Some Differences Among Various Mammalian Cell Lines in Reversal of Growth Inhibition by 5-Fluorouracil.*† (25672)

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Growth inhibition of H. Ep. #1 cells(1) in culture by 5-fluorouracil and 5-fluorodeoxyuridine and attempts to reverse this inhibition by addition of related metabolites has been reported(2,3). Thymidine completely reversed growth inhibition by 5-fluorodeoxyuridine while thymine had relatively little effect. Furthermore, inhibition of growth by 5-fluorouracil could not be reversed by either thymine or thymidine. In HeLa cells in culture, Ackermann *et al.* reported that thymine as well as thymidine reversed growth inhibition by 5-fluorouracil(4). Both of these cell lines are derived from a human cervical carcinoma. Consequently, toxicity and reversal studies were carried out for both cell lines under comparable conditions. Experiments were also carried out using several other cell lines. Reversal studies demonstrated differences among cell lines with respect to reversal of growth inhibition by 5-fluorouracil and 5-fluorodeoxyuridine.

Materials and methods. The cell lines studied were: H. Ep. #1(1) and HeLa(5), derived from human adult epidermoid carcinomas of the cervix; H. Ep. #2(1), derived from adult human epidermoid carcinoma of the larynx; Chang's conjunctiva(6), derived from normal adult, human conjunctiva; AMK(7) derived from normal, adult monkey kidney; V-1 and V-2(8), derived from normal, adult Chinese hamster lung; and L strain(9) derived from normal, adult mouse connective tissue. Stock cultures of H. Ep. #1, HeLa, AMK, V-2, and L were maintained on glass in Eagle's medium(10) containing 10% horse serum. Cultures of Chang's conjunctiva, H. Ep. #2 and V-1 were maintained in Eagle's medium supplemented with 15% horse serum and 5% human serum. The experimental medium for all cell lines was Eagle's medium supplemented with 10% horse serum. Experimental conditions were similar to those previously reported(2). Four- to 6-day stock cultures were trypsinized and subcultured in 60 mm Petri plates. After 24 hours appropriate concentrations of inhibitor and/or reverser, sterilized by Millipore filtration, were added and the plates incubated in presence of CO_2 for 7 days. Medium and appropriate compounds were renewed after 4 days. Cellular growth was measured as total protein (after triple washing) by the colorimetric method of Oyama and Eagle(11). To determine degree of inhibition and subsequent reversal, growth in control plates (no additions) at 7 days was taken as 100% with protein values at zero time representing zero growth. Control plates exhibited a 7- to 12-fold increase in protein during 7-day incubation. Unless otherwise noted, experiments were carried out twice, in triplicate. In measurement of growth rate in cell colonies 5 x 10^3 cells were plated on 60 mm Petri plates engraved on outer surface with a numbered grid. After incubation for 24 hours the position of approximately 30 cells (with respect to engraved grid) was noted and appropriate compounds were added. At 24 hour intervals, number of cells/colony for each of the 30 colonies was determined microscopically at 39 or 156 × using inverted microscope. *Chemical Procedures.* In experiments to measure incorporation of exogenous thymine, cells were grown on surface of large Blake bottles. Thymine-2-C^{14} (1.2 x 10^6 cpm/μmole) and appropriate inhibitors (5-fluorouracil and 5-fluorodeoxyuridine) were added when growth was heavy (3 days prior to confluent growth) and incubation was continued 3 days. The

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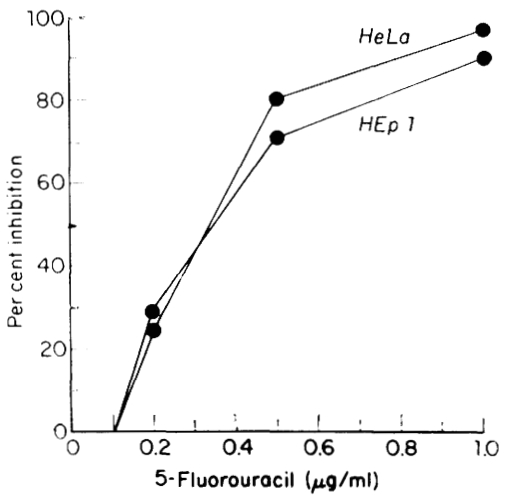

