effect on leucine incorporation, while the rate of thymidine incorporation was inhibited below control.

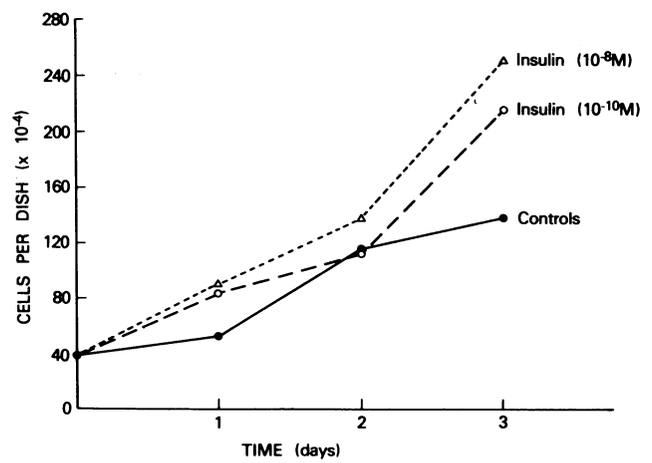

FIG. 4. Effect of insulin on growth. Insulin (10^{-8} M or 10^{-10} M) was added to cells in serum-free medium at time 0. Cells were harvested at times shown and counted on a hemocytometer. Points represent the mean of triplicate determinations. Standard deviations were between 10 and 20%.

DISCUSSION

The mechanism of action of insulin and its interaction with other hormones in target tissues are poorly understood. Binding to the cell membrane is thought to be the first step in insulin action (13), followed by stimulation of an unknown effector system which in turn stimulates a variety of cell metabolic pathways. Since other peptide and steroid hormones may influence or modulate these same pathways, it is obvious that one must consider these complex interrelationships in studying the mechanisms of action of any of these hormones. The availability of cloned cell lines maintained in long-term tissue culture has facilitated the study of hormone action in a single population of cells, in defined chemical medium, and easily controlled environment.

Table 1. Effect of actinomycin D on insulin-stimulated uridine and leucine incorporation

	dpm X 10^{-3}/mg of protein per 30 min	
Precursor	Control*	Insulin†
Urd‡	48.6 ± 2.3	86.4 ± 0.6
Urd + AD§	6.4 ± 0.4	8.4 ± 0.2
Leu¶	7.6 ± 0.2	9.4 ± 0.1 (24%)‖
Leu + AD	5.3 ± 0.3	6.9 ± 0.5 (31%)

* Mean of triplicate determination ± 1 SD.
† 10^{-8} M for 2 hr; cells previously maintained in serum-free medium for 24 hr.
‡ [^{3}H]Uridine, 5 μCi/ml, 30-min pulse.
§ Actinomycin D, 1 μg/ml, added 2 hr before insulin.
¶ [^{14}C]Leucine, 0.3 μCi/ml, 30-min pulse.
‖ Percent above control value.

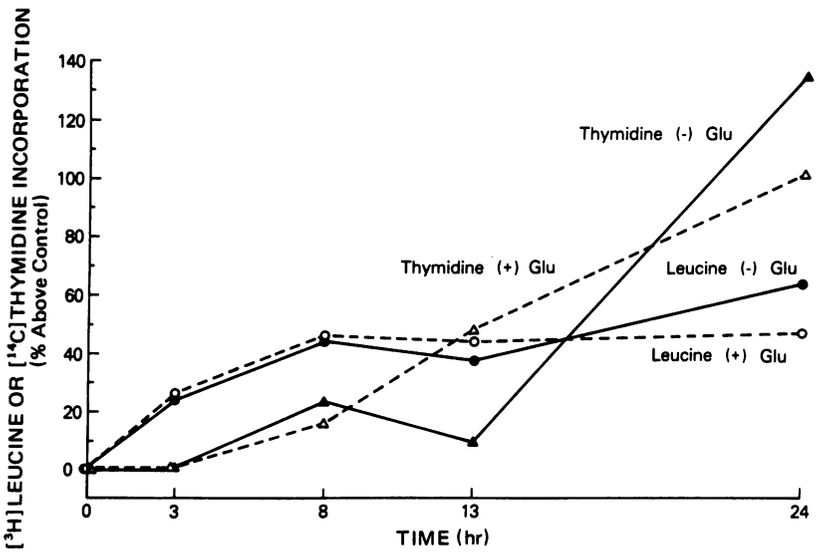

FIG. 5. The effect of insulin on the rates of thymidine and leucine incorporation in glucose-free medium. Serum-free medium without glucose or with glucose (1 mg/ml) was exchanged for serum-free medium 2 hr prior to exposure to insulin (10^{-8} M) at time 0. [^{14}C]Thymidine (0.25 μCi/ml) and [^{3}H]leucine (2.5 μCi/ml) were added for a 2-hr pulse and cells were harvested at times shown. Points represent the me ı of triplicate determinations. (- -△- -) Thymidine + glucose (1 mg/ml); (—▲—) thymidine − glucose; (- -○- -) leucine + glucose (1 mg/ml); (—●—) leucine − glucose.

