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Effects of Estrogen to Alter Amino Acid Transport in R3230AC Mammary Carcinomas and Its Relationship to Insulin Action[1]

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ABSTRACT

The effects of estrogens on transport and incorporation of amino acids into the R3230AC mammary adenocarcinoma were studied *in vivo* and *in vitro*. Dissociated tumor cells from ovariectomized rats, like those from diabetic rats, displayed elevated transport of proline, representing entry by the A system; transport of phenylalanine (L system) was unaltered, as was glucose transport and its utilization. Administration of estradiol valerate decreased the entry of proline into tumor cells from intact, diabetic, or ovariectomized animals; the response to the steroid hormone was greater in ovariectomized or diabetic rats compared to intact animals. The time course of the effects of estrogen treatment was examined in diabetic rats. By 72 hr, transport of both proline and leucine was significantly decreased; incorporation of leucine into proteins and uridine into RNA was significantly reduced by 24 hr after injection of estradiol valerate. The effects of estrogen *in vivo* to reduce transport of amino acids and their incorporation into proteins appeared to correlate with the reduced tumor growth observed.

Experiments were performed to examine the effects of 17β-estradiol *in vitro* on amino acid transport into dissociated cells from ovariectomized or diabetic rats. Under these experimental conditions, 17β-estradiol (10^{-6} M) inhibited proline transport with little or no effect on leucine transport in cells from ovariectomized rats; in cells from diabetic rats, proline transport and leucine incorporation were significantly reduced by estradiol, whereas phenylalanine transport was slightly inhibited (~20%). The effect of estradiol *in vitro* was also manifest in tumor cells obtained from diabetic rats treated *in vivo* with estradiol valerate; estradiol *in vitro* caused a further reduction in proline transport but not in leucine transport, results that imply some specificity to the action of estrogen on the A system.

Since we had earlier shown that insulin action on transport in these tumor cells was directed towards the A system, we examined the effects of insulin, estradiol, and their combination *in vitro* on proline and leucine transport. Insulin (10^{-8} M) stimulated proline transport; 17β-estradiol, at a selected lower level of 10^{-8} M, inhibited proline transport. When both were added *in vitro*, estradiol (10^{-8} M) was capable of significantly reducing the insulin (10^{-8} M)-induced increase in proline transport. Leucine transport was not altered in any of these experiments. Together, these data suggest that estrogens are capable of inhibiting amino acid transport into the R3230AC mammary carcinoma, an effect that is compatible with reduced tumor growth. The possible relationship of estrogen and insulin at the level of amino acid transport remains to be elucidated.

INTRODUCTION

The ability of hormones to influence transport of amino acids into target tissues is now well established. For example, numerous reports have shown that insulin was capable of stimulating entry of certain amino acids into muscle tissue and estrogens caused an increase in amino acid uptake into the uterus (6, 18, 21). Although the exact mechanism of these hormone-induced responses has not been completely established, the data are suggestive of an early and important role of substrate transport in the ultimate response of the target tissue. In a more general concept, Holley (16) has proposed that regulation of substrate transport is critical to cell growth, particularly in neoplasia.

Studies in this laboratory have been directed towards examination and elucidation of the effects of hormones on growth of the R3230AC adenocarcinoma, a mammary tumor that was shown to be inhibited in growth by estrogen therapy (9, 11, 12) and, more recently, by alteration of the insulin milieu of the host (9, 10). Thus, it was reported that growth of this carcinoma was faster in diabetic rats but retarded by insulin treatment; in another series of experiments, simultaneous administration of insulin and estradiol valerate were additive to inhibit growth of the R3230AC tumor (2). Because of these effects of insulin, we undertook experiments to elucidate the mechanism whereby this hormone could alter tumor growth. Briefly, we established the existence of insulin receptors the characteristics of which were similar to those in normal cells (8). In dissociated tumor cells, a passive carrier for glucose was identified (7). Carriers for amino acids demonstrated properties similar to those reported for the "A" and "L" systems, based on their kinetic properties and substrate specificity (13, 14). From these data, we concluded that insulin, or its lack, was capable of altering the entry of these substrates in a manner that was related to the growth behavior of the neoplasm, *i.e.*, increased entry in tumor cells from diabetic rats and decreased entry in animals treated with insulin.