FIG. 1. Toxicity of 5-fluorouracil for H.Ep. #1 and HeLa cells. Growth measured as cell protein after incubation for 7 days.

cells were washed 3 times with saline and removed from the glass by trypsinization. The cells were rewashed (3 times) in saline and extracted with two 5 ml volumes of cold 2% perchloric acid. The residue was extracted twice with absolute ethanol and twice with ether. Hydrolysis was carried out in 0.15 ml of hot 70% perchloric acid (boiling water bath) for 45 minutes. The free thymine was separated by descending paper chromatography (Whatman #1) using isopropanol-HCl (12) followed by ascending chromatography using water-saturated butanol(12). After elution from the paper, the specific activity of thymine was determined. Radiocarbon activity was based on flow-gas counter measurements with aluminum planchets. Concentration was calculated from optical densities at absorption maxima (pH 2). Elution from control paper strip was used as a blank. Purity of thymine was checked by optical density ratios at 250/260 and 280/260 mμ.

Results. The results shown in Fig. 1 indicate that 5-fluorouracil is equally effective in inhibiting growth of both H. Ep. #1 and HeLa cells. However, attempts to reverse growth inhibition by 5-fluorouracil gave different results for the 2 cell strains (Table I). Thymine or thymidine did not reverse growth inhibition of H. Ep. #1 cells but partially reversed growth inhibition of HeLa cells. The latter is in agreement with a report by Ackermann(4). A 10-fold increase in concentration of thymine or thymidine did not appreciably increase reversal of growth inhibition of HeLa cells.

To determine whether this 20-30% overall growth in presence of 5-fluorouracil and thymine indicated a normal growth rate by a fraction of the cell population or a reduced growth rate for the whole population, clonal growth rate studies were carried out. Results of a typical experiment are shown in Fig. 2 where each point represents a single colony. Despite the scatter it can be seen that growth rate of clones in the presence of 5-fluorouracil and thymine is less than that for untreated cells. Since each cell selected at start of experiment was accounted for, the viability of cells grown in presence of 5-fluorouracil and thymine was not significantly lower than that for cells grown in the control medium. From the curve for cells grown in 5-fluorouracil alone, it can be seen that concentration of 5-fluorouracil in this medium was sufficient to prevent growth in absence of a reverser.

In H. Ep. #1 cells, isotopic tracer studies had demonstrated that exogenous thymidine was utilized to a greater extent for DNA thymine when the pathway leading to *de novo* formation of the thymine moiety was blocked by addition of 5-fluorodeoxyuridine to the culture medium(2). It was therefore of interest to compare the extent to which exogenous thymine (supplied in the medium) is utilized in formation of DNA thymine under conditions of normal cell growth. In the experiment summarized in Table II (5-fluorouracil absent) cell growth was approximately equal for each cell line during the 3 day incubation with

TABLE I. Effect of Thymine and Thymidine on Growth Inhibition of H.Ep. #1 and HeLa Cells by 5-fluorouracil (1 μg/ml).

	Conc. (μg/ml)	% reversal of growth inhibition (± avg dev.) H.Ep. #1	HeLa
Thymine	1	0	4 ± 4
	10	0	21 ± 4
	100	0	26 ± 6
Thymidine	10	0	20 ± 5
	100	0	30 ± 12