MCF-7 cells, a cell line of human breast cancer in long-term tissue culture, has proven to be a valuable system for the study of a variety of steroid hormones because of the presence of specific receptors for and biological responses to several of these hormones (1–4). The present study demonstrates that these cells also respond to insulin at physiologic concentrations (10^{-10} to 10^{-8} M) with an increased rate of macromolecular synthesis and growth, and suggests that the MCF-7 cell line may also be a useful model for the study of insulin action and of growth control mechanisms in human breast cancer.

Insulin, in addition to glucocorticoids, prolactin, estrogen, and probably other hormones, has long been known to be essential for the growth and differentiation of normal mammary tissue (5, 6, 14) and perhaps neoplastic mammary tissue as well (7, 8). However, in most of these studies, short-term organ explant cultures were used that potentially contain nonepithelial as well as epithelial cells. Supraphysiologic concentrations of insulin were invariably used. Cloned cells in long-term tissue culture such as the MCF-7 line avoid these potential artifacts. Moreover, these cells are exquisitely sensitive to insulin, suggesting that the observed effects are indeed the result of insulin interaction with its own specific high affinity receptor and not the result of high concentrations of insulin stimulating growth through another related growth-peptide receptor such as that for nonsuppressible insulin-like activity or somatomedin. This is substantiated by our more recent characterization of a specific high affinity insulin receptor in the MCF-7 and other human breast cancer cell lines in long-term tissue culture (15), and by the fact that DNA synthesis and growth in the MCF-7 cells are 100 times less sensitive to multiplication-stimulating activity (16) than to insulin (unpublished observation).

The mechanisms by which insulin stimulates macromolecular synthesis in MCF-7 cells are unknown. Although the effect of insulin on transport of nucleotide precursors is controversial, the small increase in thymidine pool size observed here suggests that increased thymidine transport might account for part of the insulin effect on the rate of incorporation. However, inhibition of nucleoside or nucleotide exit from the cell could also augment the acid-soluble pool size. Preliminary evidence in our laboratory suggests that insulin stimulates thymidine kinase,

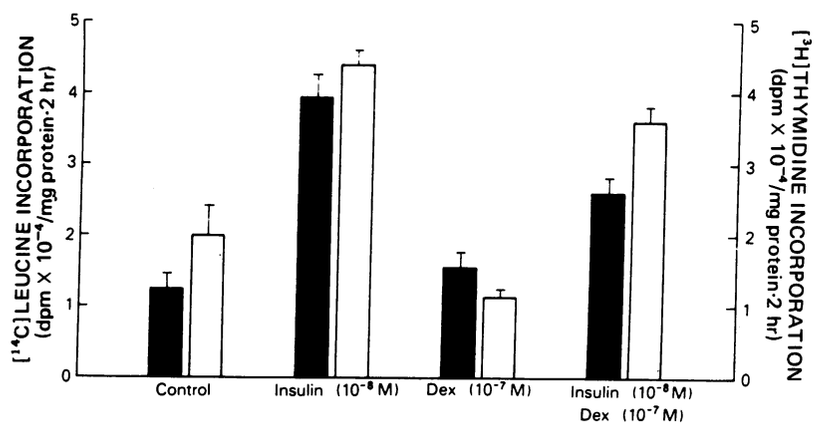

FIG. 6. The effect of dexamethasone (DeX) on insulin stimulation of the rates of leucine and thymidine incorporation. [^{14}C]Leucine (0.3 μCi/ml) and [^{3}H]thymidine (2.5 μCi/ml) were added for a 2-hr pulse 22 hr after insulin (10^{-8} M) and dexamethasone (10^{-7} M). Values represent the mean of triplicate determinations ± 1 SD. Shaded bars, leucine; open bars, thymidine.

which converts thymidine to thymidine monophosphate, which is impermeable to the cell membrane, and is, therefore, trapped.

The increased rate of protein synthesis observed in the MCF-7 cells appears to be largely an effect of insulin distal to transcription. Insulin has been shown to increase the transport of several amino acids with apparently some selectivity depending on the tissue and method of study (12, 17). However, other evidence suggests that the primary stimulation of protein synthesis by insulin occurs at a site other than membrane transport, perhaps at the level of the ribosome (12, 18). Moreover, in the present study insulin required less than 30 min to stimulate protein synthesis, and the effect was not prevented by blocking RNA synthesis, suggesting a post-transcriptional action.

Stimulation of macromolecular synthesis by insulin is clearly not the result of nonspecific stimulation of cell metabolism by enhanced glucose availability. In the present work no difference in stimulation of thymidine or leucine incorporation was observed between cells incubated with and in the absence of glucose, a result consistently observed by others (12, 19, 20).