A somewhat surprising observation was noted in our latest studies of the effects of insulin on amino acid transport (15). Insulin administered to the animal produced a decrease in transport of proline or α-aminoisobutyric acid into the R3230AC tumor, whereas dissociated tumor cells incubated with insulin *in vitro* demonstrated an increase in proline transport, the latter result agreeing with others (5). These apparently paradoxical

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results were both time and dose related, and we suggested that the presence of other hormonal factors *in vivo* could modify the effects of insulin. To test this proposal, experiments were performed to examine the influence of one ovarian hormone, estrogen, on amino acid transport by R3230AC tumor cells. The results presented here indicate that estrogens alone can inhibit transport of amino acids into this tumor and, further, that the steroid hormone can antagonize the insulin-induced increase in proline transport *in vitro*. The ability of estradiol to inhibit amino acid transport may account, at least in part, for the reduced tumor growth arising from treatment of the animal with estrogens.

MATERIALS AND METHODS

Female Fischer rats (80 to 90 g) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). The rats were offered food and water *ad libitum*. The R3230AC tumor was implanted s.c. in the axillary region on both sides by a sterile trochar technique as described by Hilf *et al.* (12).

Diabetes was induced by administration of streptozotocin into the jugular vein 2 days prior to tumor implantation (4). Streptozotocin was dissolved in 0.9% NaCl solution rapidly adjusted to pH 4.5 with 0.025 M citric acid. Due to the instability of the drug, all injections were made within 30 min of dissolving the drug. The dose administered was 60 mg/kg. Blood glucose was determined by the glucose oxidase method (Glucostat; Worthington Biochemical Corp., Freehold, N. J.), and the urinary glucose was estimated with Clinistix (Ames Co., Elkhart, Ind.). Animals were classified as diabetics if their blood glucose levels were greater than 250 mg/100 ml and if their urine glucose level exceeded 0.5 g/100 ml. Ovariectomy was performed by surgical procedures 8 days prior to implantation of the tumor. In those experiments, where indicated, animals received estradiol valerate s.c. once a week. Based on our previous report (15), experiments examining *in vitro* effects of hormones were conducted by addition of the appropriate hormone to tumor slices at the time that enzymatic dissociation was initiated. Fresh hormone was added again after the cells were prepared. Thus, total exposure of tumors to hormones *in vitro* ranged from 6.0 to 7.0 hr.

Animals were killed by cervical dislocation at 19, 20, or 21 days after tumor implantation. Tumors were excised as quickly as possible and placed in ice-cold 0.9% NaCl solution. After removal of connective and necrotic tissues, 3 g of tumor tissue were minced into 1- x 1-mm pieces on a McIlwain tissue slicer (Brinkmann Instruments, Inc., Westbury, N. Y.). The minced tissue was incubated in 250-ml Erlenmeyer flasks with 10 ml of Hanks' balanced salt solution (Ca^{2+}- and Mg^{2+}-free) containing 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) and 0.5% collagenase (type II) for 25 min at 37° in a Benco shaking water bath (approximately 50 cycles/min). At the end of the incubation period, the mixture was poured through a 100 mesh stainless steel strainer. The nondissociated tissue remaining on the strainer was returned to the flask with a fresh 10-ml portion of enzyme solution and incubated for an additional 55 min; the filtrates from the first incubation mixture were discarded due to considerable contamination by RBC and cellular debris. At the end of the second hr, the incubation mixture was again strained, and the nondissociated pieces were rinsed with HEPES[4] buffer. The filtrates and the wash mixtures were combined and centrifuged for 5 min at 80 × *g*. The cell pellet was then washed 3 more times with 10 volumes of buffer. Cell viability was estimated by trypan blue exclusion, and cell number was determined by the use of a hemocytometer. Cell preparations used had >90% viability (7).

Transport Studies. Transport was performed on 5×10^6 cells in Falcon plastic tubes in a final volume of 1 ml of HEPES buffer, pH 7.5. The buffer consisted of 25 mM HEPES, 10 mM $NaHCO_3$, 130 mM NaCl, 3 mM K_2HPO_3, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 11 mM glucose, and bovine serum albumin (1 mg/ml), with a final osmolarity of 310 mOsM. Amino acid transport reported here represented the initial velocity of entry of labeled proline, leucine, or phenylalanine at 0.1 mM substrate concentrations. Proline was used as the probe for the A system based on characterization of its entry in an earlier report (13). Either leucine or phenylalanine were used as substrates for the L system, and their transport was always measured in the presence of 50 mM proline (unlabeled proline added 30 sec prior to transport measurement), a level that saturated the A system; entry of labeled leucine or phenylalanine under these conditions represents transport by the L system. When transport of 3-*O*-methylglucose was measured, glucose was replaced by an equimolar amount of mannitol (7).