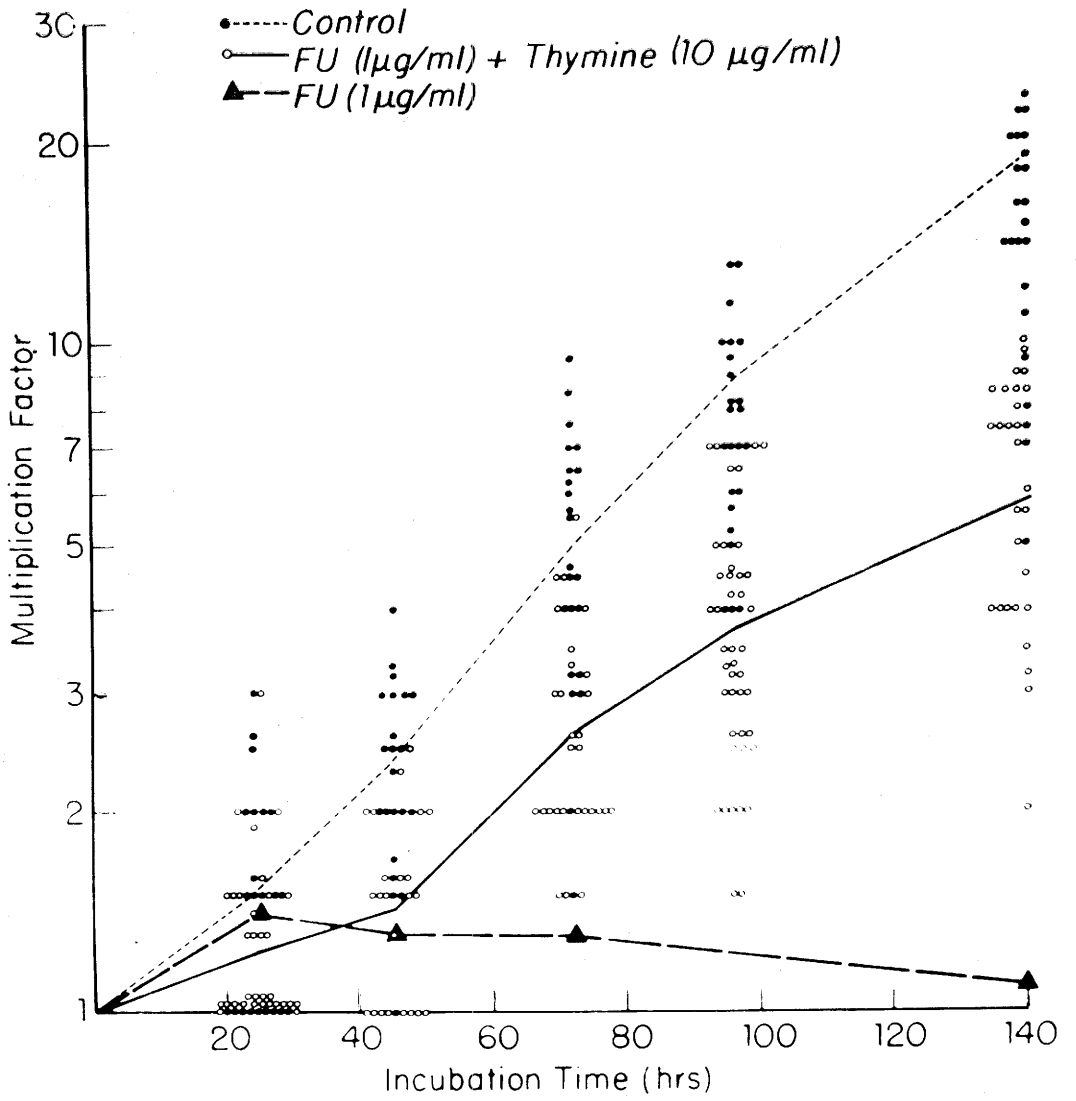

FIG. 2. Effect of 5-fluorouracil and thymine on clonal growth rate of HeLa cells. Each point represents No. of cells in a single colony. In the case of FU, only mean values are plotted. Lines are drawn through mean values for each time interval.

thymine-C^{14} present in the medium. Specific activity of DNA thymine in HeLa cells was 3 to 6 times greater than that in H. Ep. #1 cells.

TABLE II. Incorporation of Thymine-2-C^{14}* into DNA of Cells in Culture—Effect of 5-Fluorouracil.

5-Fluorouracil (μg/ml)	Specific activity of DNA thymine (cpm/μmole × 10^{-2})			
	HeLa		H.Ep. #1	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
0	231	517	74	85
1.0	2910	4170	152	168

* Concentration of thymine-2-C^{14}: 50 μg/ml. Specific activity: 1.20 × 10^6 cpm/μmole.