The present studies also suggest that the effects of insulin in the MCF-7 cells are modified by glucocorticoids. The data suggest that the inhibition of insulin's effect on protein synthesis by dexamethasone in these cells does not simply represent the sum of two opposing forces, since no effect of dexamethasone alone on leucine incorporation was observed, consistent with our previous observations (3). Whether dexamethasone induces altered insulin binding, interacts with the insulin effector mechanism distal to the receptor, or indirectly opposes an effect of insulin remains to be elucidated. Further studies of hormone interaction in these cells might provide insight into the mechanisms of altered metabolism in disease states such as glucocorticoid-induced insulin resistance.

In summary, we have shown that a human breast cancer maintained in long-term tissue culture responds to physiologic concentrations of insulin with an increased rate of DNA, RNA, and protein synthesis. The sequence of events in this action appears to be early stimulation of protein and RNA synthesis followed, in several hours, by enhanced DNA synthesis culminating in cell division and growth. The effect does not depend on the availability of glucose and is inhibited by glucocorticoids. It is hoped that further study using this model system will provide additional clues to the mechanisms of hormonal regulation of human breast cancer.

The helpful comments and advice of Dr. C. R. Kahn in the preparation of the manuscript and the technical assistance of Mr. David Rich are greatly appreciated.

1. Lippman, M. E. & Bolan, G. (1975) "Oestrogen-responsive human breast cancer in long term tissue culture," *Nature* **256**, 592–593.
2. Lippman, M. E., Bolan, G. & Huff, K. (1975) "Human breast cancer responsive to androgen in long term tissue culture," *Nature* **258**, 339–341.
3. Lippman, M. E., Huff, K. K. & Bolan, G. (1976) "Glucocorticoid and progesterone interaction in human breast cancer in long term tissue culture," *Ann. N.Y. Acad. Sci.*, in press.
4. Brooks, S. C., Locke, E. R. & Soule, H. D. (1973) "Estrogen receptor in a human cell line (MCF-7) from breast carcinoma," *J. Biol. Chem.* **248**, 6251–6253.
5. Ceriani, R. L., Contesso, G. P. & Natof, B. M. (1972) "Hormone requirements for growth and differentiation of the human mammary gland in organ culture," *Cancer Res.* **32**, 2190–2196.
6. Turkington, R. W. (1972) in *Biochemical Actions of Hormones II*, ed. Litwack, G. (Academic Press, New York and London), pp. 55–77.
7. Heuson, J. C., Legros, N. & Heimann, R. (1972) "Influence of insulin administration on growth of the 7,12-dimethylbenz[*a*]-anthracene-induced mammary carcinoma in intact, oophorectomized, and hypophysectomized rats," *Cancer Res.* **32**, 233–238.
8. Heuson, J. C. & Legros, N. (1972) "Influence of insulin deprivation on growth of the 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction," *Cancer Res.* **32**, 226–232.
9. Soule, H. D., Vazquez, J., Long, A., Albert, S. & Brennan, M. (1973) "A human cell line from a pleural effusion derived from a breast carcinoma," *J. Natl. Cancer Inst.* **51**, 1409–1417.
10. Rose, H. N. & McGrath, C. M. (1975) "α-Lactalbumin production in human mammary carcinoma," *Science* **190**, 673–674.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) "Protein measurement with the Folin phenol reagent," *J. Biol. Chem.* **193**, 265–275.
12. Wool, I. G., Stirewalt, W. S., Kurihara, K., Low, R. B., Bailey, P. & Oyer, D. (1968) "Mode of action of insulin in the regulation of protein biosynthesis in muscle," *Rec. Prog. Horm. Res.* **24**, 139–206.
13. Kahn, C. R. (1975) in *Methods of Membrane Biology*, ed. Korn, E. (Plenum Press, New York), pp. 81–146.
14. Topper, Y. T. (1970) "Multiple hormone interactions in the development of mammary gland *in vitro*," *Rec. Prog. Horm. Res.* **26**, 287–308.
15. Osborne, C. K., Monaco, M. E. & Lippman, M. E. (1976) "Insulin receptors in human breast cancer: relationship of binding, degradation and biological activity," *Diabetes* **25** (Suppl. 1), 380.
16. Smith, G. L. & Temin, H. M. (1974) "Purified multiplication-stimulating activity from rat liver cell conditioned medium: comparison of biological activities with calf serum, insulin, and somatomedin," *J. Cell. Physiol.* **84**, 181–192.
17. Manchester, K. L. (1972) "Effect of insulin on protein synthesis," *Diabetes* **21**, 447–453.
18. Wool, I. G. (1965) "Relation of effects of insulin on amino acid transport and on protein synthesis," *Fed. Proc.* **24**, 1060–1070.
19. Skarda, J., Green, C. D., Aisbitt, R. P. G. & Barry, J. M. (1974) "Insulin does not stimulate DNA synthesis in cultured mammary tissue by enhancing glucose uptake," *J. Endocrinol.* **60**, 197–198.
20. Wool, I. G. & Krahl, M. E. (1959) "Incorporation of ^{14}C-amino acids into protein of isolated diaphragms: an effect of insulin independent of glucose entry," *Am. J. Physiol.* **196**, 961–964.