Initial velocity measurements were determined by adding the amino acid (labeled and unlabeled) to cells in a Benco shaking water bath (80 cycles/min); cells were obtained at 15, 30, 45, and 60 sec after addition of the labeled amino acid. Transport was terminated rapidly by adding 8 ml of ice-cold 0.9% NaCl solution to each tube, followed immediately by centrifugation for 1 min at 3° (900 × *g*). After the supernatant was decanted, the cells were washed with another 8 ml of ice-cold 0.9% NaCl solution and centrifuged for 2.5 min (900 × *g*), and the supernatant was decanted. The tubes were allowed to stand (inverted) for 15 min to drain; any remaining droplets on the sides of the tubes were wiped. The cell pellet was dissolved in 2 successive 5-ml portions of aqueous counting scintillant (Amersham/Searle, Arlington Heights, Ill.) and transferred to vials, and the samples were counted in a liquid scintillation counter (Isocap 300; Nuclear-Chicago Corp., Chicago, Ill.). The efficiency for counting ^{14}C was 60%, and that for ^3H was 40%.

Cells prepared as described above were found to be suitable for transport studies conducted for moderately prolonged incubation periods; cells incubated at either 37 or 4° in the presence or absence of Na^+ showed no significant change in proline transport over a 4-hr incubation period (13, 15). Further, proline accumulation against a concentration gradient was maintained. The cells displayed minimal changes in viability (3 to 4% change) and in intracellular space (4.3 ± 0.13 µl (S.E.)/5 × 10^6 cells).

Incorporation of Radioactivity into Macromolecules. Incorporation of substrate into protein by tumor cells was estimated by incubating 10^6 cells in glass tubes containing 1 ml of HEPES buffer, pH 7.5, with 0.1 mM leucine plus 2 µCi [^3H]leucine. The tubes were placed in a shaking water bath and incubated at 37° for various periods of time up to 4 hr. Incorporation was terminated by adding 8 ml of ice-cold 0.9% NaCl solution to

[4] The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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each tube, followed immediately by centrifugation for 2 min at 3° (900 × g). After the supernatant was decanted, 1 ml of 10% trichloroacetic acid was added to the pellet. The contents were mixed and then allowed to stand for 10 min. The tubes and contents were agitated again and centrifuged for 5 min at 900 × g; the resulting supernatants were removed with a Pasteur pipet. The cell pellets were washed twice, first with 2 ml of 10% trichloroacetic acid and then with 2 ml of 0.9% NaCl solution. One ml of hot (95°) 5% trichloroacetic acid was added to the pellet, the contents of each tube were refluxed at 105° for 30 min, and the tubes were allowed to cool and then centrifuged for 5 min at 900 × g. After the supernatant was discarded, the pellet was washed 3 times with 2-ml portions of 0.9% NaCl solution; the remaining pellet was dissolved in 2 successive 5-ml portions of aqueous counting scintillant and transferred to vials, and radioactivity was counted. The amount of radioactivity was indicative of the labeled amino acid incorporated into proteins. Incorporation of uridine into RNA was measured in cells, utilizing 0.1 mM uridine plus 2 μCi [^{3}H]uridine. In these cases, the supernatant was obtained after centrifugation of the trichloroacetic acid extraction mixture (105° for 30 min), and a portion was transferred to scintillation vials containing 20 ml of aqueous counting scintillant. The radioactivity was then counted, and the amount of radioactivity was indicative of the amount of uridine incorporated into RNA.

Production of $^{14}CO_2$ from Labeled Glucose. Quantitative measurement of labeled CO_2 produced by the R3230AC tumor cells was determined by incubating 10^7 cells with 5 mM D-glucose and 0.2 μCi of [*U*-^{14}C]glucose (150 to 250 mCi/mol) in a final volume of 5.0 ml of HEPES buffer (without glucose), pH 7.5, in a 25-ml Erlenmeyer flask. The flasks were gassed for 30 sec (95% O_2:5% CO_2) and then sealed with a rubber serum cap; a small plastic center well was suspended from the serum cap.

The flasks were incubated at 37° in a water bath with shaking for a period of 3 hr. Under these conditions, $^{14}CO_2$ production was linear over the period of 3 hr. The reaction was stopped by injecting 1 ml of 1 N HCl into the main compartment of the flask. The flasks were swirled vigorously to assure cell disruption and liberation of the $^{14}CO_2$; 0.3 ml of NCS Tissue Solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) was placed into the suspended plastic well by injection through the serum cap. The flasks were returned to the shaking water bath (37°) for 1 additional hr, which provided maximum recovery of $^{14}CO_2$.

At the end of this time, the rubber serum caps and the attached center wells were carefully removed from the flasks, the plastic well was cut from its stem, and the well and its contents were deposited into a scintillation vial. Ten ml of aqueous counting scintillant were added to each vial, the contents were shaken vigorously, and radioactivity was measured in a liquid scintillation counter.