When 5-fluorouracil is present in the medium at 1.0 μg/ml, specific activity of DNA thymine for HeLa cells was increased by factors of 12 and 8 in Exp. 1 and 2 respectively. In these experiments, the exogenous thymine was serving as a partial reverser of growth inhibition as well as a labeled precursor of DNA thymine. In H. Ep. #1 cells specific activity of DNA thymine was increased by a 2-fold factor upon addition of 5-fluorouracil. The

TABLE III. Effect of Several Compounds on Growth Inhibition of H.Ep. #1 and HeLa Cells by 5-Fluorouracil (FU)* and 5-Fluorodeoxyuridine (FUDR).*

Compound	Ratio of conc.: Compound/inhibitor	Reversal of growth inhibition H.Ep. #1 FU	H.Ep. #1 FUDR	HeLa FU	HeLa FUDR
Thymine	10	—†	—	+	+
	100	—	—	+	+
Thymidine	10	—	+	+	+
	100	—	+	+	+
Uracil	10	—	—	—	—
	100	—	—	+	—
Uridine	10	—	—	—	—
	100	—	—	—	—
Deoxyuridine	10	—	—	—	—
	100	—	+	+	+
Cytosine	10	—	—	—	—
	100	—	—	—	—
Cytidine	10	—	—	—	—
	100	—	—	±	—
Deoxycytidine	10	—	—	±	±
	100	—	—	+	+
5-Methyldeoxycytidine	10	—	+	+	+
	100	—	+	+	+

* Concentration FU: 1 μg/ml; FUDR: 0.1 μg/ml. These concentrations of FU and FUDR completely inhibit growth of both cell lines.

† Reversal of inhibition: (—) 0-9%, (±) 10-19%, (+) 20-100%.

extent of utilization of exogenous thymine for HeLa cells in presence of 5-fluorouracil is significantly high. Specific activity of DNA thymine (4.2×10^5 cpm/μmole) was 35% of that for exogenous thymine.

Several compounds related to metabolites involved in nucleic acid synthesis were tested for ability to reverse inhibition by 5-fluorouracil and 5-fluorodeoxyuridine in H. Ep. #1 and HeLa cells (Table III). As previously reported(2) inhibition by 5-fluorouracil of H. Ep. #1 cells could not be reversed by any of the metabolites tested. Inhibition by 5-fluorodeoxyuridine could be completely reversed by thymidine or 5-methyldeoxycytidine at 10 times the concentration of inhibitor (10×) while deoxyuridine was effective at 100×. In HeLa cells, inhibition of 5-fluorouracil could be partially reversed at 10× by thymine, thymidine and 5-methyldeoxycytidine, and to a lesser extent by deoxycytidine. At 100× uracil and deoxyuridine effected a partial reversal. Uracil and cytosine at 100× did not reverse inhibition by 5-fluorodeoxyuridine, but did effect partial reversal of inhibition by 5-fluorouracil.

In view of differences between H. Ep. #1 and HeLa cells it appeared of interest to compare the response of other mammalian cell lines. The results with 6 cell lines are reported in Table IV. In all cases complete growth inhibition resulted at concentrations of 5-fluorouracil and 5-fluorodeoxyuridine of 1.0 and 0.10 μg/ml respectively.

All strains tested, except Chang's conjunctiva and V-2, exhibited a response similar to H. Ep. #1 cells in that inhibition by 5-fluorouracil could not be reversed by either thymine or thymidine. Thymine or thymidine effected an 18 to 24% reversal of growth inhibition with Chang's conjunctival cells. Addition of thymidine resulted in 35% reversal in V-2 cells. Partial reversal by thymine of inhibition by 5-fluorodeoxyuridine was observed only in H. Ep. #2 cells. Thymidine effectively reversed growth inhibition by 5-fluorodeoxyuridine in all cell lines tested.

Discussion. Studies in several laboratories strongly support the conclusion that the principal site of growth inhibition by 5-fluorode-

TABLE IV. Effect of Thymine and Thymidine on Growth Inhibition of Several Mammalian Cell Lines by 5-Fluorouracil and 5-Fluoro-2′-deoxyuridine.

Cell line	Compound tested for reversal	% reversal of growth inhibition 5-Fluorouracil*	5-Fluoro-2′-deoxyuridine*
Chang	Thymine*	±†	—†
	Thymidine*	±	+
H.Ep. #2	Thymine	—	±
	Thymidine	—	+
V-1	Thymine	—	—
	Thymidine	—	+
V-2	Thymine	—	—
	Thymidine	±	+
AMK	Thymine	—	—
	Thymidine	—	+
L	Thymine	—	—
	Thymidine	—	+

* Concentrations: 5-fluorouracil, 1.0 μg/ml; 5-fluorodeoxyuridine, 0.1 μg/ml; thymine, 100 μg/ml; thymidine, 10 μg/ml.