Incorporation of Labeled Glucose into Fatty Acids. The cell suspension remaining in the flask after removal of the center well was transferred into glass tubes and centrifuged at 2000 rpm for 5 min. The supernatant was decanted; 0.5 ml of 30% KOH plus 0.5 ml of 95% ethanol were added to the pellet, the tubes were covered by glass marbles, and the contents were refluxed overnight at 60°. After the overnight heating, the lipids were extracted by 2 successive portions of 5 ml of hexane, each followed by centrifugation at 1000 rpm for 5 min and removal of the solvent. Both extracts were combined, placed in tubes, and heated at 70° to concentrate the extract to a final volume of 1 ml. This was then added to 10 ml of aqueous counting scintillant in a scintillation vial, and the radioactivity was counted.

Chemicals. HEPES, NaCl, phenylalanine, and hyaluronidase were obtained from Sigma Chemical Co. D-Glucose was from Mallinckrodt Chemical Works, St. Louis, Mo.; bovine serum albumin (Lot 55) was from Kupits, Forked River, N. J.; collagenase (type II) was from Worthington Biochemical Corp.; NCS tissue solubilizer was from Amersham/Searle and serum caps and center wells were from Kontes Glass Co., Vineland, N. J.

L-[*U*-^{14}C]Proline (specific activity, 274 mCi/mmol), L-[*U*-^{14}C]leucine (342 mCi/mmol), and [5-^{3}H]uridine (26 Ci/mmol) were obtained from Amersham/Searle; L-[*U*-^{14}C]phenylalanine (>405 mCi/mmol), [3-*O*-methyl-^{3}H]methyl-D-glucose (4 to 5 Ci/mmol) and D-[*U*-^{14}C]glucose (150 to 250 mCi/mmol) were from New England Nuclear, Boston, Mass.

RESULTS

Effect of Ovariectomy on Substrate Transport and Utilization. In the initial experiments, tumors from ovariectomized rats were obtained, and transport measurements were made on dissociated cells. These results, summarized in Table 1, indicate that proline entry was greater in tumor cells from ovariectomized rats, compared to that measured in tumor cells from intact rats, and entry of proline was comparable in tumor cells from diabetic and ovariectomized rats. Transport of phenylalanine was not affected by removal of the ovaries or induction of diabetes. Glucose transport, assessed by entry of 3-*O*-methylglucose, and metabolism of labeled glucose were similar in tumor cells from intact, diabetic, or ovariectomized rats.

Effect of Estrogen Administration on Tumor Growth. Treatment with estrogens was shown to inhibit growth of the

Table 1

Substrate transport and metabolism in tumor cells from intact, diabetic, and ovariectomized rats

Amino acids were present at 0.1 mM; phenylalanine transport was determined in the presence of unlabeled 50 mM proline. Each experiment was performed in triplicate on samples pooled from 2 to 5 rats sacrificed 19 to 21 days after tumor implantation.

	Proline transport (pmol/min/5 × 10^6 cells)	Phenylalanine transport (pmol/min/5 × 10^6 cells)	3-*O*-Methyl-glucose transport (pmol/min/5 × 10^6 cells)	$^{14}CO_2$ evolution (μmol/min/10^7 cells)	^{14}C incorporation into fatty acids (μmol/min/10^7 cells)
Intact	153.9 ± 8.4[a] (16)[b]	2298 ± 64 (5)	335.6 ± 14.5 (5)	0.177 ± 0.039 (14)	0.404 ± 0.028 (3)
Diabetic	258.7 ± 2.5 (17)[c]	2485 ± 240 (5)	369.4 ± 32.5 (5)	0.235 ± 0.045 (14)	0.383 ± 0.008 (3)
Ovariectomized	234.2 ± 25.0 (6)[d]	2124 ± 236 (5)	319.7 ± 8.6 (5)	0.208 ± 0.034 (6)	0.415 ± 0.031 (3)

[a] Mean ± S.E.
[b] Numbers in parentheses, number of separate experiments.
[c] Significantly different ($p < 0.005$) from intact animals.
[d] Significantly different ($p < 0.01$) from intact animals.

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R3230AC carcinoma (11). The effect of this hormonal therapy on tumor weight in diabetic or ovariectomized hosts was determined (Table 2). Tumor weight was reduced in estrogen-treated animals. The effect of estrogen therapy appeared to be greater in either diabetic or ovariectomized hosts, which may be attributed to the fact that tumors from hormonally perturbed hosts were larger than those from intact animals. Body weights were lower in diabetic rats.

Effect of Estrogen Administration on Transport. Tumors from intact, diabetic, or ovariectomized animals were obtained and dissociated; transport of proline and leucine was measured. The data shown in Table 3 demonstrate that treatment with estrogen significantly reduced proline transport with the greatest reduction seen in diabetic (60%) and ovariectomized (51%) rats and a lesser effect observed in intact (31%) rats. Transport of leucine was also reduced by estrogen treatment of diabetic (44%) rats, but only a modest reduction of leucine transport was obtained in tumors from intact (13%) rats. Thus, reduction of tumor growth by estrogen treatment was accompanied by significant decreases in proline transport and in leucine transport in tumors from diabetic rats.

Time Course of Estrogen Treatment on Transport and Precursor Incorporation. Since the apparent effects of estrogen therapy were greatest in diabetic rats, experiments were conducted to examine the time course of these responses to the administration of estrogens *in vivo*. These data are summarized in Table 4. At the end of 3 days of estrogen treatment, proline transport was significantly reduced to ~40% of the control (untreated) values; continued therapy produced a further decrease such that at 16 days, proline transport into tumor cells from estrogen-treated diabetic rats was reduced to a rate that was 25% of that seen in tumor cells from untreated rats. A similar time course for inhibition of leucine transport was observed, although the decrease in leucine transport was not as great as that seen for proline transport at 8 and 16 days of treatment.

The incorporation of labeled precursors into macromolecules was examined in these neoplastic cells from estrogen-treated animals (Table 4). Using labeled leucine as the probe, a progressive, time-related decrease in incorporation of leucine into proteins was observed, with a significant decrease occurring

Table 2

Effect of estrogen treatment on tumor and body weight in intact, diabetic, or ovariectomized hosts

Animal		Estrogen treatment (days)[a]	Tumor wt (g)	Body wt (g)
Intact	(3)[b]	0	4.95 ± 0.43[c]	137 ± 7
Intact	(3)	8	2.56 ± 0.42[d]	124 ± 3
Diabetic	(3)	0	7.38 ± 0.33	106 ± 3
Diabetic	(3)	8	2.46 ± 0.17[e]	92 ± 4
Ovariectomized	(3)	0	7.07 ± 0.43	141 ± 6
Ovariectomized	(3)	8	2.11 ± 0.27[e]	130 ± 4

[a] Animals received 1 mg estradiol valerate on Days 8 and 1 prior to sacrifice; all animals were sacrificed 19 to 21 days after tumor implantation.
[b] Numbers in parentheses, number of animals.
[c] Mean ± S.E.
[d] Significantly different ($p < 0.05$) from respective untreated animals.
[e] Significantly different ($p < 0.005$) from respective untreated animals.

Table 3

Effect of estrogen therapy on proline and leucine transport

Animals[a]	Proline transport		Leucine transport	
	Untreated	Estrogen treated	Untreated	Estrogen treated
Intact	142.1 ± 11.7[b] (3)[c, d]	97.35 ± 9.9 (4)	1465 ± 54 (3)	1277 ± 41 (4)
Diabetic	277.9 ± 49.0 (3)	111.4 ± 3.6 (3)[e]	1432 ± 23 (3)	804 ± 62 (3)[e]
Ovariectomized	240.9 ± 8.0 (3)	118.6 ± 6.0 (3)[e]		

[a] Animals received 1 mg estradiol valerate on Days 8 and 1 prior to sacrifice; all animals were sacrificed 19 to 21 days after tumor implantation.
[b] Mean ± S.E.
[c] Numbers in parentheses, number of tumors assayed.
[d] Amino acids were present at 0.1 mM; leucine transport was determined in the presence of unlabeled 50 mM proline. Each assay was conducted in triplicate; transport (0.1 mM of substrate) reported as pmol/min/5 × 10^6 cells; each tumor was assayed in triplicate.
[e] Significantly different ($p < 0.05$) from respective untreated animals.

Table 4

Time course of estrogen effects on amino acid transport and precursor incorporation in R3230AC tumors in diabetic rats

Duration of estrogen treatment[a]		Tumor wt (g)	Amino acid transport[b] (pmol/min/5 × 10^6 cells)		Precursor incorporation[b] (0.1 mM) (cpm/5 × 10^6 cells)	
			Proline	Leucine	Leucine	Uridine
0	(3)[c]	7.38 ± 0.33[d]	277.9 ± 49.0	1432 ± 23	15313 ± 805	16845 ± 1167
24 hr	(3)	6.33 ± 1.47	229.8 ± 37.0	1229 ± 149	11726 ± 628[e]	2542 ± 391[f]
72 hr	(3)	3.38 ± 0.43	102.4 ± 14.0[e]	639 ± 97[f]	8041 ± 164[f]	
8 days	(3)	2.46 ± 0.17	111.4 ± 4.0[e]	804 ± 62[f]	3735 ± 859[f]	3395 ± 430[f]
16 days	(3)	1.39 ± 0.15	68.9 ± 7.0[e]	676 ± 41[f]	5163 ± 414[f]	1577 ± 69[e]

[a] Estradiol valerate, 1 mg/week, was administered for the period indicated prior to sacrifice of animals.
[b] Details are given in "Materials and Methods." Amino acids were present at 0.1 mM; leucine transport was determined in the presence of unlabeled 50 mM proline.
[c] Numbers in parentheses, number of animals sacrificed at each time period.
[d] Mean ± S.E.
[e] Significantly different ($p < 0.05$) from untreated control.
[f] Significantly different ($p < 0.005$) from untreated control.