† Scoring of reversal of growth inhibition: —, no reversal (less than 10% reversal). ±, partial reversal (less than 35% reversal). +, complete reversal (greater than 70% reversal).

oxyuridine involves interference with steps leading to *de novo* synthesis of the thymine moiety, probably at the level of thymidylate synthetase(2,13,14,15,16,17,18). Complete reversal by thymidine of growth inhibition by 5-fluorodeoxyuridine in all cell lines studied, as contrasted with partial reversal by thymine observed in one of the cell lines studied, is in accord with the demonstrated greater utilization of thymidine rather than thymine for DNA thymine synthesis in mammalian cells (19,20,21). Tracer studies in Table II suggest that thymine is utilized to a greater extent for DNA thymine synthesis by HeLa as compared with H. Ep. #1 cells. In the presence of 5-fluorouracil, thymine-C^{14} was extensively utilized for DNA thymine synthesis by HeLa cells (in one experiment specific activity of DNA thymine was 35% of that of thymine added to culture medium). Further studies may indicate the contribution of differences in catabolism of thymine and its derivatives in addition to differences in the capacities of these cells to synthesize thymidine and thymidylate from exogenously supplied thymine (22).

Although 5-fluorouracil inhibits growth of HeLa and H. Ep. #1 cells approximately equally over a range of inhibitor concentrations (Fig. 1) differences between these 2 cell lines in reversal of growth inhibition by thymine and thymidine are clearly demonstrated by results described above. While the major site of growth inhibition by 5-fluorodeoxyuridine in these experiments appears to be well established, as stated above, inhibition by 5-fluorouracil of H. Ep. #1 cells probably includes conversion to acid soluble ribonucleotides, incorporation into ribonucleic acid and interference with its synthesis, in addition to interference with *de novo* synthesis of the thymine moiety(15,16,17). The partial reversal by both thymine and thymidine of inhibition by 5-fluorouracil in HeLa cells (and not in H. Ep. #1 cells) suggests that the mechanism of inhibition involves to a lesser extent in HeLa cells those metabolic pathways not directly involving synthesis of the thymine moiety. Differences among various cell lines in catabolism of fluorouracil and its derivatives as well as anabolic steps leading to ribonucleosides and phosphorylated derivatives may underly the variations in reversal of growth inhibition among different cell lines(22).

Differences between H. Ep. #1 and HeLa cells with respect to reversal of inhibition are also reflected in attempts to reverse inhibition with other nucleic acid metabolites (Table II). Reversal of inhibition by 5-fluorodeoxyuridine in HeLa and H. Ep. #1 and 5-fluorouracil in HeLa by 5-methyldeoxycytidine suggest that this compound might be utilized in pathways leading to synthesis of DNA thymine in mammalian cells(2). A similar role in microbial systems has been demonstrated by Cohen and Barner(23).

Summary. Growth inhibition of HeLa and H. Ep. #1 cells by 5-fluorouracil and 5-fluorodeoxyuridine and reversal of inhibition by thymine and thymidine have been studied. Growth inhibition curves with 5-fluorouracil are similar in both cell lines while reversal studies reveal several differences. Thymine partially reverses inhibition by 5-fluorouracil and 5-fluorodeoxyuridine in HeLa but not in H. Ep. #1 cells. Thymidine reverses inhibition by 5-fluorodeoxyuridine in both H. Ep. #1 and HeLa cells but reverses inhibition by 5-fluorouracil only in HeLa cells. Partial reversal (about 30%) of inhibition by 5-fluorouracil in HeLa cells represents more closely a reduced growth rate by the whole cell population rather than a normal growth rate by a fraction of the population. Utilization of thymine-2-C^{14} for DNA thymine synthesis in HeLa cells was significantly increased in presence of 5-fluorouracil.

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1. Moore, A. E., Sabachewsky, L., Toolan, H. W., *Cancer Research,* 1955, v15, 598.