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within 24 hr after administration of estradiol valerate. This early decrease occurred prior to a significant reduction of leucine transport. Uridine incorporation into RNA was also measured in these cells; a striking decrease was seen at 24 hr after injection of estradiol valerate. Uridine incorporation remained markedly suppressed, showing a level of incorporation that was only 10% of that seen in cells from animals not receiving estrogen therapy. These data indicate that estrogen therapy *in vivo* produced progressive decreases in amino acid transport into R3230AC tumor cells and also reduced incorporation of precursors into proteins and RNA.

Effects of Estradiol *In Vitro*. To examine the effects of estrogen in the absence of other hormonal factors, experiments were conducted *in vitro* in which 17β-estradiol was added to dissociated tumor cells, and transport was measured. A summary of these experiments is presented in Table 5. Addition of 17β-estradiol (10^{-6} M) to dissociated tumor cells obtained from ovariectomized or diabetic hosts caused a significant reduction in proline transport. In contrast, estradiol *in vitro* did not demonstrate consistent or striking effects on transport of those amino acids that enter by the L system; leucine transport into tumor cells from ovariectomized rats was unchanged by addition of 17β-estradiol *in vitro*, and phenylalanine entry in cells from diabetic rats was slightly reduced (~12%). Leucine incorporation into proteins was reduced by 17β-estradiol *in vitro*. Thus, a direct effect of estradiol to decrease proline uptake was demonstrable *in vitro*.

We next examined the effects of 17β-estradiol *in vitro* on transport in tumor cells obtained from diabetic rats that had been treated with estrogen *in vivo* for 24 or 72 hr prior to their sacrifice and preparation of dissociated cells. These results are summarized in Table 6 and clearly demonstrate that estradiol *in vitro* was capable of inhibiting proline transport, even in cells that had a reduction in proline transport resulting from estrogen therapy *in vivo*. Estradiol *in vitro* did not significantly affect leucine transport, a result suggesting a degree of specificity of estrogens *in vitro* on amino acid transport systems.

Effect of Estradiol on Insulin-induced Responses *In Vitro*. From the foregoing data, it was concluded that estrogen *in vivo* or *in vitro* was able to inhibit proline transport (A system). In an earlier study (15), we observed that insulin *in vitro* stimulated transport of proline into the R3230AC tumor cells from diabetic rats; this response was both time and dose related. To explore the potential insulin-estrogen relationship, experiments were conducted to examine the effects of 17β-estradiol on the insulin-induced response of transport *in vitro*. The results of this study are shown in Table 7. As before, insulin (10^{-8} M) significantly increased the transport of proline, but not of leucine. The lower level of 17β-estradiol (10^{-8} M) was essentially as effective in reducing proline transport as the higher level (10^{-6} M) reported above (see Table 5). When cells were exposed simultaneously to insulin and 17β-estradiol (both at 10^{-8} M levels), the level of insulin-induced proline transport was significantly reduced by the presence of estradiol *in vitro*. However, when compared to the reduced level of transport of proline caused by addition of 17β-estradiol, insulin was still capable of inducing an increase in the entry of proline, but to a lower level than obtained with insulin alone. The specificity of these effects of insulin and estradiol on proline transport is substantiated by the lack of significant effects of any of these hormonal manipulations on leucine (L system) transport in the cells under identical conditions.

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Table 5

Effect of 17β-estradiol in vitro on transport in R3230AC tumor cells from ovariectomized or diabetic hosts

Transport and incorporation data obtained as detailed in "Materials and Methods." Amino acids were present at 0.1 mM; leucine or phenylalanine transport was determined in the presence of unlabeled 50 mM proline.

	Ovariectomized		Diabetic		
	Proline transport (pmol/min)	Leucine transport (pmol/min)	Proline transport (pmol/min)	Phenylalanine transport (pmol/min)	Leucine incorporation (cpm)
No addition	212.7 ± 13.7[a]	1,325 ± 58	260.4 ± 6.0	2,336 ± 78	16,254 ± 865
17β-Estradiol (10^{-6} M)[b]	149.3 ± 12.0[c]	1,269 ± 42	166.0 ± 11.6[d]	2,026 ± 12[c]	11,326 ± 436[d]

[a] Mean ± S.E.
[b] 17β-Estradiol (10^{-6} M) was added 6.5 hr prior to transport and incorporation measurements.
[c] Significantly different ($p < 0.05$) from control (no addition of hormones *in vitro*).
[d] Significantly different ($p < 0.02$) from control (no addition of hormones *in vitro*).

Table 6

Effect of estrogen[a] in vitro on proline and leucine transport in cells from estrogen-treated diabetic rats

Each experiment performed in triplicate. Amino acids were present at 0.1 mM; leucine transport was determined in the presence of unlabeled 50 mM proline.

Duration of estrogen treatment (hr)	Proline transport (pmol/min/5 × 10^6 cells)			Leucine transport (pmol/min/5 × 10^6 cells)		
	Control	Estrogen	% of control	Control	Estrogen	% of control
0 (3)[b]	288 ± 49[c]	195 ± 33	70	1491 ± 74	1417 ± 60	95
24 (2)	227 ± 37	96.0 ± 5[d]	42	1297 ± 150	1196 ± 118	92
72 (3)	102 ± 18[e]	57.6 ± 13[d]	57	749 ± 103[f]	683 ± 101	91

[a] Estrogen (10^{-6} M) present for 90 min.
[b] Numbers in parentheses, number of rats treated with estradiol valerate (1 mg/rat) prior to sacrifice.
[c] Mean ± S.E.
[d] Significantly different ($p < 0.05$) from control.
[e] Significantly different ($p < 0.05$) from untreated diabetic animals.
[f] Significantly different ($p < 0.01$) from untreated diabetic animals.

Table 7
Effect of insulin and estrogen on proline and leucine transport in tumor cells from diabetic rats

Amino acids were present at 0.1 mM; leucine transport was determined in the presence of unlabeled 50 mM proline.

Treatment	Proline transport[a] (pmol/min/5 × 10^6 cells)	% of control	Leucine transport (pmol/min/5 × 10^6 cells)	% of control
Control	275 ± 8	100	1466 ± 12	100
Insulin[b]	430 ± 8[c]	157	1469 ± 27	100
Estrogen[b]	167 ± 2[c]	61	1229 ± 125	84
Insulin[b] + estrogen	333 ± 5[d, e, f]	121	1347 ± 11	92

[a] Mean ± S.E.
[b] Insulin and/or 17β-estradiol (10^{-8} M) were added before and after cell preparation for a total period of 7.5 hr.
[c] Significantly different ($p < 0.001$) from control.
[d] Significantly different ($p < 0.01$) from control.
[e] Significantly different ($p < 0.005$) from insulin-treated cells.
[f] Significantly different ($p < 0.001$) from estrogen-treated cells.

DISCUSSION

The mechanisms whereby pharmacological doses of estrogen are efficacious in the treatment of breast cancer are not clearly understood. Recent studies have considered the relationship between estrogens and prolactin and mammary tumor growth; it has been proposed that estrogens may interfere with the prolactin-receptor-cell response axis (3). While this proposal may be valid for those neoplasms dependent on prolactin for growth, such as the tumors induced by 7,12-dimethylbenz(a)anthracene, it cannot fully explain the ability of estrogen to inhibit growth of the R3230AC tumor, which is not dependent on prolactin (11). The data presented here suggest that estrogens may act, at least in part, by inhibition of transport and incorporation of amino acids into cell proteins.

This suggestion is supported by the following evidence: (*a*) transport of proline was elevated in tumor cells of ovariectomized rats; (*b*) administration of estrogens *in vivo* reduced transport of proline in ovariectomized animals. Additionally, transport of both proline and leucine was inhibited by administration of estradiol valerate to diabetic rats and, to a lesser degree, intact animals; (*c*) the effects of this treatment *in vivo* were time dependent and appeared to correlate with effects on tumor growth; (*d*) 17β-estradiol caused a decrease in proline transport, and this effect *in vitro* was also observed in tumor cells from estrogen-treated rats; (*e*) 17β-estradiol *in vitro* was capable of antagonizing the insulin-induced increase in proline transport. Taken together with the data on leucine incorporation into proteins, we conclude that estrogens inhibit entry of amino acids and their incorporation into proteins in the R3230AC carcinoma, effects that are compatible with decreased tumor growth. Studies are under way to examine whether estrogens can be assigned a role of competitive inhibitors of substrate entry and whether such an effect is specific for estrogens.