2. Rich, M. A., Bolaffi, J. L., Knoll, J. E., Cheong, L., Eidinoff, M. L., *ibid.,* 1958, v18, 730.

3. Eidinoff, M. L., Rich, M. A., *ibid.,* 1959, v19, 521.

4. Ackermann, W. W., Loh, P. C., Payne, F. E., *Bact. Proc.,* 1958, 71.

5. Gey, G. O., Bang, F. B., Gey, M. K., *Ann. N. Y. Acad. Sci.,* 1954, v58, 976.

6. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

7. Parker, R. C., Castor, L. N., McCulloch, E. A., *Cellular Biology, Nucleic Acids and Viruses,* N. Y. Acad. Sci., Special Publication #5, 1957, 303.

8. Ford, D. K., Yerganian, G., *J. Nat. Cancer Inst.,* 1958, v21, 393.

9. Sanford, K. K., Earle, W. R., Likely, G. D., *ibid.,* 1948, v9, 229.

10. Eagle, H., *Science,* 1955, v122, 501.

11. Oyama, V. I., Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 305.

12. Wyatt, G. R., in E. Chargaff, and J. N. Davidson (eds.), *The Nucleic Acids,* Academic Press, 1955, v1, Chap. 7.

13. Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E., Scheiner, J., *Nature,* 1957, v179, 663.

14. Eidinoff, M. L., Knoll, J. E., Klein, D., *Arch. Biochem. Biophys.,* 1957, v71, 274.

15. Bosch, L., Harbers, E., Heidelberger, C., *Cancer Research,* 1958, v18, 335.

16. Danneberg, P. B., Montag, B. J., Heidelberger, C., *ibid.,* 1958, v18, 329.

17. Harbers, E., Chaudhuri, N. K., Heidelberger, C., *J. Biol. Chem.,* 1959, v234, 1255.

18. Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., Lichtenstein, J., *Proc. Nat. Acad. Sci.,* 1958, v44, 1004.

19. Brown, G. B., Rall, P. M., In: E. Chargaff and J. N. Davidson (eds.), *The Nucleic Acids,* Academic Press, 1955, v2, Chapter 25.

20. Holmes, W. L., Prusoff, W. H., Welch, A. D., *J. Biol. Chem.,* 1954, v209, 503.

21. Reichard, P., *Acta Chem. Scand.,* 1955, v9, 1275.

22. Chaudhuri, N. K., Mukherjee, K. L., Heidelberger, C., *Biochem. Pharmacol.,* 1959, v1, 328.

23. Cohen, S. S., Barner, H. D., *J. Biol. Chem.,* 1957, v226, 631.

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Early Permeability Effects of Estradiol on Castrate Rat Uterus.* (25673)

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The view that the primary mechanism of action of various hormones at a cellular level may involve regulation of permeability processes in target cells is being investigated in this laboratory. We previously studied cellular permeability effects of ACTH upon the adrenal(1) and have reported certain similarities between this action of ACTH and that of insulin on muscle fibers(2,3). In the present work we examined the early effects of estradiol-17β upon permeability processes in castrate uterus (using C^{14} labeled "non-utilizable" sugars, α-aminoisobutyrate (AIB), and Na^{22}) to determine whether the primary action of estrogen might be related to increasing substrate entry into the cell. Alterations in carbohydrate metabolism in isolated uteri following estrogen administration *in vivo,* have been ascribed to increased transfer of glucose into the cell(4). The effect of estrogen to increase amino acid incorporation into uterine protein under similar conditions(5) might likewise be considered as the result of increased amino acid transport, inasmuch as Noall *et al.*(6) have shown that estrogens administered 20 hours previously specifically increase accumulation of AIB in the uterus. Our results indicate that estradiol-17β does not increase uterine permeability to substrates at a time when changes in uterine metabolism of carbohydrate and protein are evident.

Methods. Sprague-Dawley rats (200-250 g), ovariectomized 4-7 weeks previously, were functionally nephrectomized at time of experiment to maintain relatively constant blood levels of radiotracers(1). In most cases they were adrenalectomized 48 hours prior to experimentation to remove possible inhibition by corticoids (liberated during stress of kidney ligation) of uterine response to estrogen(4,7). After ligation of both kidney pedicles under

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