Entry of neutral amino acids occurs by several systems, with a degree of overlap of specificity between the 2 systems studied here, *i.e.*, A and L systems. The choice of proline as the probe for the A system was based on previously reported results of kinetic and inhibition studies, which demonstrated that entry of proline was mediated exclusively by the A system at the substrate level used here (13). Although we could have used *N*-methyl-α-aminoisobutyrate as the substrate for the A system, we also wished to study incorporation of substrate into macromolecules under the same conditions, and proline was appropriate for such experiments. A greater uncertainty arises for the study of the L system, since its specificity for substrate is broader. From previous studies, we had estimated that, at low concentrations (0.1 mM) of leucine or phenylalanine, approximately 5 to 10% of their entry was mediated by the A system. To prevent this, entry of leucine or phenylalanine was measured in the presence of 50 mM proline, a level sufficient to saturate the A system; transport of leucine or phenylalanine under these conditions represents entry by the L system. Although one could measure entry of amino acids by the L system by eliminating Na^+ from the medium, the approach taken here was adopted so as to study the effects of hormones on amino acid transport in cells under the same conditions. The modest differences seen in the entry of phenylalanine or leucine could reflect slight differences in their affinity for the A system, although the presence of saturating levels of proline was felt to overcome this.

The ability of estrogens to decrease amino acid transport is somewhat at odds with an earlier report by Riggs and Walker (19), who examined the effects of estradiol disulfate and estrone sulfate on glycine and α-aminoisobutyric acid uptake by Ehrlich ascites cells. They found that the distribution ratios were increased by the estrogens, an effect attributed to an inhibition of efflux without an appreciable action on influx. The lowest level of estrogen studied by Riggs and Walker (19) was 10^{-4} mM, and at higher levels of estrone sulfate (5 to 10 mM) a decrease in the distribution ratio was observed. The data reported here indicate significant inhibition of proline entry even at 10^{-8} M 17β-estradiol, a level that more closely approximates a physiological level.

We have recently reported (15) that proline transport was greater in tumor cells from diabetic rats than in intact rats. Furthermore, insulin administration to tumor-bearing diabetic or intact rats decreased proline transport, whereas insulin *in vitro* increased proline transport in tumor cells from diabetic but not intact rats. Consequently, we proposed that the differences in the response of proline transport to insulin *in vivo versus in vitro* could be due to the presence of other hormones *in vivo*. This was further supported by the findings of Shafie *et al.* (20) that insulin binding was influenced by ovarian hormones; insulin binding was increased in tumors from ovariectomized rats and decreased by administration of estrogens. The data presented here could add additional support to an insulin-estrogen interrelationship at the level of amino acid transport. Estrogens *in vivo* consistently inhibited uptake of proline, as well as leucine in a time-dependent fashion. In contrast, 17β-estradiol demonstrated a greater specificity for the A system *in vitro*, inhibiting the transport of proline with little or no effect on the uptake of leucine or phenylalanine (L system). Furthermore, estradiol *in vitro* was capable of producing a further decrease in proline transport into tumor cells from estrogen-treated rats, which had a reduced proline transport. Finally, in the presence of both insulin and estradiol *in vitro*, proline transport was lower than that observed in the presence of insulin alone, suggesting that estradiol may have partially antagonized the maximal stimulation of proline transport by insulin; as before, no significant effects were seen on leucine transport. We interpret these results as indicative of the specificity of the steroid *in vitro*.

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Although the studies reported above suggest a possible interrelationship between estrogens and insulin on transport processes and perhaps on tumor growth, the exact mechanism or level of interaction remains to be elucidated. A direct competition for insulin receptors by steroids, such as estradiol, seems unlikely. On the other hand, a direct effect of estrogens on the transport carriers may occur in a manner analogous to that observed for estrogens and sugar transport. Phloretin, an extensively utilized agent that inhibits facilitated transport of glucose, is estrogenic (4). LeFevre (17) has shown that diethylstilbestrol was a more potent inhibitor than phloretin for preventing egress of D-glucose from erythrocytes, and hexestrol was somewhat less potent than phloretin; both diethylstilbestrol and hexestrol are potent estrogens. Thus, the structural characteristics that convey estrogenic activity may also be compatible for interaction with certain transport carriers. Data favoring such a mechanism of action of estrogens has been presented by Burk *et al.* (1) and Woods *et al.* (22) who examined the effects of diethylstilbestrol on glucolysis in a series of hepatomas and in the R3230AC tumor. They concluded that certain steroids, or steroid-like compounds, exhibited an "anti-insulin regulation of glucolysis, probably acting at the level of hexokinase." Further, these "anti-insulin" effects were diminished as this series of tumors progressed towards increasing malignancy, *i.e.*, growth rate. The level of diethylstilbestrol used in their experiments (1) was 150×10^{-6} M, considerably higher than that used here. It is possible, however, that differences in sensitivity exist among the various classes of transport carriers. Regardless, we suggest that the ability of estrogens to inhibit amino acid transport in this mammary tumor may contribute, at least in part, to their ability to inhibit tumor growth.

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